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ASP PRESIDENT 2010–2011, JANINE CAIRA: THIS IS YOUR LIFE!

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Honored guests, friends, and fellow members of the American Society of Parasitologists, welcome to the 2011 Presidential Address. I am privileged to be able to introduce Dr. Janine Caira. Before I ask her to join us for her Address, I'd like to share with you a few tidbits about her life.

She was born in Montréal, Québec, Canada November 9, 1957, in the same hospital where both her parents worked; her father, Eugene, was a surgery resident and mother, Joan, was a neonatal nurse. The world in 1957 was a tumultuous and exciting place: on October 4, the Earth's first satellite, Sputnik, a whopping sphere 59 cm in diameter, was launched into space by the (former) USSR, thus beginning the Space Age. Both the USA and the USSR successfully launched the world's first Intercontinental Ballistic Missiles (ICBMs) that year, thus beginning the Cold War, which lasted more than three decades. Gordon Gould invented the laser. Serial killer Ed Gein murdered, and ate, his last victim in Wisconsin. Former five-star general, and then President, D. D. Eisenhower ordered federal troops to integrate schools in Little Rock, Arkansas, stating, “The Federal Constitution will be upheld by me, by every means at my command.” The times they were a'changin and, for our Society, they changed that November 9th with Janine's birth. Janine has an older brother, Loren, and three younger sisters Nadia, Daren, and Martene. She also is blessed with two still-younger half-sisters, Rachel (see Fig. 1) and Leean, from Eugene’s second (well, actually third) marriage (but that’s another story).

Some of you may have noticed that Janine has lots of energy. This is not a new phenomenon, and that energy started to manifest itself at a very early age. After Montreal, the young family moved to New Brunswick, one of Canada’s three maritime provinces, and there began the “Neenie” stories which have become part of the legend and fabric of Janine’s history, both in her family and in her laboratory. The first is entitled, “Neenie all gone.”

It was a sunny, warm winter day in New Brunswick when Loren was about 3-yr-old and Janine was just 9 mo. Joan was working in the kitchen and thought the kids needed some fresh air, so she bundled them up and put them on the porch. Janine, still too young to walk, nonetheless was rambunctious and, when placed in her stroller, always moved her feet and rocked back and forth. It seems she always needed some action to stimulate herself. As the kids were on the porch, Joan was cleaning until she heard a soft ‘knock, knock’ at the door. Looking through the window, she didn’t see anyone. Opening the door, there was young Loren saying, “Mamma, Neenie all gone, Neenie all gone!” Sure enough, Neenie wasn’t on the porch; she had rocked her stroller off the porch into a snow bank and was upside down and buried with the stroller on top of her. This wasn’t the first time that big brother Loren saved Neenie. There are many “Neenie” stories that would be fun to get into, but no time. E-mail Janine and ask her about some of these:

- “Neenie up, up, and away”
- “Neenie hates family picture time”
- “Neenie and Loren near death by a large ship in Indonesia”
- “Neenie and Kirsten near death on the beaches of Senegal”
- “Neenie and the dead skunk in purse story”

... and the list goes on.

After New Brunswick, and while still reasonably young, her family moved to St. Catharines in southern Ontario and the five kids, led by Janine (organizing her first field trips), became a 5-person field crew, terrorizing the small, helpless creatures along a creek near their home. She attended Beamsville High School and took up gymnastics, a sport in which she excelled; in the summer after her senior year and the following two summers, she worked at Agriculture Canada where she got involved in a joint project between a virologist, Humburto Dias, and a nematologist, John Potter, who were studying viral transmission by a root-feeding nematode that was threatening to wipe out some wine grapes. Perhaps this experience was the ember planted in her brain that

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**FIGURE 1.** Janine and younger sister Rachael in Seward, Alaska, a few days after the ASP-2011 Annual Meeting in Anchorage, showing off the 67.13 kg (148 lb) halibut that Rachael helped Janine catch.
later would become the first spark of interest in parasitology. But after high school she was, at heart, still a gymnast and wanted to continue gymnastics in college. This led her to attend the University of Guelph (fall 1975) to do gymnastics, but when that did not work out, she bolted to the University of British Columbia (UBC) in Vancouver to ski with brother Loren, who was enrolled there doing lots of skiing.

At UBC, she entered the Honors Zoology Program and did an honors thesis with Dennis Chitty comparing territory marking in voles (1977–1979). During her second (senior) year at UBC she finally took a formal parasitology class from Jim Adams; she loved the class, was hooked (the smoldering ember burst into flame), and she asked Jim if she could do a M.S. with him. But Jim was retiring the next year and not taking any more students. Not to be deterred, she went to the top of the food chain, to Goeff Scudder, who was Head of Zoology at the time, and he agreed to be her advisor, but only if she would work on a parasite of an aquatic insect. She discussed this idea with Hilda Chung, Murray Kennedy, and Greg Norman in Jim’s research lab and ended up studying the encapsulation response to metacercariae of a digenean that encysts in the silk glands of caddisfly larvae.

While working on her M.S. (1979–1981), she participated in meetings of the BC parasitologists, which gave her the opportunity to meet Bob and Reggie Rausch, Leo Margolis, Bob Kabata, Richard Arthur, Eric Hoberg, and Dan Brooks. Dan had come to UBC to teach cladistics; Janine not only learned cladistics, she mastered it. Dan also encouraged her to attend her first ASP meeting in 1980 in Berkeley, California, a meeting that changed her life forever. There, she met Mary Lou Pritchard, who eventually invited Janine to come to the University of Nebraska–Lincoln (UNL) to work with her on a Ph.D. However, in the summer of 1981, before beginning her Ph.D. program, she and a friend were invited to California to attend a Rattlesnake Roundup near the California/Mexico border. Janine seized the moment, as she always does, as an opportunity to collect rattlesnake parasites. After all, they’re at the top of their food chain and had to have “cool” parasites. But after the long drive from Nebraska to California (remember, there were no cell phones, no e-mail, no internet then), they learned they had missed the round up by a week (!)... so they decided to trek down the Baja Peninsula to camp and play on the beach of the Northern Gulf of California (fortunately, this was before the drug lords took over). As they were setting up camp, a Mexican fishing boat was docking: Guess what? It was a load of sharks! Now Janine is legendary for always making the best of whatever situation presents itself. So this was serendipity! OK, ha, ha, ha: no rattlesnakes; how about shark parasites? And thus began the Caira—elasmobranch—tapeworm odyssey.

At UNL (1981–1985), she also had the opportunity to interact with Brent Nickol and John Janovy and their myriad of great undergraduate and graduate students and to attend four annual meetings of the Southwestern Association of Parasitologists (SWAP) in which the UNL parasitologists have been the backbone for so many years. In August, 1983, in the middle of her Ph.D. research, Janine gave an invited paper at the Society of Systematic Zoology meeting. Unbeknownst to her, Jim Slater, an entomologist at the University of Connecticut (UConn) was in the audience. During FY 1983–1984, the Department of Ecology and Evolutionary Biology at UConn opened a search for a parasitologist, but Janine was still a year away from completing her Ph.D., so she didn’t apply. A number of behind-the-scenes events were occurring at UConn at the time (D. Minchella, pers. comm.) when Jim recalled hearing Janine’s talk in 1983; he asked the chair of the search committee to call her to ask her to apply for their position. She did, but she was still >1 year away from completing her research and degree. However, Jim convinced his colleagues when he said something like, “OK, let’s wait a year and see if we can get this ‘dynamo’ to apply for our job here at UConn when she finishes next year.” The faculty agreed. The rest is history.

In August 1985, when Janine and her significant other arrived in Storrs, they had very little money, as many do in that transition from grad school to the first job. And they arrived, as many do, with all their belongings in a small U-Haul truck. In addition to not having much cash, they didn’t have a place to live. Not to worry. They moved stuff around in the back of the U-Haul and slept there for a week or so, parked in the lot behind the Torrey Life Science Building, where she began as Assistant Professor in the Department of Ecology and Evolutionary Biology.

There, her work ethic is legendary. She epitomizes the word “work-a-holic.” At UConn she typically leaves home by 6:30 a.m. and typically doesn’t return until 9 p.m. or later. In the field, she is the first one to start work and the last to stop, long after everyone else has gone to sleep. On her first trip to Baja, one of her team members had to tell her that some of the other people in the field crew might like a day off. There was initially a blank stare. It had never occurred to Janine that they didn’t all love the idea of working dawn to dusk, in 100°F temps all day, pulling tapeworms out of shark guts. It’s not that she doesn’t like to have fun or party. She just couldn’t see the point of stopping work until after the sun went down and it was too dark to do dissections!

For better or for worse, those of us who choose academia are judged by four criteria that become part of our legacy: research/scholarly activity, teaching, university/community service, and personality/ability to play nice in the sandbox with others. So how does our ‘dynamo’ stack up in these criteria?

Janine discovers, describes, and names the tapeworm parasites of sharks, skates, and rays; she is intimately involved in properly identifying the correct hosts for her tapeworms; she is keen to understand the evolutionary relationships between these vertebrates and their invertebrate passengers; and she has made substantial contributions to understanding the conceptual issues associated with the co-evolution of these cute little parasites and their very special hosts. Her interests in morphology are not restricted only to taxonomic questions, but to the much broader issues of biogeography, biological diversity, global faunistics, the improvement of both biological collections and biological databases, and even conservation biology. She’s published about 100 refereed papers, six book chapters, and three books/monographs, the most recent of which is: “A DNA sequence based approach to the identification of shark and ray species and its implications for global elasmobranch diversity and parasitology.”

A former president of our Society wrote in the Journal of Parasitology that, “Cestode research has been, and is, one of the most poorly funded areas of parasitology.” I would argue, and I’m sure Janine would concur, ‘you don’t catch no fish if you don’t put your line in the water,’ whether it be for catching sharks or federal dollars. She’s been the PI or co-PI on 14 NSF grants since 1988, totaling >US$6,327,000 and about half have had one, or two, or three REU supplements attached to them to support
undergraduate research. This funding has allowed her to take both graduate and undergraduate students on nearly 50 field trips to collect elasmobranchs from around the world including, but not limited to, such places as Australia, Borneo, Canada, Dakar, Indonesia, Mexico, Madagascar, Senegal, South Africa, Taiwan, Vietnam, and other exotic locations. These trips have truly made her our globe-trotting parasitology ambassador for ASP. And when she meets people in other countries, they quickly embrace her passion and see the infectious enthusiasm she has for her work, and later invite her back to tell them what she's learned from that work. Thus, she's given >50 invited departmental and university lectures since 1987 (~4/yr) from California to New York, from Canada to Brazil, from Madagascar, and from Japan to Malaysia to Australia. In recognition of her many early accomplishments, ASP awarded her the Henry Baldwin Ward medal at our Hawaii meeting (1998), but this just scratches the surface of her scientific accomplishments. However, Janine would never consider counts of papers or dollars as a gauge of her contributions. Rather, I believe, her argument would be that her work is simply a product of her desire to continue to learn and to transmit that new knowledge to the students whom she loves dearly. That leads us to teaching.

Her accomplishments teaching undergraduates are extraordinary. She is consistently one of the most highly-rated classroom teachers in her department, both by her chair and by the colleagues with whom she teaches. Training graduate and advanced undergraduates in the lab is also teaching and takes enormous amounts of energy and patience. Since arriving at UConn, rarely has a semester passed when there were not two to three undergraduates, often part of the UConn Honors Program, doing independent research and one to two graduates in her lab, working shoulder to shoulder with each other, and with her; all learning, all sharing, all fully-incorporated as active and equal participants. She is a woman of great passion and dignity, and she has a flair for eliciting the best from her students at every level. She listens to them and their ideas, she works with them to develop those ideas, she works with them to test those ideas, she challenges them to excel and, in doing so, she builds their confidence to levels they could not have imagined before they met her. Since arriving at UConn, 35 undergraduates have worked in her lab and 22 of them have either published their work or presented it at professional meetings as oral presentations or posters, or usually both. She also has mentored seven M.S., 11 Ph.D., and seven post-doctoral students to date and has made lifelong friends of all her former students. Perhaps it's best to end this section with statements from just 2 former students to emphasize this point: "There is a line in Chaucer's Canterbury Tales, about a character called the Clerk: 'Gladly would he learn, and gladly would he teach.' I think this phrase describes Janine as well as any could. She is a consummate scholar; she reads a lot, and I really admire how she keeps up with the field. As graduate students, we would see her keep learning and I think that helped us recognize that we also always need to keep learning. In terms of taxonomic study, I remember she would advise us to 'get intimate with your worms,' that is, learn their morphology thoroughly, but also learn everything possible about them from the literature." Florian B. Reyda, State University of New York, Oneonta, New York.

"She is the most genuine person I know, and her passion, enthusiasm, scrupulousness, and persistence are remarkable. These characteristics come through in any type of interaction with her. I think this may be why Janine is so successful at pulling large teams of people together and getting them to work intensely with her. And one of the rules she lives by is this: 'work hard and play hard.'" Claire J. Healey, Royal Ontario Museum, Toronto, Ontario, Canada.

Recognizing her accomplishments in teaching and research, Janine is one of the few faculty, if not the only one, to have received all four of the highest honors the University of Connecticut can bestow: University Teaching Fellow (1995), Alumni Association Distinguished Teaching Award (1999), Alumni Association Distinguished Professor Award (2003), and the Board of Trustees Distinguished Professor (2006). Heck, she's still got 10–15, or more, good years left. What can she possibly do for an encore at UConn?

One of the most surprising things to colleagues at UConn who work with Janine for the first time is that her days have the same number of hours as ours do. At UConn she has served on and/or chaired 80 departmental, college, and university-wide committees. She doesn't just show up for the meetings; she usually is the driving force behind them so that they accomplish the functions for which they were created (imagine, university committees that get stuff done!). And she doesn't just serve once, but has served on some standing committees for up to eight years. These extraordinary efforts have clearly established her as a campus leader. Nonetheless, such an astounding service record hasn't compromised the time she spends with her students. In fact, Tim Ruhnke, another former student, told me before this meeting that, "she has studiously avoided career-killing administrative duties." How can that be with this kind of record? I think she lives in another dimension!

Her service to our discipline of parasitology is equally impressive: She has co-chaired/organized four sessions, workshops, or symposia at ASP annual meetings; served on or chaired six standing, selection, or award committees, usually for multiple years; been on the editorial board of five of our journals, The Canadian Journal of Zoology, Systematic Parasitology, Parasitology, the Journal of Parasitology, Acta Parasitologica, and the Journal of Helminthology; she initiated and organized the First National Parasite Day (March, 1999); she is the co-founder, and later President, of the New England Association of Parasitology; in ASP she has been an elected member of the Council, Vice President, President-Elect, and this year our President; she is the chief organizer of all cestode taxonomists world-wide; she is the leader in collaborative work in elasmobranch molecular systematics; she has the best ethanol-fixed tissue collection for elasmobranchs in world (second place is not even close); she is a member of four other scientific societies in which she also is active; and she has further served our discipline by being an active member on six different NSF panels, most multiple times, continuously since 1994.

Yes, she is capable and does play nicely in the sandbox with the other kids.

Janine Caira represents the best of what a university professor can be. She sets an incredibly high bar in teaching, research, and service, a standard to which every young person in this audience should aspire. She has had a tremendous influence on parasitology in general, on the taxonomy and systematics of flatworms in particular, especially those from sharks and rays worldwide, but also on the identification of elasmobranch species and its implications for global elasmobranch diversity and parasitology. She gives credit to virtually every person with whom she has
worked and interacted in her professional journey since the balance beam but, realistically, it is Janine who has had a tremendous influence on all of us who have known and interacted with her and it is we who are better, both professionally and personally, for those opportunities. In summary, she possesses a unique combination of scholarship, integrity, morality, humility, work ethic, and sense of humor (sometimes).

Yet, Janine is a regular person. She doesn’t wear hats and is not particularly fond of cats, but she is athletic, a great cook, she loves chocolate, parties, racquetball, auto racing, and movies where stuff gets blown up, especially if they star Sly Stallone or Arnold Schwarzenegger (although she may have modified her views about Arnold in recent weeks). For those who have not yet heard her speak, I guarantee you are in for a treat. For those who have listened to her before, I know you’re hoping I’m finished so you can learn something and enjoy your journey, as one does each time Janine speaks. Ladies and gentlemen, it is my great honor in welcoming to the podium the President of our Society, Dr. Janine Caira.

ACKNOWLEDGMENTS

A number of people kindly gave me the inside details to help me put this talk together. I am very grateful to Greg Anderson, University of Connecticut, Storrs, Connecticut; Nadia Caira, younger sister; Kent Holsinger, University of Connecticut, Storrs, Connecticut; Tim Ruhnke, West Virginia State University, Institute, West Virginia; Florian B. Reyda, State University of New York, Oneonta, New York; Kirsten Jensen, University of Kansas, Lawrence, Kansas; and Claire J. Healey, Royal Ontario Museum, Toronto, Ontario, Canada. Special thanks to Lee Couch, University of New Mexico, Albuquerque, New Mexico, for her help in proofing the many drafts of this document.
THE AMERICAN SOCIETY OF PARASITOLOGISTS: WHO ARE WE NOW?*

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In contemplating possible topics for this address here today, foremost in my mind was my conviction that the mission of a president of a scientific society is not only to enhance that society’s intellectual endeavors but also to nurture its health and well-being. Had I chosen to use this opportunity to engage in the former, I would have spent this hour enlightening you, a captive audience, about the marvels of the tapeworms of elasmobranchs! However, Mike Sukhdeo, Steve Nadler, and Robert Poulin did such a terrific job of stimulating thought about the boundary (or interface!) between parasite ecology and evolution with their contributions to this year’s President’s Symposium that I feel my responsibility for intellectual enlightenment has, although vicariously, already been effectively discharged. Thus, I have chosen to focus my comments on the second aspect of a president’s mission. Indeed, this topic is particularly timely given the concerns that have been voiced over recent years about the membership, student population, and future of our Society. Ever an optimist, I have typically dismissed such concerns as unfounded but, perhaps, it is indeed time for some promotional activities. It has been quite a few years, even decades, since we have taken stock of who we are as a Society. If we are to actively promote the American Society of Parasitologists (ASP), it is important that we begin by embracing the adage “Know Thyself,” not only so that we are realistic about our current issues but, also, so that when we boast of our accomplishments, we do so accurately.

As a consequence, today I will attempt to answer the question: Who are we now? Given that an assessment of our current status and the activities and interests of our membership is more meaningful in the context of our history, I will begin with a brief review of the Society’s origins and past. The historical information I will present was drawn from all ASP meeting programs up to 2011 and the contents of the Journal of Parasitology (JP) up to 2010. The accumulation of information on our current members involved a certain amount of what would best be characterized as “cyber-stalking” using internet resources such as individual and institutional websites as well as Google searches of various funding, etc. databases. In this endeavor, I was ably assisted by Kendra Koch and Elizabeth (Beth) Barbeau. Our current graduate student representative to Council, Joanna Cielocha, provided photos of many of our student members here in Anchorage. Kirsten Jensen greatly assisted with the compilation and presentation of data. Although convenient, by its very nature, this strategy has obvious limitations and as a result, the information I present should be considered to be illustrative, rather than exhaustive. I note that our search efforts yielded data for only about 50% of our current members and the funding information obtained is primarily for our US and, to a much lesser extent, Canadian members. Although much of the material I will present is of a general nature, in some instances it seemed more appropriate to mention individual members. I apologize in advance for any inaccuracies and/or omissions and note that I alone am responsible for such errors.

HISTORICAL CONTEXT

The first volume of the Journal of Parasitology, which was edited by H. B. Ward, was published in September of 1914. This volume appeared a little over a decade before ASP was officially founded, a fact that was announced in print in March 1925 in the third issue of the 11th volume of the Journal. Not only was H. B. Ward the founding editor of our Journal but he also served as our Society’s first President. In the year of its birth, the Society had 321 members who each paid annual dues in the amount of $1. The first meeting of the Society was held in December of 1925 in Kansas City, Missouri. Over 60 members were in attendance and 38 papers were presented. With the exception of 1943, the Society has met every year since 1925. Beyond interrupting our annual meeting schedule, World War II also prompted an interesting Symposium entitled “Parasitology in Relation to the War” at the Society’s annual meeting in 1944.

Since 1925, Society membership has fluctuated substantially (Fig. 1). Our highest membership to date was reached in 1975 when we had 1,946 members. (I am guessing that this trend is not unique to our Society and that other societies experienced similar booms in membership during the 1970s.) Our lowest recorded membership since then was 810 individuals in 2007. However, a closer look at recent trends reveals that our membership now appears to be leveling out (Fig. 1 inset). Curiously, over that same 86-year period, the number of papers and posters presented, i.e., abstracts submitted, at our national meetings has remained remarkably stable, with an average of 146 abstracts per year in the 41 years for which these data are available, with the exception of a few unusually high years. These exceptions have occurred on special occasions, such as our 50th Anniversary meeting in 1975.

*Presidential address: 86th Annual Meeting, American Society of Parasitologists, 3 June 2011, Sheraton Anchorage Hotel and Spa, Anchorage, Alaska. DOI: 10.1645/GE-2980.1

FIGURE 1. Contrast between American Society of Parasitologist (ASP) membership and meeting participation (based on abstracts submitted) for years for which these data are available since the Society’s founding in 1924. *Abstract numbers are not available for years of ASP’s joint meetings with the International Congress of Parasitology (ICOPA). Inset details membership over last 10 yr.
and in instances of our joint meetings with other societies, e.g., The American Society of Tropical Medicine and Hygiene, The American Society for Protozoologists, The Society of Nematologists, and the Mexican Society of Parasitology. It is difficult to extract the number of ASP members submitting abstracts in years in which we have met concurrently with the International Congress of Parasitology (ICOPA) given the combined nature of the program in such instances. (I note that the Program of our meeting here in Anchorage includes a total of 187 abstracts, which is 42 more than the average of our nonconcurrent meetings with other societies.) It seems clear that this remarkably steady level of meeting participation attests to the existence of a core number of dedicated and faithful parasitologists who serve as the backbone of the Society. Given the duration of this trend, the constituency of this core of members has clearly changed over time. The view of the audience I currently have from the podium is of much of that core of Society members (at least those who chose to attend my talk!), for it is those of you here at this meeting in Anchorage who currently comprise that group of dedicated individuals. Of course, your numbers are complemented by the hundreds of additional members who contribute to the Society in other ways; for example, through publication in, and service to, the Journal and participation in our various committees.

PRESENT

Based on data available from the Secretary-Treasurer’s office for 2010 (given it is only June 2011, numbers are as of yet incomplete), the American Society of Parasitologists is 829 members strong and is remarkably international, for our members are distributed across 42 countries worldwide (Fig. 2). By far, the majority of these individuals reside in the United States (605) followed by Canada (47), Mexico (20), and Argentina and Japan, each with 18 members. A more detailed look at our constituencies in these 5 countries reveals the following: in the US, 6 states are each home to over 20 members. These are: California (61), Texas (40), Maryland (35), Georgia (30), Florida (28), and New York (24). Delaware, Nevada, Vermont, and Alaska each have only a single member; Rhode Island is the only state that currently lacks ASP members. In Canada, Alberta, Quebec, and Ontario each have 9 members. Newfoundland and Manitoba are each home to a single member. Saskatchewan is the only province without a member of ASP. Our 20 members in Mexico reside in 9 of that country’s 31 states. Among those 9 states, Mexico (the state) is home to the greatest number of members (7); 5 of the 9 states have only a single member, i.e., Sinaloa, Baja California Sur, Colima, Veracruz, and Chiapas. In Argentina, all 18 members reside in only 2 provinces; Buenos Aires is home to 17, with the remaining member residing in Chubut. Japan’s 18 members are distributed among 13 of the country’s 47 prefectures. Osaka has 5 members and Shiga 2 members; the remaining 11 prefectures are each home to a single member.

Our Society includes several individuals of particular note. Two, B. Campbell (elected 2002) and J. P. Dubey (elected 2010), are members of the National Academy of Sciences. One, T. Weller, although recently deceased, remained on our books in 2010 and is a 1954 Nobel Laureate. Of course, several Parasitologists in attendance here today, most as invited speakers in our various symposia, aspire to be members of ASP (the

It is illuminating (and also amusing) to compare the research approaches taken by members in our earlier years to today's standards. Among the research papers published in the first volume (1914) of the Journal of Parasitology were several contributions that would likely be considered unacceptable to modern animal use and human subjects oversight committees. For example, in a paper entitled "Experimental ingestion by man of cysticeri of carnivore tapeworms," M. Hall provided details of his self-infection (on 2 occasions!) with larvae of *Taenia pisciformis*. H. B. Ward described a case of otoacariasis from "scale" taken from the ears of a sheep from Estes Park, Colorado sent to him by G. H. Thompson, the then superintendent of the Park Fish Hatchery. Mr. Thompson's description of how he had come across the sheep was as follows: "I found a two-year-old mountain sheep that had been separated from the bunch by a mountain lion and was tired out in the deep snow; with the help of others I got it on my horse and brought it home with me, and have had it since that time. It has been very interesting but some time after I had it here I found that it had some trouble with its ears. . ." The first volume also included a plea to "American Helminthologists" from A. Mražek who, having studied the helminths of muskrats introduced into Bohemia (Slovakia), was dismayed to discover that comparative information from muskrats in their native North America was lacking. Ever an accommodating Society, not unexpectedly, the penultimate contribution to the first volume was a paper by F. D. Barker detailing the parasites of muskrats in North America.

It is interesting to compare the research interests of our current members to those of early members. For example, the 1914 volume of the Journal of Parasitology contained 27 research papers. The majority (23) dealt with what we would now consider to be typical parasitological issues. The remaining 4 indicate that the identity of our Society then deviated somewhat from its identity now, for that volume also included a paper focused on yellow fever as well as a paper discussing the then very problematic vitamin deficiency pelagra and 2 papers that considered invertebrate poisons (from brown tail moth larvae and black widow spiders). With respect to parasite taxa of interest, that first volume of JP included the following composition of papers: 22% platyhelminths, 22% nematodes, 22% arthropods, 13% protists, 13% other parasite taxa (acanthocephalans and gordion worms), and 8% on helminths overall. In comparison, internet searches yielded the following proportions of parasite taxa listed among the research interests of the approximately 50% of our 2010 members for whom such data were available: 33% are interested in platyhelminths, 21% nematodes, 10% arthropods, 31% protists, and 5% other parasite taxa (acanthocephalans, etc.). Among members interested in platyhelminths, 80 list digeneans, 49 cestodes, and 13 monogeneans among their taxa of interest; only our Australian colleague D. Blair explicitly expresses an interest in trematodes. With respect to protists, 88 members study apicomplexans (51 of these work on cermians, 33 on *Plasmodium*, and 4 on gregarines), 29 work on trypanosomes, 10 on *Giardia*, and 4 each on trichomonads and amoebae. Members studying nematodes tend to be less specific about their nematode interests (or perhaps are more general in their interests) for 45 merely list nematodes, 7 specifically filarial worms, 6 *Trichinella*, 4 ascarids, and 2 mermithids. Included among the members expressing an interest in arthropods are 19 who work on ticks, 7 on mites, 4 each on lice and barnacles, and 2 each on fleas and gnathids. The 26 members whose interests include the other miscellaneous parasite taxa consist of 9 each who work on acanthocephalans and myxozoans and 4 each who study annelids (mostly leeches) and nematomorphs. Admittedly, the comparison between published interests in 1914 and internet-articulated interests in 2011 is not quite parallel for, as discussed in more detail below, the profile of papers published in recent issues of JP differs somewhat from the profile of articulated interests of current members. Nonetheless, the comparison is interesting.

With respect to host groups of study, in 1914 70% of papers in JP dealt with parasites of mammals (equally divided between human and other mammal hosts), 9% of papers treated parasites of fishes, 9% birds, and 4% each on molluscs, amphibians, and other host taxa. Current data for 2010 members, again from internet sources, suggest that 64% of members harbor an interest in parasites of mammals. However, 71% of these individuals study parasites of medical importance and only 30% study parasites of other mammals. Of the remaining individuals, 14% focus on parasites of fishes and elasmobranchs and less than 5% work on parasites of each of the other host groups including molluscs, amphibians, birds, arthropods, and "reptiles". However, as illustrated in Figure 3, the profile of articulated host interests of our members is not reflective of the host groups that are the focus of papers published in JP. In 2010, 52% of the 195 papers focused on parasites of mammals, but only 30% of these involved humans, while the remaining 70% dealt with parasites of other mammals. With respect to other host groups, 17% of papers involved parasites of fishes, 13% birds, 8% "reptiles," and less than 4% parasites in each of the other host groups. Clearly, one of the main differences between articulated interests and those published in JP centers around human parasites for, although a substantial number of our members work on human parasites, these individuals do not necessarily publish their results in our Journal. Although this is likely largely a result of the fact that there are many other publication venues for research on human parasites (e.g., The American Journal of Tropical Medicine and Hygiene, etc.), other factors that may contribute to this situation would be interesting to explore.

Perhaps the most illuminating data generated from internet resources for the purpose of this address are those summarizing
the research areas of interest to our current members. It appears that approximately half our members (51%) conduct basic research and half (49%) conduct applied research; it is important to note that the applied aspects of parasitology investigated by our members have expanded well beyond those of medical and veterinary importance to include, for example, ecosystem health, invasive taxa and biocontrol, and climate change. The array of disciplines of interest to our members is as remarkable in its breadth as it is in depth (Table I), which brings me to a point that I think is important to emphasize, for it illustrates just how different our discipline is from those focused on all of the other categories of interspecific interactions. To review, as a result of the 3 possible effects on species partners engaging in the interactions, i.e., positive, negative, and none, 7 types of interspecific interactions are generally recognized (Fig. 4). Given the lack of effect involved in neutralism and amensalism, these 2 interactions are generally of little interest. Four interspecific interactions, i.e., commensalism, mutualism, competition, and predation/herbivory, are usually considered the purview of ecologists. Parasitism alone is the focus of its own discipline! I believe it is the combination of substantial depth and remarkable breadth (Table I) of our collective research questions that both warrants and sustains parasitology’s status as an independent discipline. This also accounts for the unfortunate fact that work involving parasites that underestimates the complexity of this field is often perceived of as naive in the eyes of parasitologists. Some of the work on sexual selection is an excellent example. Although extremely popular among ecologists for a period of time, this work failed to attract the attention of parasitologists, who were concerned about the superficial nature of many, although certainly not all, such investigations. Their lack of potential (and thus realizable) depth is likely the reason that areas such as “competitorology,” “mutualistology,” “predatorology,” and “commensalitology” have never emerged as formal disciplines.

The considerable depth and breadth of parasitology is also responsible for the remarkable heterogeneity of roles our Society’s members play in their respective institutions. A look at the affiliations of our 829 members in 2010 reveals the astonishing fact that we collectively belong to a minimum of 76 different departments and laboratories around the globe (Table II). Our ability to integrate ourselves into such a diverse suite of academic homes is a testament to both our flexibility and creativity as parasitologists. By necessity, parasitologists are broadly trained and thus are, in turn, able to provide broad training; this accounts for the further astonishing fact that the 150 parasitologists present here in Anchorage alone collectively teach a total of 72 different courses (Table III). Granted, 20 of these courses are variations on the theme of “parasitology,” but 52 of these offerings deal with other topics such as aspects of host biology, or of ecology and evolution, or all 3.

There are a number of individuals among us here in Anchorage whose particularly broad teaching contributions are of special note. M. Barger and R. Clopton each teach 7 courses; J. Harley and R. Sehgal each teach 6 courses, and B. Font, D. Minchella, and D. Woodmansee each teach 5 courses. Given that M. Barger and R. Clopton belong to the same Department at Peru State College, they alone contribute 14 courses (Biology, Ecology, Zoology, Ichthyology and Herpetology, Advanced Ecology, Wildlife Management, Limnology, Human Parasitology, Comparative Vertebrate Anatomy, Physiology, Invertebrate Zoology, Genetics, Botany, Entomology, and Experimental Biology) to the teaching offerings of their institution! Although such extensive contributions are somewhat unusual, they serve to illustrate the truly substantial breadth of the knowledge base that is an integral part of being a parasitologist. At this point, it is enlightening to pause to consider the breadth of the teaching offerings (realized or potential) of our colleagues in almost any other discipline.

With respect to the research interests of our membership published in JP, this topic became more effectively tractable by subdiscipline in 1980 when our Journal editor, Jerry Esch, instituted the concept of Associate Editors. (The exception is the subdiscipline of “Experimental” Parasitology which, although handled by its own Associate Editor in 1980 when it constituted 26% of papers, is no longer represented by a single Associate Editor; rather, papers involving experimental aspects of parasitology are now distributed among other Associate Editors.) Assuming both years are representative of their times, a comparison of papers published in JP in 1980 with those published in 2010 reveals the following trends over the last 30 or so years. While our interest in some areas, such as Ecology/Epidemiology, has remained relatively stable (11% in both 1980 and 2010), our interests in other areas have shifted. For example, over this period of time there has been an increase in the

![Figure 4. Seven categories of interspecific interactions and their corresponding fields of study.](https://example.com/figure4.png)
### Table II. Affiliations of 2010 ASP members globally.

<table>
<thead>
<tr>
<th>North America</th>
<th>Additional affiliations (other countries)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal Parasitic Diseases Lab</td>
<td>Animal Prophylaxis and Prevention Research Center</td>
</tr>
<tr>
<td>Aquatic Animal Health Lab</td>
<td>Dept. Invertebrates</td>
</tr>
<tr>
<td>Baker Institute for Animal Health</td>
<td>Dept. Natural History</td>
</tr>
<tr>
<td>Centers for Disease Control and Prevention</td>
<td>Dept. Parasitology</td>
</tr>
<tr>
<td>College of Public Health</td>
<td>Dept. Parasitology and Tropical Medicine</td>
</tr>
<tr>
<td>Dept./School of Biology</td>
<td>Dept. Science and Math</td>
</tr>
<tr>
<td>Dept. Biochemistry and Pathology</td>
<td>Dept. Science and Math</td>
</tr>
<tr>
<td>Dept. Biological Sciences</td>
<td>Dept. Zoology</td>
</tr>
<tr>
<td>Dept. Biology and Geology</td>
<td>Dept. Zoology and Fisheries</td>
</tr>
<tr>
<td>Dept. Biology and Microbiology</td>
<td>Faculty of Marine Sciences</td>
</tr>
<tr>
<td>Dept. Biological and Physical Sciences</td>
<td>Faculty of Science</td>
</tr>
<tr>
<td>Dept. Comparative Pathobiology</td>
<td>Fisheries and Oceans</td>
</tr>
<tr>
<td>Dept. Ecology, Evolution and Natural Resources</td>
<td>Institute of Biotechnology</td>
</tr>
<tr>
<td>Dept. Entomology</td>
<td>Institute of Experimental Animal Research</td>
</tr>
<tr>
<td>Dept. Fisheries</td>
<td>Institute of Parasitology</td>
</tr>
<tr>
<td>Dept. Fisheries and Allied Aquaculture</td>
<td>Institute of Systematics and Ecology of Animals</td>
</tr>
<tr>
<td>Dept. Health</td>
<td>Lab of Aquatic Pathology</td>
</tr>
<tr>
<td>Dept. Immunology and Microbiology</td>
<td>Parasitology Section</td>
</tr>
<tr>
<td>Dept. Microbiology</td>
<td>Research Center for Pacific Islands</td>
</tr>
<tr>
<td>Dept. Microbiology and Tropical Medicine</td>
<td>Research Institute for Microbial Diseases</td>
</tr>
<tr>
<td>Dept. Molecular Biology and Biochemistry</td>
<td>School of Applied Science and Engineering</td>
</tr>
<tr>
<td>Dept. Natural Sciences</td>
<td>School of Medicine</td>
</tr>
<tr>
<td>Dept. Nematology</td>
<td>School of Tropical Biology</td>
</tr>
<tr>
<td>Dept. Pathobiology</td>
<td></td>
</tr>
<tr>
<td>Dept. Pathology</td>
<td></td>
</tr>
<tr>
<td>Dept. Pathology, Microbiology and Immunology</td>
<td></td>
</tr>
<tr>
<td>Dept. Pediatrics</td>
<td></td>
</tr>
</tbody>
</table>

### Table III. Courses taught by ASP members (~150) attending 89th annual meeting in Anchorage, Alaska (2011).

<table>
<thead>
<tr>
<th>Parasite related</th>
<th>Host and/or other organismal</th>
<th>Process/methods</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced Parasitology</td>
<td>Animal Biology</td>
<td>Advanced Ecology</td>
<td>Advances in Microbiology</td>
</tr>
<tr>
<td>Biology of Disease Vectors</td>
<td>Biology</td>
<td>Cell Biology</td>
<td>Bacterial Synthetic Biology</td>
</tr>
<tr>
<td>Biology of Symbiotic Interactions</td>
<td>Botany</td>
<td>Coastal Processes</td>
<td>Biology of Human Values</td>
</tr>
<tr>
<td>Clinical Parasitology</td>
<td>Comparative Vertebrate Anatomy</td>
<td>Developmental Ecology and Behavior</td>
<td>Epidemiology of AIDS</td>
</tr>
<tr>
<td>Ecology and Evolutionary Biology of Infectious Disease</td>
<td>Diversity of Life</td>
<td>Ecology</td>
<td>Genetics</td>
</tr>
<tr>
<td>Ecological Parasitology</td>
<td>Entomology</td>
<td>Electron Microscopy</td>
<td>Human Anatomy</td>
</tr>
<tr>
<td>Ecology and Evolution of Parasitism</td>
<td>Ichthyology and Herpetology</td>
<td>Evolution/Advanced Evolution</td>
<td>Human Biology</td>
</tr>
<tr>
<td>Emerging Infectious Diseases</td>
<td>Introduction to Nematology</td>
<td>Experimental Biology</td>
<td>Immunology</td>
</tr>
<tr>
<td>Evolution of Human Health and Disease</td>
<td>Invertebrate Ecology</td>
<td>Field Biology</td>
<td>Medical Microbiology</td>
</tr>
<tr>
<td>Evolutionary Medicine</td>
<td>Invertebrate Zoology</td>
<td>Histology</td>
<td>Microbiology/Bacteriology</td>
</tr>
<tr>
<td>Experimental Parasitology</td>
<td>Limnology</td>
<td>Marine Aquaculture</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>Field Parasitology</td>
<td>Mammalogy</td>
<td>Molecular Phylogenetic Analysis</td>
<td>Physical Diagnosis</td>
</tr>
<tr>
<td>Genetic and Life History Variation in Host-Parasite Systems</td>
<td>Organismal Biology</td>
<td>Our Endangered Planet</td>
<td>Science and Religion</td>
</tr>
<tr>
<td>Great Neglected Diseases</td>
<td>Ornithology</td>
<td>Range and Wildlife Ecology</td>
<td></td>
</tr>
<tr>
<td>Marine Parasitology</td>
<td>Protozoology</td>
<td>Speciation and Macroevolution</td>
<td></td>
</tr>
<tr>
<td>Medical Parasitology</td>
<td>Vertebrate Biology</td>
<td>Systematics</td>
<td></td>
</tr>
<tr>
<td>Parasitic Protozoa</td>
<td>Zoology</td>
<td>Tropical Biology</td>
<td></td>
</tr>
<tr>
<td>Parasitology</td>
<td></td>
<td>Wildlife Management</td>
<td></td>
</tr>
</tbody>
</table>
The proportion of papers dealing with life cycles (3% vs. 16%) and taxonomy and systematics (19% vs. 28%), and new areas such as ectoparasitology (0% vs. 11%) have emerged. In contrast, there has been a decline in the number of papers dealing with such areas of parasitology as biochemistry and physiology (11% vs. 1%), immunology (15% vs. 8%) and functional morphology and ultrastructure (10% vs. 1%). The end result is that JP currently publishes more papers on basic aspects of Parasitology than on applied aspects. That having been said, the distribution of interests reflected by the abstracts submitted by the 67 students here in Anchorage is different still. This year, there is particular interest in ecology/epidemiology (31%) as compared with, for example, taxonomy/systematics/phylogeny (19%), immunity/resistance (16%), genetics/evolution (12%), biochemistry/physiology (8%), therapeutics/diagnostics (7%), and life cycles (6%).

Our membership continues to have an important presence in national funding scenes at agencies targeting medical research. In the US, with respect to the National Institutes of Health (NIH), our members have recently succeeded in obtaining funding for research on parasites that are the etiological agents of a broad spectrum of infectious human diseases. For example, work on various aspects of schistosomes (and/or their snail hosts) by C. Adema, C. Bayne, C. Caffrey, D. Dolley, P. Loverde, P. Skelly, T. Yoshino, and S-M. Zhang has received NIH support as has work on numerous questions involving nematodes including hookworms (J. Hawdon, M. Cappello), Strongyloides (S. Abraham, J. Lok), and mosquito hosts and their filarial worms (B. Christensen). Other areas receiving NIH support include malaria of humans (L. Cui) and even birds (R. Sehgal), giardiasis (J. McKerrow), fascioliasis (A. Espino), and emerging infectious diseases such as Cryptosporidium (P. Zhu), S. Loker (evolutionary and theoretical parasitology) and A. Kuris and K. LaFerty (ecological parasitology) have also received NIH support to pursue parasitological questions of a more theoretical nature.

The last few decades have seen a substantial change in attitude at the US National Science Foundation (NSF) with respect to their funding of research involving parasitological questions. Once considered to be outside their funding priorities, work on parasites of nonmedical or veterinary importance is now routinely funded by a diversity of programs at that agency. I believe this is, in large part, the result of the persistent efforts of numerous ASP members. My first encounter with NSF's antiparasite bias was in 1987 when, in a conversation with a program officer (about a proposal I had submitted to work on cestodes of elasmobranchs which, although highly ranked by its panel, was not initially funded), I was informed that “the only thing interesting about parasites is what they can tell you about their hosts.” Although I was ultimately funded in that round, the issue clearly existed. Attitudes at NSF have now changed. Examples of NSF-funded parasitological studies by our current members include work on the parasites of Australian turtles (S. Snyder and V. Tkach), elasmobranchs of Borneo (J. Caira and K. Jensen), as well as of the vertebrates of Beringia (E. Hoberg), Mongolia (S. Gardner), China (D. Clayton and S. Bush), and the Philippines (D. Clayton and S. Bush). Work on specific groups of parasites has also been supported, including for example gregarines of certain North American insects (R. Clpton) and nematomorphs (B. Hanelt and M. Bolek) as well as fish (A. Bullard and T. Cribb) and avian (S. Brant, S. Loker, and V. Tkach) blood flukes; all of the above having been supported by the Biotic Surveys and Inventories (BS&I) and/or Systematics programs. The Partnership for Enhancing Expertise in Taxonomy (PEET) program has funded monographic and associated work on coccidians (D. Duszynski and S. Upton), leeches (M. Siddall), nematodes (S. Nadler), haploporid digenans (R. Overstreet), and tapeworms (J. Caira and T. Ruhnke), and of only 9 projects funded by the Planetary Biodiversity Inventory (PBI) panel went to tapeworms of the world (J. Caira, K. Jensen, T. Littlewood and J. Mariaux). Among the select number of projects funded by the Tree of Life (ToL) program was 1 on nematodes (S. Nadler). Funds from NSF’s RAPID response program have been awarded for the assessment of the effects of the Deepwater Horizon oil spill in the Gulf of Mexico using parasites of fish as biomarkers (G. Benz and A. Bullard). The Integrative Organismal Systems (IOS) program has provided funding for investigation of mosquito immunity as it relates to host–pathogen interactions (J. Hillyer). The joint NSF and NIH Ecology of Infectious Diseases program has very recently awarded funds for work to improve ecological scaling rules by the inclusion of parasites (R. Hechinger).

In Canada, NSERC monies have been awarded for parasitological work including studies focused on aquaculture (D. Cone and M. Burt), pollution and ecosystem health (D. Marcogliese), host population genetics (M. Scott), molecular aspects of the host–parasite interface (T. Geary), immunology (M. Belosevic), and molecular characterization and control of apicomplexans (J. Barta) as well as various parasite barcoding initiatives (D. Marcogliese). Members have also been creative in seeking funds from other sources. For example, the Hawai‘i Division of Aquatic Resources has provided funds for the study of parasites of freshwater fishes of Hawai‘i (B. Font). Support for the development of a hookworm vaccine has come from the Netherlands Ministry of Foreign Affairs as well as the Bill and Melinda Gates Foundation (P. Hotz); the latter foundation has also provided support for work on drug treatment of parasitic diseases in Africa (T. Geary). Funding from the Canadian International Development Agency (CIDA) for university teacher upgrading has been successfully parlayed into an exploration of parasites of freshwater fishes in several countries in Asia (D. Cone).

Although clearly not an exhaustive list, I have made an effort to identify specific parasitologists in discussing funding for Parasitology in the hopes that these individuals might serve as a resource for other members interested in pursuing funding for their work. Given the collegial nature of our Society, I am guessing the individuals listed above will be willing to share their experiences and would thus encourage members to seek their counsel.

Fieldwork continues to be a mainstay of both the applied and basic aspects of our research. As a consequence, ours, perhaps more than any other discipline, is contributing to discoveries and advancements “on the ground” globally. Our Society includes individuals whose work is of a humanitarian nature focused, for example, in Africa, South America, and Asia and aimed at eliminating human maladies such as schistosomiasis, ascariasis, elephantiasis, onchocerciasis, and hookworm disease. Our members also include some of the most adventurous biologists, given that our hosts of interest include, but are certainly not limited to, crocodiles, large mammals, small mammals, tropical birds, sharks, stingrays, and fish. The habitats we investigate range from extremely muddy beaches, desert savannahs, and arctic plains to tropical islands, the deep oceans, and remote rivers in...
tens of countries. A visit to the image galleries on the websites of many of our members attests to the adventurous (and perhaps sometimes crazy?) nature of our group.

Our creativity is beyond dispute and, in fact, is obvious in the unusual array of items donated to our annual student auction, a tradition established by our program officer in 1989 (and the very person who introduced me here today) and which annually continues to draw a wide range of unusual items of original design. Now adorning the dwellings and/or enhancing the lives of parasitologists and their families around the world are parasitologically-themed bumper stickers, canes, cards, carvings, felt computer and i-pod covers, jewelry, paintings, photographs, pottery, prints, sculptures, shirts, pants, and undergarments. (That perhaps a larger than expected proportion of these items depicts cestodes is a tribute to the appeal of these particularly handsome platyhelminths!)

So, in closing, an informed answer to the question “Who Are We Now?” turns out to be rather impressive. We are a group of adventurous, broadly trained, capable, creative, deep, humanitarian, imaginative, knowledgeable, loyal, successful, and clearly enthusiastic group of scientists. We are The American Society of Parasitologists!
INTRODUCTION OF BRUCE M. CHRISTENSEN, RECIPIENT OF THE 2011 CLARK P. READ MENTOR AWARD

Julián F. Hillyer
Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee 37235-1634. e-mail: julian.hillyer@vanderbilt.edu

Like many others who have ventured into south-central Wisconsin to learn about tropical insects and parasites, an unlikely place for such a thing if you ask me, I am an extremely fortunate individual. “Why,” you ask? The answer is that the University of Wisconsin–Madison houses a dedicated professor who has a knack for educating graduate and undergraduate students in the fields of parasitology and entomology, while conducting cutting-edge research in topics of scientific, social, and medical importance. This professor is Dr. Bruce M. Christensen, and it is my honor to introduce him as the recipient of the 2011 American Society of Parasitologists Clark P. Read Mentor Award.

The Clark P. Read Mentor award is given to an individual who, during his or her career, has demonstrated extraordinary leadership in the training of young scientists who have successfully pursued the independent study of parasites or aspects of the host-parasite relationship. Further, the individual shall have influenced the research and/or graduate education of a department, college, or institution to significantly increase the number of students completing graduate-level training in the various disciplines of parasitology. Bruce Christensen has done all this, and more.

Dr. Christensen’s initial foray into the field of parasitology dates back to his days as an undergraduate at the University of Wisconsin–River Falls, where he studied cestode biology under the guidance of Dr. Robert Calentine. After graduating from U.W.–River Falls, Bruce spent 3 years as a biological scientist at the Fitzsimmon’s United States Army Medical Center in Denver, Colorado. Then, he trekked to Ames, Iowa, where in 1977 he completed his Ph.D. at Iowa State University under the guidance of Dr. Wayne Rowley and Dr. Martin Ulmer, where he concentrated on the interaction between filarial nematodes and their mosquito vectors. In 1978 Bruce accepted a position as an Assistant Professor in the Department of Biological Sciences of Murray State University, and after 4 years there he moved his lab to the University of Wisconsin–Madison, where he currently holds the H. Edwin Young Professorship in the Department of Pathobiological Sciences.

His scholarly record is truly impressive. He is the author of over 170 research articles, and his work has been cited more than 3,000 times. Bruce has been continuously funded by the NIH for over 30 years, and from 1990-2000 he held a prestigious MERIT award by this same funding agency. He is also a past president and past council member of our society, a former member of the editorial board of The Journal of Parasitology, and a recipient of the H.W. Ward Medal (Christensen, 1988, 2004).

Throughout a career that spans over 3 decades, Bruce has consistently placed great emphasis on student mentoring. Through his hard work, and the hard work of his students, 17 students have received an M.S. in his lab, 12 received a Ph.D., and 16 completed post-docs. These students have carried out cutting-edge research that, through the years, has shifted from ecology to organismal biology to molecular biology to genomics, and now is starting to shift back to ecology. With this plasticity, his laboratory has remained at the forefront of research innovation and has greatly increased our knowledge of mosquito immunology and the interaction between parasites and their mosquito vectors. This has been accomplished through the synergistic interaction between Bruce and his students, and his requirement that they engage in a collegial, but competitive, environment has resulted in their development into independent scientists capable of adapting novel technologies to produce new knowledge. This preparation has vaulted his students into prestigious positions at research universities, biotech industries, and government organizations. The publication record of his students is outstanding, with his Ph.D. students averaging 4.5 first-author publications and numerous other co-authorships during their tenure in the Christensen lab.

When a while back I asked Bruce what the key to successful mentoring was, he said he was not sure. But from my observations, taken both as a student and as a colleague, I gather that there are 6 key elements to his mentoring style. First, he commits to his students and demands a similar level of commitment in return. Second, within the funding framework of the laboratory, he gives students the creative freedom to craft and steer their projects. In doing so, he demands focus without micromanaging, giving plenty of leeway to explore questions that are of interest to the student even if they run tangentially to the mission of the lab. Third, he provides the student with the resources needed to succeed. These include easy access to the mentor, enabled communication with members of the scientific community as well as other members of the lab, and the accessibility of more than adequate funding for the project. Fourth, he carefully monitors progress to make sure that the correct questions are being asked and that the correct approaches are being taken. Fifth, he continuously challenges his students to think of the global question rather than just the specifics of their project. Finally, he demands that findings originating in the lab be shared with the scientific community in the form of presentations at national/international meetings and peer-reviewed publications. All in all, Bruce places his students in an ideal position to succeed, but success itself is not gifted, it must be earned.

While the training environment is exemplary, the outcomes are even more impressive. His direct mentorship has fostered the careers of 11 students who have attained faculty positions at academic institutions, most at Research I universities (Table I). Moreover, what I believe is one of Dr. Christensen’s best attributes as a mentor is his commitment to building lasting relationships with his students. At a point when they have left his lab, a time when continued mentorship does not result in personal gain, Bruce actively stays in contact with his former students and

DOI: 10.1645/GE-2885.1
Table 1. Students of Dr. Bruce M. Christensen who have attained tenured or tenure-track university professorships.

<table>
<thead>
<tr>
<th>Mentee</th>
<th>Ph.D. conferred</th>
<th>Post-doc completed</th>
<th>Current affiliation</th>
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</thead>
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<tr>
<td>Daniel R. Sutherland*</td>
<td>1987</td>
<td></td>
<td>Dept. of Biology, University of Wisconsin-LaCrosse, La Crosse, Wisconsin</td>
</tr>
<tr>
<td>Cheryl C. Courtney†</td>
<td>1988</td>
<td></td>
<td>Dept. of Biology, California State University–Northridge, Northridge, California</td>
</tr>
<tr>
<td>Jianyong Li</td>
<td>1990</td>
<td>1996</td>
<td>Dept. of Biochemistry, Virginia Tech, Blacksburg, Virginia</td>
</tr>
<tr>
<td>Brenda T. Beerntsen</td>
<td>1995</td>
<td></td>
<td>Dept. of Veterinary Pathobiology, University of Missouri, Columbia, Missouri</td>
</tr>
<tr>
<td>Michael T. Ferdig</td>
<td>1997</td>
<td></td>
<td>Dept. of Biological Sciences, University of Notre Dame, Notre Dame, Indiana</td>
</tr>
<tr>
<td>Guiyun Yan</td>
<td>1998</td>
<td></td>
<td>Program in Public Health, University of California–Irvine, Irvine, California</td>
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<td>David Severson</td>
<td>1998</td>
<td></td>
<td>Dept. of Biological Sciences, University of Notre Dame, Notre Dame, Indiana</td>
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<tr>
<td>Carl Lowenberger</td>
<td>2000</td>
<td></td>
<td>Dept. of Biological Sciences, Simon Fraser University, Burnaby, British Columbia</td>
</tr>
<tr>
<td>Chelsea T. Smartt</td>
<td>2000</td>
<td></td>
<td>Florida Medical Entomology Laboratory, University of Florida, Vero Beach, Florida</td>
</tr>
<tr>
<td>Julián F. Hillyer</td>
<td>2004</td>
<td></td>
<td>Dept. of Biological Sciences, Vanderbilt University, Nashville, Tennessee</td>
</tr>
<tr>
<td>Lyric C. Bartholomay</td>
<td>2004</td>
<td>2005</td>
<td>Dept. of Entomology, Iowa State University, Ames, Iowa</td>
</tr>
</tbody>
</table>

* Deceased.  
† Now Cheryl C. Hogue.

remains one of their strongest advocates, providing advice when it is requested without attempting to steer his former students into following his interests. In other words, he encourages and fosters the scientific independence of his former students. In addition to this, he somehow creates strong camaraderie among his former students, and this occurs even if the students’ tenure in the Christensen lab never overlapped. Personally, the longer I am removed from my graduate studies the more I understand his mentorship nuances and the more I appreciate them. As a result, a large part of my approach to mentoring is derived from my experiences in the Christensen lab.

In addition to graduate and post-graduate mentoring, for 32 years Bruce has taught parasitology-based courses, with more than 3,500 undergraduates having completed these classes. The sustained enrollment of 100–200 students per year attests to the quality of these courses, the effectiveness of the teaching methods, and the proficiency and diligence of the instructor. Having personally taken Bruce’s parasitology course, and having later served as a teaching assistant for this course, I can confirm his commitment to teaching. In today’s world of PowerPoint slides, not often does one find an individual with such mastery of the field of parasitology that he or she can completely captivate an audience without any visual aids, making them cringe and smile at the same time, while expertly describing complex life cycles using only chalk and board.

In closing, the preparation I received as a Ph.D. student in Bruce’s lab was instrumental in my success as a post-doc and now as a faculty member at Vanderbilt University. From the numerous letters supporting Bruce’s nomination for this award, it is obvious that many of his former students feel the same. His record of mentoring speaks for itself. He has done what we should all strive to do: He has introduced thousands of undergraduates to the field of parasitology, has trained countless M.S./Ph.D./post-doc students in parasitology (11 of which now train parasitologists at peer institutions), and has made seminal contributions to our current understanding of parasite biology. With that, Bruce, I thank you for all you have done for me and your other students, and congratulate you on receiving the 2011 American Society of Parasitologists Clark P. Read Mentor Award.

LITERATURE CITED


ACCESSION OF THE CLARK P. READ MENTOR AWARD: MENTORING THE MENTOR

Bruce M. Christensen
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Before I begin, I need to tell you a little story of how I got to know Julian. A long time back, when Julian only thought about slapping mosquitoes and not studying them, I met Julian’s father George. We were both serving on a study section for the NIH evaluating the merits of proposals to establish International Centers for Infectious Disease Research. We had what we used to call a knock-down, drag-em-out disagreement over one particular application, but that night in the bar of the Bethesda Holiday Inn, George came up to me and said “...great discussion today, let me buy you a beer.” It is so nice to have friends that can truly separate the business of doing science from the business of friendship. As a consequence, our friendship has grown progressively over the years. So, George introduced me to his son, Julian, a long time ago, when I think he was about this high. First, on one of my trips to San Juan, and again a number of years later when our two families went to see a Brewer’s baseball game in Milwaukee in 1991, the year UW-Madison hosted the ASP meetings. He seemed like a nice kid, but I never imagined he would someday walk into my office and ask to do his doctoral studies in my lab. Of course, I said ‘yes’ and did so without any hesitation, but then I started to think about a possible consequence—what if he wasn’t any good? Can any of you here who know George seriously contemplate telling him his son wasn’t good enough? But, as you all know, and as I knew all along, Julian turned out to be a great student and an accomplished scientist. As did the other children of colleagues I was fortunate to have as students, Steve Taft’s son Andy and Barry Beaty’s daughter Lyric Bartholomay—but I take no credit for their success; rather, I am simply more convinced that genetics works.

Julian, I know a number of my past students and post-docs helped you in your task of putting my nomination together, but I also know you were the heavy lifter and I thank you very much. I also thank all of you who wrote letters on my behalf, Ben Hanelt and the other members of the Clark P. Read Award selection committee, and my fellow members of the ASP. This is a true honor for which I am very pleased.

Standing in front of you now tells me two things. First, you now consider me an old fart and, second, that I have been very lucky to have had the opportunity to interact with some extremely gifted students and post-docs over the course of my career. But, I must tell you up front that I do not have a particular strategy for mentoring, nor can I ever remember thinking about how I should or should not “train” students and post-docs; it has all just sort of happened. I was, of course, influenced to a certain degree by my past mentors, Drs. Calentine, Ulmer, and Rowley, but they never gave me lessons in how to mentor, nor have I done so with my students—we have all just sort of learned together. So, what I am going to do for the next few minutes is to tell you a bit about my experiences in this wonderful world of parasitology research and teaching, try and convince you of a couple of things that I do think are important, and talk about some of the associations and interactions I have had since I accepted my first graduate student 32 years ago.

The one thing I know to be true in the world of graduate training is that you can hand-hold a masters student, very briefly, but no hand-holding is allowed in a doctoral program. How does one become an independent investigator if one is not given independence as a graduate student? There are, of course, a few restraints—the area of research endeavor has to fit a bit into the general area for which the lab is receiving research support—but that was never much of a restriction in my lab because the world of vector-borne diseases covers a lot of territory. And, besides, almost all of my students applied to train in my lab because of my general area of research; they really liked vectors and vector-borne parasites. There were a few exceptions along the way, like caryophyllid cestodes and aquatic oligochaetes research for Cheryl Courtney. But, it has always been hard for me to get

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those monozoic tapeworms out of my system. There have been a few cases where students could not handle the independence they were given in my laboratory, so they ended up doing something different, and some students flounder for awhile before putting things together, but I am very proud of the success seen by students who independently developed their own programs and research areas.

There is, however, another requirement if students are given the opportunity to develop their own research area—the mentor has to have adequate support so that the research proposed is not negated by a lack of funds. Really good ideas often times need much more than moral and intellectual support, especially in these times of rapidly expanding technologies that permit explorations that I could never have imagined during my graduate student days at Iowa State. And, having funding also enables students to go to whatever scientific meeting or symposia that places them in contact with established scientists who will be reviewing their papers and grant proposals and providing post-doctoral and/or faculty positions. I remember how nervous I was as a graduate student to present some of my work on filariasis at a national meeting with John Schacher, Larry Ash, Tom Orihel, and David Denham sitting in the front row. I am convinced they did so to see if I could handle the intimidation—I’m not sure if I did, but I do know, for better or worse, they all knew who I was after the presentation, and that is a wonderful feeling for a young scientist. I know my students have had similar experiences that enabled them to begin interacting, at an early stage in their career, with a fraternity of established scientists within their fields of interest.

So, that is my insight: give them independence, financial support, and expose them to the very best scientists in the field. The rest is simply a matter of conducting science. We all know that no matter how much experimentation one does, it is not science until it passes the muster of critical review and is then published for the world to scrutinize. So, it always seemed reasonable for me to have a laboratory based on critical analysis of what is being done—that is, an interacting unit where all components become involved in decision-making and scientific evaluation. As soon as someone enters the lab, they are expected to become actively involved in the scientific process. So, besides conducting their own research, they must evaluate manuscripts and grant proposals as well as works in progress by other members of the laboratory. No one is spared this task, nor is anyone spared criticism by her or his colleagues—including the major professor. New students are sometimes bothered by this, but most soon realize we operate on the ‘George principle’ of separating the business of science from the business of friendship. So, in a team approach, students are given the opportunity to become strong, critical scientists. They must, of course, be independent, but they also must interact as part of a team. From this, they are able to develop and appreciate different methodological approaches, to realize their work needs to be critically analyzed, and that grant writing always benefits from the examination by numerous, discerning eyes.

But, I now realize that, over these many years, it was I, the mentor, who was the real beneficiary and the one receiving the most mentoring. It might be possible to explain this by the simple fact that the majority of my students were, and remain, smarter than me, but actually it’s based more on how my students helped develop the scientific acumen of this mentor.

My scientific upbringing was a world of organismal biology involving parasites, medically important insects, and a few viruses. I’ve always been fascinated with the interrelationships between parasites and their intermediate hosts, and my studies in those early days were based on microscopical observations and the degree of correlation between various phenomena—it was lots of fun. But, as I kept asking questions about these various phenomena, it became apparent that more was needed than just visual analysis. So, there was a progression in my development as a scientist following grad school that began with ultrastructure studies, initially developed in my laboratory by Keith Forton and then skillfully taken to the highest level by Julian. Next came a full investment in biochemistry and cell biology from the hands and brilliant mind of Jianyong Li and elegantly continued by Nobutaka Kato. I handled these fairly well, for I had done a bit of EM work for fun at Iowa State and had dabbled in biochemistry while doing high-altitude research during my army days. But, it was a bit more daunting when Brenda Beerntsen, Mike Ferdig, Carl Lowenberger, Xueling Zhao, Chelsea Smartt, and Lingling Niu boldly carried the lab into the world of molecular biology. From there, it progressed into molecular genetics, genomics, and bioinformatics at the insistence of Guiyun Yan, Lyric Bartholomay, and Matt Aliota. And, presently, my last doctoral student, Young-Jun Choi, is showing a mathematically deficient mentor the wonders of computational biology. So the scientist I am now has been determined to a large part by the mentoring by my students and post-docs who were willing to challenge the status quo of the laboratory. And, I thank them very much, especially because they did not forget the biological basis on which they applied these new methodological approaches.

But, I would be remiss to end this discussion of mentoring the mentor with the mere evolution of my laboratory’s technologies. They have done much more. For instance, we seem to always have a tendency to criticize student’s writing abilities when they first join the lab, but I can readily admit I have learned much about creative science writing from Marc Castle and by reading anything Mike Ferdig writes—I wish I had his talent (neither I nor Dr. Janovy should take credit, because Mike’s writing ability surely is innate). In this same vein, there have been many instances where my students have provided the key statement or idea that I am sure made a difference in getting a grant proposal funded. I strongly recommend involving your students heavily in your grant writing; not only do they gain valuable experience, but you undoubtedly will produce a proposal that is more likely to receive a competitive score. A final instance I want to mention involves working in developing countries. I have always tried to provide an opportunity for my students to work in an endemic area if this is what they really wanted to do, and a major reason for doing this is for them to find out if, in reality, they really want to do this in their future careers. But, this worked in a different way with two of my students who are so good in the field, under difficult conditions, that it is impossible to be with them in these settings and not gain from the experience. One of these students was Lyric Bartholomay, who worked with me in Egypt and then replaced one of her academic grandfathers at Iowa State. The second is Sara Erickson, my next-to-last student, who will complete her doctorate this summer after spending a good part of last year working on our project in Papua New Guinea—her abilities have earned her a position at the Walter and Eliza Hall Institute in Melbourne beginning in October.
In summary, I consider my students colleagues with similar interests, traveling on the same road and, in general, having the same destination in mind. In many instances, I could be considered a more seasoned traveler, and I did have the responsibility at times to make decisions on direction, but the road I’ve traveled has been a whole lot more interesting, exciting, and productive because of my graduate students and post-docs. So, I profoundly thank all of my academic children, past and present, who are most responsible for my receipt of this award.

I want to end with a few comments about teaching undergraduates parasitology. Teaching a junior/senior level undergraduate general parasitology course every year has been one of my greatest pleasures during my career at the UW-Madison, and I am fortunate that the breadth of biological disciplines at this institution enables me to draw on a large and diverse student population. This is a grand time to be a parasitologist and to teach our discipline. I find that many of today’s students have a very real interest and concern for the health and welfare of the majority of the world’s population that is less fortunate than us. I also find these students are fascinated with parasites—both with the intricacies of their biology as well as the huge impact they have on the health and well being of human and animal populations. I have taught parasitology to thousands of students over the years, and many of them have entered our field. It is wonderfully satisfying to receive emails from past students who are in graduate school, the Peace Corps, volunteering abroad in clinics and schools, or working in public health in the tropics. It’s also great to have past students write detailed descriptions of what it is like to have dengue fever or falciparum malaria while working in Colombia or Tanzania. I say ‘great’ not because I wish them ill will, but because their descriptions serve as wonderful teaching tools. If undergraduate interests and enthusiasm at UW-Madison are any kind of predictor, then I can confidently say that parasitology should continue to fascinate students for years to come.

It was 43 years ago that my wife Linda and I, as undergraduates in Bob Calentine’s lab, attended our first ASP meeting. That meeting exposed me to the wonders and diversity of parasitology research, and I knew from then on that I wanted to pursue a career in parasitology. This Society did much to foster an aspiring young parasitologist, and now you honor me with a very meaningful award—for all of this I thank you very much.

ACKNOWLEDGMENTS

I give great thanks to all of my past and present graduate students and post-doctoral fellows—you have made my life in science a most enjoyable experience. My greatest appreciation goes to Linda, my wife of 42 years. She has prepared many dinners and other gatherings in our home for laboratory workers and family, but more importantly, has worked side by side with most of my students in the mosquito insectary. She has been a major player in this mentoring game on which this award is based.
I. OPENING REMARKS AND MOTIONS

President Janine Caira called the meeting to order at 0800 hr and noted that a quorum was present. Council members and other attendees introduced themselves, and President Caira made a few opening remarks and reviewed motion protocol. She then called for a motion to accept the published minutes of the 2010 meeting.

Motion 1: Acceptance of the minutes of the 100th Council Meeting, 2010.

The motion was made by Mark Siddall, seconded by Sam Loker, and passed unanimously. Secretary-Treasurer Dennis Minchella agreed to send the 2011 Council meeting minutes to the Council within 2 wk of the meeting for review. President Caira then called for a motion to approve new members added since the last meeting.

Motion 2: Approval of new members added since June 2010.

The motion was made by Mike Barger, seconded by Mark Siddall, and passed unanimously.

President Caira recognized the new officers for 2011-2012: President, Armand Kuris; President-Elect, Sam Loker; Vice President, John Janovy, Jr.; Council Members-at-Large, Robin Overstreet and Timothy Yoshino (2011-2015); Nominating Committee, Sara Brant (Chair), Ann Adams, Joseph Camp, Jr., Claire Healy, R. Scott Seville, and Julian Hillyer (alternate); Student Representative, Kyle Luth (2011-2012). President Caira expressed appreciation to outgoing Council Members-at-Large, Mark Eberhard and Janice Moore, outgoing Student Representative, Joanna Cielocha, and outgoing Nominating Committee, Reginald Blaylock (Chair), Derek Zelmer, Florian Reyda, Ben Hanelt, Gregory Sandland, and Benjamin Rosenthal (alternate).

President Caira also recognized the generous contributions to the Annual Meeting by Wake Forest University (Office of the Provost); the University of Connecticut; Hillsdale Animal Hospital (Karla Frazier, D.V.M); HESKA Corporation; Pfizer, Inc.; Bayer Animal Health GmbH; Merial Select, Inc.; and Intervet, Inc.

II. OLD BUSINESS

There was no old business for discussion.

III. NEW BUSINESS

Following electronic communication by Council prior to the meeting, President Caira initiated a discussion on the Ashton Cuckler New Investigator Award eligibility requirements. Scott Snyder made the following motion to open up the floor for discussion.

Motion 3: The Ashton Cuckler New Investigator Award shall be limited to doctoral students.

The motion was seconded by Mike Barger. A discussion ensued, during which a majority of Council members expressed a desire to keep the award open to deserving candidates within 2 yr of their Doctoral or Master’s degrees. President Caira then called for a vote on the motion, and the motion did not pass (5 Council members approved the motion, and 8 Council members did not approve the motion).

Kym Jacobson then proposed a discussion of the Marc Dresden Student Travel Grant guidelines. Kym Jacobson made the following motion:

Motion 4: Marc Dresden Student Travel Grant guidelines shall be made more specific, and the current Student Awards Committee shall draft the language.

The motion was seconded by Armand Kuris. During a discussion, an amendment was made by Rich Clopton and seconded by Scott Snyder to have Council review the areas in need of clarification and provide their thoughts to the Committee for their consideration. The original motion and amendment passed unanimously. Council then conducted a thorough evaluation of the Student Travel Grant guidelines. Kym Jacobson agreed that she and the other members of the Student Awards Committee would consider these suggestions and draft recommendations to change the guidelines for Council’s consideration.

In compliance with a motion passed during the 2010 Council meeting, Council discussed the topic of the annual Student Travel Grant budget for the 2012 meeting. Armand Kuris made an initial motion to return the budget to $8,000 for the 2012 meeting, and Mark Eberhard seconded it. After some discussion of the original motion, Kym Jacobson introduced the following amended motion to allow some spending flexibility if necessary:

Motion 5: The Marc Dresden Student Travel Grant Fund shall be $9,000 for the 2012 meeting in Richmond, Virginia.

The amended motion was seconded by Kirsten Jensen and passed unanimously.

President Caira then initiated a discussion of uploading NIH-funded work published in the Journal of Parasitology to PubMed Central. Since Allen Press Publishing Services is not able to perform this task for the Journal at this time, the task would ultimately be the authors’ responsibility. Council concluded that further exploration is required, including exploration of a possible timed-release option to allow immediate upload and delayed release to PubMed Central, and also concluded that the Journal may wish to consider providing explicit instructions to authors of NIH-funded work regarding the upload of their Journal articles.

Regarding Society insurance coverage, Secretary-Treasurer Dennis Minchella introduced several insurance policy options for Council consideration, including crime protection, professional liability coverage to protect Society officers, and a general liability policy to cover Annual Meeting venues. After Council reviewed the options and their financial implications, Scott Snyder made the following motion:

Motion 6: The Society shall not renew its crime liability insurance policy.

Mark Siddall seconded the motion, and it passed unanimously. Mark Siddall then made the following motion:

Motion 7: The Society shall obtain a professional liability insurance policy.

Mike Barger seconded the motion, and after a brief discussion, the motion passed unanimously. Mike Barger then made the following motion:

Motion 8: The Society shall obtain a general liability insurance policy to cover Annual Meeting venues.

Kirsten Jensen seconded the motion, and it passed unanimously.

Council also discussed Journal operations, including publishing contract negotiation, royalty payments, and the editorial stipend. Given that the current publishing contract will expire 31 December 2012, President Caira has established a committee to renegotiate the terms of the contract. Dennis Minchella confirmed that the Society will receive quarterly royalty payments of $2,000 from Allen Press in addition to the remainder of the royalty payment received in the Spring. Council decided that the editorial stipend shall continue to be received by the Secretary-Treasurer’s office with requested funds being sent to the Editorial Office.

George Cain presented his findings to Council regarding the possible financial implications of offering online-only membership for regular members. After reviewing the proposed scenarios, Mark Siddall moved for the Society to offer an online-only membership option to regular members at $50. The motion was seconded by Kym Jacobson, and a discussion ensued. Since the Society will enter into a new publishing contract with Allen Press in the near future, it was subsequently proposed that the online-only option should be postponed until the start of the new contract. Scott Snyder then suggested that the Society offer a tick box for members to opt out of receiving print copies of the Journal to test the popularity of

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an online-only option. Council voted unanimously to table the original motion, and then Scott Snyder made the following motion:

**Motion 9:** The Society shall offer the option for regular members to opt out of receiving print copies of the *Journal of Parasitology* in 2012.

Armand Kuris seconded the motion, and it passed unanimously. Discussion that ensued included clarification that this option would be offered at the same cost as the current print and online option. Karen Ridgeway, the representative from Allen Press attending the meeting, agreed to implement this option on the 2012 membership renewal form.

President Caira then initiated a discussion on the upcoming Editorial Office transfer in 2012. She commended the Editorial Selection Committee for their hard work throughout the selection process and announced that Michael Sukhdeo would succeed Gerald Esch as Editor of the *Journal of Parasitology* in the summer of 2012. Michael Sukhdeo thanked the Society of the opportunity and announced that he will keep Vickie Hennings as Editorial Assistant.

Regarding Committee member terms, President Caira recommended that future presidents consider extending terms of Committee member terms when deemed appropriate.

During the 2010 Council meeting, President George Cain charged then President-Elect Janine Caira with establishing a Web Site Assessment Committee. President Caira established this Committee and charged them with assessing the current ASP web site, and to provide Council with recommendations for its improvement and future potential directions, as well as the potential cost of the implementation of their recommendations. Committee Chair Rich Clopton presented several options for web site reconfiguration quoted by Academic Web Pages including basic redesign, conference management, member resources management, image database management, job posting board, hosting, and maintenance. Rich Clopton then made the following motion:

**Motion 10:** The Society shall negotiate a contract including basic site redesign and maintenance with Academic Web Pages. The Society web site shall be hosted commercially, and its web address shall change.

The motion was seconded by Scott Snyder and passed unanimously. It was suggested that a knowledgeable representative from ASP should help negotiate the redesign contract. Scott Snyder then made the following motion:

**Motion 11:** The current President of the Society shall appoint a contact person for web site redesign implementation.

Rich Clopton seconded the motion and it passed unanimously. President Caira suggested that Rich Clopton, Mark Siddall, and Dennis Minchella work on this implementation.

Council then discussed the possibility of creating an online membership directory for ASP members to access. Mark Siddall cautioned against establishing an online membership directory as it may lead to the accidental release of member contact information for corrupt purposes. Several members favored the dispersal of a printed membership directory instead, and Mike Barger made the following motion:

**Motion 12:** The Society shall produce and disperse a printed membership directory in the coming year.

This motion was seconded by Mark Siddall and passed unanimously. Rich Clopton and Janine Caira volunteered to implement this, and Dennis Minchella agreed to provide membership data to them.

Regarding Annual Meeting symposia budgets, President Caira suggested funds designated for the President’s discretion could be used to help defray travel and registration fees incurred by the President’s Symposium and Student Symposium speakers.

Council then discussed the reimbursement of Annual Meeting costs incurred by the Student Representative. Council members agreed that the more junior members of the Society should continue to be enthusiastically supported. After some discussion, Kirsten Jensen made the following motion:

**Motion 13:** The Society shall reimburse the current Student Representative for Annual Meeting travel and accommodations.

Scott Snyder seconded the motion, and it passed unanimously.

Mike Moser then presented the Membership Committee report to Council. He commended the Student Representative and graduate students for their work on the ASP Facebook page, which continues to be a success. Incoming Student Representative Kyle Luth offered to continue managing the Facebook page. Mike Moser then presented several ideas to increase membership and promote the Society. Sam Loker offered suggestions for targeted groups for potential members, and Mark Siddall suggested sending follow-up e-mails that would provide easy access to the renewal site to recent members who have not yet paid their membership dues.

Sam Loker raised the issue of the potential need for a local organizing committee at all future meeting venues. Scientific Program Officer Herman Eure reiterated the need for the Local Organizing Committee to be physically near the Annual Meeting site so that it is able to arrange the Meeting details in person. Mike Burger then made the following motion:

**Motion 14:** Future Annual Meeting sites must have an on-site Local Organizing Committee in place.

The motion was seconded by John Howden and passed unanimously. President Caira agreed to talk to several members about participating in the Local Organizing Committee for the 2013 meeting in Quebec City, Quebec.

Council adjourned at 1143 hr.

IV. REPORTS OF OFFICERS

A. President—Janine Caira

In my vision statement that accompanied the Presidential ballot 2 yr ago, I noted that the decline in our membership over the past decade or so, rather than being a cause for concern, should be interpreted as our transformation into a sleeker incarnation of our vibrant selves. My experiences over the last year as President of our Society have heartily reinforced this interpretation! Membership data suggest that, after a low in 2007, our numbers have risen to settle at a little more than 825 members, nearly 20% of who are students. It is my observation that this “core” of parasitologists is more engaged in the Society now than almost ever before. My invitations to over 50 members to serve on and/or chair Society Committees this year were almost unanimously accepted with enthusiasm and good cheer. In several instances, members actually contacted me volunteering to serve the Society!

The attendance at our meeting in Anchorage is among the highest at any of our national meetings over the past decade. Furthermore, as evidenced by the number of our members who currently hold research grants from the U.S. National Science Foundation (NSF), and also who have recently served on panels and/or program officers at NSF, our members have succeeded in transforming parasitology into a well-fundable discipline in a variety of NSF programs. As a consequence our voice is now being heard in important funding arenas beyond the National Institutes of Health, where we continue to have an influential presence. Our members’ engagement in our Society is also reflected in their creativity. At our last auction, parasite-themed original artistic offerings included ceramics, photographs, paintings, cuttings, and crocheted items; there are rumors of parasite-themed furniture, board games, time pieces, and felt creations in our future. Such time-consuming endeavors clearly reflect a substantial commitment to our Society that transcends membership alone.

What has precipitated this transformation to a trimmer Society? By the very nature of our field, we are multidisciplinary in our interests. We are broad of necessity—parasites transcend taxonomic boundaries, thus we work on a very wide range of organisms, applying a broad spectrum of techniques, and we have knowledge not only of our respective parasite systems, but also of the hosts in which our study organisms thrive, not to mention the expertise we possess in a variety of disciplines associated with these systems.

As a consequence, we have the luxury of being able to shift the emphasis of our research interests effectively and successfully in response to the needs of society as a whole (particularly with respect to medical and veterinary parasites), and also to take advantage of research opportunities as they arise. As I will discuss in my Presidential address, a look at our membership over the past century or so shows that our interests have fluctuated substantially over that time. Recent contributions to the *Journal*, the programs of our national meetings, and web site descriptions of our research, attest to the fact that our research interests have grown to include more work on the basic biology of parasites, which now complements the critically important applied studies in medical and veterinary areas. The membership shift seems, in part, to be the result of some whose interests are primarily medical focusing more of their attention on Societies with that orientation; I believe this is a response to the demands imposed upon us by these busy times when participation in multiple Societies is particularly challenging. Hopefully this is not an irreconcilable problem for clearly maintaining the breadth of our Society is important for all of us. In fact, as a result of the breadth of our training and research, I believe we, as parasitologists, are poised to play a key role in the integration of knowledge across major fields. My belief in the power of such integrative work is what led to my selecting “Parasites at the Boundary of Ecology and Evolution” as the topic of my Presidential Symposium. The work of the 3 speakers who have kindly agreed to contribute to that Symposium, Mike Sukhdeo, Steve Nadler, and Robert Poulin (who
although not currently a member of the New World ASP, surely aspires to be), beautifully illustrates the power of blending such fields. In my role as Secretary of the Society the past year, I have concentrated my efforts on activities that I believe will help to improve communication and streamline our Society’s basic functions. I worked to make Committee appointments in a timely fashion as possible so as to maximize the amount of time available for these groups to fulfill their charges. I worked with the Secretary-Treasurer’s office to modify the templates of the formal committee assignments, in order to include a description of the charge of the respective committees; although this information can also be found in the ASP Handbook and/or By-Laws, I felt that an explicit description of duties at the time appointments are made would foster more informed Committee membership. I also worked with the Secretary-Treasurer’s office to streamline the nomination process for the Society’s various awards. We have developed a single call for nominations and a single deadline for receipt of nominations for all of our awards, thereby unburdening the each of the Chairs of our various committees from having to seek nominations on their own. In response to input from the membership regarding a variety of issues with our Society web site, I established an Ad Hoc Web Site Assessment Committee, chaired by Rich Clopton, to assess the design, function, oversight, etc., of the web site. I look forward to hearing the report and recommendations of that Committee at the Council meeting in Anchorage. At the recommendation of the Priorities and Planning Committee, we are working to make an electronic list of all members available, possibly in PDF format, on the Society web site. Also at the recommendation of that Committee, I have asked Allen Press to look into how the Journal of Parasitology might automatically accommodate NIH’s Public Access requirement that manuscripts arising from all NIH-funded research be submitted to PubMed Central. In response to the announce- ment by our dedicated Journal editor of 17 yr, Jerry Esch, that he will step down from the Editorship in 2012, I appointed an Ad Hoc Committee to identify a new Editor for the Journal of Parasitology. I am delighted to report that the Committee, ably chaired by John Janovy, Jr., has already successfully completed its task; not only has Mike Sukhdeo agreed to assume the role of editor of the Journal beginning in 2012, but his candidacy has already been approved by Council. The timely completion of this process provides the overlap required to ensure a seamless transfer of responsibility for this important position. To take full advantage of the continuity afforded by our Society’s 4-yr term of leadership, I worked with the Secretary-Treasurer’s office to add the incoming Vice President to the Priorities and Planning Committee as a non-voting member. As a consequence, the deliberations of the Priorities and Planning Committee, whose charge is to “recommend policy and the priorities thereof for action by the Society,” will now be known to the incoming President, President-Elect, and Vice President.

With respect to the expectation that the President participate in the meetings, particularly of affiliate Societies, I am afraid my visits to affiliate Societies (with the possible exception of my own-the New England Association of Parasitologists) were limited to my participation in the momentous occasion of the celebration of the 100th Anniversary meeting of the Helminthological Society of Washington, which was efficiently organized by John Hawdon at George Washington University in Washington. In addition, I was delighted to represent ASP at the International Congress of Parasitology in Melbourne, Australia, and to participate in the selection of the venue for the next ICOPA, which will be held in Mexico City in 2014. At that meeting, I also pursued the question of eligibility of our affiliate Societies as members of the World Federation of Parasitologists (WFP) and learned that all formalized Parasitology Societies, i.e., those with officers, etc., are eligible for membership in the WFP; to join they simply need pay their dues. I also transmitted a plea from the Secretary of the WFP, Les Chappell, that our affiliate Societies consider that they are eligible to participate in the WFP. Thus, we have now established a process whereby, through a single call for nominations, we can accommodate the needs of the various Societies, not just our own. In the context of the WFP, I was also privileged to attend the Spring Meeting at the Romberg Tiburon Campus of San Francisco State University, California, as the keynote speaker at the invitation of Dr. Ravinder Sehgal. Several excellent student presentations were delivered, and I participated as a member of the student awards committee. This Campus is the only marine laboratory directly on San Francisco Bay and has additional venues to enhance the experience. I also participated in the President’s symposium of the Southeastern Society of Parasitolo- gists, hosted by Isäre de Buron. This meeting was held at the Unicoi Lodge near Helen, Georgia, providing access to an interesting state park in the southern Appalachians. This large and very active regional meeting offered 2 days of talks on a wide variety of parasitological topics. I briefly presented some ideas for increased outreach to those many researchers working on parasites who could benefit from a level of formal interactions with the American Society of Parasitologists. If we can effect this, we can enhance the role of our Society in the investigation of ecological, evolutionary, immunological, epidemiological, behavioral, and other broad areas that interact with parasitology. I also attended the Spring Meeting of the British Society of Parasitologists, where the attendees were invited to join the University of Nottingham as an invited speaker at the invitation of Andrew MacColl. The British Society has retained considerable membership working with the epidemiology, immunology, and genetics of major tropical diseases, notably malaria and schistosomiasis. It may offer a model for reinvention of our Society in these important parasitological emphases.

C. Vice President—Eric S. Loker

As one of my duties as Vice President of ASP, I attended the Annual Meeting of Rocky Mountain Conference of Parasitologists in September 2010 and will also attend the Annual Midwestern Conference of Parasitologists meeting when it is held in June 2011. My goal for attending these meetings is to solicit input from regional Societies about our Annual Meeting, or any other aspect of ASP of interest or concern to the regional groups. For example, feedback received from the Rocky Mountain group about the ASP meeting was relayed to the current scientific program officers. I will also (indirectly) request feedback from the members of the Southwestern Association of Parasitologists at their upcoming April 2011 meeting.

D. Secretary-Treasurer—Dennis Minchella

At the halfway point in our term as Secretary-Treasurer, Alice Foster and I have now encountered most of the matters associated with the position. In the event of the occasional unfamiliar issue, former Secretary-Treasurer John Janovy, Jr., has been extremely helpful in
offering his advice and insight. We would like to extend our appreciation to President Janine Caira for dealing with a number of tasks and issues in a timely and efficient manner.

**Status of the Society:** As has been the pattern in previous years, our membership and subscriptions continue to decrease. The tables below illustrate the recent trends. The number of active members is projected to increase this year despite the extensive efforts of Mike Moser and the Membership Committee (see 2011 Membership Committee report). Nevertheless, student online-only memberships remain popular (73 in 2009, 80 in 2010, 84 in May 2011). Their popularity suggests that Council may want to further consider an online-only option for regular members. Student membership in general is relatively stable, suggesting that our student-friendly approach is having a positive impact. These new members will become the lifeblood of the Society.

Our financial condition is beginning to stabilize, much like the economy did in 2010. A more favorable economic climate coupled with our determination to maintain conservative spending practices facilitated this stabilization. Following the economic crash and the Society’s subsequent overall loss of over $335,000 in 2008, our investment portfolio continues to grow with an overall gain of nearly $100,000 in 2009 and $60,000 in 2010. This growth is exclusively due to increases in the value of our investments as we have not been able to deposit any additional funds into the investment categories (that is, all funds that come into the Society are utilized). The theme of stabilization extends to the finances of the Journal as well. Although Journal expenses tend to slightly exceed Journal income, the gap between the 2 has been smaller with each passing year.

**Membership summary (by category):**

<table>
<thead>
<tr>
<th>Category</th>
<th>2009</th>
<th>2010</th>
<th>2011 (May)</th>
</tr>
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<tbody>
<tr>
<td>Regular</td>
<td>564</td>
<td>527</td>
<td>464</td>
</tr>
<tr>
<td>Student</td>
<td>162</td>
<td>140</td>
<td>129</td>
</tr>
<tr>
<td>All others</td>
<td>182</td>
<td>175</td>
<td>156</td>
</tr>
<tr>
<td>Total</td>
<td>908</td>
<td>842</td>
<td>749</td>
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**Subscriptions (by category):**

<table>
<thead>
<tr>
<th>Category</th>
<th>2009</th>
<th>2010</th>
<th>2011 (May)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Institutions</td>
<td>19</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Agencies</td>
<td>561</td>
<td>482</td>
<td>411</td>
</tr>
<tr>
<td>Complementary</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Exchange</td>
<td>8</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Foreign scientists</td>
<td>11</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>605</td>
<td>524</td>
<td>454</td>
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</table>

**Income and expenses for 2008 through 2010:**

<table>
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<tr>
<th>Year</th>
<th>Income ($)</th>
<th>Expense ($)</th>
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</thead>
<tbody>
<tr>
<td>2008</td>
<td>143,015</td>
<td>71,413</td>
</tr>
<tr>
<td>2009</td>
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<td>989</td>
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<tr>
<td>2013</td>
<td>417,14</td>
<td>7,024</td>
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<tr>
<td>2014</td>
<td>252,724</td>
<td>223,416</td>
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<tr>
<td>Margin</td>
<td>335,531</td>
<td>99,426</td>
</tr>
</tbody>
</table>

**Abbreviations:** St-St (r) = Stoll-Stunkard endowment (restricted fund), DCB = Douglas County Bank account, St-St (i) = Stoll-Stunkard endowment (interest income), B-V+B = Beuding-von Brand endowment, A C = Ashton Cuckler endowment, St Trvl = Student Travel endowment, ( ) = unrealized loss.

**Investments:** The Society has 6 categories of investments, 3 composed of stock funds and 3 that are largely cash. In addition to the income sources above, our total investment value was $448,299 in December 2008, $563,088 in December 2009, and $638,781 in December 2010. ASP’s “net worth” = (investment value + cash accounts – liabilities) at the end of 2008 was $502,244.78, the end of 2009 was $600,438.89, and the end of 2010 was $659,623.02. The March 2011 financial statement and compilation report from Mize, Houser, Inc. gave a current total asset value of $696,770.90.

**Operations:** Alice Foster continues to serve the Society as Administrative Assistant to the Secretary-Treasurer. Although we are now physically separated by the state of Illinois, we maintain nearly daily electronic contact on Society business. Alice’s dedication to the Society has been astounding, and it clearly does take both of us to effectively run the organization. Alice continues to support the operation of the Society by maintaining and reconciling financial records, preparing check requests for Mize, Houser, keeping membership spreadsheets, processing deposits and memberships with Allen Press, maintaining the ASP listserv, and communicating with Society members, Allen Press, and Mize, Houser on a regular basis.

While our financial condition and Secretary-Treasurer operations have stabilized, our primary banking institution changed this year. The majority of our banking transferred from Douglas County Bank to US Bank at the request of Allen Press at the beginning of 2011. The Douglas County Bank account remains open to allow electronic federal and state tax withdrawals as these tax agencies work to process our banking change. Assuming that all goes well, we hope to close this account in the next financial quarter.

Early in 2011 presented some temporary financial challenges. Although our investment portfolio grew, we experienced low cash flow in early 2011 while awaiting the arrival of our annual royalty check from Allen Press. Factors contributing to this low cash flow included our need to catch up on financial reviews as well as to provide funds for pre-meeting expenses. We will spend nearly $10,000 for reviews for 2008, 2009, and 2010. However, now that we have caught up, we do not expect to incur multiple review expenses in a single year. We have asked Allen Press whether they can distribute our annual royalty profits over several payments throughout the year.

The Secretary-Treasurer’s office also explored some new initiatives this year. We compiled an array of insurance policy options for the Society including insurance to protect the officers and our financial resources. These options are relatively expensive, but probably necessary for our Society. The proposals will be considered at the Council meeting in June 2011.

Electronic balloting continues to be a useful tool for the Society. In addition to using it for the fifth year for the 2011 annual election, the ASP membership voted via Allen Press electronic balloting to approve the revised by-laws in August 2010, bringing much needed clarification and updating to the Society’s rules and regulations. We thank the By-Laws and Handbook Committee and Council for their meticulous efforts throughout the process.

In summary, the economy is recovering slowly, and so is our financial condition. We will need to continue to live within our means in an environment where both memberships and subscriptions are decreasing. This does not mean that we should stifle new ideas and initiatives, but rather that we all need to work together to keep our Society moving forward.

**Communications:** Since July 2010, Officers and Council Members-at-Large considered by e-mail vote the following items:

Item 1. 30 November 2010—approved the J. W. Maritott-New Orleans in New Orleans, Louisiana, as the 2014 meeting location and 23-29 July as the 2014 meeting dates.

Item 2. 15 December 2010—approved the Hilton-Omaha in Omaha, Nebraska, as the 2015 meeting location and 24-29 June as the 2015 meeting dates.

Item 3. 3 February 2011—approved the Eminent Parasitologist Lectureship Committee recommendation that Dr. Robert Rausch be awarded the 2011 lecture.

Item 4. 10 February 2011—approved the nomination of Dr. Bruce Christensen as the 2011 recipient of the Clark P. Read Mentor Award.

Item 5. 9 March 2011—consideration of Ashton Cuckler New Investigator Award nominations.

Item 6. 15 March 2011—agreed to hold a revote on the Ashton Cuckler New Investigator Award Committee recommendation.
E. Journal of Parasitology Editor—Gerald W. Esch

Since the last meeting in Colorado Springs, the Editorial Office has been active, as usual. We are totally caught up and printing each issue on time. While the work has kept us quite busy, we have no real changes to report.

According to John Janovy, Jr., the Chair of the Editor’s Search Committee, my replacement has been identified. However, we have been asked not to reveal the person’s name until Council has approved the suggested nominee. I have discussed the situation with the new person, and we have tentatively agreed to meet in July in Lawrence, Kansas, at Allen Press, where we will review various issues pertaining to the publishing and printing of the Journal of Parasitology.

One of the important topics to be considered in Lawrence will be the publication of the Journal’s Centennial Volume in 2014. Even though I will be stepping down as Editor in July 2012, I have requested that I have a hand in developing the 100th Volume, which will pay tribute to the founders of the Journal, primarily Henry Baldwin Ward, as well as those who followed and made the Journal into what it is today. I am confident that our preparations will move fast enough over the next 12 months so that a full announcement of our plans can be made to the Society during the June meeting in Richmond, Virginia, in 2012.

Finally, as I have done in the past, I want to thank members of our Editorial Board for their hard work over the past year and to pay tribute to all of you who have faithfully served as referees and reviewers for the Journal. I think people forget sometimes that all of us, save Vickie, are volunteers, and that our contributions should be considered within that context. Vickie Hennings has been a real jewel for us again this year, just as she has been in each of the last 18 yr we have worked together. Finally, Annielaurie Seifert at Allen Press has also been a fantastic “go to” person, and so has Peter Burns.

We have one more year to go. I hope it will be our best.

F. ASP Newsletter Editor—Scott L. Gardner

Two Newsletters were sent out in 2010 in a combined effort. No funds were expended to create these and post them to the ASP web server. Because we upgraded the web server software, and then had to migrate the web site to a new machine, Newsletters have been neglected for the past few months. Perhaps someone would like to send some data and other information to the Newsletter editor for inclusion? Photos? Links to interesting stuff, lectures? Field trip data? If we are able to get contributions to the Newsletter, then it would come out more often. We are now accumulating more old Newsletters to scan and make available on the ASP web site. We have had inquiries for these, so it would be good to have the old ones made available.

G. Scientific Program Officers—Herman Eure and Kelli Sapp

The 85th meeting of the American Society of Parasitologists was held at the Crowne Plaza Hotel in Colorado Springs, Colorado, from 22–25 June 2010. The meeting was hosted by Dr. Ron Hathaway and Colorado College. Sponsors of the meeting included Colorado College, Merial Animal Health, HESKA, and JEOL, Inc.

The opening reception featured both regional and traditional southwestern cuisine and drinks of all sorts while being serenaded by a string quartet. On what has become our traditional third day offsite venue, we were treated to an evening at the Cheyenne Mountain Zoo, which included a dinner and access to the entire zoo.

Kelli Sapp and Lee Couch organized another grand Student Auction, where everything under the Colorado skies, including the shirt off of the back of President George Cain, was auctioned off by the “Three Amigos” to raise money to support student travel.

The scientific program included a Teacher Education Workshop organized for local teachers by Jim Serach from the Lawrenceville School, Lawrenceville, New Jersey, and featured presentations and demonstrations by John Janovy, Matt Bolek, and Alaine Knipes. There were 135 presentations (oral and poster collectively) by members that were scattered among 10 sessions and 5 symposia. In addition, there were several special lectures. Among them were the Presidential address in which President George Cain discussed the intersection of parasitology, civil engineering, and public health in waste treatment facilities; the Clark P. Read Mentor Award Lecture by Dr. Armand Kuris, UC Santa Barbara, who used John Wooden’s teaching and pyramid of success concept/approach to illustrate the interconnectedness of his academic family tree; and the Stoll-Stunkard Lecture delivered by Dr. Jane Carlton from the Department of Medical Parasitology, New York University, on “Parasite Genomics: The Next Generation.” The Ashton Cuckler New Investigator Award Recipient was Dr. Dawn Roellig, who could not attend the meeting due to field work being conducted in Peru during the meeting dates.

The archives of the Society continue to be located in the library of the Harold W. Manter Laboratory. Services, collections, and library are safely housed at this 30-yr location.

I. Student Council Member-at-Large—Joanna Cielocha

As western society has developed, humans have worked to eliminate pathogens, pests, and, in particular, parasites from their naturally occurring habitats, especially when they occur on or in our bodies and food. The topic for this year’s Student Symposium focuses on how parasitism has influenced current perspectives on human health in western society. The symposium is entitled “Parasites in the Developed World: Drugs, Allergies, and Cleanliness.” It will feature 3 speakers, each of whom will present a talk describing their research as it relates to the influence parasites have on human health in developed western countries. The speakers will present a broad spectrum of current parasite-related health issues—e.g., parasites in our food, how parasites may be involved in allergic responses, and overall welfare of parasites. Dr. Anna Arnot (FDA) will present aspects of her research regarding FDA regulations and drug development and treatment. Dr. T. V. Rajan (University of Connecticut Health Center) actively researches the interaction of the immune system and exposure to pathogens. His talk will present how the absence of exposure to pathogens may create exaggerated immune responses to otherwise benign stimuli. Dr. Rob Dunn (North Carolina State University) will focus his talk on the long co-evolutionary history that humans and parasites (and other symbionts) have shared. He will present the cost and benefits of these interactions and the effects of parasite elimination.

Herman Eure and Kellie Sapp, the Local Organizing Committee, were instrumental in making arrangements to secure a location for the Student Social following the Symposium. I would like to offer my sincere gratitude for all that they did in making this happen.

In 2009 ASP created a Facebook page to facilitate interaction among members of the Society that use the social network. As of 20 April 2011, 321 people were registered members of the American Society of Parasitologists Facebook group. This is twice as many members as May 2010 (159 members). This year, the Facebook page was useful for posting reminders for students and other members about the Call for Abstracts and registration. I posted reminders for students to contact me if they were searching for roommates, in addition to several people taking advantage of the Facebook Wall to post their own requests for roommates. In total, I assisted 27 students to find roommates from 21 universities and 2 countries. These arrangements have made it possible for students to share hotel costs, making the meeting more affordable. The ASP Facebook page has also served to disseminate news and links of potential interest to group members, e.g., other Society meetings and web
resources. Overall, the Facebook page has attracted many members of the Society as well as other people interested in parasites.

I spoke to both nominees for the 2011-2012 ASP Student Council Member-at-Large to explain some of the duties and responsibilities of the Student Representative. I think that either of them will make meaningful contributions to the Society as well as effectively represent the student members.

V. REPORTS OF STANDING COMMITTEES

A. Award Committees

*Henry Baldwin Ward Medal:* Steve Nadler (Chair, 12); Ronald Fayer (11), John Harley (11), Kevin Lafferty (12), Claire Healy (12).

The Committee received nominations for this award on 4 March 2011. 

*Bueding and von Brand Lectureship:* Tim Geary (Chair, 11); Eric Villegas (12), Ed Rowland (13), David Lindsay (14), Isaura de Buron (14). This is an off year for the Lectureship. The next call will be put out for 2012. 

*R. Barclay McGhee Memorial Lectureship:* Carter Atkinson (Chair, 11); Yvonne Goater (12), Michael Haskins (12), David Lindsay (14).

This is an off year for the R. Barclay McGhee Memorial Lectureship. The next lecture is scheduled for 2013. 

*Ashton Cuckler New Investigator Award:* Cheryl Davis (Chair, 11); Karl Hoffmann (11), Cam Goater (12), Julian Hillyer (12), Scott Snyder (12). The Committee received 2 nominations for the 2011 award. Nomination packets were distributed to Committee members electronically on 24 January 2011. The members of the Committee (minus Cam Goater who recused himself from deliberations) carefully reviewed and discussed the nomination packets and carefully considered the rules and criteria for the Ashton Cuckler Award before making our recommendation to the ASP Council on 4 March 2011. At that time, a majority of Committee members voted to recommend that Ms. Chelsea Matisz be the 2011 recipient of the Ashton Cuckler New Investigator Award. The following comments summarize comments provided by Committee members who voted in favor of recommending Ms. Matisz for the award. Although Ms. Matisz is at a relatively early stage in her scientific development, her CV and letters of support show that she has made important contributions in 3 areas of parasite biology within her Master's Degree. These include the role of host rodlet cells during parasitic infection, parasitic migration in a host, and analysis of the fine details of host-parasite interactions (focusing on host response to infection). This third subject area is being further pursued in Ms. Matisz's Ph.D. research at the University of Calgary. Her list of received awards, especially in light of her early stage of career development, is also very impressive. These awards and scholarships are clearly indicative of competitive potential. Ms. Matisz's contributions have also been felt within public health and governmental policy as illustrated by her time spent within the Canadian Water Network, where she helped write several regulatory or governance documents. This body of work demonstrated how Ms. Matisz's scientific background could be applied to societal impact—a subject that is highly relevant to funding bodies and public opinion. In addition, Ms. Matisz has participated in ASP as a student member and has presented her work at 2 of our Annual Meetings. One of her ASP presentations was recognized as a Meritorious Student Paper. Therefore, Ms. Chelsea Matisz has clearly met and surpassed the requirements set forth in the *ASP Handbook* for the Ashton Cuckler New Investigator Award.

The Committee forwarded Chelsea Matisz’s name to Dennis Minchella (ASP Secretary-Treasurer) on 4 March 2011, and her nomination materials were forwarded electronically on 7 March 2011. She was informed via e-mail that she had been chosen as the 2011 recipient of the Ashton Cuckler Award on 23 March 2011.

Regrettably, the Chair of the Ashton Cuckler Award Committee, Cheryl Davis, will not be able to attend the 2011 Annual Meeting of ASP to preside at the awards ceremony. Instead, Committee member Julían Hillyer has graciously agreed to present the Ashton Cuckler Award to Ms. Matisz at the Annual Meeting.

*Clark P. Read Mentor Award:* Ben Hanelt (Chair, 12); Tim Ruhnke (11), Ash Bullard (13), Janice Moore (14).

The Committee received 2 outstanding nominations for the 2011 Clark P. Read Mentor Award. Committee members reviewed the nomination materials for about 1 month and subsequently conducted a lengthy e-mail discussion and vote. On 11 February 2009, the Committee forwarded the name of Dr. Bruce M. Christensen (University of Wisconsin–Madison) to Council for consideration as the 2011 Clark P. Read Mentor Award recipient. Julían F. Huerta (Vanderbilt University) graciously agreed to introduce Dr. Christensen at the 2011 ASP meeting in Anchorage.

*Stoll-Stunkard and Eminent Parasitologist Lectureships Committee:* Bill Granath (Chair, 13); Ramon Carreno (11), Michele Klingebell (12), Rich Clopton (14).

The Committee received 2 nominations for the Eminent Parasitologist Lectureship. Both candidates have strong research records. However, after careful review of the nomination materials the Committee unanimously and enthusiastically recommended that Dr. Robert Rausch of the University of Washington (UW) receive this prestigious honor. Dr. Rausch substantially exceeds the selection criteria for this award. His career in parasitology spans more than 60 yr, during which he has been at the forefront of research in systematics, taxonomy, epidemiology, and pathology of helminths in vertebrate hosts. As Professor Emeritus at UW he continues to be active in research and teaching at the age of 89. The next Stoll-Stunkard Memorial Lectureship is scheduled for 2012.

*Distinguished Service and Extramural Awards:* Tim Goater (Chair, 12); Herman Eure (11), Mike Kent (13), Tom Platt (14).

There were no nominees and, thus, no selections for either the Distinguished Service Award or Extramural Awards this year.

B. Student Awards Committee

*Kym Jacobson (Chair, 11); Michael Hildreth (11), Agustin Jiménez-Ruíz (11), Jeffrey Laursen (11), Doug Woodmansee (11), Sarah Bush (12), Jillian Detwiler (12), Ryan Hechinger (12), Devon Keeney (12), Valerie McKenzie (12), Russell Easy (12). The Committee received a total of 56 applications. A number of these were incomplete, lacking abstracts or letters of reference. A few of these letters were accepted late following inquiries with advisors. The Committee judged abstracts and letters of 50 student applicants. Eleven of these were from countries outside of the United States, namely Canada, Australia, New Zealand, Mexico, Brazil, and Spain. Because of anticipated higher than usual costs for travel to Anchorage the ASP Council voted last year to provide more funds for travel in 2011. The Committee was given $14,000. We awarded funds to 30 students; $500 was given to the top 27 candidates, which included 3 regional winners from affiliated Societies, unless they had asked for less (4 students). The remaining funds were allotted to 3 more students as follows: $475, $450, and $425. For comparison, in 2010, 31 students applied and $8,000 was split among the students with awards between $400 and $100. In 2009, 26 students applied and awards ranged from $400 to $100. For travel to Anchorage the 2011 Committee decided that awards less than $400 may not offer enough assistance.

The following students received travel awards this year: Latasha Babcock, Kayce Bell, Inna Cañedo-Solera, Kathryn Coyne, Jessica Edwards, Rick Gerhold, Stephen Greiman, Jesús S. Hernández Orts, Kristin Herrmann, Jennifer Hogan, Sophine Jean, Brian Johnson, Anson Koehler, James Losee, Crisyda Martínez, Laura Isabel McCall, Alejandro Oceguera-Figueroa, Jason Palladino, Maria Pickering, Tamelee Roberts, Wayne Rossiter, Phil (Raymond) Scheibl, Brittany Sears, Kate Shechan, Will/Kum Shim, Leah Sigle, Anthony Stumbo, Melissa Thomson, Benjamin Walker, and Michael Zimmermann.

Finally, many questions arose during this year’s review process, for which we feel there is a need to have written guidance agreed upon by the council for future Student Awards Committees.

C. Education Committee

Scott Seville (Chair, 11); Ashleigh Smythe (12), Guy Cabrall (13), Matt Boise (14), Jim Serach (15).

The Education Committee, for the seventh year, promoted the ASP Willis A. Reid, Jr. Student Research Grant program. Requests for proposals from undergraduate and graduate students were issued via the ASP listserv and on the ASP web site during the fall of 2010. The program is intended to provide support for research being conducted by students at institutions where there are no or limited research funds available. In 2011, 6 proposals were submitted (0 undergraduate and 6 graduate students). After careful consideration by the Committee members and discussion with Dennis Minchella and because there was a virtual tie for top-ranked proposal, the $1,500 available was split, and $750 was awarded to Kayce
Bell at the University of New Mexico for her proposal “Coevolution of Chipmunks and Their Parasites” and Emily Kasl at Texas A&M for her proposal “Elucidating Patterns of Freshwater Biodiversity Using Host-Parasite Co-Phylogeny.”

The Education Committee encourages all ASP members to continue to promote parasitology through their commitment to working with K--12 students, as suggested in previous reports, and to encourage undergraduate and graduate students to apply for the ASP Willis A. Reid, Jr., Student Research Grant.

D. In Memoriam Committee

Sherman S. Hendrix (Chair), Milton Riley (11), Bernard Fried (12).

It is with sadness and regret that the In Memoriam Committee reports the deaths of members and former members of the Society: Dr. Edward Ogden ‘Ted’ Hooghkirk, 88, a well-known medical educator in Maine, in January 2009; Dr. John Frederick Adrian Sprent, of Brisbane, Australia (Honorary Member of the Helminthological Society of Washington); Dr. Steve J. Upton, 57 (1996 Henry Baldwin Ward medal recipient), on 29 July 2010; Dr. John H. Cross (Past-President of the Helminthological Society of Washington and 2001 Anniversary Award recipient), on 19 November 2010; Dr. David Richard Lincicome, 98 (Past-President, 1975 Anniversary Award recipient, and Life Member of the Helminthological Society of Washington since 1976), on 9 March 2011; and Dr. Jeffrey Bier, on 2 April 2011.

E. Business Advisory Committee

Ann Adams (Chair), Stephen Kayes (11), Lillian Mayberry (11), Don Munson (13), Dennis Minchella (ex officio).

Trying to utilize references from the humanities, we are stymied in our efforts to provide a suitable clarion for our report. Perhaps the best choice would be “Dueling Banjos” to represent the economy and its effects on the Society’s investments. The economy continues to improve for global companies and those invested in their stock. However, debt (especially governmental), rising costs in commodities, and the continuing drag on real estate have a strong dampening effect.

Our Society’s investments closely mirror the activity in the economy, reaching the lowest point of value in February 2009 and steadily increasing in value since then. As of the end of 2010, the Society’s investments have increased 65% from the lowest level and 13.5% in the past year. The total value of the Society’s investments in the 6 categories at the end of 2010 was $638,781.

The Society has 6 categories of investments, 3 of which are primarily made up of stock funds. These 3 have increased with the improvement of the economy, with actual earnings including dividends and capital gains, as well as unrealized gains (increases in market value). The Stoll Stunkard Restricted Fund, Category I, had earnings of $3,081 and an overall increase in value of $44,877 or 14.2% from the previous year. The Ashton Cuckler Fund, Category V, has the lowest overall increase in value (8.5%) because it is not entirely invested in stock funds, but also in the Plum Creek REIT, Enron stock (no value), and 16% in cash. The Student Travel Fund, Category VI, had the greatest increase in value at 18.3% from the previous year. The fund had fewer actual earnings, $2,224, than the Category I, but greater overall increase in value. The components of Category VI consist of domestic and international funds, which are recovering their value at a faster rate.

The 3 remaining categories are made up entirely of cash and are earning negligible interest. Category II, the General Fund, has the smallest amount of monies and has remained stable for numerous years. Category III, the Stoll Stunkard Fund, can be used for operating expenses but has a generally small balance. Some funds were used in the past year for an increase in value of $26,522. These monies relieve pressure on the Society’s investments to help fund the day-to-day operations.

Recommendation: As recommended previously, the Society should continue to hold the investment positions as the economy slowly recovers.

We anticipate the rate of increase in value will moderate over the next several years as the markets respond to various pressures imposed by domestic and international debt, by governmental policy and budgetary decisions, and by changes in the commodity markets. We also recommend that after evaluation of the cash flow for operations, cash in funds such as Category I (currently $25,000) be considered for further investment in stock or bond funds.

F. Meeting Site Selection Committee

David Marcogliese (Chair, 11); Kirsten Jensen (12), Joe Camp (13), Don Duszyński (Ex Officio).

Future meetings approved by Council in 2010-2011 include 23-29 July 2014 to be held at the J. W. Marriott Hotel in New Orleans, Louisiana; 24-29 June 2015 to be held at the Hilton Hotel, Omaha, Nebraska. Currently our Committee is exploring Corvallis, Oregon, and Charleston, South Carolina, as potential meeting sites for 2016 and 2017. The Committee thanks Joe Camp for serving an extra term.

G. Nominating Committee 2010–2011

Reginald Blaylock (Chair), Derek Zelmer, Florian Reyda, Ben Hanelt, Gregory Sandland, Benjamin Rosenthal (alternate).

The list of nominees submitted for the 2011 ballot was as follows: Vice President: Gerald W. Esch, John Janovy, Jr.; Council Members-at-Large: Vincent Connors, Timothy Goater, Robin Overstreet, Timothy Yoshino; Nominating Committee: Ann Adams, Sara Brant, Joseph Camp, Jr., Brian Fredensborg, Christopher Hall, Claire Healy, Ryan Hechinger, Julian Hillyer, R. Scott Seville; and Student Council Member-at-Large: Kyle Luth, Raymond (Phil) Scheibl.

H. Tellers Committee 2010–2011

Samuel Black (Chair), Barbara Burleigh, Patrick Skelly.

189 ballots were received; 4 of the total ballots were paper ballots. There were no invalidated ballots in any category.

The election results are as follows: Vice President: Gerald W. Esch, John Janovy, Jr.; Council Members-at-Large, 2011–2015—Robin Overstreet and Timothy Yoshino; Nominating Committee members, 2011–2012—Sarah Brant (Chair), Ann Adams, Joseph Camp, Jr., Claire Healy, R. Scott Seville, Julian Hillyer (alternate); and Student Representative to Council, 2011–2012—Kyle Luth.

I. Priorities and Planning Committee

Armand Kuris (Chair, 11); Cynthia Church (11), John Hawdon (11), Susan Perkins (12), Charles Criscione (13), Sam Loker (Vice President ASP, non-voting member), John Janovy, Jr. (Vice-President-Elect ASP, non-voting member).

Following the style of the 2010 Chair, the present Chair believes that the charge of the Committee to “recommend activities to the Council that the Society might undertake to further the purpose of the organization” will be most effective if pursued in an actual rather than a virtual discussion. Thus, the P&P will meet at the 2011 meeting in Anchorage. At the 2010 meeting in Colorado Springs we discussed the impact factor of the Journal and decided to hold off further discussion of that issue until the new Editor was in place. The membership of the Committee to select the next Editor was considered, and that has led to the appointment of Michael Sukhdeo as the next Editor of the Journal.

Issues concerning the ASP web site were discussed, and Richard Clopton was appointed by President Cairns to chair ad hoc committee for web site assessment. The possibility of offering workshops at national meetings, focused on a diversity of aspects of parasitology was discussed, and will be raised again at the Anchorage P&P Committee meeting.

We also discussed an initiative to increase access to, and membership in, ASP for young parasitologists (graduate students, postdoctoral fellows, new faculty) who come from academic backgrounds that are not grounded in parasitology (such as ecology, evolutionary biology, immunology), but who are working with parasites, often in isolation from parasitologists. This might best be initiated by parasitology graduate students, led by the ASP Student Representative, acting on an outreach basis. President Cairns assigned Mike Barger as an ad hoc member of the Membership Committee with the specific task to identify students with NSF
Dissertation Improvement Grants to look into whether they would like to join the Society. We will extend this discussion at the 2011 meeting.

**J. Honorary Members and Distinguished Members Emeriti Committee**

Tami Cook (Chair, 13); Mark C. Jenkins (11), Tim Yoshino (12). There were no nominees and no selections for Honorary Members and Distinguished Members Emeriti.

**K. Industrial Liaison Committee**

Tim Geary (Chair, 11); Sharon Patton (11), David Lindsay (13). No report as of publication date.

**L. Membership Committee**

Mike Moser (Chair, 11); Hugo Mejia-Madrid (11), Marius Fuentes (12), Mike Barger (14), Joanna Cielocha (ad hoc).

The Membership Committee would like to thank Alice Foster (ASP) and Jeff Mercer (Allen Press) for their help.

<table>
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<th>Category</th>
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<td>X</td>
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<td>158</td>
<td>323</td>
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</tbody>
</table>

* Based on a 7% increase in memberships in the last 7 months of 2010.  
** Based on a 17.5% increase in memberships in the last 7 months of 2010.  
*** Based on a 13.7% increase in membership in the last 7 months of 2010.

**Discussion of the Membership Status:** We anticipate that in 2011 there will be an approximate 12% decrease in the Active Members Print and Online category and an approximate 4% decrease in Total Student membership. It is anticipated the Student Online Only category will remain the same. Other projects could not be calculated due to lack of past data.

In 2010 we contacted the Secretaries of 12 Latin American parasitology Societies and select Latin American individuals and invited them to share their research and academic ideas with our members. We described the Consortium membership option and the economic advantage of publishing in the *Journal* as a member. Perhaps this effort contributed to the increase in Consortium Online Only members from 4 in 2009 to 14 in 2010.

Unfortunately, the enrollment is currently low.

Last year we contacted approximately 950 non-members who had published in the *Journal*. We explained the economic advantages of membership when publishing in the *Journal* and the academic benefits and collegiality in ASP. In spite of these efforts it is anticipated that Active Members Print and Online may decrease 12% in 2011.

We contacted the Secretaries of 47 international parasitological Societies. Our goal was not primarily recruitment but rather to make them more aware of ASP and invite their participation in our activities. We received replies from several Secretaries asking us to post information about their Society on our web page and stating they would do the same for us.

In an effort to contact non-members working in parasitology we contacted 39 principal investigators (PI) who had received Dissertation Improvement Grants from NSF for research involving parasites. These projects were varied in their focus, but all topics are routinely published in the *Journal*. We received a couple replies saying the PI would forward our message to the graduate student involved.

Student membership is the most stable of all the above categories. We believe that we should continue to actively recruit and accommodate students and make every effort to retain them.

Our Facebook membership has doubled during the last year. This is the result of the hard work and attention of the Student Representatives.

**Current and Proposed Activities:**

1. The ASP Facebook page has been a successful means to communicate among ourselves and with others.

   (a) We will continue to contact ASP’s regional Societies and affiliates and encourage them to maintain their own Facebook pages. Posting the events and abstracts of the presentations at their local meetings is a good way to keep people connected.

   (b) We will continue to join other Societies’ Facebook pages and post brief messages about ASP. Since Facebook postings continually change, this is an ongoing effort. The message will be directed to the interests of the members of that Society. The message is short and introduces them to ASP. Here is an example of a message to the Western Society of Naturalists:

   **GOT PARASITES?**

   Did you know that for every host species there may be up to 10 parasites species associated with that host? You can learn more about parasites from the American Society of Parasitologists on our web page, http://asp.unl.edu, and Facebook, http://www.facebook.com/group.php?gid=122311337389.

2. It is important to stay in touch with the Secretaries of foreign parasitology Societies, non-member authors, and members who have dropped during the last year. We do not want to alienate them with spam, but we want to remind them we are still here. One possible way is to send them the Table of Contents from the most recent issue of the *Journal* and invite them to check out our web page and Facebook. This would be done after each issue of the *Journal.* Is that OK?

3. ASP—More than just a great journal.

   (a) Our approach has been largely to direct the people we contact to the ASP web site and Facebook. This is an efficient means introduce ourselves and provide information to them. The ASP web page should continue to be a source of information about parasites. It should not be redundant with other sources but a link to them and an expansion of our own resources. For example, the ASP web site could be a host and a link to databases for DNA barcoding and host-parasite lists. The ASP web page could become a primary source when searching for this type of information. If there was support by the members a Wikipedia-type host-parasite list could be maintained. For example, about 30 yr ago a host-parasite list was published for fish from the West Coast. There were only a limited number of copies printed. If this publication was hosted on our web site, it could be revised and expanded by qualified members. The need for such reliable sources is seen in the recently published, large, *10 Year Census of Marine Life (2000-2010)* (http://www.coml.org; go to the link http://www.iobis.org/mapper/, in the left box “Data Search,” open “Taxa,” then open the Taxonomic Tree, then Animalia under Biota, which leads to some parasite phyla). They published all the marine fish species discovered or reported. They also reported a host-parasite list. A brief literature search by a parasitologist would have recovered many more parasites. The Membership Committee believes that the more people that become aware of ASP as a source of information, the more interest there will be in becoming members.

VI. REPORTS OF AD HOC COMMITTEES

**A. Information and Technology Committee**

Scott Gardner (Chair), John Barta, Gerald Esch, Agustin Jiménez-Ruiz, Mark Siddall.

The ASP web server, a Sun dual SPARC system running UNIX with mirrored data drives, had a software upgrade during early 2010. A database-
At the 2010 meetings in Colorado Springs, Janine Caira assembled an ad hoc committee for the purpose of selecting a new editor for the *Journal of Parasitology*, with duties to begin in 2012. The Committee received several nominations and recommendations. All individuals whose names came before the Committee were contacted, sometimes more than once. Based on these conversations, the ad hoc Editor Selection Committee has forwarded a recommendation to Council for consideration.

### E. Web Site Assessment Committee

Rich Clopton (Chair), Pete Olson, John Barta, Joe Camp.

The consensus of the Committee is that although the current ASP web site is nice and has served us well over a number of years, a highly professional and effectively commercial web site is now the de facto norm for a Society of our size. As we expect more and more out of the web site, it is unreasonable to expect a single member to maintain and update the site. In our judgment, it is time to hand the ASP web site over to a professional company. This becomes even more of an issue as we continue to integrate our web site with services from Allen Press and move our membership and meeting registration functions online.

As a general outline, ASP needs a fairly static, commercial web site that incorporates or provides linkage with the Annual Meeting, the Allen Press ASP site, the Journal archive, and the Newsletter/Newsletter Archive. In talking to members, they use the site primarily to get information about joining the Society or renewing their existing membership, Journal format, the ASP Newsletter, and to try to get a membership list. We should consider having an annual membership list in PDF format that our members can access and download. (This raises minorbot issues, but there are ways to deal with that in the implementation.)

Organizational things to do with the Journal should be handled by Allen Press as is apparently the case now, whereas everything to do with ASP should ideally exist under a single aegis, i.e., one web site. The Committee suggests that meeting registration should be incorporated into our web site. This is easy for a company to implement, but often difficult for an individual. Commercial oversight will also deal with cleanup and archive issues surrounding an independent Annual Meeting site.

The web site will require updates 2-4 times each year, primarily to deal with issues surrounding the Newsletter and Annual Meeting. These are readily available through commercial web maintenance companies. The main site is currently hosted out of the University of Nebraska–Lincoln. We may be able to continue using that host site but are investigating the costs of paying for host service as an alternative. Passwords and logging in have been perennial issues and should be addressed. This is best left to a professional company.

The Committee has requested an estimate from a commercial developer called Academic Web Pages (http://www.academicwebpages.com), and we expect to have a proposal and estimate for Council to consider in Anchorage.

### F. Local Arrangements Committee for the 87th Annual Meeting

Submitted by Ghislaine Mayer (Local Organizing Committee Chair for 2012 meeting).

The site for the 2012 meeting will be the Omni Richmond Hotel located near the fashionable Shockoe Bottom of downtown Richmond and a few blocks away from the Virginia State Capitol designed by Thomas Jefferson and Charles-Louis Clerisseau in 1785. It offers 3 dining facilities, a heated indoor pool and outdoor sundeck, children’s program, and complimentary shuttle service to downtown Richmond within a 5-mile radius.

For your travel convenience, Richmond is served by 7 domestic airlines and 1 international airline. The weather in Richmond for the month of June is pleasant, with warm days and cool evenings. Richmond is located approximately 50 miles away from colonial Williamsburg and Jamestown to the east and 60 miles away from Charlottesville to the west. Virginia boasts over 190 wineries, many within 70 miles of Richmond.

Richmond offers a diversity of fun family activities, all within 10 miles of the Omni Hotel. Visit the Maymont Foundation, St. John’s Church where Patrick Henry made his famous “Give me liberty or give me death” speech, the Edgar Allan Poe Museum, which features many of his writings and other objects of his life, the Hollywood Cemetery, which is the burial grounds of 2 U.S. Presidents as well as many Civil War officers and soldiers, take a wine tour, see historic monuments along Monument Avenue, shop in Carytown,
stroll through Shockoe Bottom, visit Bell Isle island in the James River, or visit the Virginia Museum of Fine Arts, home of the largest collection of Fabergé Easter eggs outside of Russia.

As usual, the scientific program will be diverse, rich, and stimulating. Plan to attend the 2012 meetings in Richmond. Bring your family and plan to stay a few days after the meeting. The hotel rates are great and can be extended beyond the conference.

V. REPORTS OF AFFILIATE SOCIETIES

A. Annual Midwestern Conference of Parasitologists

Submitted by Douglas Woodmansee, Secretary-Treasurer, AMCOP. The 63rd Annual Midwestern Conference of Parasitologists will meet 23–25 June 2011 at St. Mary’s College, Notre Dame, Indiana.

B. Canadian Society of Zoologists, Parasitology Section

Report submitted by Todd Smith, Chair, Parasitology Section of the Canadian Society of Zoologists.

Executives for 2010–2011:

Chair: Dr. Todd Smith, Acadia University (todd.smith@acadiau.ca)
Chair-Elect (Vice Chair): Dr. Lucy Lee, Wilfrid Laurier University (llee@wlu.ca)
Past Chair: Dr. Michael Duffy, University of New Brunswick (mduffy@unb.ca)

CSZ, 2011 at University of Ottawa: The Parasitology Section symposium, organized and hosted by our Vice Chair, Lucy Lee, will start at 0830 hr on Thursday, 19 May. The title of the symposium is “Comparative Aquatic Parasitology and Immunology” and features Brian Dixon (University of Waterloo), John Lumsden (University of Guelph), and Spencer Greenwood (University of Prince Edward Island).

This year’s winner of the Robert Arnold Wardle Medal is J. Daniel (Dan) McLaughlin, Professor Emeritus at Concordia University. On Thursday, 19 May at 1630 hr, following an introduction by Michael (Mick) Burt, Dan will be presenting the Wardle Lecture, entitled “Digeneans, Diversity and DNA.” Congratulations, Dan!

There are 2 sessions of oral presentations, both on Tuesday, 17 May. The first session, starting at 1100 hr, chaired by Lena Measures (Maurice Lamontagne Institute), is entitled “Protozoa, Ecology and Control” and features 6 papers. The second session, starting at 1400 hr, chaired by Michael Duffy (University of New Brunswick), is entitled “Microsporidia, Trematodes and Nematodes” and features 7 papers.

Of the 13 papers, fully 10 will be given by students who are in the running for the Murray Falls Prize for the best oral presentation in parasitology. We have retained the services of 3 judges for the competition; these individuals will remain anonymous so as not to stress the students!

Membership status: The current membership in the Section stands at 69. This breaks down to 30 who are members of PAR only, and 39 who are members of PAR and 1, or more, other sections of CSZ. This also breaks down to 25 regular members, 6 post-doctoral fellows, 26 students, and 12 honorary, emeritus, or associate members. At the Section AGM on Thursday, 19 May, we will be having a candid discussion about the state of the Section. In particular, we will be discussing how to increase membership in the Section, in particular how to attract former members who have left CSZ and become members of regional parasitology groups or other Societies such as the CSEE back to parasitology.

C. Helminthological Society of Washington


The Helminthological Society of Washington celebrated our Centennial Year with our 70th meeting, held at The George Washington University on 8–9 October 2010. For this momentous event, the Society combined the Anniversary Dinner and our annual Spring Meeting into a single gala event. The Anniversary Dinner was held at Circle Bistro in Washington, D.C., on Friday evening, 8 October. Following a buffet dinner, the keynote speaker, Dr. John Janovy, presented an engaging talk entitled “The Field Station: A Parasitological Incubator.” Dr. Janovy was also awarded the 2010 Anniversary Award. Dr. Janovy is a world-renowned parasitologist and educator from the University of Nebraska–Lincoln and a longtime member of the Society. We congratulate Dr. Janovy on receiving this prestigious award from the Society for his long service to the field of parasitology.

The Plenary Session and Student Paper Presentation were held Saturday morning, 9 October. Winner of the first Stirewalt-Lincicome Student Award was Matthew Gardner of George Washington University (student of John M. Hawdon) for his paper entitled “Cloning of the Helix-Loop-Helix 13 (HLH-13) Transcription Factor from the Hookworm Ancylostoma caninum.” Second place went to Camille Dowling (student of John M. Hawdon) for her talk entitled “Starvation-Induced Developmental Arrest of Ancylostoma caninum in the First Larval Stage,” and Honorable Mention went to Kelly Fitzsimmons of Hamilton College (student of Ashleigh B. Smythe) for the paper entitled “Patterns of Lecith Parasitism on Freshwater Turtles in Three Upstate New York Counties.” In addition to cash awards, the students received a free membership to the Society. We thank them for their participation, and congratulate them on winning the Stirewalt-Lincicome Student Awards.

The General Scientific Session followed immediately and consisted of the following papers:

“Regulation of Heat Shock Response Factors in Infectious L3 Larvae of Ancylostoma caninum” by Verena Gelmedin (presenting), and John M. Hawdon, Department of Microbiology, Immunology and Tropical Medicine, The George Washington University Medical Center, Washington, D.C.;

“Identification of Hookworm DAF-16/FOXO Response Elements and Direct Gene Targets” by Xin Gao (presenting), Zhengyu Wang2, John Martin3, Sahar Abubucker3, Xu Zhang2, Makedonka Mitreva4, and John Hawdon2, The George Washington University, Washington, D.C., and “Washington University School of Medicine, St. Louis, Missouri;

“Some Observations on Leptothyphloides thecatus (Acanthocephala)” by Dennis J. Richardson5 (presenting), Kristen E. Richardson6, and Richard E. Clopton7, Quinnipiac University, Hamden, Connecticut, and “Peru State College, Peru, Nebraska;

“Helmint Parasites of Muskrats from Central New York, USA” by Ashleigh B. Smythe (presenting), Calvin Johnson, and Alyssa Kanagaki, Department of Biology, Hamilton College, Clinton, New York;

“Ultrastuctural and Histopathological Studies of the Digenetic Trematode Siphodera ghanseni (Cryptogonimidae), Parasite of Chrysichthys nigrodistatus (Lacepede, 1802) from Lekki Lagoon, Lagos, Nigeria” by Akinsanya Bamidele (presenting) and A. A. Hassan, University of Lagos, Lagos, Nigeria;

“Insect Immune Responses to Entomopathogenic Nematodes and their Symbiotic Bacteria” by Ioannis Eleftherianos, Department of Biological Sciences, The George Washington University, Washington, D.C.;

“Hidden in a Shell: Parasite Biodiversity in Turtles” by S. D. Snyder1 (presenting) and Vasily V. Tkach2, 1Department of Biology, University of Nebraska at Omaha, Omaha, Nebraska, and 2Department of Biology, University of North Dakota, Grand Forks, North Dakota.

A lunch and social hour followed the presentations.

Current officers of the Society are Allen L. Richards (President), William E. Moser (Vice President), John M. Hawdon (Immediate Past-President), Tami Cook (Editor, Comparative Parasitology), Richard Clopton and Dennis Richardson (Associate Editors, Comparative Parasitology), Sherman Hendrix (Corresponding Secretary-Treasurer), Ashleigh Smythe (Recording Secretary), and Eric P. Hoberg, Scott Snyder, Ellen Andersen, and Ronald Neafie (Executive Committee Members-at-Large). We are currently gathering nominations for a slate of officers for 2011–2012.

Finally, we are deeply saddened to announce that Helminthological Society of Washington members Dr. Jeffrey Bier, Dr. David Richard Lincome, Dr. John H. Cross, and Dr. John F. A. Sprent passed away this year.
D. New England Association of Parasitologists

Submitted by Jon Vermeire, Secretary, NEAP.

The 18th meeting of the New England Association of Parasitologists was hosted by NEAP members from the University of Massachusetts–Amherst on 13 November 2010. Welcoming remarks were given to the attendees by NEAP President Dr. Lisa Ganley-Leal. The scientific program included keynote presentations by Dr. Dyann Wirth from the Harvard School of Public Health entitled “From Genes to Genomes: Genetic Diversity in Malaria, Implications for Biology and Pathogenesis” and Dr. George Cross from the Rockefeller University entitled “New Ways of Thinking about VSG Switching.” Other members of NEAP also made compelling 15-min presentations on their current research, and a poster session was held between sessions. Association President Dr. Lisa Ganley-Leal from the Boston University School of Medicine moderated the NEAP business meeting. Dr. Eugenia Zavras, NEAP Treasurer, addressed the assembled membership to report on NEAP financial business. During the business meeting Dr. Jon J. Vermeire (Yale University) was nominated and unanimously elected as NEAP President-Elect. Almost simultaneously, Dr. Marc-Jan Gubbels (Boston College) was nominated and elected President-Elect-Elect. This meeting marked our first student oral and poster presentation contest. The winners for oral presentations were Brooke Anderson-White (First Place, Boston College) and Daria Van Tyne (Runner-up, Harvard School of Public Health). The winners for poster presentations were Tiffany Sun (First Place, Yale University) and Jessica Barowski (Salve Regina). We would like to thank our sponsors, IDEXX Laboratories, Krakeler Scientific, Inc., A&G Applied Biosystems, Agilent Technologies, Boston University, Epsilon Therapeutics, New England Biolabs, and Promega, as well as our other hosts and the UMass Amherst NEAP members who made this meeting possible. The 2011 NEAP meeting will be hosted by NEAP members at Salve Regina University in Newport, Rhode Island, on 19 November 2011. For further information please contact NEAP Secretary Dr. Jon J. Vermeire, Yale School of Medicine, 464 Congress Avenue, New Haven, Connecticut 06510, or e-mail jon.vermeire@yale.edu, or visit our web site, http://neap.yale.edu.

E. Northern California Parasitologists

Submitted by Ravinder Sehgal, Ph.D., President of Northern California Parasitologists.

The winter Northern California Parasitologists (NCP) meeting took place on 18 January 2011 at UC Berkeley, Dan Salkeld, Ph.D., from the California Department of Public Health was the keynote speaker. The title of his presentation was “Of Squirrels, Mice and Men: Biodiversity and Infectious Diseases.”

The NCP met for their annual Spring Meetin 12 March 2011 at the Romberg Tiburon Center for Environmental Studies. This venue is much less expensive than the Marconi Center where we had previous meetings. This resulted in higher attendance. Approximately 35 members, students, and guests participated in the one-day Spring Meetin.

At the Spring Meetin, Professor Armand Kuris, from UC Santa Barbara, President-Elect of ASP, provided an inspirational plenary talk entitled “Parasites Approach the Darwinian Demon: Adaptations, Evolutionary Wormholes and Their Ecological Visibility.”

At the Spring Meetin, 7 presentations were given by graduate students. Subject areas included topics on Cryptosporidium, avian blood parasites, acanthocephalans, and trematodes. Ravinder Sehgal also introduced the meeting with research highlights in parasitology from the past year.

The winner of the Walter Carr Award was Criseyda Martinez from San Francisco State University for her presentation “Identification of MAEBL, an Erythrocyte Binding Protein, in Plasmodium gallinaceum.” Criseyda received an award of $100. The award qualified her for the Marc Marquardt Award for Best Paper Presentation by a Graduate Student—Sarah A. Orlofske, University of Colorado; William C. Marquardt Award for Best Paper Presentation by an Undergraduate Student—Nicole Searcey, University of Nebraska–Lincoln; William C. Marquardt Award for Best Poster Presentation by an Undergraduate Student—Emily Rees, Metropolitan State College of Denver.

The 2011 Annual Meeting of the Rocky Mountain Conference of Parasitologists will be held again at the University of Nebraska, Cedar Point Biological Station, on 8–10 September.

F. Rocky Mountain Conference of Parasitologists

Submitted by Ron Hathaway, Secretary-Treasurer, RMCP.

The 41st Annual Meeting of the Rocky Mountain Conference of Parasitologists was held 9–11 September 2010 at the University of Nebraska, Cedar Point Biological Station, located on the south shore of Lake Ogallala in beautiful western Nebraska.

Following the reception on Thursday evening, paper and poster sessions were held all day Friday and Saturday morning. A total of 35 people attended the meeting, and 12 papers and posters were presented. Besides the scenery and excellent paper presentations, highlights of the meeting included an invited paper by Scott L. Gardner, University of Nebraska–Lincoln; the fourth Newton Kingston Memorial Lecture presented by Eric S. Loker, University of New Mexico; and the Gerald D. Schmidt Memorial Lecture given by Daniel R. Brooks, University of Toronto.

Following the Saturday morning paper session, President Rick McKown called the annual business meeting to order. The membership unanimously elected Scott L. Gardner to the office of President-Elect. At the end of the business meeting, the Marquardt gavel was passed to President Bill Granath, University of Montana.

Student awards of $100 and an engraved plaque were given as follows: Datus M. Hammond Award for Best Paper Presentation by a Graduate Student—Sarah A. Orlofske, University of Colorado; William C. Marquardt Award for Best Paper Presentation by an Undergraduate Student—Nicole Searcey, University of Nebraska–Lincoln; William C. Marquardt Award for Best Poster Presentation by an Undergraduate Student—Emily Rees, Metropolitan State College of Denver.

The 2011 Annual Meeting of the Rocky Mountain Conference of Parasitologists will be held again at the University of Nebraska, Cedar Point Biological Station, on 8–10 September.

G. Southeastern Society of Parasitologists

Submitted by Vince Connors, Secretary-Treasurer, SSP.

The Southeastern Society of Parasitologists (SSP) held its Annual Meeting at the Unicoi Lodge, Unicoi State Park, Helen, Georgia, 6–8 April 2011. The meeting was sponsored in part by the College of Arts and Sciences, University of South Carolina, Upstate. Dr. Michael Yabsley, ably assisted by a cadre of energetic graduate students, was in charge of local arrangements. Dr. Isaure de Buron, President-Elect, served as Chair, and Dr. Alexa Rosypal, Secretary-Treasurer Dr. Vince Connors. Dr. Derek Zelmer recruited judges and presided over the annual Byrd-Dunn Student Paper Competition. Approximately 50 people attended the meeting. Of the 41 presentations, 27 were by students, of which 22 competed in the Byrd-Dunn Student Paper Competition. The Byrd-Dunn Award for the best student paper presented at the Annual Meeting was presented to Carrie Umberger, College of Charleston, Department of Biology, for her paper entitled “Effects of the Parasitic Nematode, Philometroides paralichthydis, on the Swimming and Burying Performance of the Southern Flounder, Paralichthys lethostigmus.”

The Presidential Symposium on 6 April, entitled “Parasites: From Pathogens to the Bizarre to Good Fellows” and organized by Dr. Isaure de Buron, included 3 presentations: “Parasites as Pathogens”—Dr. Cheryl Davis, Western Kentucky University; “Parasites and Behavior: The Good, the Bad, and the Bizarre”—Dr. Janice Moore, Colorado State University; and “Parasites Approach the Darwinian Demon, Adaptations, Evolutionary Wormholes, and Metabolic Scaling”—Dr. Armand Kuris, University of California, Berkeley. The symposium was co-sponsored with the Presidential Symposium and the following reception and social at the lakeside “Beach House” on the shores of beautiful Unicoi (Smith) Lake were well attended and enjoyed by all. After a day of excellent student papers on the 7th, Dr. Kuris updated the Society on the events and business of the American Society of Parasitologists in its role as ASP President-Elect, after which the members and guests enjoyed a BBQ dinner replete with liquid refreshments in the cool and delightful Georgia mountain air.
On the 8th the members enjoyed a full morning of papers, followed by a Society-hosted lunch and business meeting. The Society welcomed several new full or student members. Officers of the Society for 2011–2012 are President—Dr. Isare de Buron, College of Charleston; President-Elect and Program Officer—Dr. Chris Hall, Berry College; Vice President—Dr. Andrea Varela-Stokes, Mississippi State University; Secretary-Treasurer (continuing)—Dr. Vince Connors, University of South Carolina Upstate; and ASP Council Representative (continuing)—Dr. Bruce Conn (Berry College).

In 2012 SSP will meet in Cumberland Gap, Tennessee, 21–23 March. Dr. Vina Diderrich, Lincoln Memorial University, and Dr. Charles Faulkner, University of Tennessee, will co-chair the local arrangements committee. Persons interested in submitting abstracts or obtaining other information about the meeting should contact either Dr. Hall (chall@berry.edu), Dr. de Buron (deburon@cofc.edu), or Dr. Varela-Stokes (stokes@cvm.msstate.edu).

H. Southwestern Association of Parasitologists

Submitted by Jerry L. Cook, Secretary-Treasurer, SWAP.

The 44th Annual Meeting of SWAP was held 16–18 April at the University of Oklahoma Biological Station. President Matt Bolek presided over the Annual Meeting, which had 74 participants, 26 oral presentations (22 of which were given by student members), and 12 poster presentations. Student award winners at the 2010 meeting were Joanna Cielocho, University of Kansas (David A. Becker Memorial Award), Kate Trout, Peru State College (Marc H. Dresden Memorial Award), Victoria Polito, Oklahoma State University (Honorable Mention Graduate Student), and Nicole Searcy, University of Nebraska (Honorable Mention Undergraduate Student). Student research awards were given to Heather Robinson and Crystal Wiles, both from Oklahoma State University. Officers for the next year will be Alan Fedynich (President), Charles Blend (President-Elect), Matt Bolek (Past-President), Rich Clpton (ASP Representative), and Debra Clpton (Secretary-Treasurer). SWAP membership includes 92 regular members from 13 states.

I. Southern California Society of Parasitologists

No report as of publication date.

VII. REPORTS OF ASP REPRESENTATIVES

A. American Association for Zoological Nomenclature

Please see the Association web site (http://www.nhm.ac.uk/hosted_sites/iczn/AAZN.htm) for recent activities.

B. American Institute of Biological Sciences

Submitted by Scott Seville, ASP Education Committee.

ASP does not currently have a representative to the American Institute for Biological Sciences (AIBS). However, ASP has been directly and indirectly involved in the continuing efforts by AIBS, NSF, and AAAS to examine and reform current practice in higher education biology education.

ASP (Lee Couch and Scott Seville) participated in the AIBS-NSF Conversation on Undergraduate Biology and the AIBS Education Summit in Washington, D.C. (2008). Scott Seville served on the advisory board for the NSF-AAAS-sponsored conference “Vision and Change in Undergraduate Biology Education: A View for the 21st Century Initiative” (2009), and both he and Dennis Minchella attended the meeting. Results of the conference are available on the web (http://visionandchange.org/). The final report “Vision and Change in Undergraduate Education: A Call to Action” is available at the web site and in hardcopy. Please visit the web site or talk with Dennis or Scott for additional information.

C. Council of Scientific Society Presidents

Submitted by David Bruce Conn, ASP representative to CSSP.

For several years, ASP has been an active participant in the Council of Scientific Society Presidents (CSSP), which is a council of current and former presidents of international and national scientific societies throughout the United States, which include all of the physical and life sciences, engineering, and science education organizations. Together the council represents more than 1.4 million member scientists and engineers across the country, from the academic, corporate, and government sectors.

As Past President and the Delegate from ASP, designated by Siting President Janine Caira, I attended the Semiannual Meeting of CSSP, which convened at the American Chemical Society Building in Washington, D.C., from 3–6 December 2010. The council was attended by presidents from other member societies, as well as invited guests from the U.S. Government and various tech corporations.

CSSP holds regular council meetings in Washington, D.C., in May and December each year. The purpose is to meet with congressional and executive branch leaders in Washington who have substantial influence regarding the scientific and technological interests of the United States, and to help them connect with corporate and academic leaders in science. Some highlights of this year’s Council included a scheduled meetings with Bart Gordon (D-Tennessee), Chairman of the House Science and Technology Committee, who has been among the strongest supporters of science on the Hill for many years. Also, David Hayes, Deputy Secretary of the U.S. Department of the Interior, met with us and discussed the work of DOI related to several areas of science, including DOI’s role in surveillance of disease among wildlife and migratory birds. Martha J. Kanter, Under Secretary of U.S. Department of Education, gave a perspective of needed reforms in U.S. science/math education. Steven E. Koonin, Under Secretary for Science, U.S. Department of Energy, discussed mostly green alternatives and future energy needs. Cathie E. Woteki, Under Secretary for Research, Education, and Economics, U.S. Department of Agriculture, discussed food safety and security for the future, which touched upon several parasite contaminants.

Much of the Council meeting was devoted to break-out committee meetings with defined action agendas. As in previous years, I served on the International Science Committee. Our primary actions involved drafting recommendations to the White House Office of Science and Technology Policy (OSTP) and other federal agencies regarding technological export controls, measures to ensure access to rare earth elements through bilateral engagement, and issues regarding visas and immigration status of foreign scientists. The latter point is especially important to parasitologists, as the subjects of our research cross borders, frequently with important international implications for human and animal health.

In my current role at the State Department, I agreed to reach out to the Bureau of Consular Affairs and my own Bureau of Oceans, Environment, and International Science, to discuss the issues of expedited short-term visas for foreign scientists attending scientific conferences in the United States, and facilitated applications for green cards for foreign scientists completing Ph.D. programs at U.S. universities. These issues are considered important to maintain U.S. competitiveness in science and technology. Again, this is a very important issue for parasitologists who collaborate internationally.

My recommendation is to continue ASP’s involvement with CSSP, as this group does combine our voice with those of other scientific Societies and does get attention of many elements of the U.S. Federal Government. Also, our participation elevates the awareness of parasitology as a discipline with much to offer in the arenas of medicine, public health, agriculture, wildlife, fisheries, and environmental health. As I reported last year, all of these areas of keen interest to the Obama Administration. Furthermore, as the United States’ Global Health Initiative now includes major commitment to malaria and neglected tropical diseases, the need for more input from parasitologists is widely recognized and will continue beyond the current administration.

D. National Council for Science and the Environment

Please see the Alliance web site (http://ncseonline.org) for recent activities.

E. American Association of Veterinary Parasitologists

Submitted by Dr. Bob Arther, Secretary-Treasurer, AAVP.

The 55th annual AAVP meeting was held 31 July–2 August 2010 at the Loews Hotel, Atlanta, Georgia. The theme for the meeting was “Parasite Evolution—Veterinary Parasites and Their Strategies to Survive Human Intervention.” The AAVP Distinguished Veterinary Parasitologist Award for contributions leading to significant advancements in the field of veterinary parasitology was presented to Dr. George A. Conder, Pfizer Animal Health-Retired, Kalamazoo, Michigan. The AAVP-Intervet/
Schering-Plough Outstanding Graduate Student Award was presented to Stephanie Heise from Oklahoma State University, Stillwater, Oklahoma. The AAVP-CAPC Graduate Student Research Award in Zoonotic Disease was presented to Sriveny Dangoudoubiyam from Purdue University. Best student presentations sponsored by Bayer Animal Health were presented to Lindsay Starkey from Oklahoma State University for First Place, and to Richard Gerhold from the University of Georgia for Second Place. Fourteen AAVP-sponsored Young Investigator Travel Grants were awarded to students and post-docs to help pay a portion of their travel expenses to the meeting. Outgoing AAVP President Dr. Lora Ballweber was presented a plaque in recognition of her exceptional service to AAVP. Outgoing Secretary/Treasurer Dr. Alan Marchiondo was presented a plaque and recognized for 6 yr of exceptional service to AAVP.

Newly Elected or Installed Officers: The AAVP officers for 2010–2011 are as follows:

President: Dr. Karen Snowden, Texas A&M University, College Station, Texas
Past-President: Dr. Lora R. Ballweber, Colorado State University, Fort Collins, Colorado
President-Elect and 2011 Program Chair: Dr. Patrick F. M. Meeus, Pfizer Animal Health, Kalamazoo, Michigan
Secretary/Treasurer: Dr. Bob Arther, Bayer Animal Health, Shawnee, Kansas
Vice President: Dr. Alan A. Marchiondo, Pfizer Animal Health, Kalamazoo, Michigan

The 56th Annual Meeting of AAVP will be held at the Sheraton Westport Chalet Hotel, St. Louis, Missouri, 16–19 July 2011, in conjunction with the 55th Livestock Insect Workers’ Conference (LIWC), and the 11th International Symposium on Ectoparasites of Pets (ISEP). The theme for the 2011 meeting is “Sustainable Control of Parasites and Vectors.” Individuals with questions about AAVP should contact the Secretary-Treasurer (bob.arther@bayer.com) or visit the AAVP web site (www.aavp.org).

F. Clinical and Laboratory Standards Institute

Please see the Institute web site (http://www.clsi.org/) for recent activities.

G. American Board of Medical Microbiology

Please see the Board web site (http://www.asm.org/Academy) for recent activities.

H. The Natural Science Collections Alliance

No report as of publication date.

I. American Type Culture Collections

Please see the ATCC web site (http://www.atcc.org/) for recent activities.
I. CALL TO ORDER

The business meeting was called to order by President Janine Caira at 2:48 PM on Saturday, 4 June 2011 in the Howard Rock Ballroom of the Sheraton Anchorage Hotel and Spa. President Caira made her opening remarks and recognized the new Officers and Nominating Committee members for 2011-2012. She then thanked the outgoing Officers and Nominating Committee for their service to the Society. See Council meeting minutes for lists of these individuals. President Caira also thanked the organizations that donated funds for the meeting; see the Council meeting minutes for the list of sponsors.

II. CLARK P. READ MENTOR AWARD

Julian Hillyer introduced Bruce M. Christensen, University of Wisconsin-Madison, as the 2011 recipient of the Clark P. Read Mentor Award. Dr. Christensen accepted the award and delivered his acceptance speech entitled "Mentoring the Mentor."

III. ASHTON CUCKLER NEW INVESTIGATOR AWARD

Julian Hillyer also introduced Chelsea Matisz, University of Calgary, as the 2011 winner of the Ashton Cuckler New Investigator Award. Ms. Matisz accepted the award and thanked the Society.

IV. OTHER AWARDS ANNOUNCEMENTS

Scott Seville announced the recipients of the Willis A. Reid, Jr. Student Research Grant Awards: Kayce Bell, University of New Mexico (graduate) and Emily Kasl, Texas A&M University (graduate). Kym Jacobson then announced the winners of the Student Paper Awards and Marc Dresden Student Travel Grants; Kristin Herrmann, University of Otago, and Anthony Stumbo, University of Lethbridge, received the Outstanding Student Paper awards and Kayce Bell, University of New Mexico, and James Losee, Oregon State University/National Oceanic and Atmospheric Administration (NOAA), received the Meritorious Student Paper awards. Kym Jacobson then recognized the Student Travel Grant recipients (see Council minutes for a list of students receiving travel grants).

V. SOCIETY BUSINESS

President Caira announced that Council had approved the Council minutes from 2010 and voted to accept new members since the last meeting. She then called for the membership to vote on the new members. A motion was made to accept the new members since the last meeting, the motion was seconded, and it passed unanimously. President Caira also informed the membership of significant actions taken by Council during the 2011 meeting:

- voted to provide the option for regular members to opt out of receiving print copies of the Journal in 2012;
- voted to have the Society website hosted commercially and undergo a change in website address;
- voted to negotiate a contract including basic site redesign and maintenance for the ASP website with Academic Web Pages;
- voted to produce and disperse a printed membership directory in the coming year;
- voted to provide reimbursement to current student representatives for annual meeting travel and accommodations; and
- voted to ensure that future annual meeting sites have an on-site Local Organizing Committee in place.

President Caira then reviewed the 2011 award recipients including Eminent Parasitologist Lecturer Robert L. Rausch, University of Washington School of Medicine, Clark P. Read Mentor Award recipient Bruce M. Christensen, and Ashton Cuckler New Investigator Award winner Chelsea Matisz. She also recognized the winners of the Student Paper Awards and Student Travel Grants.

VI. SECRETARY-TREASURER REPORT

Dennis Minchella presented the Secretary-Treasurer’s report including membership and subscription data, cash flow data, and performance of investment accounts. Secretary-Treasurer Minchella indicated that membership and subscriptions continue to decline, but mentioned the high quality of the Society members, echoing the sentiment expressed by President Caira during her Presidential Address. He then indicated that the Society’s financial condition is stabilizing and encouraged the Society to work together to keep the Society moving forward as it pursues new ideas and initiatives. Secretary-Treasurer Minchella also made special mention of Alice Foster and her many contributions to the Society.

VII. THE JOURNAL OF PARASITOLOGY EDITOR

In Gerald Esch’s absence, President Caira recognized the incoming Editor of the Journal, Michael Sukhdeo, Rutgers University, effective in the summer of 2012. She expressed her gratitude for Michael Sukhdeo’s willingness to accept the position of Editor. President Caira also invited members to review Gerald Esch’s report in the upcoming December issue of the Journal.

VIII. SCIENTIFIC PROGRAM OFFICER

Herman Eure gave a report of the events and successes of the 2010 meeting in Colorado Springs, Colorado. He expressed his appreciation to all who helped make the meeting possible, including Local Organizing Committee Chair Ron Hathaway.

IX. IN MEMORIUM

The committee received notice of the death of the following individuals: Dr. Edward Ogden “Ted” Hooghkirk, Dr. John Frederick Adrian Sprent, Dr. Steve J. Upton, Dr. John H. Cross, Dr. David Richard Lincicome, and Dr. Jeffrey Bier. A moment of silence was held in memory of these individuals.

X. OLD BUSINESS

President Caira opened the floor for old business items. There were no old business items in need of discussion.
XI. NEW BUSINESS

President Caira then called for new business items. In response to the positive reception of the 43 parasite haikus created at the Society outing the previous night, President Caira stated that the haikus should be placed on the ASP website for all members to enjoy. A colleague from the Mexican Society of Parasitology then announced that the 2014 International Conference of Parasitologists will be held in Mexico City, and she invited ASP members to attend the meeting.

XII. RESOLUTIONS

Susan Perkins, John Hawdon, and Derek Zelmer delivered an entertaining and clever list of "whereases," including accolades for the individuals who helped plan and set up the meeting, especially Herman Eure and Kelli Sapp; the individuals who helped make the student auction a success; and the presenters, speakers, and attendees; all of whom made the 86th meeting an "unequivocal, unwavering, invariant, unambiguous success.

XIII. CLOSING REMARKS

In her closing remarks, President Caira thanked the Society for giving her the opportunity to serve as President. She then passed the ASP gavel to incoming President Armand Kuris, who presented President Caira with a plaque in recognition of her service. After a few remarks, incoming President Kuris entertained a motion to adjourn. Such a motion was made, seconded, and passed unanimously. The meeting adjourned at 3:24 PM.
HOST SIZE- AND HABITAT-DEPENDENT INTENSITY OF HELICONEMA LONGISSIMUM (NEMATODA: PHYSALOPTERIDAE) IN THE JAPANESE EEL (ANGUILLA JAPONICA)

Hirotaka Katabira, Kouki Mizuno*, and Kazuya Nagasawa

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ABSTRACT
Heliconema longissimum (Ortlepp, 1923) is an ecologically poorly known nematode found in the stomach of anguilliform and synbranchiform fishes in India and East Asian countries (e.g., Moravec et al., 2007). In Japan, the nematode is known to parasitize the Japanese eel Anguilla japonica Temminck and Schlegel (see Nagasawa et al., 2007), but the host–parasite relationship is poorly understood.

Japanese eels are known as a catadromous fish (McDowall, 1988), which migrate to an oceanic breeding area west of the Mariana Islands (Tsukamoto, 1992), after spending a significant part of their lifetime in freshwater. However, a recent chemical analysis of the strontium and calcium concentration in otoliths has demonstrated that some individuals are catadromous while others primarily reside in coastal marine or brackish-water habitats (Tsukamoto et al., 1998; Tsukamoto and Arai, 2001).

Tsukamoto and Arai (2001) reported that non-catadromous eels, living in coastal marine and brackish-water habitats, are present in Misho (as Mishou) Cove in Ehime Prefecture, western Japan. In and around this cove, we have also found that eels are heavily infected with H. longissimum. In the present paper, we examined the occurrence of H. longissimum and then used a regression analysis to assess the relationship between the nematode intensity and eel size and sampling location. We then discuss the feeding habits of Japanese eels in the study area in relation to the parasite’s intensity of infection.

MATERIALS AND METHODS

Study area

The study area was conducted in the brackish-water area of both Misho Cove (32°57’N, 132°33’E) and the lower reaches of the Renjoji River that flows into the cove, in Ehime Prefecture, western Japan. The bottom of the cove consists of a mud, sand, and gravel substratum. Water depth and bottom salinity of the cove fluctuated between 30–190 cm and 3.6–30.7% at low tide, respectively. Bottom water temperature varied from 11.6 to 31.3 °C during April 2008 to March 2009. The lower Renjoji River is tidal, with salinity varying between 0 and 1.8% at low tide. This area has a gravel substratum, and bottom water temperature was 14.2–24.5 °C during the study period.

Sampling and dissection

Eels were caught with covert traps and eel pots at low tide from April 2008 to March 2009. The eels were kept alive in oxygenated containers and brought to the laboratory of Hiroshima University, where they were measured for total length (mm), then killed within several days and necropsied; eel stomachs and other organs were examined using a stereomicroscope.

Nematodes were collected from the host’s stomach, washed in physiological saline, and fixed in 70% ethanol. Identification was based on the description of Moravec et al. (2007). Prevalence and mean intensity were calculated for each sampling. Prevalence, intensity, and mean intensity are used in accordance with the definitions of Bush et al. (1997).

Model selection

To detect which variables affect the nematode intensity in Japanese eels, generalized additive models (GAM; Hastie and Tibshirani, 1990) were constructed. GAM is a semi-parametric extension of generalized linear model with a linear predictor involving a sum of smooth functions of covariates (Wood, 2006). Since this method provides a robust and straightforward analytical result (Swartzman et al., 1992; Venables and Dichmont, 2004), its application has been increased in fisheries science to understand and predict stock abundance or distribution associated with geographical and environmental variables (see Venables and Dichmont, 2004). Using this approach, Agnew et al. (2003) demonstrated an association between the 7-yr periodicity of oceanographic fluctuation of sea-ice duration and sea-surface temperature with the occurrence of the myxosporean parasite Kudoa alliaria Schukman and Kovaljova, 1979, in southern blue whiting Micromesistius australis australis Norman, 1937, from the southeast Atlantic.

In our study, intensity was assumed to originate from a negative binomial error distribution, because most of the aggregation by macroparasites could be described by a negative binomial distribution in their host populations (Shaw et al., 1998). Prior to model building, correlations between any pair of variables were checked and excluded from the full model to prevent excessive co-linearity. The tested variables included the total length of host, sampling location, season, and their interactions. Total length of the host was regarded as a continuous variable, while sampling location and season were used as categorical data. The sampling location consisted of 2 categories, i.e., Misho Cove and the lower Renjoji River. Season was represented as 4 categories combining several samplings (April–June, July–September, October–December, February–March), since each sample size was small. The model selection was performed using the stepwise procedure based on Akaike information criterion (AIC; Burnham and Anderson, 2002).

The GAM analyses were performed using R 2.8.1 (R development core team 2008) and mgcv-package (version 1.5–5, simon.wood@r-project.org).

RESULTS

Eel data

In total, 86 A. japonica were collected from the study area during the sampling period (Table 1). Sixty-three eels were caught in the cove, and their total length ranged from 281 to 534 (mean ± SD = 383 ± 54.2) mm. The other 23 eels were caught in the lower
TABLE I. Seasonal occurrence of the parasitic nematode *Heliconema longissimum* in *Anguilla japonica* collected from 2 brackish-water habitats (Misho Cove and the lower reaches of the Renjoji River) in Ehime Prefecture, western Japan.

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>No. of eel examined</th>
<th>TL of eel (mm) mean ± SD (range)</th>
<th>No. of eel infected</th>
<th>Prevalence (%)</th>
<th>Total no. of worms found</th>
<th>Intensity min.–max. (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cove reaches</td>
<td>Lower reaches</td>
<td>Cove reaches</td>
<td>Lower reaches</td>
<td>Cove reaches</td>
<td>Lower reaches</td>
</tr>
<tr>
<td>2008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 Apr.</td>
<td>8</td>
<td>356 ± 62.4 (254–437)</td>
<td>6</td>
<td>75</td>
<td>168</td>
<td>1–87 (28.0 ± 33.0)</td>
</tr>
<tr>
<td>11 May</td>
<td>2</td>
<td>432 ± 94.0 (366–499)</td>
<td>1</td>
<td>50</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>25 May</td>
<td>6</td>
<td>384 ± 82.1 (321–534)</td>
<td>6</td>
<td>100</td>
<td>429</td>
<td>3–250 (71.5 ± 93.4)</td>
</tr>
<tr>
<td>22 Jun.</td>
<td>4</td>
<td>329 ± 74.3 (264–405)</td>
<td>4</td>
<td>100</td>
<td>44</td>
<td>2–36 (11.0 ± 16.7)</td>
</tr>
<tr>
<td>21 Jul.</td>
<td>7</td>
<td>370 ± 64.0 (285–482)</td>
<td>7</td>
<td>100</td>
<td>558</td>
<td>1–196 (76.9 ± 71.7)</td>
</tr>
<tr>
<td>4 Aug.</td>
<td>5</td>
<td>378 ± 70.4 (281–454)</td>
<td>5</td>
<td>100</td>
<td>349</td>
<td>2–219 (69.8 ± 87.3)</td>
</tr>
<tr>
<td>30, 31 Aug.</td>
<td>13</td>
<td>379 ± 62.7 (311–509)</td>
<td>12</td>
<td>92.3</td>
<td>983</td>
<td>9–318 (81.9 ± 86.8)</td>
</tr>
<tr>
<td>9 Sep.</td>
<td>9</td>
<td>378 ± 37.9 (341–462)</td>
<td>2</td>
<td>100</td>
<td>750</td>
<td>6–166 (83.3 ± 48.2)</td>
</tr>
<tr>
<td>4 Oct.</td>
<td>4</td>
<td>394 ± 77.1 (329–506)</td>
<td>4</td>
<td>100</td>
<td>392</td>
<td>9–244 (98.0 ± 101.8)</td>
</tr>
<tr>
<td>18 Oct.</td>
<td>9</td>
<td>386 ± 20.0 (341–415)</td>
<td>9</td>
<td>100</td>
<td>946</td>
<td>6–389 (105.1 ± 123.4)</td>
</tr>
<tr>
<td>15 Nov.</td>
<td>2</td>
<td>402 ± 48.1 (266–334)</td>
<td>2</td>
<td>100</td>
<td>13</td>
<td>1–12 (6.5 ± 7.8)</td>
</tr>
<tr>
<td>21, 22 Dec.</td>
<td>5</td>
<td>402 ± 54.8 (345–493)</td>
<td>5</td>
<td>100</td>
<td>396</td>
<td>1–236 (79.2 ± 94.4)</td>
</tr>
<tr>
<td>2009</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 Feb.</td>
<td>2</td>
<td>433 ± 27.6 (413–452)</td>
<td>2</td>
<td>100</td>
<td>82</td>
<td>17–65 (41.0 ± 33.9)</td>
</tr>
<tr>
<td>8 Mar.</td>
<td>3</td>
<td>361 ± 25.1 (346–390)</td>
<td>3</td>
<td>100</td>
<td>115</td>
<td>15–77 (38.3 ± 33.7)</td>
</tr>
<tr>
<td>Total</td>
<td>63</td>
<td>383 ± 54.2 (281–534)</td>
<td>62</td>
<td>99.2</td>
<td>5024</td>
<td>1–389 (80.3 ± 82.5)</td>
</tr>
</tbody>
</table>

*Table showing the seasonal occurrence of the parasitic nematode *Heliconema longissimum* in *Anguilla japonica* collected from 2 brackish-water habitats (Misho Cove and the lower reaches of the Renjoji River) in Ehime Prefecture, western Japan.*
The total length of the host had a nonlinear relationship with the nematode intensity (Fig. 2a). The partial effect on the intensity increased with host size and exhibited a convex curve. This effect also turned into plus when the total length exceeded about 350 mm. The partial effect on the nematode intensity in the lower-river eels was significantly lower than that in the cove eels ($P < 0.05$, Fig. 2b).

**DISCUSSION**

*Heliconema longissimum* showed high prevalence and intensity throughout the study period. This high level of infection implies that the study area was a suitable habitat for transmission of *H. longissimum* to eels. While we only dealt with the occurrence data in this study, the year-round presence of the nematode also suggests that decline and recruitment into the nematode population may be seasonally overlapping.

The eels collected from the cove were heavily infected with *H. longissimum*, compared with the eels from the lower reaches of the river. This suggests that the nematode infection occurs mainly in the cove, because most of the eels in the cove primarily reside there (Tsukamoto and Arai, 2001). Furthermore, the difference in infection level between 2 areas can be treated as an aspect of the nematode distribution, i.e., higher infection in the cove suggests that the nematode biomass is skewed toward the cove. Despite poor biological information, *H. longissimum* has previously been regarded as a brackish-water parasite (e.g., Nagasawa et al., 2007). The difference observed here supports this conventional assumption.

The GAM analysis detected a higher intensity in the larger eels. This tendency probably reflects an increase in the eels' consumption of the intermediate hosts, since the larger eels can be expected to consume much more food. However, many investigators have noted that infection levels of fish helminths vary with fish size (e.g., Skorping, 1980; Buchmann, 1995). *Anguilla japonica* spp. are known to change their diet from small-sized prey, including a mysid crustacean (*Neomysis* sp.), to fishes and benthic animals as the fishes increase in size. Therefore, the observed patterns in the nematode infection related to the body size of eels are possibly reflected by their ontogenetic changes in diet.

Additionally, some eels larger than 350 mm were infected with remarkably high numbers of *H. longissimum* (up to 389 parasites). These extreme intensities of infection probably indicate that the

**Prevalence and intensity**

*Heliconema longissimum* occurred in 82 (95.3%) of the 86 eels examined (Table I). A total of 5,419 *H. longissimum* was collected from the infected eels, of which 5,024 were collected from the cove eels and the remaining 395 from the lower-river eels. The nematode occurred throughout the sampling period, with nearly 100% prevalence in both areas. However, the overall mean intensity in the cove eels (mean ± SD = 80.3 ± 82.5) was significantly higher than that in the lower-river eels (mean ± SD = 22.0 ± 30.6) ($t = 2.98; P < 0.05$).

**Model selection**

Since any pair of variables represented low Pearson correlation (maximum; $r^2 = 0.35$, total length of host vs. sampling locations), all variables were used for model building. As a consequence of the model selection, the most supported model included the total length of the host and the sampling location as predictor variables (Table II); the model explained 23.6% of the variations in nematode intensity. Other variables (season and interactions of the 3 variables tested) were deleted from the most supported model.
The present study used several kinds of variables for the selection of GAM. The model employing 2 variables (body size of the larger eels in the study area).

intermediate host of H. longissimum is an important prey animal for the larger eels in the study area.

Since the life cycle of H. longissimum has yet to be determined, intermediate hosts are still uncertain. However, the white shrimp, Penaeus setiferus, has been reported as a probable intermediate host of Helicenema brooksi (Crites and Overstreet, 1991). Moreover, the Chilean crab, Cancer plebejus, is also considered to be an intermediate host of a nematode, Proleptus sp. (George-Nascimento et al., 1994), which belongs to the Proleptinae in which Helicenema spp. is also known to occur. It is thus highly possible that decapod crustaceans are the source of H. longissimum infection in Japanese eels. As far as we know, 17 decapods species occur in Misho Cove (Anonymous, 2005). To clarify the life cycle of H. longissimum, further research is needed.

The present study used several kinds of variables for the selection of GAM. The model employing 2 variables (body size of host and sampling location) was conclusively identified as the most supported. However, the combination of these 2 variables represented low precision (23.6%) for the H. longissimum intensity. Shaw et al. (1998) indicated that host effect (behavioral, physiological, or immunological) is 1 of the causes for the overdispersion in macroparasite populations. In the present case, such an effect may exert an influence on intensity as an additional factor. More specifically, individual differences in eel feeding, such as variations in diet and/or differences in infection level of H. longissimum in prey animals (= intermediate hosts), are possibly related to the dispersion.

The local migration of Japanese eels is also likely to have contributed to the observed low precision of the model. As discussed above, most of the eel coves stay in the cove (Tsukamoto and Araki, 2001), and H. longissimum may be acquired there. For these reasons, the eel coves are likely to comprise a large number of non-migratory individuals (heavily infected) and a small number of individuals migrating from the river (lightly infected). It is also possible that the lower-river eels may include infected-individuals migrating up from the cove, although we have no information on the local migratory pattern of these eels. To use H. longissimum as a biological indicator for the local migration of eels from the marine and/or estuarine to freshwater habitats, it is first desirable to identify migratory history of eels using the otolith microchemistry data and then to study the nematode occurrence corresponding to the migratory patterns.

**ACKNOWLEDGMENTS**

We are grateful to the local fisherman, Mr. Susumu Takamori, and his family for assistance in the field, and members of the Laboratory of Aquaculture, Department of Graduate School of Biosphere Science, Hiroshima University, for support and encouragement. Thanks also to Hokuto Shirakawa, Laboratory of Breeding Science, Division of Marine Biosciences, Graduate School of Fisheries Sciences, Hokkaido University, for useful advice on the statistical analysis.

**LITERATURE CITED**


PARASITE VOLUME AS AN INDICATOR OF COMPETITION: THE CASE OF ACANTHOCEPHALUS TUMESCENTS AND PSEUDOCORYNOSOMA SP. (ACANTHOCEPHALA) IN THEIR INTERMEDIATE HOST

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ABSTRACT: In Lake Mascardi (Patagonia), 2 acanthocephalan species, Acanthocephalus tumescens and Pseudocorynosoma sp., share an amphipod intermediate host but have different definitive hosts. Because both acanthocephalan species are potentially capable of manipulating amphipod behavior, one of the parasites may, therefore, have no opportunity to complete its life cycle; accordingly, negative interactions between them can be expected. The purpose of the present work was to examine the possibility of competition in the intermediate host through a comparison of A. tumescens and Pseudocorynosoma sp. cystacanth volume. Specimens of the amphipod Hyalella patagonica were collected monthly over almost 2 yr. Amphipods were measured (total length), necropsied, and their volume was calculated. Size of both acanthocepahan species was positively associated with amphipod total length. Competition, during 3 different infection periods, was assessed: high level of Pseudocorynosoma sp. infection (HP), high level of A. tumescens infection (HA), and high level of mixed infection (HM). In Pseudocorynosoma sp., intra-specific competition in HM was the only interaction found. In contrast, in A. tumescens, inter-specific competition in HP, intra-specific competition in HA, and intra- and inter-specific competition in HM were found. We suggest that Pseudocorynosoma sp. is a non-plastic species mostly found in single infections, while A. tumescens is a more variable species occurring more frequently in co-infections.

Larvae of acanthocephalans are transmitted by predation of the intermediate host by the definitive host. In some cases more than 1 larva of the same, or another, species may share an intermediate host, establishing conditions for positive, or negative, interactions between them. For parasite species transmitted via similar trophic pathways, i.e., sharing intermediate and definitive hosts, association between parasites can be expected and has been reported for the acanthocephalans Pomphorhynchus laevis and Acanthocephalus clavula in the amphipod Echinogammarus stammeri and for the acanthocephalan Profiticollis spp. and the trematode Maritrema sp. in the crab Macrophthalmus hirtipes (Dezfuli et al., 2000; Poulin, Nichol, et al., 2003). However, if 2 parasite species are manipulators of host behavior and infect different definitive hosts, a potential for conflict between them could happen, and negative interactions, such as competition in the intermediate host, could occur. Parasite species can develop mechanisms to prevent co-infections; e.g., microhabitat partitioning in the acanthocephalans Leptorhynchoides thecatus and Pomphorhynchus bulbocelli allows these species to infect different subsets of the same amphipod populations (Barger and Nickol, 1998). Despite this segregation, negative interactions in the amphipod intermediate host between the 2 parasite species (such as a slower development in co-infections) have also been reported (Barger and Nickol, 1999).

Intermediate hosts are obviously required as transport for parasite transmission but also as a supply from which parasites acquire resources for their development (Crompton and Nickol, 1985). Thus, the size of parasites can be potentially affected by their interactions with other individuals. Parasite volume previously has been used as an indicator of competition. For instance, intra-specific competition was observed when parasite volumes were smaller in individual acanthocephalans found in multiple infections compared to those found in single infections (Poulin, Nichol, et al., 2003; Cornet, 2011). Additionally, reduced volumes of P. laevis in infections with conspecifics, and with individuals of A. clavula, were perceived as evidence for intra- and inter-specific competition (Dezfuli et al., 2001).

In Lake Mascardi (Patagonia), the amphipod Hyalella patagonica is parasitized by 2 acanthocephalan species, Acanthocephalus tumescens and Pseudocorynosoma sp., which use freshwater fishes and aquatic birds as definitive hosts, respectively (Rauque and Semenas, 2007). At this site, both acanthocephalans are segregated by season, size, sex, and developmental stages of amphipods (Rauque and Semenas, 2007). Considering that in co-infections one species has no chance to complete its life cycle, competitive interactions are likely.

Since both A. tumescens and Pseudocorynosoma sp. are potential manipulators of host behavior and use H. patagonica as an intermediate host, yet employ different definitive hosts, the purpose of the present study was to examine intra- and inter-specific interactions in the amphipod host using cystacanth volume as an indicator of competition.

MATERIALS AND METHODS

Specimens of H. patagonica were collected monthly with sieves along the shoreline of Lake Mascardi, Patagonia, Argentina (41°17'S, 71°38'W). Amphipods were fixed in the field in 5% formalin and taken to laboratory. Crustaceans were measured (total length) and necropsied using a dissecting microscope. Fully developed larvae, as judged by their inverted proboscis (cystacanths), were measured using light microscopy; their volume was estimated using the formula for an ovoid, $V = \frac{4}{3}\pi L_{\max} W_{\max}^2$, where $L_{\max} =$ maximum length and $W_{\max} =$ maximum width.

Two different comparisons were made. The first contrasted cystacanth volume during all months sampled, while the second compared parasite volume in 3 different infection periods, i.e., (HP) high level of Pseudocorynosoma sp. infections (December 2002–February 2003), (HA) high level of A. tumescens infections (March 2003–September 2003), and (HM) high level of mixed infections (October 2003–February 2004) (Fig. 1).

A Spearman Correlation Rank Test was used to evaluate co-variations between amphipod total length and cystacanth volume. A Mann-Whitney test, Kruskall Wallis, and Multiple Comparisons of Mean Ranks Test were used to compare cystacanth volume in single, multiple, and mixed infections. Tests were performed with a significance level of 5%.

RESULTS

Comparison of Pseudocorynosoma sp. cystacanths

A total of 804 cystacanths (mean 0.104 mm$^3$; SE = 0.001 mm$^3$) was collected from October 2002 to May 2004. Cystacanth
volume showed a weak positive correlation with amphipod total length ($r = 0.09; P = 0.009; n = 804$) (Fig. 2). Cystacanths were classified into 3 categories of infection, i.e., 1 Pseudocorynosoma sp. cystacanth per amphipod (single infection), between 2 and 3 Pseudocorynosoma sp. cystacanths per amphipod (multiple infections), and 1 Pseudocorynosoma sp. larvae co-occurring with A. tumescens (mixed infections). No significant differences in cystacanth volume were found among these categories ($H = 1.77; P = 0.41; n = 804$) (Fig. 3A).

**Comparison of Pseudocorynosoma sp. cystacanths in the 3 infection periods**

In the period of high infection levels of Pseudocorynosoma sp., no significant differences in cystacanth volume were found among single, multiple, and mixed infections ($H = 3.85; P = 0.15; n = 281$). In the period of high infection levels of A. tumescens, no multiple infections were recorded, and no significant differences in volume were found between single and multiple infections ($Z = 0.14; P = 0.89; n = 20$). In the period of high levels of mixed infections, however, cystacanth volume was significantly lower in multiple infections than in single infections ($H = 6.59; P = 0.04; n = 421$) (Fig. 4A).

**Comparison of Acanthocephalus tumescens cystacanths**

A total of 362 cystacanths (mean 0.268 mm$^3$; SE = 0.005 mm$^3$) was collected between December 2002 and May 2004. Cystacanth volume was positively correlated with amphipod total length ($r = 0.27; P < 0.001; n = 362$) (Fig. 2). Cystacanths were classified into 3 categories, i.e., single A. tumescens larva per amphipod (single infections), between 2 and 4 A. tumescens larvae per amphipod (multiple infections), and between 1 and 2 A. tumescens larvae co-occurring with Pseudocorynosoma sp. (mixed infections). Cystacanth volume was significantly lower in multiple infections compared with single infections ($H = 12.49; P = 0.001; n = 362$) (Fig. 3B).

**Comparison of Acanthocephalus tumescens cystacanths in the 3 infection periods**

In the period of high infection levels of Pseudocorynosoma sp., no multiple infections were recorded, and cystacanth volume was significantly lower in multiple infections ($Z = -2.05; P = 0.04; n = 32$) (Fig. 4B). In the period of high infection levels of A. tumescens, cystacanth volume was significantly lower in multiple infections compared with single infections ($H = 13.89; P = 0.001; n = 151$) (Fig. 4C). In the period of high levels of mixed infections, cystacanth volume was significantly lower in multiple infections ($H = 13.58; P = 0.02; n = 154$) and in mixed infections ($H = 13.58; P = 0.001; n = 154$) than in single infections (Fig. 4D).

**DISCUSSION**

Although competition has been observed in several freshwater host-parasite systems and even between different stages of the same species (Cézilly et al., 2000; Sparkes et al., 2004; Lagrue and Poulin, 2008), no information regarding temporal variation of these interactions has been compiled. In our study, when cystacanth volume of Pseudocorynosoma sp. larvae was analyzed
over the 3 infection groupings (high infection of *Pseudocorynosoma* sp., high infection of *A. tumescens*, and high infection of mixed infections), no variation in the first 2 states was observed, but a reduced volume was seen in multiple infections, indicating intra-specific competition in high mixed infections. This difference in cystacanth volume during only 1 infection state suggests a lower variability of *Pseudocorynosoma* sp. In contrast, when cystacanth volume of *A. tumescens* was assessed during the same 3 infection groupings, larvae were more variable in size, suggesting competition. In the period of high infection by *Pseudocorynosoma* sp., *A. tumescens* exhibited inter-specific competition. In the period of high infection by *A. tumescens*, the species exhibited intra-specific competition, while in the period of high levels of mixed infection, *A. tumescens* reflected intra- and inter-specific competition. These patterns point to high vulnerability of *A. tumescens*, with its cystacanth being negatively affected by interactions that reduced their volume. This species, less prevalent than *Pseudocorynosoma* sp., was more common in multiple and mixed infections than *Pseudocorynosoma* sp., indicating a more intense competition in the amphipod intermediate host. The reasons of these variations of competition in *A. tumescens* are unclear, but an influence of a small sample size could be involved considering the absence of *A. tumescens* cystacanths in multiple infections during the period of high infection levels of *Pseudocorynosoma* sp. and the low numbers of cystacanths (only 5) in mixed infections during the period of high infection levels of *A. tumescens*.

In analyses including the total number of cystacanths collected, *Pseudocorynosoma* sp. showed no change in cystacanth volume, whereas *A. tumescens* exhibited a decrease in cystacanth volume, a pattern consistent with intra-specific competition. The results show that competition is the driving force for size variation in *Pseudocorynosoma* sp. and *A. tumescens* and that this competition varies according to the environmental abundance of individuals of the same and of the other species.

The greater variation in size of *A. tumescens* may suggest a higher allocation of resources to growth, as was previously pointed out for *Acanthocephalus lucii* females (Benesh and Valtonen, 2007).

In our study, *A. tumescens* were found to share their hosts frequently, whereas *Pseudocorynosoma* sp. occurred more commonly in single infections. How can this pattern be explained? Several scenarios seem plausible. First, there could be an aggressive strategy by *Pseudocorynosoma* sp., including effects preventing the establishment and growth of other parasites as were described for Pomphorhynchus bulbocolli and Leptorhynchoides thecatus in *Hyalella azteca* (Barger and Nickol, 1999). Alternatively, failing establishment when other parasites are previously infecting the amphipods is a possibility. Our research, however, fails to give us enough information to choose one scenario over another. Nonetheless, avoiding conspecifics seems to be an effective strategy for a common parasite such as *Pseudocorynosoma* sp., but not for less prevalent ones such as *A. tumescens*.

In Lake Mascardi, both *A. tumescens* and *Pseudocorynosoma* sp. cystacanth volume increased with amphipod total length. Similar results have been recorded in acanthocephalans from other freshwater systems (Dezfuli et al., 2001). These findings can be attributed to the greater availability of space for development and to increased food resources for parasites in larger amphipods. Comparing both parasite species, *A. tumescens* showed a stronger correlation with amphipod total length than *Pseudocorynosoma* sp. (Fig. 2). This could be associated with the greater *A. tumescens* cystacanth volume and agrees with our previous suggestion of elevated variability in volume and superior allocation to growth in *A. tumescens* compared to *Pseudocorynosoma* sp. Variations in cystacanth volume could have a strong impact on the fitness of individual parasites; i.e., it has been suggested that cystacanth volume could influence the transmission rate to definitive hosts (Steinauer and Nickol, 2003), determine the size of adults (Poulin,
Wise, et al., 2003), affect the speed of maturation (Amin et al., 1980), and increase the numbers of eggs as was reported for some digeneans (Fredensborg and Poulin, 2005).

In Lake Mascardi, interactions between the 2 acanthocephalan species vary according to the conditions of infection, indicating different parasite strategies: *A. tumescens* has more variability and is more frequently found in co-infections, while *Pseudocorynosoma* sp. shows less variation in size, being found mainly in single infections. To the best of our knowledge, this is the first study to show temporal variation of competition among parasites in an intermediate host.

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LITERATURE CITED


SPATIAL AND TEMPORAL PATTERNS OF INTRASPECIFIC MORPHOLOGICAL VARIATION IN DACTYLOGYRUS SIMPLEXUS FROM FATHEAD MINNOWS IN NEBRASKA

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ABSTRACT: Dactylogyrus simplexus Monaco and Mizelle, 1955, occurs on the gills of fathead minnows (Pimephales promelas). Previous research on parasites of fathead minnows from 3 converging Nebraska streams, Elk Creek, Oak Creek, and West Oak Creek, shows that fish in each stream constitute distinct populations. To determine whether their parasites had diverged structurally in response to such isolation, or in response to seasonal change, we searched for patterns of intraspecific morphometric variation among D. simplexus. Over 3 collection dates in fall 2007, spring 2008, and fall 2009, 203 D. simplexus were collected from Elk and West Oak Creeks. We ran 1-way ANOVA to compare differences in 15 distinct point-to-point measurements of sclerotized parts across sites and collection dates. Significant differences were found in some D. simplexus measurements between Elk and West Oak Creeks for all 3 collection dates, but the characteristics that differed and the trend of variation between the creeks were not consistent over time. Dactylogyrus simplexus from both Elk and West Oak Creeks showed consistent patterns of variation over time for 5 measurements, including hamulus gap width, bar length, marginal hook length, sickle length, and sickle width. In conclusion, D. simplexus demonstrate consistent patterns of seasonal variation, but not spatial.

Monogeneans are ideal organisms for studying the evolutionary mechanisms behind biological diversity because of their diversity and wide distribution. Monogeneans possess a broad diversity of specialized attachment organ structures, resulting in a high degree of host specificity. Examples of attachment organ structures include hamuli, marginal hooks, and, in some cases, suckers and clamps. The anatomy and morphometric measurements of these structures are of taxonomic significance, but can vary over time and space. For example, Olstad et al. (2007) found significant intraspecific differences in these measurements between 10 populations of Gyrodactylus salaris and Gyrodactylus thymalli. With the use of morphometric measurements of attachment organs, Shinn et al. (2004) correctly assigned specimens of G. thymalli and G. salaris from 4 separate populations to their correct populations. Ferdig et al. (1993) found significant differences in morphometric variations of Salsuginus yutanensis attachment organs from 3 separate locations. These variations in attachment structure morphology could arise from phenotypic plasticity or could represent true genetic differentiation of an evolutionary origin. In addition to site-related differences, monogeneans also experience seasonal variation in attachment structure morphology, presumably caused by temperature (Mo, 1991a, 1991b, 1991c; Ferdig et al., 1993).

The present study examined seasonal and spatial variations in attachment structures of Dactylogyrus simplexus Monaco and Mizelle, 1955 (simplex Mizelle, 1937 renamed), from fathead minnows, Pimephales promelas Rafinesque, 1820, in 3 diverging streams in eastern Nebraska, i.e., Elk, Oak, and West Oak Creeks.

In these streams, the fathead minnow serves as host for 3 species of dactylogyrids, i.e., D. simplexus, Dactylogyrus bychowski Mizelle, 1937, and D. pectenatus Mayes, 1977. Previous work suggests that these fathead minnow populations are isolated and exhibit little or no cross-stream movement based upon parasite community structures of dactylogyrids and other parasite species (Weichman and Janovy, 2000; Knipes and Janovy, 2009). In addition, the streams exhibit differing environmental characteristics, most notably flow-rate variability. These findings suggest that the worms are also isolated and may show divergence in their attachment organ morphology in response to the different environments of the 3 creeks. The present study focused on D. simplexus because its prevalence was high enough to produce sample sizes adequate for statistical analysis.

The goals of this study were to determine whether D. simplexus populations in Elk, West Oak, and Oak Creeks are similarly isolated as their hosts and are structurally divergent, as evidenced by attachment organ morphology, and if these dactylogyrids show consistent trends of temporal variation in attachment-organ morphology. The null hypotheses are that D. simplexus attachment organs will not show significant variation with respect to collection site or collection date.

Seining of fathead minnows was conducted in mid-September to late October 2007, mid-March to mid-May 2008, and late August to early November 2009. Fathead minnows were transported to the University of Nebraska–Lincoln laboratory in buckets with aerators and were necropsied within 48 hr of the collection date.

Gills were excised and stored in 1:4,000 formalin (Kritsky et al., 1986). Monogeneans were removed using Pasteur pipettes, flattened on microscope slides under cover slips, and examined using a compound microscope. Attachment structures were photographed with the use of the 100× magnification oil immersion lens and a Nikon 990 Coolpix camera with a 10× microscope adapter, for a total of 1,000× magnification. Images were adjusted to 114 × 152 mm, 100 pixels/inch, and auto levels, and saved as a bitmap with the use of Adobe Photoshop 7.0.

Monogenean species identity was determined with the use of qualitative characteristics of their copulatory organs according to the descriptions of Mizelle (1937). The digital images were analyzed and measurements obtained with the use of ScionImage Alpha 4.0.3.2 (Scion Corporation, 2000–2001; Frederick, Maryland). Fifteen unique measurements from sclerotized attachment structures were included according to the protocols of Murith and Beverley-Burton (1985). Hamuli measurements included overall length (OL), base length (BL), deep root length (DRL), superficial root length (SRL), blade length (BL), gap length (GW), gap length (GL), and root angle (RA). Bar measurements included length (BIL) and width (BILW). Marginal hook measurements included overall length (OHL), handle length (HL), sickle length (SL), handle width (HW), and sickle width (SW).
For each collecting period, *D. simplexus* sample means from Elk and West Oak Creeks were compared with the use of 1-way analysis of variance (ANOVA) to determine the presence of site-related variation for each of the 15 measurements. A *P* value of less than 0.05 was considered significant. Analyses were conducted in Microsoft Excel 2007.

**RESULTS**

Ninety-six fathead minnows were collected from Elk, West Oak, and Oak Creeks. From these fathead minnows, 228 *D. simplexus* were analyzed, including 120 from Elk Creek, 89 from West Oak Creek, and 19 from Oak Creek. Because of the low sample size, Oak Creek was excluded from analyses. In total, 998 marginal hooks were measured from Elk Creek and West Oak Creek, for an average of 4.8 marginal hooks per worm. Table I presents the numbers of fish, *D. simplexus*, and marginal hooks collected or measured by collection date and site.

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Table I. Numbers of fathead minnows and *Dactylogyrus simplexus* collected by collection date and site.

For all of these characteristics, Elk Creek *D. simplexus* were larger on average than West Oak Creek *D. simplexus*.

In the spring 2008 sample, 3 of 15 characteristics differed significantly between collection sites, including superficial root length (*P* = 0.001), blade length (*P* = 0.024), gap length (*P* = 0.019), and marginal hook sickle width (*P* < 0.000). For all of these characteristics except marginal hook sickle width, West Oak Creek *D. simplexus* were larger on average.

In the fall 2009 sample, 2 of 15 characteristics different significantly between collection sites, including base length (*P* = 0.048) and marginal hook handle length (*P* = 0.008). For both of these characteristics, West Oak Creek *D. simplexus* were larger on average.

No single characteristic showed significant variation between collection sites during all 3 collection dates. Marginal hook length showed statistically significant variation between Elk Creek and West Oak Creek populations in both fall samples, but the trends of variation between the populations were reversed. Gap length and marginal hook sickle width showed significant site variation in both the fall 2007 and spring 2008 samples, with marginal hook sickle width showing the same trend of variation in both dates and gap length showing reverse trends.

**FIGURE 1.** Hamuli and bar measurements from *Dactylogyrus simplexus*. Abbreviations are as follows: OL, overall hamuli length; BL, base length; DRL, deep root length; SRL, superficial root length; BL, blade length; GW, gap width; GL, gap length; RA, root angle; BaL, bar length; BaW, bar width.

**FIGURE 2.** Marginal hook measurements from *Dactylogyrus simplexus*. Abbreviations are as follows: OHL, overall hook length; HL, handle length; SL, sickle length; HW, handle width; SW, sickle width.
Table II. Comparison of *Dactylogyrus simplexus* measurements between Elk and West Oak Creeks for 3 collection dates. Mean values are given in micrometers with standard deviations in parentheses. *P* values are given for 1-way ANOVA. Bolded measurements indicate a *P* value of less than 0.05. Abbreviations are as follows: OL, overall hamuli length; BL, base length; DRL, deep root length; SRL, superficial root length; BL, blade length; GW, gap width; GL, gap length; BaL, bar length; BaW, bar width; RA, root angle; OHL, overall hook length; HL, handle length; SL, sickle length; HW, handle width; SW, sickle width.

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</thead>
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<tr>
<td></td>
<td>Elk</td>
<td>West Oak</td>
<td>Elk</td>
</tr>
<tr>
<td></td>
<td><em>n</em></td>
<td></td>
<td><em>P</em></td>
</tr>
<tr>
<td><strong>Hamuli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>OL (µm)</em></td>
<td>40</td>
<td>17</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>29.16 (1.39)</td>
<td>27.90 (1.46)</td>
<td>0.003</td>
</tr>
<tr>
<td><em>BL (µm)</em></td>
<td>24.49 (1.15)</td>
<td>23.80 (1.41)</td>
<td>0.057</td>
</tr>
<tr>
<td><em>DRL (µm)</em></td>
<td>3.07 (0.57)</td>
<td>3.10 (0.57)</td>
<td>0.877</td>
</tr>
<tr>
<td><em>SRL (µm)</em></td>
<td>7.72 (1.06)</td>
<td>8.18 (0.85)</td>
<td>0.116</td>
</tr>
<tr>
<td><em>BIL (µm)</em></td>
<td>7.82 (0.82)</td>
<td>7.92 (1.27)</td>
<td>0.727</td>
</tr>
<tr>
<td><em>GW (µm)</em></td>
<td>11.19 (0.94)</td>
<td>10.93 (1.16)</td>
<td>0.391</td>
</tr>
<tr>
<td><strong>Marginal hooks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N</em></td>
<td>183</td>
<td>55</td>
<td>312</td>
</tr>
<tr>
<td><em>OHL (µm)</em></td>
<td>17.44 (1.79)</td>
<td>16.80 (1.77)</td>
<td>0.021</td>
</tr>
<tr>
<td><em>HL (µm)</em></td>
<td>5.91 (1.33)</td>
<td>5.31 (1.45)</td>
<td>0.004</td>
</tr>
<tr>
<td><em>SL (µm)</em></td>
<td>5.66 (0.65)</td>
<td>5.23 (0.56)</td>
<td>10^-4</td>
</tr>
<tr>
<td><em>HW (µm)</em></td>
<td>1.59 (0.31)</td>
<td>1.45 (0.30)</td>
<td>0.004</td>
</tr>
<tr>
<td><em>SW (µm)</em></td>
<td>1.74 (0.35)</td>
<td>1.55 (0.30)</td>
<td>10^-4</td>
</tr>
</tbody>
</table>

**Dactylogyrus simplexus** attachment organ measurements from Elk and West Oak Creeks were also evaluated for structural variation over time with the use of 1-way ANOVA (Table III). In the Elk Creek sample, 8 of 15 characteristics showed significant variation across collection dates, including gap width (*P* < 0.000), gap length (*P* = 0.006), bar length (*P* = 0.000), bar width (*P* = 0.005), marginal hook total length (*P* < 0.000), handle length (*P* < 0.000), sickle length (*P* = 0.040), and sickle width (*P* < 0.000).

Table III. Comparison of *Dactylogyrus simplexus* measurements between collection dates for Elk and West Oak Creeks. Mean values are given in micrometers with standard deviations in parentheses. *P* values are given for 1-way ANOVA. Bolded measurements indicate a *P* value of less than 0.05. Abbreviations are as follows: OL, overall hamuli length; BL, base length; DRL, deep root length; SRL, superficial root length; BL, blade length; GW, gap width; GL, gap length; BaL, bar length; BaW, bar width; RA, root angle; OHL, overall hook length; HL, handle length; SL, sickle length; HW, handle width; SW, sickle width.

<table>
<thead>
<tr>
<th>Number/ measurement</th>
<th>Fall 2007</th>
<th>Spring 2008</th>
<th>Fall 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elk</td>
<td>West Oak</td>
<td>Elk</td>
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<td></td>
<td><em>n</em></td>
<td></td>
<td><em>P</em></td>
</tr>
<tr>
<td><strong>Hamuli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>OL (µm)</em></td>
<td>29.16 (1.39)</td>
<td>28.58 (1.66)</td>
<td>29.28 (1.15)</td>
</tr>
<tr>
<td><em>BL (µm)</em></td>
<td>24.49 (1.15)</td>
<td>24.57 (1.51)</td>
<td>24.97 (1.08)</td>
</tr>
<tr>
<td><em>DRL (µm)</em></td>
<td>3.07 (0.57)</td>
<td>2.87 (0.44)</td>
<td>3.15 (0.94)</td>
</tr>
<tr>
<td><em>SRL (µm)</em></td>
<td>7.72 (1.06)</td>
<td>7.58 (0.88)</td>
<td>7.92 (1.16)</td>
</tr>
<tr>
<td><em>BIL (µm)</em></td>
<td>7.82 (0.82)</td>
<td>7.53 (0.62)</td>
<td>7.81 (0.80)</td>
</tr>
<tr>
<td><em>GW (µm)</em></td>
<td>11.19 (0.94)</td>
<td>11.71 (0.84)</td>
<td>11.97 (0.74)</td>
</tr>
<tr>
<td><strong>Marginal hooks</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>N</em></td>
<td>183</td>
<td>312</td>
<td>167</td>
</tr>
<tr>
<td><em>OHL (µm)</em></td>
<td>16.80 (1.79)</td>
<td>18.59 (2.00)</td>
<td>17.74 (1.80)</td>
</tr>
<tr>
<td><em>HL (µm)</em></td>
<td>5.91 (1.33)</td>
<td>6.61 (1.56)</td>
<td>5.89 (1.53)</td>
</tr>
<tr>
<td><em>SL (µm)</em></td>
<td>5.66 (0.65)</td>
<td>5.79 (0.64)</td>
<td>5.70 (0.50)</td>
</tr>
<tr>
<td><em>HW (µm)</em></td>
<td>1.59 (0.31)</td>
<td>1.55 (0.54)</td>
<td>1.53 (0.31)</td>
</tr>
<tr>
<td><em>SW (µm)</em></td>
<td>1.74 (0.35)</td>
<td>1.40 (0.21)</td>
<td>1.35 (0.22)</td>
</tr>
</tbody>
</table>

BI AND JANOVY—DACTYLOGYRUS SIMPLEXUS MORPHOLOGICAL VARIATION 1005
In the West Oak Creek sample, 9 of 15 characteristics showed significant variation across collection dates, including base length \((P < 0.000)\), superficial root length \((P = 0.023)\), gap width \((P = 0.002)\), bar length \((P = 0.003)\), bar width \((P = 0.027)\), marginal hook length \((P < 0.000)\), handle length \((P < 0.000)\), sickle length \((P < 0.000)\), and sickle width \((P < 0.000)\). Seven characteristics showed significant variation between collection dates in both creeks, and, of these, 5 showed a similar trend of variation in both locations, including gap width, bar length, marginal hook length, sickle length, and sickle width.

**DISCUSSION**

The present study demonstrates a lack of consistent, stable, site-related variation in attachment-organ morphology between 2 populations of *D. simplexus* on populations of minnows isolated in different streams of a single drainage. During individual collection dates, specific measurements showed significant variation between locations, but the characteristics that showed site-related differences and the nature of these relationships between the 2 populations did not remain stable over separate collection dates. In contrast, *D. simplexus* in both Elk Creek and West Oak Creek showed patterns of attachment organ morphology variation across time that were similar in both creeks and that match earlier studies by Mo (1991a, 1991b, 1991c) conducted on populations of *Gyrodactylus salaris*. Notably, *D. simplexus*, like *G. salaris*, shows larger marginal hook measurements in spring than in fall and winter.

The lack of stable, site-related differences in attachment-organ morphology argues against the existence of isolated, evolutionary divergent *D. simplexus* populations in Elk and West Oak Creeks, regardless of the evidence that their hosts are isolated (Weichman and Janov, 2000; Knipes and Janov, 2009). Other studies that observed significant morphological differences between geographically separated monogenean populations studied populations that were often separated by hundreds of kilometers (Ferdig et al., 1993; Shinn et al., 2004). As previously described, Elk and West Oak Creeks are tributaries of the same stream and converge a few km downstream of the collection sites. Given the proximity of these locations, one expects a high degree of gene flow. The observed variations between collection sites during individual collection dates may be due to phenotypic plasticity in response to differing environmental conditions. Environmental characteristics of Elk and West Oak Creeks, especially flow rate, have been shown to vary from year to year (Knipes and Janov, 2009), which may account for the lack of concordance in variation over time. Further studies in similar settings are warranted to confirm our conclusions and to clarify the mechanism of site-specific morphological variation.

**ACKNOWLEDGMENT**

The authors would like to acknowledge the assistance of Alaine Knipes in technical advice and fish collecting.

**LITERATURE CITED**


NEW SPECIES OF ACANTHOCHONDRIA (COPEPODA: CHONDRACTHIDAE) INFECTING THE LONGTAIL SOUTHERN COD, PATAGONOTOTHEN RAMSAYI (PERCIFORMES: NOTOTHENIIDAE), FROM PATAGONIAN WATERS, ARGENTINA

Delfina Maria Paula Cantatore, Ana Laura Lanfranchi, and Juan Tomás Timi

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ABSTRACT: Acanthochondria lilianae n. sp. (Copepoda: Chondracanthidae) is described and illustrated based on specimens of both sexes collected from the operculum of the longtail southern cod, Patagonotothen ramsayi (Regan) (Perciformes: Nototheniidae), from the Patagonian Shelf, Argentina (42°-48°S, 60°-63°W). Acanthochondria lilianae is characterized by the combination of a Type B-III antennule and Type A leg 2, in addition to both the cephalosome and the trunk being wider than long. The new species most closely resembles Acanthochondria incisa Shiino, 1955, Acanthochondria ophidii (Kroyer, 1863), Acanthochondria priscanithi Shiino, 1964, and Acanthochondria tasmaniae Heegaard, 1962, but differs from these species in the general measurements and proportions of the body, shape and size of head, shape and size of genitoabdomen, and fine details of appendages such as the armature of antennule, ornamentation of both pairs of legs, number of teeth on mandible and maxilla, and ornamentation on maxillipeds. This is the first Acanthochondria species recorded from a nototheniid and the second southernmost record of a species of this genus in the southwestern Atlantic Ocean.

Acanthochondria Oakley, 1927, is the largest genus among species of Chondracanthidae Milne Edwards, 1840 (Ostergaard, 2003). A species key for the genus was provided by Kalman (2003), in which 46 accepted species were listed. Five additional species have been described since, e.g., Acanthochondria triangularis Alves, Luque and Paraguassu, 2003, Acanthochondria serrani Braicovich and Timi, 2009, Acanthochondria alteni Tang, Kalman and Ho, 2010, Acanthochondria helicoleni Cantatore and Timi, 2010, and Acanthochondria sagitta Alarcos and Timi, 2011 (Alves et al., 2003; Braicovich and Timi, 2009; Alarcos and Timi, 2010; Cantatore and Timi, 2010; Tang et al., 2010). The last authors have made justified additional taxonomic changes to the genus, including the transfer of Acanthochondria zebrinae Ho, Kim and Kumar, 2000, to Heterochondria Yü, 1935, based on a combination of apomorphic features that they felt excluded the species from Acanthochondria. Moreover, by slightly expanding the generic diagnosis of Acanthochondria to include “head with or without outgrowths (in the form of processes, protrusions or knobs),” a monotypic Pterochondria Ho, 1973, was considered a junior synonym of Acanthochondria, and Pterochondria alatalonigollis (Heegaard, 1940), was moved to Acanthochondria (Tang et al., 2010). Thus, Acanthochondria presently includes 51 valid species.

During a parasitological survey carried out on Patagonotothen ramsayi (Regan) (Perciformes: Nototheniidae) captured on the Patagonian Shelf, Argentina (42°-48°S, 60°-63°W), in November 2007, were examined for parasites. Copepods were removed from inner surface of the operculum, fixed in 10% formalin, and transferred to ethanol for storage before being studied and measured. A subsample of selected specimens was cleared in lactic acid for a minimum of 24 hr; appendages were dissected and examined using a light microscope, and illustrations were drawn with the aid of a drawing tube. Measurements were based on all specimens examined unless otherwise indicated and given in mm as mean ± standard deviation, with ranges in parentheses. The terms prevalence and mean intensity of infection were used according to Bush et al. (1997). Criteria for identifying the new species followed Ho and Kim (1995) and Kalman (2003); morphological terminology used herein follows Boxshall and Halsey (2004), and host taxonomy is in accordance with FishBase (Froese and Pauly, 2010).

DESCRIPTION

Acanthochondria lilianae n. sp. (Figs. 1–28)

Diagnosis (based on 7 ovigerous females and 6 adult males): Female body (Figs. 1–3) divided into head, short neck, and stout trunk bearing moderately large posterior processes, and small genitoabdomen. Total body length 6.88 ± 0.38 (6.51–7.66) (from anterior margin of head, excluding antennules, to distal end of posterior processes of trunk). Head (Figs. 1–3) composed of cephalosome only, with 2 pairs of lateral outgrowths (1 antero-dorsal and 1 postero-ventral), slightly wider than long; 1.59 ± 0.06 (1.53–1.71) long, 1.75 ± 0.05 (1.69–1.84) wide; triangular in lateral view, with conspicuous oral region (Fig. 3). Neck region (Figs. 1–3) composed of first and second pedigers, 1.20 ± 0.21 (0.96–1.46) long, 1.10 ± 0.05 (1.03–1.19) maximum width, length/maximum width 1.09 ± 0.19 (0.88–1.34). Leg 1 located immediately posterior to head; leg 2 located 0.70 ± 0.10 (0.53–0.83) from head. Trunk (Figs. 1–3) dorsoventrally depressed, 2.96 ± 0.18 (2.75–3.18) long (excluding processes), slightly constricted at about middlelength, 3.52 ± 0.34 (3.25–4.28) wide at constriction and slightly narrower anteriorly, 3.65 ± 0.37 (3.25–4.28) wide, than posteriorly, 3.79 ± 0.27 (3.48–4.20) wide; posterolateral processes 1.13 ± 0.17 (0.90–1.41) long, blunt, extending beyond distal limit of genitoabdomen. Pair of nuptial organs (Ostergaard and Boxshall, 2004) on posteroverentral surface of trunk near junction with genitoabdomen. Genitoabdomen (Fig. 4) almost as long as wide, 0.66 ± 0.07 (0.58–0.75) (n = 6) long, 0.70 ± 0.00 (n = 5) wide; genital somite with pair of short setae on anterior margin of genital apertures; abdominal somite small, pear shaped, with rounded posterior margin. Caudal ramus (Fig. 5) small, conical, longer than wide, swollen basal portion armed with 2 setae on ventral surface, 1 seta on dorsal surface, and 1 knob near junction with terminal process; terminal process trifurcate. Egg sac (Fig. 3) cylindrical, curving ventrally then anteriorly to lie beneath trunk, 8.66 ± 0.95 (7.58–9.40) (n = 4) long, 1.13 ± 0.10 (1.00–1.37) wide, with multisierate arrangement of eggs.

Antennule (Fig. 6) unsegmented, cylindrical (Type B-III); basal portion armed with 3 isolated stout setae along anterior margin, tip with patch of spinules on ventral margin and 12 elements (Fig. 7), including subdistally 2 stout anterior setae and 2 slender ventral setae; 6 slender apical setae (2 small) and 2 knobs. Antenna (Fig. 8) 2 segments; basal segment unarmed; terminal segment a heavily sclerotized, curved claw with pointed tip and band of transverse surface striations near tip. Labrum (Fig. 9) with row of spinules along posterior margin and small knobs on lateral margins.

Mandible (Fig. 10) 2 segments, with 45–47 teeth on convex margin and 34–36 teeth on concave margin of terminal falcate process. Paragnath lobate, ornamented with spinules (Fig. 11). Maxillule (Fig. 11) suboval, with 2 unequal setae, and 2 patches of spinules on outer surface. Maxilla (Fig. 12) 2 segments; first segment squat and unarmed; terminal segment claw-like, armed with row of 4–5 denticles in addition to single subterminal denticle, and seta near base. Maxilliped (Fig. 13) 3 segments; first segment stout, covered with patches of spinules; second segment bearing 2 patches of denticles on inner and inner-distal surfaces in addition to patch of spinules on surface; terminal segment a curved claw-like structure bearing hooklet on inner surface and 2 basal rows of denticles on outer surface. Leg 1 (Fig. 14) slightly smaller than leg 2 (Fig. 15); both legs bilobate, type A, carrying outer seta on protopod and on exopod, and ornamented with patches of spinules.

Male body (Figs. 16–17) divided into globose cephalothorax and ventrally flexed genitoabdomen; 0.66 ± 0.04 (0.60–0.68) long (measured from base of antennae to distal end of genital segment, excluding caudal rami), 0.44 ± 0.03 (0.40–0.46) maximum width. Genital segment with pair of ventral ridges. Caudal ramus (Fig. 18) with spinules on terminal processes and 3 setae on basal portion, 1 dorsal and 2 ventral (1 small).
Antennule (Fig. 19) unsegmented, with armature formula 1, 1, 2, 2, 6. Antenna (Fig. 20) robust; basal segment unarmed, terminal claw with minute hyaline seta on outer surface. Labrum (Fig. 21) with row of spinules along posterior margin, small knobs on lateral margins and anterior median knob. Mandible (Fig. 22) 2 segments, with 24–27 teeth on convex margin and 15–19 teeth on concave margin of terminal falcate process. Paragnath lobate, armed distally with spinules (Fig. 23). Maxillule (Fig. 24) robust, bearing 2 setae. Maxilla (Fig. 25) similar to that of female except terminal process devoid of teeth. Maxilliped (Fig. 26) 3 segments, first segment unarmed; second segment bearing patch of denticles on inner and distal surfaces; terminal segment a curved claw-like structure bearing hooklet on distal inner surface, and 2 basal denticles. Legs bilobate, leg 1 (Fig. 27) and leg 2 (Fig. 28) similar in size; protopod with long outer setae; endopod reduced to conical lobe; exopod in leg 1 bearing trifurcated element; exopod in leg 2 bearing bifurcated element in addition to rounded knob at base.

**Taxonomic summary**

*Type host:* Longtail southern cod, *Patagonotothen ramsayi* (Regan) (Perciformes: Nototheniidae).

*Site of infection:* Inner surface of operculum.
Type locality: Patagonian Shelf, Argentina (42°48'S, 60°63'W).

Date of collection: November 2007.

Prevalence: Nine fish infected of 58 fish examined (12.06%).

Mean intensity (range): 1.29 (1-2).

Specimens deposited: Holotype No. MLP 26731 (female), allotype No. MLP 26732 (male), and paratypes (2 females each with attached male) No. MLP 26733 are deposited in the Carcinological Collection of the Museo de La Plata (CCMLP), La Plata, Argentina.

Etymology: The species is named in honor of Liliana B. Degrandi, the first author's mother.

Remarks

The identification of Acanthochondria species is based on adult female morphology because males do not show species specific features (Ho and Kim, 1995; Kalman, 2003). The criteria widely used for the identification of Acanthochondria species are (1) proportion and structure of head; (2) general appearance of antennule; (3) structure and general appearance of legs 1 and 2; (4) fine structure of oral appendages; (5) structure of genitoabdomen; and finally, (6) host and geographic distribution data (Ho, 1970; Kabata, 1984; Ho and Kim, 1995), despite the latter having been deeply criticized by Poly and Mah (2001).

Acanthochondria lilianae n. sp. is characterized by having a short neck consisting of first and second pedigers, the combination of a Type B-III antennule (with inflated basal region) and a Type A Leg 2, in addition to a cephalosome and trunk both wider than long. Considering these features, the new species closely resembles 4 congeners, i.e., Acanthochondria incisa Shiino, 1955, Acanthochondria ophidi Shiroy, 1863, Acanthochondria pricanthi Shiino, 1964, and Acanthochondria tasmani Heegaard, 1962. The original descriptions of these 4 species are old and inadequate. Fortunately, A. ophidi and A. pricanthi were redescribed by Ho (1977) and Ho and Kim (1995), respectively, providing detailed and useful information for appropriate comparisons. Indeed, A. ophidi, a parasite of Ophidion sp. (Ophidiodae), from the South Pacific Ocean (Ho, 1977), differs from A. lilianae n. sp. in having a larger body size and head and trunk distinctly longer than wide, in fine details of appendages, such as the antennule and maxillule armature, number of mandibular teeth (32 in concave side and 40-42 in convex side), and maxilla teeth (14-16), in having a less ornamented maxilliped, and in the number caudal rami setae and the proportional size of caudal rami relative to abdomen. Acanthochondria pricanthi was redescribed based on specimens found at the base of the gill arch of a Japanese sandfish, Arctiscus japonicus (Steindacher) (Trichodactylidae), collected in the Sea of Japan (Ho and Kim, 1995). This species differs from the newly described species by the smaller body size, the shape of the head, the antennule armature, fine details regarding the oral appendages such as number of mandibular teeth (41 on both sides of terminal blade) and maxilla teeth (11), and the ornamentation of the maxilliped, and the distribution of spines in both legs.

In contrast, the only descriptions available for A. incisa and A. tasmani are the original ones; therefore, close comparisons with these species cannot be made with accuracy based on criteria mentioned above. We assert that these species need to be redescribed in detail in accord to appropriate comparisons. Indeed, the only descriptions available for A. incisa and A. tasmani are the original ones; therefore, close comparisons with these species cannot be made with accuracy based on criteria mentioned above. We assert that these species need to be redescribed in detail in accord to appropriate comparisons.

Acanthochondria incisa was described based on specimens found in the buccal cavity of Helicolenus dactylopterus (Delauroche) (Sebastidae) taken on the east coast of Tasmania, Australia (Heegaard, 1962). It is known from various localities, including stations between latitude 41°29'S and longitude 147°32'W, on the Patagonian Shelf, Argentina (Heegaard, 1962).

The species is named in honor of Liliana B. Degrandi, the first author's mother.

DISCUSSION

Five species of Acanthochondria have so far been reported from fishes of southwestern Atlantic Ocean. They are A. phycidis parasitic on "trout" (Salmonidae) and "mullet" (Mugilidae) from Malvinas Islands (Ho, 1971), A. triangularis parasitic on the Brazilian codling, Urophycis brasilensis (Kaup) (Phycidea), and the Gulf hake, Urophycis mystaceus Ribeiro (Phycidea), from the Brazilian coastal zone (Alves et al., 2003), A. serrani parasitic on Serranops auriga (Cuvier) (Serranidae) (Braicovich and Timi, 2009), A. helicoleni parasitic on the rubio, Helicolenus lalileil Norman (Sebastidae) (Canetore and Timi, 2010), and A. sagitta infecting the flounder, Xysteureys rasilis (Jordan) (Paralichthyidae) (Alarcos and Timi, 2011), from Argentinean waters. Acanthochondria lilianae n. sp. is the first species in this genus to be reported from a nototheniid (Nototheniidae), and this record is the second southernmost record of Acanthochondria in the southwestern Atlantic Ocean.

ACKNOWLEDGMENTS

We thank Claudio C. Buratti and Claudia V. Dato (Instituto Nacional de Investigación y Desarrollo Pesquero [INIDEP]) for kindly providing fish samples. This study was partially supported by grant EXA 442/08 from Universidad Nacional de Mar del Plata, PICT 02199/07 from CONICET, and PIP 112-20001-00024 from CONICET. This work is part of the doctoral thesis of D.M.P.C.

LITERATURE CITED


DETECTION OF ANAPLASMA BOVIS IN AN UNDESCRIBED TICK SPECIES COLLECTED FROM THE EASTERN ROCK SENGI ELEPHANTULUS MYURUS

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ABSTRACT: Ticks are important vectors of numerous pathogens causing illness, fatalities, and economic loss worldwide. Infectious disease episodes are increasing, and novel tick-borne pathogens are described frequently. Identification of novel reservoir hosts and vectors of tick-borne pathogens is essential if control measures are to be successful. In South Africa, the eastern rock sengi, Elephantulus myurus, hosts a number of tick species of veterinary importance. Despite this, there remains a paucity of information regarding the tick fauna of this species, the pathogen associations of ticks that it hosts, and its role as a reservoir host of tick-borne pathogens. The current study documents the tick fauna of E. myurus and sympatric small mammal species in Limpopo Province, South Africa. The pathogen associations of ticks hosted by elephant shrews were also investigated by PCR screening of engorged nymphs for a broad range of bacterial and protozoan tick-borne infections, including Borrelia burgdorferi sensu lato and members of Apicomplexa and the order Rickettsiales. There were marked differences in tick species and abundance among host species. Elephantulus myurus was heavily, and predominantly, parasitized by an as-yet undescribed tick species that we identify as Rhipicephalus sp. near warburtoni. PCR and sequence analysis revealed the presence of Anaplasma bovis in this tick species, which may have consequences for livestock production and conservation efforts in the area where this tick species occurs.

Ticks are vectors of some of the most important pathogens affecting humans and livestock throughout the world (Jongejan and Uilenberg, 2005). Recently, the number of human infectious disease episodes attributed to tick-borne pathogens has increased (Spach et al., 1993; Jensonius et al., 2003; Randolph, 2004), and novel tick-borne pathogens are being discovered regularly (Parola and Raoult, 2001). Economic losses to agriculture through mortality and the treatment of tick-borne infections are also substantial (Kivaria, 2006). In order to identify the risk posed to animal and human health, and as a starting point for control, it is important to identify novel reservoirs and vectors of emerging infectious diseases.

Elephant shrews, or sengis, of the Macroscelidae are hosts for the immature stages of approximately 27 species of ixodid ticks in Africa (Fourie et al., 1995). Eastern rock sengis, Elephantulus myurus, are of particular interest because they host large numbers of the immature stages of 3 tick species that transmit toxins causing paralysis in domestic animals. Ixodes rubicundus is responsible for numerous annual mortalities of sheep in South Africa (Spickett and Heyne, 1988). Rhipicephalot muttilli causes paralysis in dogs (Norval and Colborne, 1985), while Rhipicephalus warburtoni has been implicated in the paralysis of goats (Fourie et al., 1988). Despite the potential for E. myurus to host ticks of medical and veterinary importance, there remains a paucity of information regarding the tick associations of this species. Moreover, the vector competence and disease associations of many of the tick species that infest E. myurus have not been established (Walker et al., 2000).

Rodents are important hosts for immature ixodid ticks across the world (Sonenshine, 1991), but the limited data available from locations in South Africa suggest that elephant shrews carry much larger tick loads than sympatric rodent species (MacLeod, 1970; Colbo and MacLeod, 1976; Fourie et al., 1992). The reason for this disparity in tick loads between small mammal species is unclear, and the extent of this phenomenon is currently unknown.

The present study aims to document the tick fauna of the eastern rock sengi, E. myurus, and sympatric small mammal species in Limpopo Province, South Africa. We also aim to assess the potential for elephant shrews to act as reservoir hosts of tick-borne pathogens by screening engorged ticks collected from these animals for a wide range of bacterial and protozoan pathogens using PCR.

MATERIALS AND METHODS

Small mammals were trapped across 20 consecutive nights between 24 April and 13 May 2009 at Goro Game Reserve (25°00‘12‘S, 29°26‘03‘E), located in the Southpansberg mountain range, Limpopo Province, South Africa. Two-hundred Sherman live traps, baited with an oats, peanut butter, and sardine mix, were deployed in lines at 10-m intervals on rocky outcrops. Depending on the terrain, either 2 trap lines of 100 traps or 4 trap lines of 50 traps were set in parallel rows 10 m apart. All traps were set after 1600 hr and collected before 0800 hr the next day. Animals were identified to species, and their sex, age, and body mass were recorded. The body of each individual was searched for ticks, and particular attention was given to the ear margins, legs, and the base of the tail, where ticks aggregated. The rest of the body was searched by back-combing the fur. All ticks were removed using fine-tipped forceps and placed in 70% ethanol, and the animals were released. Ticks were identified to species or species group by I.G.H. using descriptions provided by Thelher (1961), Walker et al. (2000), and Apanaskevich et al. (2007) and by comparison with reference material. Developmental stage was recorded as larva, nymph, or adult. The study was conducted with the ethical approval of the Animal Care and Use Committee, University of Pretoria, under permit number EC015-09.

The correlation of sex, body size, and their interaction on total tick loads of E. myurus and Micaelamyss namaquensis was investigated using generalized linear models (GLMs) with a negative binomial error distribution and a log link function. To avoid the confounding effect of host age on body mass and immune status, all models were conducted on a subset of data containing adults only. Analyses were performed using the glm.nb procedure in package MASS in the R software package available under GNU license at http://www.r-project.org.

Five engorged nymphs collected from each E. myurus individual were placed in 2-ml Sure-Lock microcentrifuge tubes, and 0.5-ml aliquots of 1.25% ammonia solution were added. The nymphs were macerated using a single-use pipette tip, and DNA was extracted as previously described (Bown et al., 2003). Ten-microliter samples of extract from each of the 5 ticks were then combined to give a 50-ml pooled sample representing a single host animal.
To assess the reservoir potential of E. myurus for tick-borne pathogens, a range of non-specific diagnostic tests was used. Pooled tick samples were tested for the presence of Borrelia burgdorferi sensu lato, species of Apicomplexa, (including Babesia spp.), and for the order Rickettsiales (including species of Rickettsia, Anaplasma, and Ehrlichia) by PCR as described previously (Pancholi et al., 1995; Courtney et al., 2004; Simpson et al., 2005). Positive samples were identified via electrophoretic resolution of PCR products on a 1.0% agarose gel stained with ethidium bromide and examined under UV light. All PCRs included negative controls in a ratio of 1 to 5 and positive controls. Amplification products were purified using QiAquick kit (Qiagen, Valencia, California), and sequences were determined using a commercial sequencing service (Macrogen, Seoul, Korea). Successfully sequenced amplification products were used to search for other closely related sequences using the NCBI nucleotide BLAST database, and other known species within the genus were included in the analysis. Sequences were aligned and compared using BioEdit v.7.0.9.

**RESULTS**

**Tick data**

In total, 172Namaqua rock mice (Micromys namaquensis), 57 eastern rock elephant shrews (Elephantulus myurus), 16 spiny mice (Acomys spinnosissimus), and 7 red rock rats (Aethomys chrysophilus) were caught across 2,600 trap nights. Marked differences were evident among the tick fauna of each host species. *Elephantulus myurus* was heavily parasitized, hosting tick abundances many orders of magnitude greater than other hosts. Altogether, 22,739 ticks (21,648 larvae and 1,091 nymphs) (Table I) were collected from *E. myurus*, the vast majority of which (22,729, 99.95%) belonged to an undescribed tick species that we have designated as *Rhipicephalus* sp. near *warburtoni*. This tick was remarkably abundant on *E. myurus*, with mean infestations of 379.7 ± 33.8 larvae and 19.1 ± 1.3 (±SE) nymphs, but it was virtually absent from sympatric rodent species. Although in low numbers, ticks from the *Rhipicentor nuttalli/bicornis* group were only found on *E. myurus*. Small numbers of ticks belonging to the *Rhipicephalus simus/follis* group were collected from all host species but were present on *E. myurus* at lower abundances than on the other small mammals. Small numbers of *Rhipicephalus lunulatus* and ticks belonging to the *Haemaphysalis elliptica* group were also present on hosts. None of the characteristics recorded, i.e., sex, mass, and their interactions, significantly affected tick loads on *E. myurus* or *M. namaquensis* (Table II). In addition, an incidental collection of ticks found that questing on the tips of grass within Goro Game Reserve yielded 10 males and 8 females of *Rhipicephalus appendiculatus* and 5 males and 7 females of *R. sp. near warburtoni*.

**Pathogen detection**

Of the 57 tick pools tested, none was positive for *B. burgdorferi* sensu lato or any species of Apicomplexa. Four tick pools of *R. sp. near warburtoni* tested positive for the presence of *Rickettsiales*. This tick was remarkably abundant on *E. myurus*, with mean infestations of 379.7 ± 33.8 larvae and 19.1 ± 1.3 (±SE) nymphs, but it was virtually absent from sympatric rodent species. Although in low numbers, ticks from the *Rhipicentor nuttalli/bicornis* group were only found on *E. myurus*. Small numbers of ticks belonging to the *Rhipicephalus simus/follis* group were collected from all host species but were present on *E. myurus* at lower abundances than on the other small mammals. Small numbers of *Rhipicephalus lunulatus* and ticks belonging to the *Haemaphysalis elliptica* group were also present on hosts. None of the characteristics recorded, i.e., sex, mass, and their interactions, significantly affected tick loads on *E. myurus* or *M. namaquensis* (Table II). In addition, an incidental collection of ticks found that questing on the tips of grass within Goro Game Reserve yielded 10 males and 8 females of *Rhipicephalus appendiculatus* and 5 males and 7 females of *R. sp. near warburtoni*.

### Table I. Total abundances and mean infestations (±1 standard error) of tick species or species group parasitizing small mammals.

<table>
<thead>
<tr>
<th>Species</th>
<th>M. namaquensis (n = 172)</th>
<th>E. myurus (n = 57)</th>
<th>Acomys spinnosissimus (n = 16)</th>
<th>Aethomys chrysophilus (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rhipicephalus sp. near warburtoni</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae</td>
<td>3 (0.017 ± 0.010)</td>
<td>21,645 (379.737 ± 33.843)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nymphs</td>
<td>0</td>
<td>1,084 (19.018 ± 1.335)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3 (0.017 ± 0.010)</td>
<td>22,729 (398.754 ± 33.962)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Rhipicephalus simus/follis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae</td>
<td>74 (0.430 ± 0.715)</td>
<td>2 (0.035 ± 0.025)</td>
<td>2 (0.125 ± 0.085)</td>
<td>2 (0.286 ± 0.286)</td>
</tr>
<tr>
<td>Nymphs</td>
<td>77 (0.448 ± 0.092)</td>
<td>1 (0.018 ± 0.018)</td>
<td>5 (0.313 ± 0.176)</td>
<td>3 (0.429 ± 0.297)</td>
</tr>
<tr>
<td>Total</td>
<td>151 (0.878 ± 0.126)</td>
<td>3 (0.053 ± 0.030)</td>
<td>7 (0.438 ± 0.182)</td>
<td>5 (0.714 ± 0.565)</td>
</tr>
<tr>
<td><strong>Rhipicephalus lunulatus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nymphs</td>
<td>2 (0.012 ± 0.008)</td>
<td>1 (0.018 ± 0.018)</td>
<td>0</td>
<td>2 (0.286 ± 0.286)</td>
</tr>
<tr>
<td>Total</td>
<td>2 (0.012 ± 0.008)</td>
<td>1 (0.018 ± 0.018)</td>
<td>0</td>
<td>2 (0.286 ± 0.286)</td>
</tr>
<tr>
<td><strong>Haemaphysalis elliptica</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nymphs</td>
<td>4 (0.023 ± 0.142)</td>
<td>1 (0.018 ± 0.018)</td>
<td>1 (0.143 ± 0.143)</td>
<td>1 (0.143 ± 0.143)</td>
</tr>
<tr>
<td>Total</td>
<td>4 (0.023 ± 0.142)</td>
<td>1 (0.018 ± 0.018)</td>
<td>1 (0.143 ± 0.143)</td>
<td>1 (0.143 ± 0.143)</td>
</tr>
<tr>
<td><strong>Rhipicentor nuttalli/bicornis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae</td>
<td>0</td>
<td>1 (0.018 ± 0.018)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nymphs</td>
<td>0</td>
<td>5 (0.088 ± 0.072)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>6 (0.105 ± 0.074)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown larvae</td>
<td>17 (0.099 ± 0.049)</td>
<td>0</td>
<td>1 (0.063 ± 0.063)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>19 (0.109 ± 0.135)</td>
<td>21,648 (379.790 ± 33.843)</td>
<td>3 (0.188 ± 0.101)</td>
<td>2 (0.286 ± 0.286)</td>
</tr>
<tr>
<td>Nymphs</td>
<td>83 (0.483 ± 0.094)</td>
<td>1,091 (19.140 ± 1.331)</td>
<td>5 (0.313 ± 0.176)</td>
<td>6 (0.857 ± 0.705)</td>
</tr>
<tr>
<td>Combined</td>
<td>177 (1.029 ± 0.135)</td>
<td>22,739 (398.930 ± 33.952)</td>
<td>8 (0.500 ± 0.224)</td>
<td>8 (1.143 ± 0.986)</td>
</tr>
</tbody>
</table>

* Ticks identified only to species group because of current classification ambiguity.
TABLE II. Generalized linear models investigating the effect of sex and mass on tick loads of *Elephantulus myurus* and *Micaelamys namaquensis*. Parameter coefficients ± 1 standard error; *z*-values and associated *P*-values are presented.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β coefficient</th>
<th>SE</th>
<th>z-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. myurus (n = 56)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>5.139</td>
<td>1.252</td>
<td>4.104</td>
<td>0.000*</td>
</tr>
<tr>
<td>Sex</td>
<td>−0.435</td>
<td>1.526</td>
<td>−0.285</td>
<td>0.775</td>
</tr>
<tr>
<td>Mass</td>
<td>0.019</td>
<td>0.024</td>
<td>0.763</td>
<td>0.446</td>
</tr>
<tr>
<td>Sex × mass</td>
<td>0.006</td>
<td>0.030</td>
<td>0.189</td>
<td>0.850</td>
</tr>
<tr>
<td>M. namaquensis (n = 148)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>−0.431</td>
<td>1.149</td>
<td>−0.376</td>
<td>0.707</td>
</tr>
<tr>
<td>Sex</td>
<td>−0.421</td>
<td>1.610</td>
<td>−0.262</td>
<td>0.793</td>
</tr>
<tr>
<td>Mass</td>
<td>0.010</td>
<td>0.027</td>
<td>0.394</td>
<td>0.694</td>
</tr>
<tr>
<td>Sex × mass</td>
<td>0.014</td>
<td>0.039</td>
<td>0.364</td>
<td>0.716</td>
</tr>
</tbody>
</table>

* Indicates significance at the *P* < 0.001 level.

Identification of *Anaplasma bovis* isolated from *Haemaphysalis longicornis* ticks in South Korea, domestic livestock in China, from an unknown host in South Africa, and 2 uncultured *Anaplasma* spp. from Japan. The next most closely related sequence was a strain of *A. bovis* isolated from an eastern cottontail rabbit in the United States (99.5%). The most closely related strains not belonging to *A. bovis* were *Anaplasma platys* and *Anaplasma phagocytophilum* (99.0%), while *Anaplasma marginale* and *Anaplasma ovis* were 96.0% related.

**DISCUSSION**

**Identification of tick species**

The taxonomic status and specific identification of the tick species collected from eastern rock sengis and other small mammals in this study are problematic. By making use of the morphology of immature stages, Walker et al. (2000) identified 6 species groups within *Rhipicephalus*. The *Rhipicephalus pravus* group contains 6 species, including *R. warburtoni* sensu stricto and a further 2 species that have not been described (*Rhipicephalus* sp. near *pravus* and *Rhipicephalus* sp. near *punctatus*). Based on the morphological similarities of the larvae, nymphs, and adults of this species to each other and to those of other tick species in the *R. pravus* group, we now add another undescribed species to the group. Until a thorough taxonomic study is conducted, we have chosen to name this tick *R. sp. near warburtoni*, due to its close morphological similarity to *R. warburtoni* sensu stricto, as described and illustrated by Walker et al. (2000). With the exception of *Rhipicephalus oculus*, the immature stages of the ticks within the *R. pravus* group feed on sengis (Walker et al., 2000). Because it is extremely difficult to distinguish between the immature stages of the *Rhipicephalus sinuus* and *Rhipicephalus fowlis* groups (Walker et al., 2000), as well as those of *Rhipicentor bicornis* and *Rhipicentor nuttalli* (Theiler, 1961), and of the *Haemaphysalis elliptica* group (Apanaskevich et al., 2007), the immature stages were designated to the appropriate group.

**Pathogen detection**

There are currently no known pathogen associations of *R. sp. near warburtoni*; therefore, the present study provides the first record of the presence of *A. bovis* in this tick. *Anaplasma bovis* (previously *Ehrlichia bovis*) was first described in 1936 during transmission experiments involving *Hyalomma* from Iranian and French cattle (Donatien and Lestoquard, 1936). Its presence in South Africa was confirmed in 1937 in blood and lymph node smears from cattle on which adult *Rhipicephalus appendiculatus* collected in Limpopo Province were allowed to feed (De Kock et al., 1937). Since then, it has been isolated from deer, goats, and buffalo, but most commonly domestic cattle in Africa, Brazil, Japan, and the Middle East (Uilenberg, 1997; Kawahara et al., 2006; Santos and Cavalho, 2006; Ooshiro et al., 2008). It has also been recovered from *Haemaphysalis* spp. ticks in Korea and cottontail rabbits, *Sylvilagus floridanus*, in North America (Goethert and Telford, 2003; Kim et al., 2003). The bacteria infect monocytes within the blood of their hosts, resulting in debility, fever, anorexia, and up to 40% mortality of cattle in some cases (Stewart, 1993; Santos and Carvalho, 2006). Adults of *R. warburtoni* sensu stricto feed on scrub hares, *Lepus saxatilis*, as well as on a range of large mammals, including domestic stock and wild ungulates (Walker et al., 2000). If adults of *R. sp. near warburtoni* have a similar host preference that includes domestic stock, and are competent vectors of *A. bovis*, the discovery of *A. bovis* in this tick could have important implications for livestock production and conservation.

Because only engorged nymphs were tested for the presence of *A. bovis*, it is unclear whether *E. myurus* was the reservoir host of this pathogen or if the nymphs had acquired infection while feeding on an alternative host as larvae. Given the virtual absence of this tick on the other small mammal hosts in the current study, it is unlikely that they were the source of infection. Goethert and Telford (2003) found that *Haemaphysalis leporispalustris* ticks could maintain enzootic infections of *A. bovis* in cottontail rabbits in the United States and suggested that leporids may be a natural reservoir host of this pathogen. In the present study, it is possible that *E. myurus* were simply feeding nymphs that had acquired the infection from scrub hares while feeding as larvae. Moreover, while *A. bovis* DNA was detected in engorged nymphs of *R. sp. near warburtoni*, we cannot assume that this tick species is a vector until transmission experiments are conducted using live animal models.

The geographic distribution of *R. warburtoni* sensu stricto is confined to the southwestern and central regions of Free State Province, South Africa. Within the latter region, large numbers of larvae and nymphs of this tick are found on scrub hares and eastern rock sengis (Horak and Fourie, 1991; Fourie et al., 2005), while adults are present on scrub hares and domestic and wild ruminants (Walker et al., 2000). In Limpopo Province, only the larvae and nymphs of *R. sp. near warburtoni* have been collected from eastern rock sengis, and the hosts of the adults are unknown, but may be scrub hares. Although surveys have been conducted on scrub hares in Limpopo Province, no individuals of *R. warburtoni* sensu stricto or *R. sp. near warburtoni* have been found on these animals (Horak et al., 1993), whereas other ticks belonging to the *R. pravus* group have. Therefore, the importance of scrub hares as hosts of *R. sp. near warburtoni* and their reservoir status for *A. bovis* are currently unknown.

**Tick-host relationships**

There were marked differences in the tick fauna of the host species examined in the present study, and tick loads encountered on *E. myurus* were much higher than those present on rodents, as
reported elsewhere (MacLeod, 1970; Colbo and MacLeod, 1976; Fourie et al., 1992). Whereas the underlying cause is currently unknown, there are a number of mechanisms that could explain the differences in parasitism between these hosts. *Elephantulus myurus* is a solitary species (Ribble and Perrin, 2005), while *M. namaquensis* is communal, living in family groups of up to 10 individuals (Smithers, 1971; Choate, 1972). Thus, it may be expected that an increase in sociality could lead to increased transmission rates for direct-transmitted parasites (Cote and Poulin, 1995). Social animals can also benefit from allogrooming or the dilution of ectoparasites amongst potential hosts, thus inducing reduced levels of parasitism (Marshall, 1981; Bordes et al., 2007; Hillegass et al., 2008). Differences in activity patterns may also play a role. *Micaelamys namaquensis* is nocturnal (Fleming and Nicolson, 2004), while *E. myurus* is active throughout the day and night (Ribble and Perrin, 2005). Ticks may also exhibit daily detachment rhythms (Rechav, 1978; Du Toit et al., 1994b) and may have daily activity patterns while questing (Semtnner and Hair, 1973; Belozerov, 1982; Madden and Madden, 2005). Thus, differences in the daily movements of hosts will determine the frequency with which they encounter ticks. Similarly, differences in home range size and mobility (Morand et al., 2004) or microhabitat selection may influence tick contact rates. It has been suggested that *M. namaquensis* may use trees as nest sites (Skinner and Chimimba, 2005); in this case, they would not encounter ticks questing on vegetation or from the soil surface as frequently as strictly ground-dwelling species such as *E. myurus*. Ticks also display different patterns of appetite behavior (Fourie et al., 1993), may be attracted to specific chemical compounds associated with hosts (Rechav, 1978), or to ticks that have already attached (Norval, Perry, and Young, 1992). Host immunity could also contribute to differences in tick load; some species of small mammals develop immunity to ticks (Dizij and Kurtenbach, 1995), although others, including western rock sengis, may not (Du Toit et al., 1994a).

*Rhipicephalus nuttalli* has been recovered previously from *E. myurus* in the northern provinces of South Africa (Fourie et al., 2002, 2005). *Elephantulus myurus* is thought to be an important host for the immature stages of *Rn. nuttalli* (Fourie et al., 2002). Although quite rare, this tick is potentially important because it produces a toxin that causes paralysis in domestic dogs (Perchman, 1976; Norval and Colborne, 1985). To our knowledge, immature stages of *R. lunulatus* and of the *R. simulifollis* group have not been reported on *E. myurus* previously. *Rhipechippus lumilatus* is the suspected vector of *Babesia traumanni*, the causative agent of piroplasmosis in pigs (Tendeiro, 1952, cited in Walker et al., 2000) and has been associated with toxic paralysis in lambs and calves (Theiler, 1962; Lawrence and Norval, 1979). The role of *E. myurus* in the life cycle of this tick species is unknown, but in the current study, only a single nymph was recovered. *Rhipechippus simus* transmits *Anaplasma marginale* and *Anaplasma centrale*, the causative agents of bovine anaplasmosis (Norval, Peter, and Meltzer, 1992; Jongejan and Uilenberg, 2005), and it is also a vector of *Rickettsia conorii*, the cause of Mediterranean spotted fever in humans (Gear, 1992). However, compared to the 3 rodent species examined in this study, *E. myurus* is a poor host for the larvae and nymphs of *R. simus* and is unlikely to play an important role in the life cycle of this tick.

Nothing is known about the pathogen associations of *R. fowlis* (Walker et al., 2000). Future work should focus on the screening of blood from elephant shrews in order to identify the natural reservoir of *A. bovis* in this system and on the description of *R. sp. near warburtoni*. The ability of *R. sp. near warburtoni* to transmit *A. bovis* should also be investigated. The present study documents the presence of *A. bovis* DNA in a previously unknown species, *R. sp. near warburtoni*, for the first time, providing much needed information regarding the tick-pathogen associations of small mammals in Africa. The study also highlights the importance of continued surveillance for novel wildlife reservoirs and vectors of tick-borne pathogens.

**ACKNOWLEDGMENTS**

This work was supported by a British Ecological Society Small Ecological Project Grant (2353/2909) awarded to A. Harrison and a National Research Foundation grant to I. G. Horak. We thank Nigel Bennett for access to facilities at the University of Pretoria, Heike Lutermann, Katarina Medger, and Joseph Mtiombeni for assistance in the field, and Goro Game Reserve for kindly providing access to the study site. We also thank Ian Montgomery for access to facilities at Queen’s University Belfast and for constructive discussion on the manuscript.

**LITERATURE CITED**


PRESENCE OF Ctenocephalides canis (Curtis) AND Ctenocephalides felis (BOUCHÉ) INFESTING DOGS IN THE CITY OF AGUASCALIENTES, MÉXICO

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ABSTRACT: Prevalence and seasonal distribution of Ctenocephalides canis (Curtis) and Ctenocephalides felis (Bouché) infestations in urban dogs of the city of Aguascalientes, Mexico, were studied. Between January and December 2007, 863 dogs in the Municipal Canine and Feline Control Center were examined. Overall prevalence of infestation was 12% (95% CI 10–14%). Seasonal distribution revealed that prevalences in spring and summer were highest, while autumn and winter had lower prevalences. Two infestation peaks were observed, i.e., in April (17.7%) and July (18.9%). A positive correlation was detected between prevalence and temperature during the winter season (P < 0.05). Prevalence in relation to gender showed that males were more frequently infested, 14% (95% CI 11–17), than females, 9.4% (95% CI 7–13); hair length did not affect differences in prevalence. Six hundred twenty-nine fleas were examined; 62% were C. canis and 38% C. felis. Dogs infested with only C. canis were 48% (95% CI 38–58), while 18% were infested only with C. felis (95% CI 11–27); the remainder, 34% (95% CI 24–44), had mixed infestations.

The dog flea, Ctenocephalides canis (Curtis, 1826) and the cat flea Ctenocephalides felis (Bouché, 1835), are hematophagous ectoparasites that have worldwide distribution; nevertheless, C. canis is widely associated with dogs and has a geographically restricted distribution (Marchiondo et al., 2007), while the presence of C. felis has increased and, in some areas, replaced C. canis on dogs for unclear reasons (Durden and Traub, 2002).

Flea infestation is a common problem in dogs, and it is responsible for various health problems caused by its feeding habits, such as the inoculation of allergen substances that cause pruritus, skin irritation, and eventually flea allergy dermatitis (FAD). They are also intermediate hosts of internal parasites and vectors of other microorganisms responsible for important medical and veterinary diseases (Durden and Traub, 2002; Blagburn and Dryden, 2009).

In Mexico, C. felis and C. canis are widely distributed, but their presence is more important in tropical and subtropical geographical areas where they are present throughout the year (Quintero, 1992; Cruz-Vázquez et al., 2001). However, there is no epidemiological information on these ectoparasites in temperate areas.

The purpose of the present study was to determine the prevalence and seasonal distribution of infestations by C. canis and C. felis in stray dogs within the urban area in the city of Aguascalientes, Mexico.

MATERIALS AND METHODS

The study was carried out in the city of Aguascalientes, capital of the state with the same name; it is located in the north-central region of Mexico at an altitude of 1,870 m above sea level within the coordinates 22°27' and 21°37'N latitude and 101°49' and 102°52'W longitude. The climate is semi-arid temperature, and warm with extremes; the annual mean precipitation reaches 526 mm, with a rainy season during summer.

In total, 863 dogs from the Municipal Canine and Feline Control Center of the Ministry of Public Services and Ecology of the Municipality of Aguascalientes were examined during weekly visits between January and December 2007. This Municipal Center houses stray dogs that are picked up from streets within the urban area; it also receives dogs that are voluntarily abandoned by their owners.

On each visit to the Municipal Center, 18 to 20 dogs were randomly selected for examination, and their gender and coat length were recorded. A meticulous inspection of the head, neck, body, flanks, tail, and ventral region was carried out for each animal in order to establish the presence/absence of fleas. From each positive dog, fleas (1–10) were collected and deposited in glass vials with 70% ethanol and retained until they were identified. All specimens were cleared in 10% potassium hydroxide, dehydrated through an ethanol series, transferred to xylene, and slide-mounted in Canada balsam. The species and sex identification was based on microscopic examination, using descriptions and/or taxonomic keys (Quintero, 1992; Acosta and Morrone, 2003).

Monthly data on average temperature (C) and total rainfall (mm) were obtained from the weather station located within the city of Aguascalientes (Fig. 1). The number of infested dogs per week and month were recorded in order to estimate the general prevalence, as well as seasonal distribution. Similarly, the prevalence by gender and coat type (long/short) was established. The proportions were analyzed with chi square tests (P < 0.05) in order to detect differences between them. Seasonal infestation percentages were correlated with temperature and total rainfall for each season using Pearson’s coefficient (P < 0.05).

RESULTS

Fleas were present throughout the year, with a 12% (104/863) general infestation prevalence (95% CI 10–14%). Season distribution showed higher infestation prevalence in spring and summer, while autumn and winter had the lowest prevalence (Table I). Two infestation peaks were observed, the first in April with 17.7% prevalence, and the second in July with 18.9% prevalence.

Infestation prevalence in the studied population by gender showed that males 14% (95% CI 11–17) were infested more frequently than females, 9.4% (95% CI 7–13). There was no statistically significant difference between the observed prevalence in dogs with long hair coats (10%) versus those with short hair (9%).

A total of 629 fleas was examined, of which 62% (n = 390) were C. canis (88 male and 302 female), while 38% (n = 239) were C. felis (75 male and 164 female); no other flea species was identified.

We found that 48% of the dogs (50/104) (95% CI 38–58) were infested only with C. canis, while 18% (19/104) (95% CI 11–27) possessed only C. felis. The remaining 34% (35/104) (95% CI 24–44) had mixed infestations.
No statistically significant relationship could be found between prevalence and either temperature or total rainfall in spring, summer, or autumn. Nevertheless, for winter, a positive correlation \( P < 0.05 \) was seen between prevalence and temperature; none was observed between prevalence and total rainfall.

**DISCUSSION**

Presence of *C. canis* and *C. felis* in domestic canines represents an animal health, and occasionally a public health, problem, since these fleas have characteristics in their life cycle that differentiate them from other ectoparasites, i.e., low host specificity and interrelationships with the environmental conditions in which they develop (Blagburn and Dryden, 2009).

In the present study, it was found that infestation by *C. canis* and *C. felis* occurs throughout the year, although there is a clear seasonal distribution, i.e., a higher prevalence in spring and summer, with peak infestation prevalence in April and July, and a lower prevalence in autumn–winter. Similar to this study, other investigations carried out in temperate climate regions have shown that summer is when highest infestation prevalence can be found, while prevalence is generally lower in winter (Beck et al., 2006; Rinaldi et al., 2007). Nevertheless, to our knowledge, none of these studies has reported high infestations in the spring such as that observed here. In warm and humid environmental conditions, infestation can be high throughout the year (Cruz-Vázquez et al., 2001). In the study area during the summer of 2007, average temperature reached 20°C, with an average relative humidity of 59%, while in spring the average temperature was 21°C, with an average relative humidity of 41%. Temperature and humidity are the 2 environmental parameters that overwhelmingly influence the survival, development, and reproduction of fleas. Extreme temperatures, >35°C or <3°C, together with low (<33%) relative humidity limit population development (Harwood and James, 1979; Silvermann and Rust, 1981, 1983). A positive correlation between prevalence and temperature can be seen in winter, confirming that prevalence is low during this season mainly because of the influence of environmental temperature, a finding that is in agreement with other studies that identified winter as the season with the least infestation frequency (Cruz-Vázquez et al., 2001; Durden et al., 2005; Beck et al., 2006; Rinaldi et al., 2007).

The infestation prevalence, as well as its intensity, are influenced by various environmental factors, apart from climate, which can be very different between geographical regions and localities, e.g., housing and handling of dogs can have an influence on infestation (Durden and Traub, 2002; Blagburn and Dryden, 2009). Infestation prevalence of up to 30.3% has been reported for Mexico in a warm climate area; nevertheless, the general prevalence in the present study reached 12%, which is attributable to the influence of the prevailing climatic conditions that include a semi-dry temperate climate with well defined seasons. In studies carried out in Europe, infestation prevalence ranges between 5% and 18% (Beck et al., 2006; Bond et al., 2007; Rinaldi et al., 2007; Farkas et al., 2009), while in tropical and subtropical regions in Western Hemisphere, the prevalence is considerably higher (Harman et al., 1987; Danta-Torres et al., 2009). In our study, male dogs had a higher prevalence than females, while no differences could be found relative to coat length. To our knowledge, there are no reports of infestation prevalence differences between genders; nevertheless, it is possible that male dogs included in our study had more opportunities to remain in open environments such as streets, public parks, or gardens, as well as have contact with other dogs, and, therefore, be more exposed to infestations, although this does not preclude the existence of established colonies in homes. The absence of chemical control of the infestation is probable, since these were stray dogs or animals abandoned by their owners. Other factors such as the type of housing, urban or rural environment where they live, coexistence with other dogs or cats, and zootechnical qualities have been identified as possible risk factors (Koutinas et al., 1995; Beck et al., 2006; Rinaldi et al., 2007).

Fleas were present with 12% (104/863) general infestation prevalence. The dogs infested only with *C. canis* in Aguascalientes, Mexico, reached 48%; and those with *C. felis* were 18%; while the remaining 34% of dogs had mixed infestations. Moreover, 62% of the fleas analyzed corresponded to *C. canis*, with a 1:3.4 male/female ratio, while the remaining 38% corresponded to *C. felis*, with a 1:2.2 male/female ratio. A study carried out in Cuernavaca, Mexico, reported that 81.8% of dogs were infested only with *C. felis*, 16.8% with *C. canis*, and only 2% had mixed infestations; furthermore, *C. felis* were predominant (84%) over *C. canis* (16%) (Cruz-Vázquez et al., 2001).

The presence of *C. canis* in North America is scarce, with *C. felis* being considered to have virtually displaced it (Halliwell, 1979; Harman et al., 1987; Durden and Traub, 2002; Durden et al., 2005). In contrast, in central Europe both species are present, although *C. felis* tends to be predominant (Baker and Hatch, 1972; Beck et al., 2006; Rinaldi et al., 2007), while in warm climates the absence of other ectoparasites, i.e., low host specificity and interrelationships with the environmental conditions in which they develop (Blagburn and Dryden, 2009).
climate regions of that continent, i.e., Greece, the most common species is *C. canis* (Koutinas et al., 1995). Both species have been observed in South America, with *C. felis* generally the predominant species, although not uniformly (Alcaino et al., 2002; Rodriguez et al., 2008; Dantas-Torres et al., 2009). Several authors agree that *C. canis* is most common in rural environments, whereas *C. felis* is most common in urban settings (Alcaino et al., 2002; Beck et al., 2006), although this is not consistent (Durden et al., 2005).

These observations suggest that the geographical distribution of *C. canis* can be influenced by climate and environmental factors that have not been clearly identified, but it is evident that their presence is less intense and restricted to certain geo-ecological areas. More studies are needed on this subject.

The flea male/female ratio found here, notably female skewed, has been documented elsewhere (Durden et al., 2005). This finding is probably due, along with other causes, to their need to feed, forcing them stay for longer periods of time on their host, thereby facilitating their collection and quantification in this type of study (Marshall, 1981).

In conclusion, the present study has shown that *C. canis* and *C. felis* are present throughout the year, with differing frequency, and that infestation is influenced by the climatic factors prevailing during each season. The dog flea *C. canis* predominated, although a high proportion of mixed infestations was found, which shows the polyxenous feeding capacity of *C. felis*. More studies are needed in order to better understand the biology and interrelations of these 2 flea species, as well as their geographic distribution tendencies.

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FIRST RECORDS OF KNOWN ENDOPARASITIC SPECIES OF *PSEUDEMPLEUROSOMA* YAMAGUTI, 1965 (MONOGONOIDEA: DACTYLOGRYRIDAE) OF TETRAODONTID AND RACHYCENTRID FISH OFF THE NORTHERN COAST OF THE YUCATAN PENINSULA, MEXICO

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**ABSTRACT.** Monogenoideans infecting the rectum of the wild checkered puffer fish, *Sphoeroides testudineus* (Tetraodontidae), and the pyloric ceca of the cultured cobia, *Rachycentron canadum* (Rachycentridae), from the northern coast of the Yucatan Peninsula, Mexico, were morphologically identified as *Pseudempleurosoma carangis* Yamaguti, 1965 and *Pseudempleurosoma gibsoni* Santos, Mourão and Cárdenas, 2001 (Dactylogyridae), respectively. Morphometric comparison between the paratypes of *P. carangis* and those from *S. testudineus* showed that the latter differ only in the length of the body, germarium, and dorsal anchors. Similarly, a small form of *P. gibsoni* based on body size was detected in the present study. These metric differences may be attributable to the host effect, i.e., *S. testudineus/R. canadum* versus *Caranx lugubris* (Carangidae) (type host of *P. carangis*) from Hawaii and *Paralochclus brasiliensis* (Schiæidae) (type host of *P. gibsoni*) from Brazil, or by the degree of maturity, or both. In view of these considerations, new illustrations and several supplemental observations for *P. carangis* and *P. gibsoni* are provided. The present findings also represent new geographical records, and new sites of infection, e.g., rectum and pyloric ceca, for species of *Pseudempleurosoma*, and the first known endoparasitic monogenoideans infecting tetraodontid and rachycentrid fishes in Mexico.

Most monogenoids are ectoparasites on the skin or gills of fish. However, there is a restricted number of endoparasitic monogenoideans of freshwater and marine fish, e.g., species of *Acopentor* Fischthal and Allison, 1940 in fish of the families Centrarchidae and Cyprinidae; *Diplectanotrema* Johnston and Tiesg, 1922 in Carangidae; *Enterogyrus* Paperna, 1963 on Cichlidae; *Kritskyia* Kohn, 1990 in Anostomidae, Characidae, and Prochilodontididae; *Pavaniellia* Kritsky and Boeger, 1998 in Heptapteridae; *Neodiplectanotrema* Gerasev, Gayevskaya and Kovaleva, 1994 in Cichlidae. These endoparasitic species have been reported to infect several microhabitats within the same host, such as the urodes, nose, esophagus, pharynx, and eyes (*Gerasov et al., 1987, Kohn, 1990; Bilong-Bilong et al., 1994; Boeger et al., 1995; Edward and James, 1995; Kritsky et al., 1996; Kritsky and Boeger, 1998; Viozzi and Gutiérrez, 2001; Cepeda et al., 2011*). Very few endoparasitic monogenoideans have been reported from Mexico, namely, *Enterogyrus malinbergi* Bilong-Bilong, 1988 (Dactylogyridae) from the stomach of the introduced tilapia *Oreochromis niloticus* (L.) and native cichlids (*Jiménez-García et al., 2001*, and *Pavaniellia scaphioptylus* Kritsky and Mendoza-Franco, 2003 (Dactylogyridae) from the nasal cavity of the catfish *Rhamdia guatemalensis* (Günther) (Heptapteridae) in southeastern Mexico (Kritsky and Mendoza-Franco, 2003). Here, we report for the first time the occurrence of *Pseudempleurosoma carangis* Yamaguti, 1965 from the rectum of the wild checkered puffer fish, *Sphoeroides testudineus* (Linnaeus, 1758) (Tetraodontidae), and *Pseudempleurosoma gibsoni* Santos, Mourão and Cárdenas, 2001 from the pyloric ceca of the cultured cobia *Rachycentron canadum* (Linnaeus, 1766) (Rachycentridae), both off northern coast of the Yucatan Peninsula, Mexico. New illustrations, morphometric data, and a brief discussion about the biogeography of both monogenoidean species are provided.

**MATERIALS AND METHODS**

Between March 2007 and February 2008, specimens of *S. testudineus* and *R. canadum* were captured by hook and line or casting nets from Chuburná, (21°16'0"N, 89°49'0"W), Progress (21°18'N, 90°08'W), and the Ixoye fish farm on the coast of Dzilam Bravo (21°19'N, 88°35'W), respectively, along the northern coast of Yucatan, Mexico. Fish were transported alive to the laboratory where they were killed and examined for monogenoids. Worms found were isolated and individually fixed using hot 4% formaldehyde solution and then stained with Gomori’s trichrome, mounted in Canada balsam, and examined as permanent mounts. Additional specimens were mounted unstained using Gray and Wess’s medium or a mixture of lactic acid (LA) and glycerin-ammonium picrate (GAP) to obtain measurements and line drawings of sclerotized structures; processed worms were remounted in Canada balsam (see Mendoza-Franco et al., 2009). All other measurements were obtained from unflattened specimens stained in Gomori’s trichrome. Drawings were made with the aid of a drawing attachment for a microscope (Olympus, Tokyo, Japan) with differential interference contrast microscopy. Measurements, all in micrometers, represent straight-line distances between extreme points and are expressed as the mean followed by the range and number (n) of structures measured in parentheses. Body length represents the length of the body proper with the haptor. The measurement of the copulatory organ represents the longest straight distance between the base and the end of the penis. Voucher specimens were deposited in the Colección Nacional de Helmintos, México (CNHE), Institute of Biology, National Autonomous University of Mexico, Mexico; the Parasitology Laboratory, CINVESTAV, Mérida, Mexico (CHCM); and the U.S. National Parasite Collection, Beltsville, Maryland (USNPC), as indicated. For comparative purposes, 8 paratypes (USNPC 63505) of *P. carangis* also were examined. Scientific and common names of hosts are consistent as indicated.
REDESCRIPTIONS

Pseudempleurosoma carangis Yamaguti, 1965
(Figs. 1–12)


Taxonomic summary

Host: Checkered puffer fish, Spherooides testudineus (Linneaus, 1758) (Tetraodontiformes, Tetraodontidae).

Site of infection: Rectum.

Locality/collection date: Chuburna (21°16’O, 89°49’W), Progreso (21°18’N, 90°08’W), northern coast of Yucatan, Mexico; March 2007.

Previous records: Esophasus and/or pharynx of Caranx lugubris Poey, 1860 (Perciformes, Carangidae) (type host), Caranx sexfasciatus Quoy and Gaimard, 1825 and Myrissiris berndti Jordan and Evermann, 1903 (Beryciformes, Holocentridae) from Hawaii (see Santos et al., 2001).

Specimens deposited: Three voucher specimens CNHE (6282).

Remarks

Examination of paratypes of P. carangis (USNPC 63505) and metric data for the present specimens, which have 2 pairs of dissimilar anchors, a single quadrangular dorsal bar, vitellarium divided into numerous, transversally elongate follicles, haptor truncate and shallowly constricted from the body proper, a muscular disc at the end of ootype, and a sclerotized tubular copulatory organ (Figs. 1–8), indicates that these latter specimens found on S. testudineus belong to Pseudempleurosoma and are conspecific with P. carangis. All these specimens possessed a twisted copulatory organ and an accessory piece with a proximal muscular sheath and small terminal spiniform projections (Fig. 3). Santos et al. (2001) redescribed and illustrated P. carangis based on the sclerotized structures from the copulatory complex, haptor, and reproductive system. Here, we provide a whole-mount drawing and supplemental observations of P. carangis based on comparison between its paratypes from C. lugubris from Hawaii (northern Pacific Ocean), a host species with a circumtropical distribution, including the Gulf of Mexico (see Smith-Vaniz, 1955), as well as those found in S. testudineus. Although we noted slight morphologic differences in the base of the copulatory organ (ovate vs. subspHERical) between specimens from S. testudineus (see Fig. 10) and C. lugubris (Figs. 11, 12), we do not consider this character as sufficient to designate a different species of Pseudempleurosoma. We were unable to trace the course of the vas deferens and the vaginal opening (“close to genitral atrium, at level of the muscular opening of uterus” in Santos et al., 2001) in all specimens; the prostatic reservoir at base of the copulatory organ was only visible in a few specimens (Fig. 9). Present specimens metrically differ from those paratypes of P. carangis only in the size of the body length (695–862 vs. 848–1,304), germarium (47–57 vs. 48–90) (Figs. 9–12), dorsal anchors (43–49 vs. 48–55), and by the infection site on host (rectum vs. esophagus and pharynx), different hosts, and geographical distribution. These differences found in the length of the body, germarium, and anchors of specimens of P. carangis (see measurements in this study) may reflect the environmental influences placed on the parasite by their host (S. testudineus vs. C. lugubris), reflect the maturity degree of worms, i.e., most type specimens from C. lugubris were gravid with a larger uterus (Figs. 11, 12), or both. Similar morphometric variation has been observed among specimens of other monogeneoid species, e.g., Uroleoides spp. (Dactylogyridae) infecting the gills of the freshwater host species Hoplias malabaricus (Bloch, 1794) (Characiformes) from Brazil (Rosim et al., 2011). The present finding represents a new site of infection (rectum) for the host and a new locality record for P. carangis.

Pseudempleurosoma gibsoni Santos, Mourão and Cárdenas, 2001
(Figs. 13–23)

Supplemental observations (measurements based on 14 specimens stained [small form in brackets] with Gomori’s trichrome and on 19 specimens removed from LA and GAP mixture): Body 1,384 (1,100–1,630; n = 8) (695 [550–810]; n = 6) long, greatest width 261 (245–290; n = 8) (205 [165–262]; n = 6). Pharynx spherical 73 (63–84; n = 8) (51 [39–65]; n = 6) in diameter. Haptor 102 (81–112; n = 4) (91) wide. Ventral anchors 13 (12–14; n = 16) long, base 8 (7–13; n = 11) wide; each one with two ventral bars: attached (on deep root) ventral bar 18 (15–21; n = 8) long, free ventral bar 18 (16–21; n = 7) long. Dorsal anchor 51 (45–55; n = 38) (48 [46–49]; n = 3) long, base 31 (28–34; n = 21) (31) wide. Dorsal bar 14 (11–17; n = 15) (11) long, 17 (14–20; n = 15) (12) wide. Hooks similar, each with elongate depressed thumb, curved shaft and fine point, slightly dilated proximally; FH loop one-third shank length; hooks 13 (13–14; n = 20) long. MCO 48 (40–60; n = 18) (36 [30–40]; n = 3) long. Accessory piece 9 (n = 19; n = 11) long, 19 (19–20; n = 43 [19–40]; n = 4) long, 72 (51–89; n = 8) (49 [40–62]; n = 4) wide. Testis 62 (57–70; n = 6) (36 [32–40]; n = 5) long, 41 (33–45; n = 7) (36 [26–48]; n = 4) wide. Ootype gives rise to uterus, ovate, with thick wall which ends as muscular disc of 21 (25) long, 24 (23 [19–27]) wide. Egg subovate with short filament, 69 long, 48 wide.

Taxonomic summary

Host: Cobia Rachycentron canadum (Linneaus, 1766) (Periformes, Rachycentridae).

Site of infection: Pyloric ceca.

Locality/collection date: Ixoye fish farm from the coast of Drizamo Bravo in the Yucatan Peninsula; 8 February 2008.

Previous record: Esophasus of Paralonchurus brasiliensis (Periformes, Sciaenidae) from the coast of Ubatuba, Sao Paulo, Brazil (see Santos et al., 2001).

Specimens deposited: Thirty-three voucher specimens CNHE (8082), CHCM (250), USNPC (104569).

Remarks

Shape and size of features of present specimens from P. canadum ranged accordingly to those of P. gibsoni from their original description from P. brasiliensis from Brazil (Santos et al., 2001), confirming their conspecificity with the latter monogeneoid species. All these specimens from P. canadum from Mexico and those of P. gibsoni from P. brasiliensis distinctly possess a tubular MCO that is not twisted on its midportion and an egg with a short filament, specific characteristics for P. gibsoni. Although our specimens of P. gibsoni are smaller than those of Santos et al. (2001), all other features were similar in proportion. The present finding represents a new site of infection, e.g., pyloric ceca, for the host and locality record of P. gibsoni.

DISCUSSION

There is not doubt that our specimens from S. testudineus and R. canadum from the northern coast of the Yucatan Peninsula represent 2 known species of Pseudempleurosoma, i.e., P. carangis and P. gibsoni, respectively. Without considering new host records, it is noteworthy that the present specimens were found in new sites of infection, i.e., rectum and pyloric ceca versus gills, esophagus, and pharynx, on their respective hosts. The present study indicates the same monogeneoid species infects different sites of different host species occurring in distant geographical areas. It suggests that diversification of Pseudempleurosoma spp. has lagged behind that of their hosts because speciation is not currently evident, at least with respect to the comparative morphology of P. carangis and P. gibsoni.

gibsoni from Mexico, Hawaii, and Brazil. Other techniques, i.e., molecular analyses, would be useful in determining possible cryptic differences in the populations of these monogenoids. High speciation rates in monogenoids has been observed at a much faster pace when high numbers of potential new hosts are available and vice versa (see Mendoza-Franco et al., 2004).

Consistent with this observation, a study of new potential hosts, i.e., carangids and sciaenids, of *Pseudempleurosoma* spp. is
Figures 9–12. *Pseudempleurosoma carangis* from different host species. (9–10) Enlargement of individual specimens at level of reproductive organs from *Sphoeroides testudineus* (9, ventral; 10, dorsal composite). (11–12) Enlargement of type specimens (USNPC 63505) at level of reproductive organs from *Caranx lugubris* (11, ventral; 12, dorsal composite). Scale bars in μm. Ba, base of the copulatory organ; Md, muscular disc; Pr, prostatic reservoir; Ut, uterus; Vi, vitellaria.

necessary to evaluate speciation and to understand the diversification pattern of these endoparasitic forms in the region (see Remarks for *P. carangis*). For example, *P. carangis* and *P. gibsoni* seem to parasitize unrelated and geographically distant host species, i.e., *S. testudineus* (Tetraodontidae), *C. lugubris* *C. sexfasciatus* (Carangidae), *Myripristis berndti* (Holocentridae), and *R. canadum* (Rachycentridae), whereas *P. brasiliensis* occurs in sciaenids. It is unclear which modes of colonization have produced the current zoogeographical distribution of these monogenoids. It is known that carangid and sciaenid hosts are naturally dispersed in the Gulf of Mexico and the Caribbean Sea; thus, the possibility exists that *P. carangis* invaded *S. testudineus* or vice versa in Mexico by host switching. Although carangid host species have not been reported as host of any species of *Pseudempleurosoma* in the Gulf of Mexico, further sampling could support this hypothesis, e.g., the occurrence of *Pseudempleurosoma* spp. from tetraodontid (*S. testudineus*) and carangid fishes (*C. lugubris*). Thus, the morphometric differences showed 2 different size classes for *P. carangis*. The deliberate introduction, accidental introduction, or both of these hosts, along with their monogenoideans, into the fish farms could favor transmission of these worms to new hosts. This latter notion would explain, in part, the occurrence of *P. gibsoni* in the cultured *R. canadum* in the north of Yucatan Peninsula.

An alternative for the presence of *P. carangis* in both the Pacific (Hawaiian Islands) and Atlantic oceans (off the Yucatan Peninsula) is that the original distribution of the ancestors of scobrids, and probably carangids, could have been continuous while the Tethys Sea was open. However, with the closure of the Tethys Sea 20 mya, this continuity could have been interrupted due to the convergence of Africa and Eurasia and, in turn, isolated the Indo-Pacific region from the Atlantic. A similar explanation for the monogenoideans and parasitic copepods of scobrid fishes has been suggested by Rohde and Hayward (2000).

To date, there are 4 species of *Pseudempleurosoma*, namely, *P. carangis* (type species) from *C. lugubris*, *C. sexfasciatus* (Carangidae), and *M. berndti* (Holocentridae) off Hawaii, and *S. testudineus* (Tetraodontidae) (present study); *Pseudempleurosoma caranxi* (Gerassov, Gayevskaya and Kovaleva, 1987) Santos, Mourão and Cárdenas, 2001 from *Caranx ruber* (Bloch, 1793) off Cuba; *P. gibsoni* from *P. brasiliensis* from Brazil and *R. canadum* (Rachycentridae) (present study); and *Pseudempleurosoma myripristi* (Gerassov, Gayevskaya and Kovaleva, 1987) Santos, Mourão and Cárdenas, 2001 from *Myripristis jacobus* Cuvier, 1829 (Holocentridae) off Cuba (see Santos et al., 2001). Santos et al. (2001) considered *Metadiplectronotrema* as 2 valid species, *M. caranxi* and *M. myripristi*, as a junior synonym of *Pseudempleurosoma* based on presence of 2 linked bars to each ventral anchor (1 anchor attached and the other anchor detached), a long polar filament on the eggs, and a larger dorsal bar. Another species of *Pseudempleurosoma*, apparently new (but
not illustrated or formally described), has been reported from the esophagus of the deep sea fish *Hoplichthys citrinus* Gilbert (Scorpaeniformes, Hoplichthyidae) from the Chesterfield Islands (Coral Sea, approximately halfway between New Caledonia and Australia; see Rehulková et al., 2009). Currently accepted species of *Pseudempleurosoma* share very similar features, i.e., a thick tegument with transverse ridges, 2 pairs of dissimilar anchors, a single quadrangular dorsal bar, a truncate haptor and shallowly constricted from the body proper, a muscular disc at the end of ootype, a sclerotized tubular copulatory organ, and 7 pairs of similar hooks with a protruding thumb and slender shaft. However, the number of ventral and dorsal bars associated with the anchors seems to be a controversial diagnostic characteristic. For example, 3 dorsal bars instead of 1 (as amended by Santos et al., 2001) have been reported in undescribed species of *Pseudempleurosoma* found on *Chlorophthalmus punctatus* Gilchrist, 1904 (Chlorophthalmidae) and *Hoplichthys acanthopleurus* Regan, 1908 (Hoplichthyidae) from the southwestern Indian Ocean off the coast of East Africa, and *Sillago ingenua* McKay, 1985 (Sillaginidae) from the coast of Indonesia and Malaysia (Hayward, 1997; Lim, 1998; Santos et al., 2001). Clearly, this latter characteristic should be verified, preferably based on newly collected fresh material, because in many cases, type specimens have been destroyed, lost, or are not available from collections (see Kritsky et al., 2004; Chisholm and Whittington, 2007; Mendoza-Franco et al., 2008). Moreover, specimen assignment from new findings depends on a comparison with respective published accounts, e.g., as for *P. caranxi* (see Santos et al., 2001). Currently, geographic distribution of species of *Pseudempleurosoma* along with their hosts (Carangidae, Chlorophthalmidae, Holocentridae, Hoplichthyidae, Rachycentridae, Sciaenidae, Sillaginidae, and Tetraodontidae) ranges from the western Indian Ocean, e.g., East Africa, to subtropical and tropical areas (southern Mexico, Cuba, and Brazil). Among all other endoparasitic monogenoidean genera included here, *Pseudempleurosoma* is the only genera reported here with species parasitizing tetraodontid and rachycentrid fish in the Gulf of Mexico.

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**LITERATURE CITED**


NEW BENEDEANIA SPECIES (MONOGENEA: CAPSALIDAE) FROM DIAGRAMMA LABIOSUM (PERCIFORMES: HAEMULIDAE) ON THE GREAT BARRIER REEF, AUSTRALIA, WITH ONCOMIRACIDIAL DESCRIPTIONS AND A REPORT OF EGG ATTACHMENT TO THE HOST

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ABSTRACT: The slate sweetlips, Diagramma labiosum Macleay, 1883 (Perciformes: Haemulidae), off Heron Island, Great Barrier Reef, Queensland, Australia, hosts 2 new species of Benedenia Diesing, 1858 (Monogenea: Monopisthocotylea: Capsalidae). Benedenia beverleyburtoniae n. sp. infects proximal regions of the primary gill lamellae and gill arches. The adult is characterized by a dorsal vaginal pore anterior to the common genital pore and a voluminous, highly coiled vas deferens. This species also has fine muscle fibrils concentrically arranged in the haptor. Its ciliated oncomiracidium differs little from larvae of other Benedenia species, with the exception of at least 4 gland cells containing a granular secretion on each side of the body at the level of the excretory bladders, with fine ducts opening anterior to the eyes. Benedenia disciliata n. sp. infects gill arches and gill rakers, and the adult is characterized by its small total length, anterior hamuli with a small proximal notch, posterior hamuli with a broad, triangular shape proximally, a conspicuous internal fertilization chamber, and asymmetrical eggs. None of the 5 specimens collected had testes, but their definite absence is undetermined. Their absence and the fact that the vas deferens was discernable only distally in the specimens of B. disciliata examined may represent atrophying of the male organs. Benedenia disciliata is unique among Benedenia species because eggs are attached to the host’s gill arches by tight wrapping of the appendages around spines on the gill rakers and the larva is not ciliated. Among Capsalidae species, these features are shared only with species of Dioncus Goto, 1899. Recent molecular evidence has indicated Benedenia is polyphyletic, but with no clear morphological characters available to divide the genus, the 2 new species fit the current concept for the genus more closely than other capsalid genera. Proposal and description of these taxa and accounts of their oncomiracidia and other aspects of their biology indicate potentially useful characters for division of the genus in the future.

In April 1989, examination of the gills and gill rakers of slate sweetlips Diagramma labiosum Macleay, 1883, from Heron Island, Great Barrier Reef, Queensland, Australia, in collaboration with Professor Mary Beverley-Burton (then of the Department of Zoology, University of Guelph, Ontario, Canada) revealed specimens of a species of Benedenia Diesing, 1858. During a subsequent visit to Heron Island, further study of D. labiosum specimens showed this fish species hosted a second Benedenia species. The checklist of parasites from Heron Island by Lester and Sewell (1989) lists only “Capsulidae 2 spp.” attributed to Klaus Rohde from this fish species as Diagramma pictum (Thunberg; see Kuiter, 1993). A species of Metabenedeniella Yamaguti, 1958, reported from the dorsal fin of D. labiosum (as D. pictum) was described by Horton and Whittington (1994). The present paper reports 2 new species of Benedenia from the gills, gill arches, and gill rakers of D. labiosum off Heron Island, one named in honor of Professor Beverley-Burton. Availability of live adults provided an opportunity to collect eggs and hatch and describe larvae. Attachment of eggs to the host by 1 of the new species is also reported.

MATERIALS AND METHODS

Live specimens of D. labiosum caught by handline at Heron Island Research Station of the University of Queensland at the southern end of the Great Barrier Reef were identified using Grant (1987) and Randall et al. (1990). Protocols to examine fish, collect, and prepare Monogenea for study follow Horton and Whittington (1994). Measurements made using an eyepiece micrometer are presented in micrometers as the range followed by the mean in parentheses and the number of measurements. Terminology for anatomical structures and hooklet numbering follows Deveney and Whittington (2010). Eggs collected from adult specimens and hatched oncomiracidia were handled as described in Whittington et al. (1994). Larval anatomy was drawn free-hand from live specimens, while photography contributed to recording shapes of median haptor sclerites.

Collections in which new taxa are deposited are denoted as follows: NHMUK, Natural History Museum, Cromwell Road, London, SW7 5BD, U.K. (contact: Eileen Harris, e.harris@nhm.ac.uk); QM, Worms, Biodiversity Program, Queensland Museum, P.O. Box 3300, South Brisbane, Queensland 4101, Australia (contact: Robert Adlard, Robert.Ad@qm.qld.gov.au); SAMA, Australian Helminthological Collection (AHC) of the South Australian Museum, Parasitology Section, Adelaide, South Australia 5000, Australia (contact: Leslie Chisholm, leslie.chisholm@samuseum.sa.gov.au); USNPC, United States National Parasite Collection, United States Department of Agriculture, Agricultural Research Service, Building 1180, BARC-East, 10300 Baltimore Avenue, Beltsville, Maryland 20705-2350 (contact: Eric Hoberg, eric.hoberg@ars.usda.gov, or Pat Pilitt, Pat.Pilitt@ars.usda.gov).

RESULTS

Two separate collections of specimens of D. labiosum (April 1989; November–December 1992) caught directly from waters of the lagoon of Heron Island (23°27’S, 151°55’E) and dissected within 4 hr of capture revealed natural infections by 2 previously uncharacterized Benedenia species.

DESCRIPTIONS

Benedenia beverleyburtoniae n. sp. (Figs. 1–8)

Diagnosis of adult (based on type series of 15 partially flattened, preserved, stained, and mounted specimens collected in April 1989, some studied alive by phase contrast microscopy before preservation; 32 heavily flattened, preserved, unstained, and mounted voucher specimens collected in November–December 1992, and some studied alive by phase contrast microscopy before preservation, also contributed to the description and drawings): Benedenia Diesing, 1858 sensu Whittington et al., 2001, Benedeninae Johnston, 1931 sensu Whittington et al., 2001 (Capsalidae Baird, 1853 sensu Yamaguti, 1963). Total length including haptor 1,030–1,756 (1,425) (n = 11); maximum
FIGURES 1–5. *Benedenia beverleyburtonae* n. sp. (1) Whole animal, ventral view (composite drawing mostly from type specimens). Bar = 250 μm. (2) Accessory sclerite. (3) Anterior hamulus. (4) Posterior hamulus. Bars for 2, 3, and 4 = 50 μm. (5) Schematic diagram of anterior extremity (dotted box in Fig. 1) showing elevated anterior protuberances of body proper and associated gland cells (gc) dorsal and median to anterior attachment organs visible clearly only in live specimens. Abbreviations: a, male accessory gland reservoir; aa, anterior attachment organ; ah, anterior hamulus; as, accessory sclerite; b, excretory bladder; bm, body musculature; cp, common genital pore; cm, concentric muscle fibrils; e, pigmented eye; g, germarium; G, gland of Goto; h, hooklet; m, marginal valve; ml, marginal lobe; o, ootype; p, pharynx; pe, penis; ph, posterior hamulus; t, testis; te, tendon; v, vagina; vd, vas deferens; vf, vitelline follicle; vl, vaginal lobe; vp, vaginal pore; vr, vitelline reservoir.

breadth 363–810 (531) (n = 12) at level of testes (Fig. 1). Haptor slightly elliptical, 365–690 (508) long (n = 12); 381–604 (503) wide (n = 12) (Fig. 1). Accessory sclerite 74–114 (95) long (n = 17), slightly curved with pointed distal tip (Fig. 2). Anterior hamulus 66–110 (96) long (n = 20), distal tip rounded with subterminal, ventrally directed, spike-like process at right angles to shaft (Fig. 3); proximal shaft of anterior hamulus not overlapping posterior (proximal) tip of accessory sclerite (Fig. 1). Posterior hamulus 68–93 (78) long (n = 19), with hooked distal tip (Fig. 4). Fourteen hooklets at haptor periphery, each 7–9 (8) long (n = 10). Tendons from extensive body musculature passing through proximal notch of accessory sclerites (Fig. 1). Fine concentric muscle fibrils in haptoral tissues (Fig. 1). Marginal valve scalloped with consistent number of lobes between hooklets on each side of haptor (n = 3 specimens): 1 large lobe between hooklets of pair II on posterior border of haptor; 1 large lobe between hooklets II and distal...
FIGURES 6–7. _Benedenia beverleyburtonae_ n. sp. (6) Reproductive system, ventral view. Vitellarium (except vitelline reservoir and associated ducts), gut and excretory bladders omitted. Bar = 200 μm. (7) Egg (full appendage length not shown). Bar = 50 μm. Abbreviations: a, male accessory gland reservoir; aa, anterior attachment organ; ag, duct from male accessory gland reservoir; cp, common genital pore; e, pigmented eye; G, gland of Goto; g, germarium; ic, internal (fertilization?) chamber of germarium; M, ducts of Mehlis' gland; ma, ducts of male accessory gland; ml, marginal lobe; o, ootype; ov, ovovitelline duct; p, pharynx; ps, penis sac; sr, seminal receptacle; sv, seminal vesicle; t, testis; u, uterus; v, vagina; vd, vas deferens; vl, vaginal lobe; vp, vaginal pore; vr, vitelline reservoir.

Position of posterior hamuli; 1 large lobe between distal position of posterior hamuli and hooklets III; 1 large lobe between hooklets III and IV and between hooklets IV and V; 4 or 5 smaller lobes between hooklets V and VI; 7 to 8 small lobes between hooklets VI and VII; 10 or 11 small lobes between hooklets VII and VIII; 10 small lobes on anterior border of haptor between hooklets of pair VIII (Fig. 1).

Anterior attachment organs approximately circular or elliptical, 97–205 (168) long n = 22, 77–184 (116) wide (n = 22); 3 distinct adhesive zones (reported in other _Benedenia_ species, e.g., Whittington and Kearn, 1993; Whittington et al., 1994; Deveney and Whittington, 2010) at anteroventral region (Fig. 1). Pair of anterior median gland cells observed in live specimens, each on elevated anterior protuberance, dorsal to median region of body proper between attachment organs (Fig. 5); difficult to locate in preserved material. Pharynx 87–151 (110) long (n = 10), 103–214 (131) wide (n = 10). Two pairs of eyespots, dorsal, between anterior margin of body and pharynx; lense remnants usually associated with pigment cups in live specimens, but lenses seem to disappear on preservation. Gut caeca branched, mostly obscured by vitellarium; unclear whether caeca unite posteriorly.

Large glands of Goto in posterior angle between testes (Figs. 1, 6). Testes 145–235 (198) long (n = 24), 96–185 (130) wide (n = 24). Vas deferens swollen slightly to form seminal vesicle between testes and germarium. Vas deferens forms voluminous coils immediately anterior to vitelline reservoir filling available space around seminal receptacle (Figs. 1, 6). Vas deferens narrows before looping, dorsal to distal region of ootype, to enter penis canal dorsally distal to male accessory gland reservoir. Vas deferens and duct from male accessory gland reservoir spiral together along length of penis, joining near distal tip (Figs. 1, 6). Wall of penis canal slightly thickened. Male accessory gland reservoir filled with granular contents occupies proximal end of penis canal. Single tract of fine male accessory gland ducts entering proximal end of penis canal (Figs. 1, 6). Penis muscular, protrusible via common genital duct and submarginal, dorsal common genital pore. Two prominent marginal lobes on left body margin at level of pharynx ventral to common genital pore (Figs. 1, 6).

Germarium globular, compact, with internal fertilization chamber from which oviduct arises to pass dorsal to vitelline reservoir into area between proximal region of penis canal and voluminous vas deferens (Fig. 6). Oviduct receives common vitelline duct from dorsal aspect of vitelline
reservoir-transverse vitelline duct. Anteriorly, ovovitelline duct receives several fine ducts from Mehls' glands. Ootype comprises thin-walled, coiled proximal region and bulbous, thick-walled, muscular distal region. Uterus short opening into penis canal (Fig. 6). Vaginal pore simple, on left side of body, dorsal to posterior half of left anterior attachment organ; single lobe adjacent to vaginal pore, dorsal to attachment organ (Fig. 6).

Vagina narrow, tubular, sinuous, runs posteriorly passing dorsal to penis canal. In some specimens, vagina accommodating sperm. In 1 specimen (SAM A paratype AHC 35109), remains of what appears to be a spermatophore visible. Posteriorly, seminal receptacle partially obscured by voluminous coils of vas deferens; proximal vaginal connection to vitelline reservoir not observed. Vitelline reservoir of variable size. Vitelline follicles extend from level anterior to eyes to posterior end of body proper, coextensive with gut. Eggs tetrahedral (Fig. 7), sides 76–109 (92) from (n = 7) free eggs; appendage from 1 non-opercular pole slender, sinuous, in excess of 1,000 long from (n = 7) free eggs.

Observations on behavior of live specimens: Specimens on gill arches in Petri dishes containing sea water during parasite collection observed to undulate as described for the capsalid Entodobella soleae (Van Beneden and Hesse, 1864) Johnston, 1929, by Kearn (1962).

Embryonation period (at 25 C) of freely deposited eggs: Five to 6 days; hatching occurred spontaneously.

Description of oncomiracidium (based on 10 live specimens): See Figure 8. Total length 152–210 (189) (n = 3). Haptor selerite lengths: accessory selerite 15–16 (n = 11); anterior hamulus 19–23 (21) (n = 7); posterior hamulus 17–20 (19) n = 10; hooklet 8–10 (9) (n = 19). Tendons associated with proximal notch of accessory selerites not seen. Ciliated epidermal cells containing small refringent droplets bearing locomotory cilia in 3 zones: 1 zone on head, 1 zone on posterior region of body proper, 1 zone on posterior region of haptor. General anatomy including number and distribution of flame bulbs (Fig. 8) resembles in most respects larvae of Benedenia serialia (Yamaguti, 1934) Meserve, 1938 (compare to Kearn et al., 1992), Benedenia lutjani Whittington and Kearn, 1993 (see Whittington and Kearn, 1993), and Benedenia rohdei Whittington, Kearn and Beverley-Burton, 1994 (see Whittington et al., 1994). Differences between oncomiracidia of B. beverleyburtonae and larvae of other Benedenia species mostly concern glandular elements. In addition to lateral head glands containing needle-like secretion, larva of B. beverleyburtonae bears at least 4 other lateral head glands containing granular secretion on each side of body at level of excretory bladders; fine ducts lead anteriorly, terminating in at least 4 swellings (Fig. 8). Additional lateral head glands containing granular secretion not reported for larvae of B. serialia, B. lutjani, and B. rohdei. Median granular secretions at anterior extremity more conspicuous than in B. lutjani and B. rohdei. Granular secretion reported in posterior larval body for B. serialia and B. rohdei and in haptor. In larvae of B. serialia, B. lutjani, and B. rohdei not observed in oncomiracidium of B. beverleyburtonae. Glands of Goto, described for larvae of B. lutjani and B. rohdei, not observed.

Taxonomic summary

Host: Slate sweetlips, Diagramma labiosum Macleay, 1883 (Perciformes: Haemulidae).

Type locality: Off Heron Island, Queensland, Australia (23°27’S, 151°55’S E).

Specimens: Type material collected in April 1989. Holotype QM G 211388 (1 specimen, 1 slide). Paratypes QM G 211389-211390 (2 specimens, 4 slides), NHMUK 31151-31152 (4 specimens, 4 slides); SAM A HHC 35109-35112 (4 specimens, 4 slides); USNPC 104734 (1 specimen, 1 slide), USNPC 104735 (3 specimens, 3 slides). Voucher material collected in November–December 1992: SAM A HHC 35113 (field no. TH92-12; 14 specimens, 14 slides); SAM A HHC 35115 (field no. TH92-19; 15 specimens, 15 slides); SAM A HHC 35114 (field no. TH92-28; 3 specimens, 3 slides).

Infection details: In April 1989, each of 2 fish (100%) examined (total length range 53.5–59.5 cm) were infected with up to 20 specimens of B. beverleyburtonae. In November–December 1992, each of 3 fish (100%) examined (total length range 34–49 cm) were infected with up to 15 specimens.

Etymology: The name beverleyburtonae honors Mary Beverley-Burton (erstwhile of the Department of Zoology, University of Guelph, Guelph, Ontario, Canada) for her contributions to monogenean systematics and who was involved in discovering this species during a visit to Heron Island with the senior author last century (April 1989).

Remarks

Of the 25 described species of Benedenia, 3 possess a vagina that opens anterior to the common genital pore like that reported for B. beverleyburtonae: see 4a and couples 5 and 6 in “Key to the described species of Benedenia” in Deveney and Whittington (2010); Benedenia acanthopagri (Hussey, 1986) Whittington, Deveney, and Wyborn, 2001; Benedenia anticauvigatinae Byrnes, 1986; and B. lutjani. Only B. anticauvigatinae and B. lutjani possess a dorsal vaginal pore as described for B. beverleyburtonae, but neither shares a conspicuous, highly coiled vas deferens, a consistent feature in all specimens from several host individuals. Benedenia anticauvigatinae has posterior hamuli invested in muscle fibers (see Fig. 9H in Whittington et al., 2001) and a single large lobe associated with the common genital pore and vaginal pore (see Fig. 10B in Whittington et al., 2001), neither of which is reported for B. beverleyburtonae or B. lutjani. Benedenia lutjani and B. beverleyburtonae are similar because in addition to a dorsal vaginal pore anterior to the common genital pore, these species are of similar size, have similarly shaped median haptoral sclerites, and possess a pair of marginal lobes associated with the common genital pore and a single dorsal lobe near the vaginal pore (compare descriptions in Whittington and Kearn, 1993, and present study). Since its description predominantly from pelvic fins of Lutjanus carponotatus (Richardson, 1842), larger adult specimens of B. lutjani were recovered from the branchiostegal membranes of the same host species (see Table V in Whittington et al., 2001, for comparative measurements). Benedenia lutjani is slightly larger for all dimensions provided by Whittington et al. (2001) than B. beverleyburtonae, especially the anterior attachment organs: length, 173–332 (204) versus 97–205 (168); width, 151–319 (243) versus 77–184 (116), respectively. Benedenia beverleyburtonae adults are best characterized by a combination of the dorsal vaginal pore anterior to the common genital pore, a voluminous, highly coiled vas deferens that fills the region around the seminal receptacle and fine haptoral muscle fibers arranged concentrically, which are more apparent in some specimens than others. Benedenia beverleyburtonae is the first Benedenia species described from the Haemulidae (Perciformes), although Whittington et al. (2001; see their Table VIII) drew attention to a Benedenia sp. from the “epidermis covering the premaxillae” of 1 specimen of Haemulon scirius (Shaw, 1803) (Haemulidae) off Bermuda (Cone and Beverley-Burton, 1981).

The cited oncomiracidium of B. beverleyburtonae differs little from larval types of other Benedenia species. We report at least 4 gland cells containing granular secretion on each side of the body with fine ducts that open anterior to the eyes, which appear to be unique (compare with larval descriptions for B. serialia, B. lutjani, and B. rohdei by Kearn et al., 1992, Whittington and Kearn, 1993, and Whittington et al., 1994, respectively). While glands of Goto were noted in adult specimens, they were not observed in the larva of B. beverleyburtonae (compare Fig. 8 with larval drawings for B. lutjani by Whittington and Kearn, 1993, and for B. rohdei by Whittington et al., 1994).

Benedenia discillata n. sp.

(Figs. 9–15)

Diagnosis of adult (based on 5 heavily flattened, preserved, unstained, and mounted adult type specimens): Benedenia Diesing, 1858 sensu Whittington et al., 2001, Benedeniinae Johnston, 1931 sensu Whittington et al., 2001 (Capsalidae Baird, 1853 sensu Yamaguti, 1963). Total length including haptor 592–1,030 (840) (n = 5), maximum breadth 324–520 (395) (n = 5); at level of vitelline reservoir (Fig. 10). Haptor slightly elliptical 209–288 (236) long (n = 5), 189–233 (216) wide (n = 5) (Fig. 10). Accessory selerite 43–64 (52) (n = 10) long; broad, rounded proximal region; stout with tapering distal tip (Fig. 11). Anterior hamulus 43–61 (50) long (n = 10) tubular, with small proximal notch, distal tip strongly recurved (Fig. 12); proximal selerite of anterior hamulus ending in a hooklet 8–10 long (n = 5), posteriorly rounded, with posterior (proximal) tip of accessory selerite (Fig. 10). Posterior hamulus 40–56 (46) long (n = 10) with broad, triangular proximal region, strongly recurved distal tip (Fig. 13). Fourteen hooklets at haptor periphery, each 7–9 (9) long (n = 10). Tendons entering haptor from body musculature passing through proximal notch of accessory selerites. Median valvule scalloped bearing small lobes, but detail of number, arrangement, and relative size of lobes undetermined due to damage.

Benedenia discillata n. sp.
Anterior attachment organs slightly elliptical, 57–98 (71) long (n = 8), 97–161 (112) wide (n = 10); 3 adhesive zones at anteroventral region of each organ (Whittington and Kearn, 1993; Whittington et al., 1994; Deveney and Whittington, 2010) (Fig. 10). Pair of small gland cells at anterior body margin often obscured ventrally by anterior attachment organs (Fig. 10). Pharynx 59–102 (84) long (n = 4), 79–158 (119) wide (n = 4). Two pairs of eyespots, dorsal, just anterior to pharynx. Gut caeca branched, largely obscured by vitellarium; unclear whether caeca unite posteriorly.

Glands of Goto not observed. Testes not observed despite all 5 type specimens appearing to be sexually mature adults characterized by presence of vitelline follicles and egg in ootype of 2 specimens. Region of body where testes and glands of Goto expected well provided with vitelline follicles. Vas deferens observed only approaching penis canal in region to left of ootype; contents sparse; passing dorsal to distal region of ootype, entering penis canal dorsally at level of anterior region of ootype. Vas deferens travels posteriorly to proximal end of penis canal, turning through 180° to pass toward distal tip of penis. Wall of penis canal slightly

**Figures 8-9.** Anatomy of the oncomiracidia of (8) *Benedenia beverleyburtonae* n. sp. and (9) *B. disciliata* n. sp. Whole larvae, ventral view. Bars = 50 μm. Abbreviations: alg, additional lateral gland cells with granular contents; ah, anterior hamulus; am, anterior median head gland; as, accessory sclerite; b, excretory bladder; c, cilia; ce, ciliated epidermis; d, domus; e, pigment-shielded eye; f, flame bulb; h, hooklet; i, intestine; l, lateral head glands; le, lens of eye; o, oesophageal gland; p, pharynx; ph, posterior hamulus; pm, posterior median head gland; rd, refringent droplet.
thickened. Male accessory gland reservoir slender, inconspicuous, with dense, fine, darkly granular contents, located near proximal end of penis canal (Fig. 10). Single tract of few, fine male accessory gland ducts entering proximal end of penis canal (Fig. 10). Duct from male accessory gland reservoir and vas deferens travel side by side along length of penis joining close to distal tip (Fig. 10). Penis protrusible via common genital duct and submarginal, dorsal common genital pore at left body margin on slight protuberance, posterior and lateral to left anterior attachment organ. A second slight lateral protuberance present, anterior to site where common genital pore opens, but no marginal lobes observed (cf., Figs. 6, 10).

Germarium globular, compact; conspicuous internal fertilization chamber containing large prominent oocytes, from which oviduct arises, right of vitelline reservoir, travels anteriorly towards ootype (Fig. 10). Transverse vitelline duct and common vitelline duct not observed. Mehlis’ glands not observed. Ootype with long, coiled proximal tubular region
constricting anterior to vitelline reservoir before joining small seminal
appendages (arrow) on appendages expanding into thin-walled distal chamber. Uterus short, opening into
of 3 eggs (eg). Note the egg appendages (arrow) that encircle the
egg bunches attached to gill arches (Figs. 16–18). Bunches of eggs could not be dislodged easily, and removal from gill spines required
either breaking egg appendages or slipping appendage loops from “spine.”
Many eggs in most attached bunches contained active, fully embryonated
oncomiracidia bearing prominent pigmented eyes (Fig. 18). Fully embryonated egg bunches kept in dishes of clean sea water hatched spontane­ously.
On collection, some egg bunches contained few empty shells with opercula missing, indicating hatching had taken place on host.

Embryonation period (at 23–24 °C) for unembryonated eggs removed from host gills: Approximately 6 days.

Description of oncomiracidium (based on 5 live specimens; see Fig. 9): Total length 179–248 (213) (n = 2). Haptor sclerite lengths: accessory sclerite 16–18 (17) (n = 4); anterior hamulus 18–20 (20) (n = 4); posterior hamulus 20 n = 4; hooklet 9 (n = 9). Tendons associated with proximal notch of accessory sclerites not seen. General anatomy including number and distribution of flame bulbs (Fig. 9) resembles larvae of B. lutjani, B. rohdei, and B. seriolae (see above). Additional lateral head glands containing granular secretion and anteromedian granular secretion reported in larva of B. beverleyburtonae not observed. No secretion observed posteriorly in body and haptor. Most important difference between oncomiracidium of B. disciliata and all previously described larvae of Benedenia species is absence of epidermal cells bearing locomotory cilia. Unciliated hatched larvae capable of leech-like looping movements across glass surfaces. Pigment cup of posterior eyes of B. disciliata with conspicuous, posteriorly directed spine.

**Taxonomic summary**

**Host:** Slate sweetlips, *Diagramma labiosum* Macleay, 1883 (Perciformes: Haemulidae).

**Site:** Gill arches and gill rakers.

**Type locality:** Off Heron Island, Queensland, Australia (23°27'S, 151°55'E).

**Specimens:** Holotype QM G 211383 (1 specimen, 1 slide). Paratypes QM G 211384–211387 (4 specimens, 4 slides).

**Infection details:** In November 1992, 2 of 3 fish (67%) examined (total length range 34–49 cm) infected: 1 with 3 specimens; I with 2 specimens. This parasite species was not encountered in April 1989.

**Etymology:** The name *disciliata*, derived from the Latin *dis* meaning “without” and *ciliata* meaning “cilia,” refers to the unciliated oncomiracidium of this species (Fig. 9), the first *Benedenia* species reported to have a non-ciliated larva.

**Remarks**

Of the 26 previously described species of Benedenia, *Benedenia fieldi* Deveney and Whittington, 2010, and *B. disciliata* are the smallest (mean total length <1 mm). No specimens of *B. disciliata* bearing testes were collected, and their definite absence is undetermined. The vas deferens had sparse contents detectable only distally. If specimens with testes are encountered, other features that distinguish this species include its small total length, anterior hamuli with a small proximal notch (Fig. 12), posterior hamuli with a broad, triangular proximal region.
conspicuous internal fertilization chamber, and an asymmetrical egg shape when turned through 90° (Figs. 14, 15). Egg attachment to the host and the unciolated oncomiracidium are unique biological features among all capsalids except for species of Dioncus Goto, 1899 (see Whittington, 1990; Bullard et al., 2000).

Note our use of Dioncus, not Dionchus Goto, 1899, which deserves clarification at this point. Dionchus agassizii Goto, 1899, was proposed as a new genus and new species (Goto, 1899). Yamaguti (1963) used Dioncidae Bychowsky, 1959, and Dioncus, providing footnotes stating “correct transliteration” without further comment. Goto (1899) gave no etymology for his new genus, but it is presumably derived from the Greek, di, for 2, and onkos, for hook based on “1 pair of strong hooks” on the haptor. The cestode taxa Onchobothrium Blainville, 1828, and Onchobothriidae Braun, 1900, similarly derived, received comparable treatment without comment by Yamaguti (1959), and Oncobothrium and Oncobothriidae are adopted (e.g., Schmidt, 1986). Wardle and McLeod (1952, p. 54) pointed out that the oncosphere of tapeworms is derived from the Greek onkos for tumor or swelling and “is sometimes, though erroneously, spelled ‘oncho-sphere.”’ Oncospheres also bear 3 pairs of hooks. Whether derived from swelling or hook, the Greek root, onkos, is spelled without an h in English, as onco (Brown, 1956, p. 572). Oncomiracidium, proposed for the hooked larval stage of Monogenea by Llewellyn (1957), is occasionally misspelled as onchomiracidium. We trust this explains the actions of Yamaguti transliteration”.

Each new species may indicate potentially useful oncomiracidial characters and details that may be valuable for future revisions of Benedictina to divide the genus, we follow the generic concept for Benedictina of Whittington et al. (2001) in describing these 2 new species. Anatomical features of adults such as 3 distinct adhesive zones on the anterodorsal surface of each anterior attachment organ, anteromedian gland cells located dorsally in the body proper, e.g., B. beverleyburtonae and B. discilata, whether the proximal ends of the anterior hamuli overlap the proximal ends of the accessory sclerites (see Fig. 9 in Whittington et al., 2001), and the presence of fine concentric muscle fibrils in the haptor, e.g., B. beverleyburtonae may prove fruitful characters to explore in future studies to divide the genus. The opportunity to describe the larvae of the 2 new taxa contributes additional morphological characters and details that may be valuable for future revisions of Benedictina and closely related capsalid genera. Additional lateral head glands containing granular secretion observed in the larva of B. beverleyburtonae and the absence of glands of Goto in larvae of each new species may indicate potentially useful oncomiracidial features on which to focus.

Of the 4 Benedictina species that possess a vaginal pore anterior to the common genital pore, only B. anticavaginata and B. acanthopagiri were included in the molecular phylogeny of Perkins et al. (2009), but these species were not each other’s closest relative. This supports the suggestion by Whittington et al. (2001) that the vaginal pore position in Benedictina species and, indeed, among capsalids is a specific morphological variable displaying a continuum of variation that may have evolved independently on several occasions (refer to Fig. 12 in Whittington et al., 2001).

Diagramma labiosum is the third teleost species reported from Heron Island to host 2 species of Benedictina. The lutjanid L. carponotatus (Richardson, 1842) is infected by B. lutjani on body surfaces, especially the pelvic fins and branchiostegal membranes (Whittington and Kearn, 1993; Whittington and Ernst, 2002), and by B. rodeoi on the gills (Whittington et al., 1994). Another lutjanid, Symphysorus nematophorus (Bleeker, 1860), is host to Benedictina ernstii Deveney and Whittington, 2010, from the gills and Benedenia haywardi Deveney and Whittington, 2010, from the skin (Deveney and Whittington, 2010). In each of these species pairs, except those described in the current paper, the Benedictina species are reported from different microhabitats on the same host species. It is also noteworthy that the location of the vaginal pore reported in each Benedictina pair differs markedly (cf. Whittington and Kearn, 1993; Whittington et al., 1994; Deveney and Whittington, 2010). Benedictina beverleyburtonae and B. discilata also display some spatial segregation on the gills (proximal gill lamellae, gill arches, and gill rakers versus gill rakers only, respectively). Diagramma labiosum at Heron Island also hosts the capsalid Metabenedeniella parva Horton and Whittington, 1994, on the dorsal fin (Horton and Whittington, 1994) and other undescribed capsalids from external surfaces (I. Whittington, pers. obs.).

Deveney and Whittington (2010) drew attention to larger specimens of B. fieldsi possessing small or no testes, with atrophy of the male reproductive system a likely cause of absence in individuals from dense parasite populations on serranid hosts in captivity. No individuals of B. discilata possessing testes were found. Like other capsalids, presumably B. discilata is protandrous, and, if so, atrophy of the male reproductive system was observed at low infection intensity. Degeneration of the male system does not seem to affect the reproductive biology of B. discilata; all 5 specimens observed were sexually mature, capable of laying viable eggs, and 2 preserved specimens had an egg in the ootype.

The oncomiracidium of B. discilata is not ciliated, cannot swim, and presumably hatches from eggs attached to the spines of the host’s gill rakers (Figs. 16–18). In the Capsalidae, only Dioncus species share non-swimming larvae hatching from eggs attached to the host. Whittington (1990) reported that eggs of Dioncus remorae (MacCallum, 1916) Price, 1938, are firmly anchored to primary gill lamellae of its remora host, Echeneis naucrates L., and that hatching occurred in situ on gills releasing unciiliated larvae. Bullard et al. (2000) proposed a life cycle for Dioncus species based on the recovery of postoncomiracidia from the skin of a shark that supported remoras. The low infection intensity of B. discilata raises the possibility that this species may lay 2 types of eggs that release unciiliated and ciliated larvae as reported for the polyopisthocotylean, Eupolystoma alluaudi (de Beachamp, 1913) Euzet and Combes, 1967, by Fournier and Combes (1979). In E. alluaudi, it was suggested that unciiliated larvae hatch from eggs to autoinf ect their anuran host when infrapopulation density is low, whereas ciliated larvae emerge to disperse broadly throughout a host population when the parasite infrapopulation is high and crowded (Kearn, 1986). Further study may demonstrate whether B. discilata lays 2 types of eggs that hatch to release unciiliated and ciliated larvae related to parasite population density or whether its oncomiracidia are always unable to swim. If the latter is the case, how new hosts are infected remains unknown, but cleaner wrasse, Labroides dimidiatus Valenciennes, 1839, may be appropriate vectors to transmit specimens between individual D. labiosum attending cleaner stations. Chisholm et al. (1996) reported that the polyopisthocotylean monogenean, Tonkinopsis transfretanus.
Lebedev, 1972, which also infects gills of *D. labiosum* at Heron Island, also lay eggs that release unciliated larvae, but, as far as is known, its eggs are not attached to the host. This suggests that the behavior of this haemulid species may be conducive under some circumstances to invasion by non-swimming, crawling monogenean larvae from freely deposited eggs of *T. transfreterans*, as well as eggs of *B. disciliata* attached to gill rakers so that their larvae can gain access to, and infect, the host gills. Why larvae of *B. beverleyburtonae* have a distinctly different invasion strategy via swimming oncomiracidia is unknown.

**ACKNOWLEDGMENTS**

The senior author IDW thanks Professor Mary Beverley-Burton (formerly of the University of Guelph, Ontario, Canada) and Dr. Rob Adlard (Queensland Museum) for comradeship and field experiences at Heron Island Research Station (HIRS) in April 1989. Marnie Horton provided field assistance in November–December 1992. The Director and staff of HIRS provided excellent field facilities for this research. Tim Benson, Leslie Chisholm, and Marnie Horton prepared mounted material. We thank Eileen Harris (NHMUK), Rob Adlard and Mal Bryant (QM), Leslie Chisholm (SAM), and Pat Plitt (USNPC) for accessioning museum material. Funding was provided by Australian Research Council grants (nos. A19130175 for 1992–1994 and A19231545 for 1993–1995) awarded to IDW and DP0556780 awarded to IDW and Professor Steve Donnellan of SAM for 2005–2007. MRD is supported by the Marine Innovation South Australia (MISA) Biosecurity node.

**LITERATURE CITED**


DEVELOPMENT OF ASCAROPHIS SP. (NEMATODA: CYSTIDICOLIDAE) TO MATURITY IN GAMMARUS DEUBENI (AMPHIPODA)

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ABSTRACT: Experimentally transmitted Ascarophis sp. (Spirurida) developed to adult worms in the invertebrate host, Gammarus deubeni (Amphipoda), collected in the intertidal zone in Passamaquoddy Bay, New Brunswick, Canada. The morphological development and growth of larval stages is very similar to other cystidicolids, which are found as adults in fish. Unlike virtually all other Spirurida, which require a vertebrate definitive host, infective larvae of Ascarophis sp. migrate from the invertebrate host musculature into the hemocoel where they molt twice to become adults. Gravid females appear at 80 days and 69 days post-infection at 10–12 C and 18–20 C, respectively. While there is little evident host reaction to the parasite within the muscle tissue, within the hemocoel there is hemocytic reaction to shed nematode cuticles, released eggs, and sometimes the worm itself, including some melanization. The worms are morphologically similar to Ascarophis sp. from G. oceanicus in the Baltic and White seas and among Ascarophis species from fish is most similar to A. arctica. It is suggested that Ascarophis sp. no longer requires a vertebrate host and is transmitted between amphipods either through death and disintegration of infected amphipods and dispersal of the nematode eggs, or more likely through cannibalism or necrophagy.

Parasitic nematodes of the order Spirurida are with some rare exceptions heteroxenous; adult worms are present in a vertebrate host, and infective third-stage larvae develop in various arthropods (Anderson, 2000). The Cystidicolidae (Nematoda: Habronematoida), for instance, are typical spirurids adapted to an aquatic habitat. Adult worms are present in the digestive tract or swim bladder of fishes, and infective third-stage larvae develop in crustaceans or aquatic insects (Moravec, 2007). Among the cystidicolids, Ascarophis is the predominant genus that utilizes marine fishes as the definitive host and is widely distributed in littoral, to deep, ocean fishes (see Ko, 1986; Moravec, 2007; Moravec and Klimpel, 2009).

While the heteroxenous life cycle of Ascarophis sp. has been deduced from the records (Fagerholm and Butterworth, 1988), in some instances, sub-adult and adult specimens of Ascarophis have also been found in crustacean hosts (Petter, 1970; Tsimbalyuk et al., 1970; Valter and Valovaya, 1987; Valter et al., 1987; Fagerholm and Butterworth, 1988; Valter and Valovaya, 1990; Jackson et al., 1997a, 1997b). In particular, the advanced stage of Ascarophis sp. has been documented in the wild from Gammarus oceanicus Segerstrale, 1947 (Amphipoda) in the White Sea (Valter et al., 1987), from G. oceanicus from the Baltic Sea, and Gammarus spp. from the Canadian Atlantic (Fagerholm and Butterworth, 1988; Jackson et al., 1997a). The present study documents the development of Ascarophis sp. to maturity in experimentally infected amphipod G. deubeni Liljeborg, 1851, and describes the morphogenesis and migration of the parasite within its host.

MATERIALS AND METHODS

Infected and uninfected Gammarus deubeni (Amphipoda) used in experimental studies were collected in February 1983, intertidally in Passamaquoddy Bay, New Brunswick, Canada, and air shipped to the University of Guelph, Ontario, Canada, where they were kept at 10–12 C in aquaria containing water of approximately 15% salinity. Amphipods infected with Ascarophis sp. were collected intertidally at localities where 2 small streams enter Brandy Cove. Uninfected G. deubeni were collected where Pottery Creek enters the intertidal zone in Passamaquoddy Bay.

Gammarus species collected intertidally at Pottery Creek were previously found to be free of natural infections with cystidicolid larvae (Appy and Dadswell, 1983). Thirty G. deubeni collected in Pottery Creek during the present study were also free of natural infections. In addition, juvenile G. deubeni stripped from the marsupium of adult female amphipods from Pottery Creek were raised in isolation. When these amphipods reached a length of approximately 3 mm, they were also used in experimental infections.

Uninfected adult amphipods were placed individually in vials containing freshly collected eggs of Ascarophis sp. Juvenile amphipods were placed en masse into a watch glass containing eggs of Ascarophis sp. After 12 hr, amphipods were removed from vials or watch glasses and placed in aquaria at either 10–12 C or 18–20 C. Amphipods were examined at various intervals after infection (Table I; Fig. 1). Nematodes collected from experimentally infected amphipods were killed in hot glycerin-alcohol and cleared in glycerin. A few first-stage larvae were vitally stained in a drop of saline on a microscope slide, which had been smeared with a drop of 1% methylene blue and air dried. Specimens used for scanning electron microscopy were fixed in 1.25% gluteraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in an ethanol series, critical point dried, and viewed with a Jeol (Model JSM 35-C) scanning electron microscope. For histology, amphipods were fixed in Carnoy’s as modified by DeGiusti and Ezman (1955), embedded in paraffin, sectioned at 6 μm, and stained with Delafield’s hematoxylin and eosin. Measurements and drawings were made with the aid of a camera lucida. Unless stated otherwise, measurements are given in micrometers (μm) and are of specimens fixed in hot glycerin-alcohol.

RESULTS

Development inside amphipods

All 5 stages of Ascarophis sp. were present in experimentally infected G. deubeni. At 10–12 C, 89% of 31 amphipods examined at 1 to 100 days post-infection (PI) were infected with a mean of 9.9 (1–35) nematodes. At 18–20 C, 69% of 39 amphipods examined at 5 to 72 days PI were infected with a mean of 5.3 (1–16) nematodes. At 10–12 C, size of larvae increased after the first molt (Fig. 1). The increase in size continued thereafter in only those worms that continued molting. Some third- and fourth-stage larvae apparently did not molt immediately after reaching maximum size but remained as third- and fourth-stage larvae up to 80 and 100 days PI, respectively (Fig. 1; Table I), resulting in significant overlap in the presence of third- and fourth-stage larvae and adult worms. Female worms with eggs containing larvae were first observed at 80 days PI. At 18–20 C, the pattern of growth rate was generally similar to that at 10–12 C, with molts occurring more rapidly (Table I). However, despite the more rapid molts and initial rapid growth, fifth-stage nematodes kept at the warmer temperature were
and muscular and glandular esophagus is faintly visible at the level of conical point. Two posterolateral branches of the excretory system are visible. The genital primordium is present at approximately 72% of the body length and is composed of approximately 12 cells. The elevation, with a minute tooth-like refractile structure. The esophagus and at approximately 29% of the body length from the anterior end. There is only a single row of hypodermal cells extending anteriorly and posteriorly from the attachment site of the primordium to the hypodermis. The posterior end is usually concave on one side. The female genital primordium is attached to the hypoderms at its mid-point; both the anterior and posterior extremities of the primordium are narrow. The mucron, which is in various stages of development, is visible beneath the second stage cuticle of the tail.

**First-stage larva (Fig. 2; Table II)**

The anterior end of early first-stage larvae have a small conical elevation, with a minute tooth-like refractile structure. The buccal cavity is threadlike. The esophagus is narrow, except at the club-shaped extremities; there is a slight bend and swelling present just posterior to the nerve ring. The excretory cell is located at the posterior third of the esophagus and connected to the excretory pore by a short, anteriorly directed duct. The excretory pore is located at approximately the mid-point of esophagus and at approximately 29% of the body length from the anterior end. The intestinal lumen is lined by a narrow wall with only a few cells. The anal opening is located at approximately 86% of body length from the anterior end. There is a single genital cell and 3 rectal cells visible between the anus and the level of intestinal lumen. The rectum is narrower and difficult to discern. The tail narrows abruptly and ends in a sharp conical point.

**Larva at the second molt (Figs. 6, 7, 24, 25)**

A distinct junction of the muscular and glandular esophagus is present at the level of the excretory pore. The ratio of the muscular to the glandular esophagus is a mean of 1:1.8 (1:1.1-1:2.4) (n = 8). The male genital primordium is not visibly attached to the hypoderms. The posterior end of the primordium is wider than the anterior end, and a prominent terminal cell at the posterior end is usually concave on one side. The female genital primordium is attached to the hypoderms at its mid-point; both the anterior and posterior extremities of the primordium are narrow. The mucron, which is in various stages of development, is visible beneath the second stage cuticle of the tail.

**Third-stage larva (Figs. 8-12, 26, 27, 30; Table II)**

The cuticle has transverse striations. The anterior end has 4 cephalic papillae and 2 lateral amphids. The mouth is dorsoventrally elongate and has poorly defined submedian labia. The pseudolabia are well developed, and each has a prominent anterior conical process. The medial portion of the pseudolabia is slightly wider than the anterior portion. The sub-labia are well developed; sometimes each sub-labium has a slight indentation in the mid-apical surface. The walls of the buccal cavity are well sclerotized and dilated dorsoventrally behind the oral opening. A slight flange is present in the wall of the buccal cavity where it joins the muscular esophagus. Small deirids are present midway between the level of the nerve ring and the level of the anterior margin of the muscular esophagus. The nerve ring and excretory pore are present at approximately 25 and 41% of the length of the muscular esophagus, respectively. The tail has a prominent mucron.

In male worms the ratio of lengths of muscular to glandular esophagus is a mean of 1:2.8 (1:2.0-1:3.4) (n = 10). The genital primordium is elongate; the anterior portion is narrow, is directed anteriorly, and is sometimes convoluted; the posterior portion of the primordium is wider than anteriorly. Primordial cells of the spicular pouch are visible on the dorso-lateral margins of the rectum. The tail is relatively long. In female worms, the ratio of lengths of the muscular to the glandular esophagus is a mean of 1:3.0 (1:2.1-1:3.8) (n = 9). The primordium of the vagina vera is a single row of hypodermal cells extending anteriorly and posteriorly from the attachment site of the primordium to the hypoderms. The genital primordium is attached to the hypoderms at a mean of 64% (59-69%) (n = 9) of the total body length from anterior end. The tail is relatively short.

**Larva at the third molt (Figs. 13-17, 28)**

In male worms, the ratio of the lengths of the muscular to the glandular esophagus is a mean of 1:3.3 (1:3.0-1:3.5) (n = 3). The testes is variable in length and, in some cases, extends to the posterior third of the body. Spicule pouches are visible, with spicule primordial cells present at the anterior margin of the forming pouch. The tail is slightly bent ventrally. In female worms, the ratio of the muscular to the glandular esophagus is a mean of 1:3.4 (1:2.9-1:3.7) (n = 4). The rudiments of the vagina vera are present at the attachment site of the genital primordium.

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**Table I.** Comparison of development of Ascarophis sp. in Gammarus deubeni held at 10-12°C and 18-20°C and minimum longevity of larval stages in G. deubeni at 10-12°C.

<table>
<thead>
<tr>
<th>Stage*</th>
<th>Minimum longevity</th>
<th>Days PI†</th>
<th>Minimum longevity</th>
<th>Days PI†</th>
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<tbody>
<tr>
<td>L1</td>
<td>1-16</td>
<td>14</td>
<td>1-14</td>
<td></td>
</tr>
<tr>
<td>ML1</td>
<td>20-24</td>
<td>6</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>20-25</td>
<td>5</td>
<td>14-18</td>
<td></td>
</tr>
<tr>
<td>ML2</td>
<td>25-33</td>
<td>8</td>
<td>18-20</td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td>33-80</td>
<td>47</td>
<td>18-60</td>
<td></td>
</tr>
<tr>
<td>ML3</td>
<td>46-70</td>
<td>24</td>
<td>40-52</td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>46-100</td>
<td>&gt;54</td>
<td>40-61</td>
<td></td>
</tr>
<tr>
<td>ML4</td>
<td>59-100</td>
<td>&gt;41</td>
<td>45-61</td>
<td></td>
</tr>
<tr>
<td>L5</td>
<td>64-100</td>
<td>&gt;36</td>
<td>52-81</td>
<td></td>
</tr>
<tr>
<td>Gravid‡</td>
<td>80</td>
<td></td>
<td>69</td>
<td></td>
</tr>
</tbody>
</table>

* L = larva; M = molting; 1-5 = development stage.
† PI = post-infection.
‡ Female with larvated eggs.
The ovaries are variable in length, extending to the anterior and posterior third of the body. The tail is straight.

**Fourth-stage larva (Table II)**

Worms have prominent striations. The sub-labia have well-defined apical notches forming 2 lobes. A well-developed flange is present in the buccal cavity wall at its junction with the muscular esophagus. The nerve ring and excretory pore are present at approximately 29 and 51% of the length of the muscular esophagus, respectively. The tail is bluntly rounded and sometimes has a slight terminal depression.

In male worms, the ratio of the length of the muscular to the glandular esophagus is a mean of 1:3.9 (1:3.3–1:4.9) ($n = 7$). The genital primordium is attached to the rectum. The spicules are in various states of formation and are slightly sclerotized; the left spicule is usually longer than the right. Rudiments of the caudal papillae are sometimes visible. The tail is relatively long and usually has a slight ventral curl.

In female worms, the ratio of the lengths of the muscular to the glandular esophagus is a mean of 1:4.1 (1:3.8–1:4.4) ($n = 8$). The vagina uterina is present in various stages of development. The anterior and posterior ovaries are present near the level of the glandular esophagus and the anus, respectively. A non-patent vulva is present at a mean of 63% (57–68%) ($n = 8$) of the total body length. The tail is relatively short and straight or bent slightly dorsally.

**Larva at the fourth molt (Figs. 18–22a, 29)**

In male worms, the seminal vesicle has sperm. The spicules are the same as in adult worms, but more lightly sclerotized. The area rugosa, caudal papillae, and phasmids are visible under the fourth-stage cuticle. The caudal end is in 2 or 3 coils. In female worms, the reproductive system is complete, although the vulva is not patent and the uterus is without eggs or ova. The tail is often bent dorsally.

**Fifth-stage worms (Fig. 22b; Table II)**

Worms have prominent striations. The nerve ring and excretory pore are present at approximately 26 and 49% of the length of the muscular esophagus.

In male worms, the ratio of lengths of muscular to glandular esophagus is a mean of 1:5.3 (1:4.9–1:5.7) ($n = 5$). The spicules are moderately sclerotized. The ratio of the right to the left spicule is a mean of 1:6.1 (1:5.5–1:6.6) ($n = 5$). The ratio of the left spicule shaft to the blade is a mean of 1:1.8 (1:1.5–1:1.9) ($n = 5$). The area rugosa and 4 pairs of precloacal and 6 pairs of postcloacal papillae are present. Phasmids are present just posterior to the last 2 pairs of postcloacal papillae. The caudal end is coiled ventrally. The tail is bluntly rounded.

In gravid female worms, the ratio of the lengths of the muscular to the glandular esophagus is a mean of 1:4.8 (1:4.0–1:5.6) ($n = 5$).
Table II. Dimensions (in micrometers) of *Ascarophis* sp. in experimentally infected *G. deubeni* held at 10-12°C.

<table>
<thead>
<tr>
<th>Sample size</th>
<th>L1</th>
<th>L2</th>
<th>L3M</th>
<th>L3F</th>
<th>L4M</th>
<th>L4F</th>
<th>L5M</th>
<th>L5F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days post-infection</td>
<td>13, 16</td>
<td>20, 25</td>
<td>33-80</td>
<td>33-80</td>
<td>46-100</td>
<td>46-100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Total length†</td>
<td>219 (156-275)</td>
<td>738 (580-938)</td>
<td>1,772 (812-2,585)</td>
<td>1,777 (1,481-2,140)</td>
<td>2,690 (2,369-3,385)</td>
<td>3,679 (2,900-4,340)</td>
<td>4,909 (4,710-5,050)</td>
<td>8,844 (7,460-9,700)</td>
</tr>
<tr>
<td>Maximum width</td>
<td>17 (10-22)</td>
<td>31 (26-36)</td>
<td>37 (27-49)</td>
<td>32 (26-37)</td>
<td>54 (44-66)</td>
<td>61 (45-106)</td>
<td>88 (84-95)</td>
<td>136 (118-159)</td>
</tr>
<tr>
<td>Buccal cavity length</td>
<td>29 (25-35)</td>
<td>32 (27-37)</td>
<td>66 (50-79)</td>
<td>64 (53-70)</td>
<td>72 (64-82)</td>
<td>83 (71-95)</td>
<td>98 (91-102)</td>
<td>111 (95-125)</td>
</tr>
<tr>
<td>Deirid‡</td>
<td>—</td>
<td>—</td>
<td>78 (56-94)</td>
<td>72 (60-79)</td>
<td>83 (73-91)</td>
<td>94 (78-106)</td>
<td>106 (101-111)</td>
<td>118 (96-136)</td>
</tr>
<tr>
<td>Nerve ring‡</td>
<td>47 (43-55)</td>
<td>80 (72-92)</td>
<td>108 (94-131)</td>
<td>99 (77-109)</td>
<td>121 (111-130)</td>
<td>129 (115-137)</td>
<td>152 (144-157)</td>
<td>161 (144-177)</td>
</tr>
<tr>
<td>Excretory pore‡</td>
<td>64 (53-88)</td>
<td>101 (92-109)</td>
<td>136 (92-175)</td>
<td>130 (111-152)</td>
<td>160 (150-170)</td>
<td>165 (151-176)</td>
<td>203 (193-217)</td>
<td>205 (176-229)</td>
</tr>
<tr>
<td>Muscular esophagus length</td>
<td>—</td>
<td>68 (60-81)</td>
<td>135 (116-171)</td>
<td>135 (120-147)</td>
<td>150 (138-168)</td>
<td>178 (157-204)</td>
<td>188 (172-202)</td>
<td>221 (209-237)</td>
</tr>
<tr>
<td>Glandular esophagus length</td>
<td>—</td>
<td>117 (78-148)</td>
<td>377 (293-488)</td>
<td>403 (282-528)</td>
<td>576 (480-687)</td>
<td>694 (556-785)</td>
<td>1,001 (840-1,110)</td>
<td>1,087 (860-1,265)</td>
</tr>
<tr>
<td>Total esophagus length</td>
<td>87 (78-96)</td>
<td>185 (161-211)</td>
<td>511 (427-659)</td>
<td>538 (407-670)</td>
<td>730 (640-827)</td>
<td>890 (789-989)</td>
<td>1,189 (1,012-1,304)</td>
<td>1,238 (1,069-1,490)</td>
</tr>
<tr>
<td>Genital primord/gonad length</td>
<td>6 (3-9)</td>
<td>33 (20-45)</td>
<td>466 (197-908)</td>
<td>345 (123-910)</td>
<td>—</td>
<td>2,264 (1,850-2,470)</td>
<td>—</td>
<td>4,830 (4,390-5,320)</td>
</tr>
<tr>
<td>Vulva‡</td>
<td>—</td>
<td>—</td>
<td>1,118 (716-1,385)</td>
<td>—</td>
<td>—</td>
<td>108 (103-114)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Right spicule length</td>
<td>—</td>
<td>—</td>
<td>50 (24-72)</td>
<td>—</td>
<td>—</td>
<td>108 (103-114)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Left spicule length</td>
<td>—</td>
<td>—</td>
<td>150 (44-307)</td>
<td>—</td>
<td>—</td>
<td>657 (617-690)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tail length</td>
<td>31 (18-39)</td>
<td>37 (32-44)</td>
<td>40 (27-60)</td>
<td>30 (24-34)</td>
<td>51 (25-64)</td>
<td>27 (23-34)</td>
<td>111 (95-119)</td>
<td>34 (30-38)</td>
</tr>
<tr>
<td>Egg length</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>535 (50-57)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Egg width</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>25§ (23-26)</td>
<td>—</td>
</tr>
</tbody>
</table>
FIGURES 13–22. Developmental stage of Ascarophis sp. in G. deubeni kept at 10–12°C. Drawings are in lateral view unless stated otherwise. (13) Anterior end of larva at the third molt. (14) Posterior extremity of male larva at third molt. (15) Posterior extremity of female larva at third molt. (16) Anterior extremity of larva at third molt. (17) Anterior extremity of larva at third molt, ventral view. (18) Anterior extremity of larva at fourth molt. (19) Anterior extremity of larva at fourth molt, ventral view. (20) Anterior end of larva at fourth molt. (21) Posterior extremity of male larva at fourth molt. (22a) Posterior extremity of female larva at fourth molt. (22b) Egg containing larva. Abbreviations: ar, area rugosa; d, deirid; Is, left spicule; ph, phasmid; pop, postcloacal papillae; prp, precloacal; rs, right spicule.
Figures 23-29. Development of the reproductive system of *Ascarophis* sp. in *G. deubeni* kept at 10-12 C. Drawings are in lateral view. (23) Genital primordium at the first molt. (24) Male genital primordium at the second molt. (25) Female genital primordium at second molt. (26) Anterior (a) and posterior (b) portion of the genital primordium of a male third-stage larva. (27) Anterior (a), middle (b), and posterior (c) portions of the genital primordium of a female third-stage larva. (28) Vaginal primordium of a female larva at third molt. (29) Vagina of female worm at fourth molt.
Form both poles (Fig. 22b). The tail is short, straight, and with an amphid; bc, buccal cavity; cp, cephalic papilla; pl, pseudolabia; sl, submedian labium; sml, submedian labium.

A patent vulva is present at 55% (52–59%) (n = 5) of the total body length. The egg-filled uterus takes up most of the posterior 75% of body. The eggs containing larvae have filaments extending form both poles (Fig. 22b). The tail is short, straight, and with a button present at the tip.

**Histopathology (Figs. 31–41)**

At 1 day PI, first-stage larvae are present in muscle tissues in various parts of the amphipod, particularly the muscles surrounding the digestive tract in the posterior dorsal region. Larvae usually lie parallel to the muscle fibers (Fig. 31). No visible host reaction to first-stage larvae is evident within the muscle, although muscle fibers appear frayed and disrupted adjacent to the larvae (Fig. 32).

At 11 days PI, larvae are still present within the muscle bundles, and a space exists between the larvae and the surrounding muscle tissue (Fig. 33). Host cells within the muscle bundle often appear degenerate and muscle fibers stain more weakly with eosin than adjacent uninfected muscle fibers. By 21 to 28 days PI, an enlarged space is sometimes evident around the larvae, and the host muscle cells are often pyknotic (Figs. 34, 35). At 38 days PI, infected muscle bundles are occupied by third-stage larvae, pockets of degenerating host nuclei, and very little host muscle fiber (Fig. 36). A thin host tissue wall containing presumptive muscle cell nuclei is present around the larvae. Third-, and more advanced, stage larvae are present in the hemocoel of the amphipods (Fig. 37). The intestinal contents of these worms are often orange in color, as are lipid deposits within the amphipod.

Host hemocytes are also present in the intestinal lumen of the nematodes. Within the hemocoel, masses of hemocytes and melanin-like deposits are present around the shed larval cuticles (Fig. 38). Live fourth-stage larvae may be present within shed third-stage cuticles, which have been surrounded by hemocytes and presumptive melanin deposits (Fig. 39). Hemocyte conglomerations are also present on sinus walls adjacent to worms in the hemocoel, and throughout the body wherever nematode eggs are present (Figs. 40, 41). No first- or second-stage larvae were ever found in the hemocoel. Infrequently, dead, or moribund, worms are present in amphipods. These worms usually have masses of hemocytes attached to the cuticle and melanin-like deposits around the anus and oral opening. Dead adult worms covered in melanin deposits have been found in natural infections of *G. deubeni*.

**DISCUSSION**

A heteroxenous life cycle in *Ascarophis* spp. has been deduced from the records of infective larvae in crustaceans (see Fagerholm and Butterworth, 1988; Martorelli et al., 2000) and experimental studies on other cystidicolid nematodes, including *Salmonema* (Moravec, 1971), *Cystidicola* (Black and Lancaster, 1980), *Capillospirura* (Appy and Dadswell, 1983), and *Spinicicuta* (Jilek and Crites, 1982). There is also evidence of the predisposition of *Ascarophis* spp. and other cystidicolidis to undergo precocious development to fourth-stage or sub-adults in the intermediate host (see Fagerholm and Butterworth, 1988, for a summary; see also Jackson et al., 1997a, 1997b). However, to date, *Ascarophis* sp. appears to be the only cystidicoloid that is regularly found as adult in an invertebrate host. Valter et al. (1987) identified adult *Ascarophis* sp. from *Gammarus oceanicus* Seegerstrale, 1947 (Amphipoda) in the White Sea, while Fagerholm and Butterworth (1988) described ovigerous *Ascarophis* sp. from natural infections of *G. oceanicus* in the Baltic and *G. deubeni* in eastern Canada. Valter and Valovaya (1990) experimentally infected *G. oceanicus* from the White Sea with *Ascarophis* sp., observed the third and fourth molt, and obtained 1 adult male and 3 adult females. They also exposed an isopod, *Jaera ischiotetosa* Forsman, 1949, but worms did not develop beyond the third stage. More recently Jackson et al. (1997a) reported adult *Ascarophis* sp. from *Gammarus lawrencianus* Bousfield, 1956, and *G. oceanicus* in the Gulf of St. Lawrence, Canada.

Development stages of *Ascarophis* sp. in *G. deubeni* from the present study are morphologically typical of the Spirurida and substantially identical to those of the heteroxenous cystidicoloid *Capillospirura pseudodagmenteosa* (Appy and Dadswell, 1978), which utilizes *Gammarus tigrinus* Sexton, 1939, and the shortnose sturgeon, *Acipenser brevirostrum* Lesueur, 1818, as intermediate and definitive hosts, respectively (Appy and Dadswell, 1983). However, unlike *C. pseudodagmenteosa* and nearly all other species of spirurid nematodes, where development is arrested at the third stage in the invertebrate host (Anderson, 2000), *Ascarophis* sp. reaches the adult stage in an invertebrate host, *G. deubeni*. Based on sectioned material from this study, the worm passes from the muscle into the hemocoel at the third stage where they undergo a third and fourth molt to become adults. In the hemocoel, the worms feed and presumably mate, and females release eggs with larvae just as heteroxenous cystidicolidis would in the digestive tract of the fish host. Based on compression of...
infected amphipods, Valer and Valovaya (1990) also documented the migration of *Ascarophis* sp. into the hemocoel of *G. oceanicus* at the third molt and their subsequent development to adults, with adult worms present at day 73 PI at 6–8°C.

Similar to *C. pseudoargumentosa* in *G. tigrinus*, there is little overlap in the presence of the first 3 larval stages with the first 2 molts. Following the appearance of third-stage larvae, there is significant overlap of developmental stages of *Ascarophis* sp. in *G. deubeni* (Fig. 1). Third-stage larvae are present for at least 47 days (Table 1) and may take from 13 to 47 days to initiate molting. No third-stage larvae were observed in the muscles of amphipods at 61 days PI, which suggests that the seemingly prolonged occurrence of third-stage larvae is due to factors within the hemocoel and not related to the timing of their migration from the muscle into the hemocoel. Such factors could include inadequate parasite nutrition or resistance from the host. The shorter length of adult *Ascarophis* sp. at days 92 and 100 (Fig. 1) may be due to the continued addition of younger, more recently molted, adult worms.

In most cases, macro parasites in arthropods elicit an encapsulation response that involves deposition of eumelanin and adhesion of hemocytes to the parasite (Helluy and Thomas, 2010). Third-stage larvae of some *Ascarophis* species occurring in decapod crustaceans become encapsulated at the third stage by hemocytic reaction or by muscle cells (Poinar and Thomas, 1976; Poinar and Hess, 1977). In *G. deubeni*, there is little apparent host reaction to first-, second-, and third-stage *Ascarophis* sp. in the muscle (Figs. 31–36), but once within the hemocoel, there is a pronounced hemocytic reaction to molting worms, shed larval cuticles, nematode eggs, and, infrequently, adult worms.

*Figures 31–36.* Histology of *Ascarophis* sp. in tissues of *G. deubeni* kept at 10–12°C. (31) First-stage larva in muscle tissue 24 hr. (32) First-stage larva in muscle tissue 5 days PI. (33) Cross section of first-stage larva in muscle tissue 11 days PI. (34) Cross section of presumptive second-stage larva 21 days PI. (35) Late second-stage larva in muscle tissue 28 days PI. (36) Third-stage larva in degenerate muscle tissue 38 days PI.
(Figs. 37–41). Melanization of adult worms has also been observed in natural infections. The attachment of hemocytes to, and melanization of, molting worms and their shed cuticles indicates that they are more recognizable as foreign material by the host. Crompton (1967) found that wounded or damaged acanthocephalan larvae in *Gammarus* sp. were subjected to host reaction, while undamaged larval cysts did not elicit hemocyte congregation or melanization. It may be that continued molting past the third stage provides the parasite some protection or sequestration (Anderson, 2000) from host reaction as evidenced by the presence of live worms within melanized shed cuticles (Fig. 39). The host response to *Ascarophis* sp., the continued release of filamented eggs into the hemocoel of the amphipod where they attract hemocytes, and the mass of the parasite and nutritional requirements, must affect the longevity and activity of infected *G. deubeni*. Poinar and Kuris (1975) suggested that increased mortality or reduced growth rate in shore crabs was a consequence of infection with encapsulated third-stage *Ascarophis* sp. larvae.

The mechanism that allows *Ascarophis* sp. to develop beyond the third-stage larvae in different species of *Gammarus* is not known. Fagerholm and Butterworth (1988) suggested that osmotic pressure from reduced salinities in the intertidal/littoral zone might compromise the amphipods defense reaction to the parasites. While *G. oceanicus* is the common host of *Ascarophis* sp. in the Baltic and White seas and has been found in low numbers in this host in the Northwest Atlantic (Jackson et al., 1997a), it has not been found to be infected in Passamaquoddy...
### TABLE III. Comparison of adult *Ascarophis* sp. from experimentally and naturally infected *Gammarus* spp.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>New Brunswick*</th>
<th>New Brunswick†</th>
<th>Baltic Sea†</th>
<th>White Sea‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>G. deubeni</em></td>
<td><em>G. deubeni</em></td>
<td><em>G. oceanicus</em></td>
<td><em>G. oceanicus</em></td>
</tr>
<tr>
<td></td>
<td>Natural</td>
<td>Experimental</td>
<td>Natural</td>
<td>Experimental</td>
</tr>
<tr>
<td>Sample size</td>
<td>Male 5</td>
<td>Female 5</td>
<td>Male 6</td>
<td>Male 1</td>
</tr>
<tr>
<td>Total length</td>
<td>4.9 mm (4.7–5.1)</td>
<td>8.8 mm (7.5–9.7)</td>
<td>6.4 mm (5.5–7.2)</td>
<td>5.1 mm (5.1–8.3)</td>
</tr>
<tr>
<td>Width</td>
<td>88 (84–95)</td>
<td>118 (118–159)</td>
<td>91 (72–117)</td>
<td>99 (93–110)</td>
</tr>
<tr>
<td>Buccal cavity length</td>
<td>98 (91–102)</td>
<td>111 (95–125)</td>
<td>106 (101–111)</td>
<td>105 (100–115)</td>
</tr>
<tr>
<td>Deirid§</td>
<td>106 (101–111)</td>
<td>118 (96–136)</td>
<td>108 (87–148)</td>
<td>154 (140–168)</td>
</tr>
<tr>
<td>Nerve ring§</td>
<td>152 (144–157)</td>
<td>161 (144–177)</td>
<td>172 (140–194)</td>
<td>209 (193–230)</td>
</tr>
<tr>
<td>Muscular esophagus length</td>
<td>188 (172–202)</td>
<td>221 (209–237)</td>
<td>217 (177–250)</td>
<td>274 (237–300)</td>
</tr>
<tr>
<td>Glandular esophagus length</td>
<td>1.0 mm (0.8–1.1)</td>
<td>1.1 mm (0.8–1.3)</td>
<td>1.3 mm (0.9–1.2)</td>
<td>1.4 mm (1.0–1.6)</td>
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<tr>
<td>Total esophagus length</td>
<td>1.2 mm (1.0–1.3)</td>
<td>1.2 mm (1.1–1.5)</td>
<td>1.3 mm (1.1–1.5)</td>
<td>1.8 mm (1.4–2.0)</td>
</tr>
<tr>
<td>Vulva§</td>
<td>4.8 mm (4.4–5.3)</td>
<td>6.4 mm (4.5–8.8)</td>
<td>6.4 mm (4.5–8.8)</td>
<td>8.4 mm (6.9–10.1)</td>
</tr>
<tr>
<td>Vulva % of body</td>
<td>55 (55–59)</td>
<td>52 (49–55)</td>
<td>54 (51–61)</td>
<td>58 (53–69)</td>
</tr>
<tr>
<td>Right spicule length</td>
<td>108 (103–114)</td>
<td>105 (92–129)</td>
<td>114 (100–128)</td>
<td>116 (104–130)</td>
</tr>
<tr>
<td>Left spicule length</td>
<td>165 (161–670)</td>
<td>653 (572–712)</td>
<td>749 (710–813)</td>
<td>670</td>
</tr>
<tr>
<td>Spicule ratio</td>
<td>6.1 (6.0–6.1)</td>
<td>6.3 (5.4–6.8)</td>
<td>6.7 (5.8–7.5)</td>
<td>7.8</td>
</tr>
<tr>
<td>Tail length</td>
<td>111 (95–119)</td>
<td>34 (30–38)</td>
<td>133 (90–200)</td>
<td>167 (143–192)</td>
</tr>
<tr>
<td>Egg length</td>
<td>53 (50–57)</td>
<td>52 (50–55)</td>
<td>51 (45–53)</td>
<td>86 (51–54)</td>
</tr>
<tr>
<td>Egg filaments</td>
<td>Both poles</td>
<td>Both poles</td>
<td>Both poles</td>
<td>Both poles</td>
</tr>
<tr>
<td>Cuticular striations</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Present study.
† Fagerholm and Butterworth, 1988.
‡ Valier and Valevaya, 1990.
§ Distance from anterior end.
Bay in the same stream areas where infected G. deubeni have been collected (E. W. Butterworth, pers. obs.). At this location G. oceanicus is generally found lower in the tidal zone under more saline conditions and may not overlap in distribution with G. deubeni, which is more commonly found where freshwater seeps or springs result in lower salinities.

Despite being found in different amphipod species at distant locations, the morphology of Ascarophis sp. from G. deubeni in the Canadian Atlantic indicates it is the same species that infects G. oceanicus from the Baltic and White seas, although there are some minor differences (Fagerholm and Butterworth, 1988; Table III). Worms from natural infections and those from G. oceanicus are somewhat larger than worms from experimental infections, and from G. deubeni. A comparison of the growth of Ascarophis sp. under experimental conditions compared to natural infections shows that worms continue to grow as adults, particularly female worms, so larger worms in natural infections may have been present in amphipods for a longer period of time (Fig. 42). The right spicule size described by Valter and Valovaya (1990) is significantly smaller than the present material and may be an anomaly since this description was based on a single male worm.

Among species of Ascarophis present in North Atlantic waters, Ascarophis sp. from G. deubeni is very similar in morphology to Ascarophis arctica Poljansky, 1952, which is found as an adult in a variety of marine fishes in the North Atlantic Ocean and Baltic Sea (Poljansky, 1952, Appy, 1981, Fagerholm and Berland, 1988). Both Fagerholm and Butterworth (1988) and Valter and Valovaya (1990) identified some morphological differences between Ascarophis sp. taken from amphipods and A. arctica, which do not appear related to overall worm size. These include a longer right spicule relative to the left spicule, somewhat longer eggs, and the presence of a notch in the sub-labia. The notched sub-labia identified by Fagerholm and Butterworth (1988) as distinguishing Ascarophis sp. from A. arctica is evident in the third stage of Ascarophis sp. in the present study (Fig. 30). While Valter and Valovaya (1990) believed that these morphological differences provide evidence that Ascarophis sp. is distinct from A. arctica, a preliminary nuclear rDNA study of populations of Ascarophis sp. infecting the amphipod G. oceanicus and A. arctica infecting the fish Triglopsis (Myxocephalus) quadricornis Linnaeus, 1758, in the Baltic (Pullinen et al., 2005), suggests that these worms are conspecific. Zander (2005) considered the worms in amphipods in the Baltic to be A. arctica and suggested that direct development in the amphipod is an adaptation, which guarantees that the parasites continued existence in extreme conditions in brackish waters where availability of the fish host is uncertain. Additional work will be required to determine if Ascarophis sp. is a separate species or represents progenetic development of A. arctica.
Anderson (1988) and Anderson and Bartlett (1993) believed the direct development of *Ascarophis* sp. in the invertebrate host to be “extreme precocity,” where development beyond the infective third-stage larvae is a strategy to accelerate gamete production in the final host. While it is possible that the presence of adult *Ascarophis* sp. in *Gammarus* spp. is an adaptation to enhance transmission to a vertebrate host, evidence suggests that it is self-sustaining in the invertebrate host population. Valter and Valovaya (1990) believed that the life cycle of *Ascarophis* sp. in the White Sea amphipods was self-sustaining as no species of vertebrate host, and cannibalism/necrophagy replaces predation. Black, G. A., and M. W. Lancaster. 1980. Migration and development of swim-bladder nematodes, *Cystidicola* spp. (Habronematoidea), in their definitive hosts. Canadian Journal of Zoology 58: 1997–2005.


SURFACE ULTRASTRUCTURE OF JUVENILE AND ADULT ACANTHOPARYPHIUM TYOSENENSE (DIGENEA: ECHINOSTOMATIDAE)

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ABSTRACT: The tegumental ultrastructure of juvenile and adult Acanthoparyphium tyosenense (Digenea: Echinostomatidae) was observed by scanning electron microscopy. One- to 3-day-old juveniles and 10-day-old adults were harvested from chicks experimentally fed metacercariae from a bivalve, M. veneriformis. The juvenile worms were minute, curved ventrally, and had 23 collar spines characteristically arranged in a single row. The lips of the oral sucker had 7 single aciliated sensory papillae and 4 grouped unaciliated sensory papillae. The ventral sucker had 25 aciliated round swellings on its lip. The anterolateral surface between the 2 suckers was densely packed with tongue-shaped tegumental spines, and the ventral surface just posterior to the ventral sucker was covered with peg-like spines. Retractile, peg-like spines were seen on the anterolateral surface, whereas scale-like spines with round tips and broad bases were sparsely distributed posterior to the ventral sucker. The cirrus was characteristically protruding and armed with minute spines. The surface ultrastructure of A. tyosenense was unique, especially in the number and arrangement of collar spines, shape, and distribution of tegumental spines and in distribution of sensory papillae.

MATERIALS AND METHODS

Metacercariae of A. tyosenense were collected from the muscle of the marine bivalve, M. veneriformis, purchased from a local market near Kyelwa-myon, Buan-gun, Jeollabuk-do where the human infections were discovered (Chai et al., 2001). The metacercariae were divided into groups of 50 to 100 and fed to 1- to 3-wk-old hatchery-raised broiler chicks as described previously (Han, Kim, and Chai, 2003). The chicks were killed at day 1-3 and day 10 post-infection and their whole guts were resected. The small intestine was opened along the mesenteric border in pH 7.4 phosphate-buffered saline (PBS). After washing the intestinal contents several times with PBS, juvenile and adult A. tyosenense flukes were recovered from the sediment using a dissecting microscope. The worms were washed with 0.2 M cacodylate buffer (pH 7.3) and fixed in 2.5% glutaraldehyde. They were dehydrated with a graded series of ethanol and stubs and coated with gold using an ion sputtering coater (IB-3, Eiko Engineering Co., Tokyo, Japan). The specimens were observed using a scanning electron microscope (SIU DS-130C, Akashi Co., Tokyo, Japan) at an accelerating voltage of 10 kV.

RESULTS

Juvenile flukes (Figs. 1–6)

Small 1- to 3-day-old worms were ventrally curved (Fig. 1) with their ventral sucker near the middle of the body. The oral sucker was subterminal (Fig. 2). The tegument of the whole body was wrinkled transversely. Tegumental spines were distributed densely from the anterior to the ventral sucker level (Fig. 3), rarely up to the posterior part of the worm. The lips of the oral sucker had 7 aciliated round swellings anteriorly and 2 groups of 4-5 unaciliated sensory papillae posteriorly. In the lateral portion of the oral sucker, several grouped, unaciliated sensory papillae were distributed symmetrically (Fig. 2). The head collar (=head crown) was surrounded by a total of 23 collar spines, each of which was embedded in a cytoplasmic pocket (Fig. 4). The spines were arranged in a single row without interruption dorsomedially (Fig. 4). The ventral sucker was dome-shaped at the median level of the body. There were 25 equidistant round swellings on the lip of the ventral sucker (Fig. 5). Tongue-shaped spines were densely distributed on the ventral surface from the oral to ventral suckers (Fig. 6). Peg-like tegumental spines were arranged on the ventral
Figures 1-6. Scanning electron micrographs of 1- to 3-day-old juvenile *A. tyosense*. (1) Ventral view of a worm showing ciliated sensory structures and swellings, probably sensory, on the head collar, oral and ventral suckers, and the excretory pore. Note that the tegumental spines are distributed mainly on the tegument of the anterior part of the body. Bar = 30 μm. (2) The oral sucker showing the unciiliated and aciliated sensory papillae and round swellings on its lip. Bar = 7 μm. (3) Dorsal view of the anterior part of the body showing the tegumental spines. Bar = 20 μm. (4) The oral sucker and the head collar with collar spines which are retracted under the tegument. Groups of sensory papillae are seen on the surface of the head collar. Bar = 12 μm. (5) The ventral sucker showing single and clumped unciiliated papillae on the tegument of each lateral side of the ventral sucker. Bar = 10 μm. (6) Tegumental spines on the ventral surface between the oral and ventral suckers. Note the tongue-shaped spines and unciiliated sensory papillae with 1 cillum. Bar = 1 μm.
surface just posterior to the ventral sucker. The tegument of the ventral and dorsal surfaces near the posterior extremity was devoid of spines and transversely wrinkled.

**Adult flukes (Figs. 7–17)**

The oral and ventral suckers were located close together at the anterior part of the body (Figs. 7, 8). The genital pore was located ventrally and distant from the anterior margin of the ventral sucker. The cirrus was protruding and armed with small spines over the entire area (Fig. 8). The rim of the ventral sucker had a wrinkled border and was equipped with 25 randomly distributed and equidistant round swellings (Fig. 8). Unciliated sensory papillae were distributed on the surface under the head collar and protruding collar spines (Fig. 9). Unciliated sensory papillae were also found near the lateral collar spines (Fig. 10). Two different sizes of unknown sensory-like organs, with round concave bases and bud-like structures on them, were located on the lip of the oral sucker (Fig. 11) and between the oral sucker lip and collar spines (Fig. 12). The cirrus had densely distributed spines and aggregated spines were seen on the median part of the cirrus (Figs. 13, 14). Wrinkled, tongue-shaped tegumental spines were located between the oral and ventral suckers. Tongue-shaped spines were distributed near the ventral sucker (Fig. 15). The uterine eggs had a smooth shell surface; the operculum was recognizable with difficulty (Fig. 16). The tegument near the excretory pore was devoid of spines (Fig. 17).

**DISCUSSION**

Hosts of *Acanthoparyphium* are usually migratory aquatic birds. However, in 2001, 10 human cases naturally infected with *A. tyosenense* were reported (Chai et al., 2001). Life cycle studies were performed on *Acanthoparyphium spinulosum, Acanthoparyphium paracharadrii, Acanthoparyphium macracanthum*, and *A. tyosenense* (Martin and Adams, 1961; Velasquez, 1964; Rybakov and Lukomskaya, 1988; Chai et al., 2001; Kim et al., 2004). In the present investigation, surface ultrastructure of *Acanthoparyphium* species was observed for the first time.

Longitudinal ventral depressions on the surface between the oral and ventral suckers have been seen in various echinostome species, including *Echinostoma caprioni* (Fried et al., 1990), *Echinostoma trivolvis* (Fried et al., 1990), *E. paraensei* (Maldonado et al., 2001), *E. recurvatum* (Sohn et al., 2002), and *H. alincia* (Han, Han, and Chai, 2003). Such a depression was also found on *A. tyosenense* in our study. On the surface of the ventral depression, tegumental spines were lacking in *E. japonicus* (Lee et al., 1987), *M. denticulatus* (Koie, 1987), *E. caproni* (Fried et al., 1990), and *E. trivolvis* (Fried et al., 1990). However, spines were found on the ventral depression in *E. revolutum* (Smals and Blakemanspoor, 1984), *E. cinetorhisch* (Lee et al., 1992), *E. hortense* (Lee et al., 1986), *E. malayanum* (Tesanap et al., 1987), *E. paraensei* (Maldonado et al., 2001), and *E. recurvatum* (Sohn et al., 2002). Tegumental spines were also observed on the ventral depression in *A. tyosenense*.

Sensory papillae, with short or long cilia, have been observed over the tegumental surface of various monogenean and digenean trematodes, from cercariae to adult stages, in both single and grouped forms (Morris and Threadgold, 1967; Fujino et al., 1979; Koie, 1987). This type of sensory papillae appears mainly on the anterior half of the ventral body and is densely distributed around the oral and ventral suckers. These papillae may function as mechanico-, rheo-, tango-, and chemoreceptors (Smals and Blakemanspoor, 1984). In *A. tyosenense*, unciliated sensory papillae were distributed densely around the oral sucker in both the juvenile and adult worms, and it is suggested that they may sense and recognize nutrients and perceive microenvironment in the host intestine. Another type of papillae, acilated round swellings, was seen on the lip of the oral sucker in juvenile worms and on the rim of the ventral sucker in juvenile and adult *A. tyosenense*. This type of papillae is commonly found in various trematode species, including echinostomes, and was suggested to function as a contact or stretch receptor and have a secretory function (Smals and Blakemanspoor, 1984). In our study, a third, unknown-type papillae, in 2 different sizes and shapes, was found on and around the oral sucker of adult worms. Their function and significance in taxonomy remain uncertain.

The differentiation of the tegumental spines of digeneans is closely related to development, parasitic niche, and migration status (Bennett and Threadgold, 1975; Fujino et al., 1979; Lee et al., 1982, 1985; Han, Han, and Chai, 2003). *Clonorchis sinensis* larval flukes, after excystation from metacercariae in the host duodenum, have double- or triple-pointed tegumental spines at the anterior half of the body (Fujino et al., 1979; Lee et al., 1982). However, the spines gradually disappear as the worms grow into adults, and fully grown adults are completely devoid of spines (Fujino et al., 1979; Lee et al., 1982). Conversely, in *Fasciola hepatica* larval stages, single-tipped tegumental spines metamorphose into multi-pointed spines just prior to their entry into the bile duct (Bennett and Threadgold, 1975). The conversion of simple spines into digitated ones during development is also known in *Metagonimus takahashii* (Chai et al., 2000) and *Neodiplostomum seoulense* (Lee et al., 1985). In *A. tyosenense*, there was not much difference in the tegumental spines between juvenile and adult worms, except in that peg-like spines appeared in juveniles but not in adults. However, the tegumental spines of *A. tyosenense* revealed no digitations in both juvenile and adult flukes. This was comparable with *H. alincia*, in which the tegumental spines between the oral and ventral suckers have 4–7 digitations in 1- to 5-day-old worms and 8–10 digitas in 20-day-old adults (Han, Han, and Chai, 2003). It is to be determined whether this is a generic character of *Acanthoparyphium* or a specific character among different species of echinostomes.

Using light microscopy, species of trematode parasites can be distinguished by the adult and egg morphologies and by the presence or absence of spines on the cirrus. The morphology of the cirrus is important in the differentiation of *Himasthla* spp., i.e., the cirrus of *H. alincia* is armed with spines (Lumsden, 1962; Han et al., 2009) and that of *Himasthla limnodromi* has an aspinous cirrus (Didyk and Burt, 1997). In our study, the cirrus of *A. tyosenense* was characteristically armed with minute spines. This is contrasting with other echinostome species, such as *E. caproni* (Fried et al., 1990), *E. trivolvis* (Fried et al., 1990), *H. alincia* (Han, Han, and Chai, 2003), *E. paraensei* (Maldonado et al., 2001), and *E. recurvatum* (McCarthy, 2011), which have no spines on the cirrus tegument.

In conclusion, the surface ultrastructure of *A. tyosenense* was generally similar to that of the other echinostomes but was unique in the number and arrangement of collar spines, the shape and distribution of tegumental spines, and the type and distribution of sensory papillae.
FIGURES 7–11. Scanning electron micrographs of 10-day-old adult *A. tyosense*. (7) Ventral view of an adult fluke showing the tegumental spines covering the anterior half of the body surface, with an oral sucker, head collar, and ventral sucker. Bar = 140 μm. (8) Ventral view of the anterior part of the worm showing the head collar, ventral sucker, and cirrus. Bar = 12 μm. (9) Dorsolateral view of the head collar and the collar spines. Bar = 10 μm. (10) Collar spines and sensory papillae on the ventrolateral surface of the head collar. Bar = 7 μm. (11) Unknown sensory organ-like structures near the lip of the oral sucker. Bar = 7 μm.
FIGURES 12-17. Scanning electron micrographs of 10-day-old adult *A. tyosenense*. (12) Another type of unknown sensory-like structure between the oral sucker lip and collar spines. Bar = 1 μm. (13) The cirrus showing densely distributed, minute spines and the aggregated sperm. Bar = 12 μm. (14) Sperms on the cirrus; magnification of Figure 13. Bar = 4 μm. (15) Tegumental spines near the ventral sucker; their morphology is tongue-shaped. Bar = 2 μm. (16) Uterine eggs showing their ovoid to elliptical shape and smooth shell surface. Bar = 25 μm. (17) The excretory pore near the end of the body without tegumental spines. Bar = 10 μm.
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LITERATURE CITED


EXPERIMENTAL EXPOSURE OF HELISOMA TRIVOLVIS AND BIOMPHALARIA GLABRATA (GASTROPODA) TO RIBEIROIA ONDATRAE (TREMATODA)

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ABSTRACT: Experimental infections provide an important foundation for understanding host responses to parasites. While infections with *Ribeiroia ondatrae* cause mortality and malformations in a wide range of amphibian second intermediate host species, little is known about how the parasite affects its first intermediate hosts or even what species can support infection. In this study, we experimentally exposed *Helisoma trivolvis*, a commonly reported host of *R. ondatrae*, and *Biomphalaria glabrata*, a confamilial snail known to host *Ribeiroia marini*, to increasing concentrations of embryonated eggs of *R. ondatrae* obtained from surrogate definitive hosts. Over the course of 8 wk, we examined the effect of parasite exposure on infection status, time-to-cercariae release, host size, and mortality of both snail species. *Helisoma trivolvis* was a highly competent host for *R. ondatrae* infection, with over 93% infection in all exposed snails, regardless of egg exposure level. However, no infections were detected among exposed *B. glabrata*, despite previous accounts of this snail hosting a congeneric parasite. Among exposed *H. trivolvis*, high parasite exposure reduced growth, decreased time-to-cercariae release, and caused marginally significant increases in mortality. Interestingly, while *B. glabrata* snails did not become infected with *R. ondatrae*, individuals exposed to 650 *R. ondatrae* eggs grew less rapidly than unexposed snails, suggesting a sub-lethal energetic cost associated with parasite exposure. Our results highlight the importance of using experimental infections to understand the effects of parasite exposure on host- and non-host species, each of which can be affected by exposure.

*Ribeiroia ondatrae* is a trematode found in wetland habitats across much of North America (Johnson and McKenzie, 2008). This parasite uses rams horn snails (Planorbidae) as first intermediate hosts, fishes and amphibians as second intermediate hosts, and birds or mammals as definitive hosts (Price, 1931; Beaver, 1939; Johnson et al., 2004). *Ribeiroia ondatrae* can have deleterious effects on amphibian intermediate hosts, in which infections cause increased mortality and severe limb malformations, ranging from missing limbs to multiple supernumerary limbs (Johnson et al., 1999, 2001; Szuroczyki and Richardson, 2009; Johnson et al., 2010; Rohr et al., 2010). These malformations are hypothesized to increase the susceptibility of infected amphibians to definitive host predators (Rohr et al., 2009). In avian definitive hosts, *R. ondatrae* infection can produce necrotic lesions in the mucosa of the proventriculus (Newsom and Stout, 1933; Beaver, 1939; Leibovitz, 1961; Dyer et al., 2002), sometimes leading to increased mortality (Newsom and Stout, 1933; Leibovitz, 1961; Basch and Sturrock, 1969).

Although *R. ondatrae* can infect a wide range of amphibians and birds (Johnson et al., 2004), less is known about its use of first intermediate hosts, including what species are susceptible and how infection influences host fitness. As with many trematode species (Baudoin, 1975; Crews and Esch, 1987; Negovetich and Esch, 2008), *Ribeiroia* spp. infections cause complete castration in snail hosts (Harry, 1965; Huizinga, 1973), which can reduce population size. For instance, in a large-scale field experiment, Nasi et al. (1979) found that the introduction of 7–9 million *Ribeiroia marini* eggs caused a temporary collapse in a *Biomphalaria glabrata* population, thereby reducing available hosts for the human blood fluke *Schistosoma mansoni*. These results highlight the potential consequences of *Ribeiroia* spp. infection on snail host population dynamics. However, because few studies have experimentally examined the effects of different *Ribeiroia* sp. exposure levels on snail hosts, it is unclear how many miracidia are required to induce infection successfully and what other effects occur in suitable first intermediate host species. Given the potential negative effects of *R. ondatrae* on its hosts, including amphibians, birds, and snails, there is a need for more mechanistic studies that examine the effects of infection on host fitness.

*Ribeiroia ondatrae* has been reported in the United States and Mexico, where its first intermediate hosts are planorbid snails (Beaver, 1939; Kuntz, 1951; Malek, 1977; Fried et al., 1998; Johnson et al., 2004; Peterson, 2007). Species of *Helisoma* (=*Planorbella*) are among the most commonly reported first intermediate hosts of *R. ondatrae* (9 of 12 records in Johnson et al., 2004). In the southern United States, the Caribbean, and South America, species of *Biomphalaria* have also been recorded to support different species of *Ribeiroia*, e.g., *B. glabrata* is the only known snail host of *R. marini* (Basch and Sturrock, 1969; Huizinga, 1973; Johnson et al., 2004). Native to the Neotropics (Campbell et al., 2000), *B. glabrata* also serves as a host of the human parasite *S. mansoni* (Loker, 1982; Théron and Moné, 1986). *Ribeiroia marini* is antagonistic with *S. mansoni*, such that rediae of *R. marini* will actively consume schistosome sporocysts within infected snails (Page and Huizinga, 1976). Indeed, *R. marini* has historically been used as a control agent against *B. glabrata* and *S. mansoni* (Nasi et al., 1979; Paraense, 1987; Pointier, 1989). In the southern United States, *Biomphalaria obstricta* has also been recorded as a host for *R. ondatrae* (Malek, 1977). Despite these observations, there is no information about whether *B. glabrata* can host *R. ondatrae*, which could provide a useful experimental system for further studies into the ecology and pathogenesis surrounding *Ribeiroia* spp. infections.

In the current study, we experimentally exposed *Helisoma trivolvis* and *B. glabrata* to a range of *R. ondatrae* exposure levels to compare host competency for the 2 species under controlled conditions and quantify the effects of exposure on snail survival, growth, and the maturation of infection. In 2 separate experiments, we quantified the effects of *R. ondatrae* exposure on the survival, growth, and maturation of infection within *H. trivolvis* (Experiment 1) and *B. glabrata* (Experiment 2). In Experiment 2, which was conducted a separate year from Experiment 1, we also included a small number of *H. trivolvis* to confirm *R. ondatrae* egg viability and to test susceptibility to *R. ondatrae* among 2 different

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**Materials and Methods**

**Parasite culturing**

We obtained embryonated eggs of *R. ondatrae* from experimentally infected Holzman rats (75-124 g). Male rats (n = 10–15) were raised in the laboratory and exposed to *R. ondatrae* metacerariae (n = 50–75) isolated from infected amphibians (*Lithobates catesbeianus* and *Pseudacris regilla*). After 2 wk, rat fecal matter was collected on wet paper towels, soaked in spring water for 24 hr at 5 C, and then filtered through a sieve series (largest: 2 mm, 425 mm, 106 mm, 45 mm). The remaining rat fecal matter was placed in 2 mason jars. 2 L each, filled with 1.9 L commercial spring water and incubated at 23–27 C in the dark for 3 wk. During this time, spring water was changed weekly and aerated continuously to prevent bacterial buildup. Eggs were re-filtered through the 45-μm sieve during each water change. After 3 wk, we determined *R. ondatrae* egg density by quantifying the number of viable eggs in at least 10 sub-samples, 10 μl each, examined at >200 magnification. Only eggs that appeared viable were included in this count; any eggs that had already hatched or no longer contained a developing miracidium were excluded from the count. We used these *R. ondatrae* egg density estimates to determine the amount of rat fecal material each snail should be administered based on their treatment.

**Experiment 1: Helisoma trivolvis**

Helisoma trivolvis were obtained from the Art Oehmke Fish Hatchery in Woodruff, Wisconsin, and maintained for several generations under laboratory conditions. We placed 50 uninfected adult *H. trivolvis* individually into 1-L tubs filled with 0.75 L of filtered lake water. We measured the shell width of each snail before placing it into the tub (mean shell width ± 1 SE = 11.4 ± 0.1 mm). Tubs each contained 1 snail and were randomly assigned to 1 of 4 treatments (0, 50, 100, or 200 mg) of filtered rat feces. Each treatment was replicated 20 times. We administered the treatments by slowly lowering a 60 × 15 mm Petri dish filled with the appropriate amount of filtered material based on the sub-sample examinations into the bottom of each tub. A comparable amount of fecal material from uninfected rats was added to snails in the control treatment.

Snail water was changed weekly, and any snails that died were necropsied to determine infection status. During the first 2 wk, snails received a partial (50%) water change to prevent the loss of unhatched *R. ondatrae* eggs or miracidia. Thereafter, snails received complete (100%) water changes. Throughout the experiment, we fed snails romaine lettuce and 1:1 mixture of ground TetraMin tropical flakes and ground TetraVege Spiralina Enhanced Flakes ad libitum. During the experiment, air temperature was 28 ± 2 C, and the photoperiod followed a 16:8 light:dark cycle. On days 32, 38, and 43 post-exposure, we placed each snail into a 50-ml centrifuge tube filled with 40 ml treated water to determine whether snails were releasing cercariae and confirm patent infections. Given that *R. ondatrae* releases cercariae at night (Beaver, 1939), snails were left in a dark room overnight, and in the morning the centrifuge tube water was examined for cercariae. The experiment was terminated on day 44 post-exposure, and final shell width was measured. All snails that did not release cercariae on day 32, 38, or 43 were necropsied to determine infection status.

**Experiment 2: Biomphalaria glabrata**

We obtained *B. glabrata* that were known to be susceptible to *S. mansoni* from the National Institutes of Health and raised them through multiple generations in the laboratory. For this experiment, *H. trivolvis* snails were raised through multiple generations in the laboratory and originally obtained from a wetland in Santa Clara County, California. We placed 60 uninfected juvenile *B. glabrata* (mean shell width ± 1 SE = 2.98 ± 0.09 mm) and 15 uninfected juvenile *H. trivolvis* (mean shell width ± 1 SE = 7.10 ± 0.28 mm) individually into 1-L tubs filled with 0.75 L of dechlorinated tap water. Owing to a limited number of available *R. ondatrae* eggs, in this experiment each tub containing 1 snail was randomly assigned to 1 of 5 treatments (0, 65 ± 11, or 650 ± 108 mg *R. ondatrae* embryonated eggs). Each treatment was replicated 20 times for *B. glabrata* and 5 times for *H. trivolvis*. We administered the treatments by slowly lowering a 60 × 15 mm Petri dish filled with the allocated amount of infected rat feces (0, 0.273, and 2.73 ml of infected rat feces for the 0-, 65-, and 650-egg treatment, respectively) into the bottom of each tub. Snails assigned to the control treatment, i.e., 0 eggs, were given 1.5 ml of uninfected rat feces.

Because differences in snail sizes can influence infection likelihood (Huizinga, 1973), we placed 20 *B. glabrata* and 10 *H. trivolvis* snails of varying sizes (3–12 mm shell width) in a group within a 11.3 L-container filled with dechlorinated tap water and exposed them to a large quantity of infected rat feces (>1,000 eggs). This group exposure treatment was used to test infection results when varying sizes of *B. glabrata* and *H. trivolvis* were exposed concurrently to large concentrations of *R. ondatrae* eggs.

We changed snail water weekly and dissected all snails that died to determine *R. ondatrae* infection. During the first 2 wk, snails received a partial (50%) water change to prevent the loss of unhatched *R. ondatrae* eggs or miracidia. Thereafter, snails received complete (100%) water changes. Every 3–4 days during the experiment, all snails were fed a small (~125 mm³) cube containing ground TetraMin tropical flakes solidified with agar. The food was prepared by mixing 40 ml of treated tap water, 0.5 g of agar, and 3 g of ground TetraMin tropical flakes. Throughout the experiment, air temperature was 23 ± 2 C, and the photoperiod followed a 14:10 light:dark cycle. We accidentally damaged 2 *B. glabrata* (1 snail each from the 65 and 650 treatments) and 1 control *H. trivolvis* during the first water change, and their data were omitted from the analysis. Shell width was measured 23 and 50 days post-exposure to estimate growth in all *B. glabrata* snails, except for those snails in the group exposure treatment. On days 29 and 50 post-exposure, we placed each *B. glabrata* snail into an individually labeled 50-ml centrifuge tube filled with 40 ml of water in a dark room overnight to determine whether snails were releasing cercariae and confirm patent infections. After 51 days, all snails that had not released cercariae were dissected to determine infection status.

**Analyses**

We evaluated the effects of different *R. ondatrae* exposure levels on the survival, growth, infection prevalence, and time-to-cercariae release of *H. trivolvis* (Experiment 1) and *B. glabrata* (Experiment 2). We used logistic regression to assess the relationship between *R. ondatrae* exposure level and the infection status, time-to-cercariae release, and mortality of *H. trivolvis* and *B. glabrata*. For time-to-cercariae release, a logistic regression was performed for each sampling date. For all logistic regression tests, we used a Wald's chi-square test to compare among treatments. Because *Ribeiroia* sp. requires 1–2 wk to achieve detectable infection in snails after exposure (Huizinga, 1973), snails that died earlier than 3-wk post-exposure were not included in the infection analysis. To examine the effects of the parasite exposure on snail survival, we used analysis of variance (ANOVA) for *H. trivolvis* and repeated-measures ANOVA for *B. glabrata* with treatment as a fixed effect. We used repeated-measures ANOVA for *B. glabrata* because growth was assessed twice during the experiment (1–3-wk post-exposure and 4–7-wk post-exposure). We used Tukey's pairwise comparisons to identify significant differences among groups. All analyses were conducted using SPSS 19 (SPSS, Chicago, Illinois).

**Results**

**Experiment 1: Helisoma trivolvis**

Exposure to *R. ondatrae* did not significantly affect *H. trivolvis* survival (logistic regression, $\chi^2 = 3.3$, df = 3, $P = 0.352$). However, mortality tended to be higher in the 1,000-egg treatment.
and egg treatment, such that growth tended to be greatest in the 400-egg exposure, with cercariae increased monotonically with cercariae than 400-egg exposure, compared with the control treatment treatments varied by days post-exposure with significantly more releasing cercariae day 43 post-exposure than day 32 post-exposure. There were no differences in infection status among the egg exposure treatments (P = 0.236; Fig. 1A). Among snails that died before wk 3 in the 50-, 400-, and 1,000-egg treatments (n = 4, 4, and 3 respectively), none was infected with R. ondatrae. R. ondatrae exposure significantly affected the proportion of H. trivolvis that released cercariae on each sample date (logistic regression, \( \chi^2 = 12.9, df = 3, P = 0.005 \); Fig. 2). On all sample dates, exposure to parasite eggs significantly increased time-to-cercariae release compared with the control treatment (P ≤ 0.004). Differences between R. ondatrae exposure levels varied by days post-exposure with significantly more H. trivolvis releasing cercariae day 43 post-exposure than day 32 post-exposure (P < 0.003, Fig. 2). On day 32 post-exposure, 31.9% of all exposed snails released cercariae, but there were no differences among the 3 different egg exposure treatments (Fig. 2). However, by day 38 post-exposure, the proportion of snails releasing cercariae increased monotonically with egg exposure level, with a significantly greater proportion of 1,000-egg snails releasing cercariae than 50-egg snails (P < 0.003, Fig. 2). On the final sampling date (43 days post-exposure), 100% of surviving exposed snails released cercariae (Fig. 2), at which point the experiment was terminated.

**Helisoma trivolvis** growth exhibited a concave relationship with exposure, such that growth tended to be greatest in the 50- and 400-egg exposure treatment but lower in the control and 1,000-egg treatments (F = 3.619, df = 3, P = 0.019, Fig. 3). Snails in the 1,000-egg treatment grew significantly less than snails in the 50-egg treatment (P = 0.032, Tukey’s test, Fig. 3), while there were no significant growth differences among the other treatments (P ≥ 0.0956, Tukey’s test, Fig. 3). Host egg mass data were not collected in this study because H. trivolvis tend to produce few eggs when isolated into individual containers (Escobar et al., 2011).

**Experiment 2: Biomphalaria glabrata**

B. glabrata exhibited high survival (>89%), with no significant differences in mortality among treatments (logistic regression, \( \chi^2 = 2.9, df = 2, P = 0.236 \)). None of the 58 surviving B. glabrata became infected with R. ondatrae, regardless of egg exposure level (Fig. 1B). Correspondingly, no B. glabrata released cercariae and, upon dissection, no evidence of trematode rediae was noted. However, and in support of the results of Experiment 1, all H. trivolvis exposed to R. ondatrae in Experiment 2 that lived for at least 17 days were infected (n = 9), as determined from necropsies and cercariae release (Fig. 1B). Furthermore, within the group exposure treatment involving 20 B. glabrata and 11

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**Figure 1.** Percentage of *Helisoma trivolvis* in Experiment 1 (A) and *H. trivolvis* and *Biomphalaria glabrata* in Experiment 2 (B) that became infected with *Ribeiroia ondatrae* and lived for at least 3-wk post-exposure in each treatment.

**Figure 2.** Percentage of *Helisoma trivolvis* within each treatment that released cercariae within each sampling event. Treatments at each sampling event sharing letters are not significantly different based on based on Wald’s chi-square tests (P > 0.05).
H. trivolvis exposed to >1,000 eggs concurrently, all of the H. trivolvis were infected, whereas none of the B. glabrata was infected.

The repeated-measures ANOVA on snail growth exhibited significant effects of time (F(1,52) = 196.4, P < 0.001) and the time × exposure treatment interaction (F(2,52) = 10.2, P < 0.001), but not the main effect of exposure treatment (F(2,52) = 0.7, P = 0.524). Given the significant interaction, we examined the effects of the parasite exposure treatment on growth for each observation date. Biomphalaria glabrata growth was significantly different among exposure treatments during the first 3-wk post-exposure (F = 7.210, df = 2, P = 0.002, Fig. 4) and from wk 4–7 post-exposure (F = 6.025, df = 2, P = 0.004, Fig. 4). During the first 3-wk post-exposure, snails in the control treatment grew significantly more than snails in the 650-egg treatment (P < 0.001, Tukey’s test, Fig. 4). There were no differences in growth of B. glabrata between the 0-egg and 65-egg or between the 650-egg treatment (all P > 0.14, Tukey’s test, Fig. 4). From wk 4 through 7, growth of B. glabrata in the 650-egg treatment was greater than the 0-egg treatment (P = 0.0039, Tukey’s test, Fig. 4) and marginally greater than the 65-egg treatment (P = 0.0591, Tukey’s test, Fig. 4). However, there was no difference in growth between the 0-egg and 65-egg treatments (P = 0.62, Tukey’s test, Fig. 4).

**FIGURE 3.** Daily growth rate (mm/day) of Helisoma trivolvis exposed to increasing numbers of Ribeiroia ondatrae eggs. Growth rate was assessed at wk 6 post-exposure. Treatments sharing letters are not significantly different based on Tukey’s test (P > 0.05). Average initial sizes of H. trivolvis in each treatment were all within ±1 standard error of each other. Data are means ± 1 SE.

**FIGURE 4.** Daily growth rate (mm/day) of Biomphalaria glabrata exposed to increasing numbers of Ribeiroia ondatrae eggs. Growth rate was assessed at weeks 3 and 7 post-exposure. Within the same growth period, treatments sharing letters within weeks 1–3 and numbers within weeks 4–7 are not significantly different based on Tukey’s test (P > 0.05). Data are means ± 1 SE.

**Discussion**

Growing attention has focused on the ecology and pathology of R. ondatrae, which causes severe malformations in amphibian hosts. However, little is known about its infection patterns or the ability of snail species to infection by R. ondatrae. The high infection prevalence (>93%) of H. trivolvis exposed to 50–1,000 R. ondatrae embryonated eggs demonstrates that both H. trivolvis from California and Wisconsin are susceptible to R. ondatrae. In contrast, none of the 58 surviving B. glabrata exposed to R. ondatrae became infected. Moreover, when H. trivolvis and B. glabrata representing a range of sizes (3–12 mm) were exposed concurrently to >1,000 eggs (‘group exposure treatment’), all of the H. trivolvis became infected, while none of the B. glabrata became infected. Taken together, these findings provide compelling evidence of variation in competency among planorbid snail species, with H. trivolvis functioning as a highly competent host for R. ondatrae and B. glabrata being resistant to infection.

Given that B. glabrata has been recorded as a host for R. marini and B. obstructa as a host for R. ondatrae, the lack of susceptibility of B. glabrata to R. ondatrae is rather surprising. Native to the Neotropics (Campbell et al., 2000), B. glabrata is the only snail host of R. marini and R. marini guadeloupensis (Harry, 1965; Huizinga, 1973). Moreover, in Louisiana, Malek (1977) reported B. obstructa supported what was identified as R. ondatrae. Ribeiroia ondatrae and R. marini are closely related species (Wilson et al., 2005), such that apparent differences in usage of snail hosts, even within the same family, is striking. Considering that we exposed 60 B. glabrata to viable R. ondatrae embryonated eggs, which included a range of snail sizes, and the majority were the size typically most susceptible to infection (Richards, 1977), we are confident that the strain of B. glabrata used was not susceptible to infection. However, we cannot rule out different strains of B. glabrata, and we emphasize future work using field-collected strains. These results highlight the importance of host-by-parasite compatibility (Combes, 2001; Théron and Coustau, 2005). As one of the first studies to examine the effects of R. ondatrae infection on snail hosts under controlled laboratory conditions, this experiment furthers our understanding of the life cycle of R. ondatrae, including both time to infection maturation and amount of exposure needed to cause infection, as well as the consequences of infection on snail fitness of both host and non-host species.

Results of our experimental infections involving H. trivolvis revealed that mature infections can occur within 32 days of exposure to embryonated R. ondatrae eggs, as evidenced by the 32% of exposed H. trivolvis that released cercariae. Furthermore, within 43 days of exposure, all exposed H. trivolvis had released...
cercariae. This time course of infection is similar to other snail-parasite interactions (Basch and Sturrock, 1969; Gérard and Théron, 1997). For instance, in an experimental study, Basch and Sturrock (1969) found *B. glabrata* began to release *R. marini* cercariae 25–27 days after exposure to miracidia. Interestingly, while all surviving *H. trivolvis* exposed to *R. ondatrae* released cercariae by day 43 post-exposure (Fig. 2), a greater proportion of *H. trivolvis* exposed to higher dosages, e.g., 1,000-egg, released cercariae on day 38 post-exposure than those exposed to lower dosages, e.g., 50-egg (Fig. 2). These results suggest that initial exposure levels influenced the time-to-cercariae release, but did not affect the final infection prevalence.

Although not significant, high *R. ondatrae* exposure, e.g., 1,000-egg, potentially increased mortality in *H. trivolvis*. Increased mortality of infected snails compared with control snails has been observed in other snail-parasite systems (Nassi, 1978; Kuris, 1980; Krist et al., 2004; Blair and Webster, 2007). For example, Nassi (1978) found that *B. glabrata* exposed to *R. marini* experienced higher mortality relative to uninfected individuals. Additional research that uses larger sample sizes and longer experimental durations will help to determine whether parasite-induced mortality occurs in *H. trivolvis*.

Among *H. trivolvis*, *R. ondatrae* had mixed effects on growth; high dosages (1,000-egg) had no effect on growth, while low to moderate dosages (50- and 400-egg) increased growth (Fig. 2). Parasite-induced growth responses have been known to vary depending on when snails become infected (Sousa, 1983; Krist and Lively, 1988). Trematode infections in juvenile snails generally stunt or have no affect on growth, while infections in adult snails can enhance growth (Sousa, 1983; Krist and Lively, 1988). These growth differences in response to trematode infections are the result of differences in the investment to reproductive tissue, which is the target site for infection. Given that juvenile snails lack reproductive tissue, parasites draw their resources from the maintenance energy of the snail, resulting in reduced snail growth. In contrast, parasites are able to use the reproductive tissue of adult snails, thereby enabling the snail to allocate resources to growth rather than reproduction following infection. In addition to snail maturity level at time of exposure, previous research has found that trematode induced snail growth, known as 'snail gigantism', is also influenced by parasite pathogenicity (Gorbushin, 1997), snail food availability (Eisenberg, 1970; Fernandez and Esch, 1991), and snail life history strategy (Sousa, 1983; Minchella, 1985; Gorbushin, 1997).

However, no study to our knowledge has reported threshold effects in snail growth, with increases up to a certain level of trematode exposure, followed by decreases in growth. Our growth results suggest that low levels of infection, i.e., stress, may enhance growth, i.e., horesis, while higher levels suppress, or have no effect on, growth in adult *H. trivolvis*. Further experimentation is needed to fully explain this trend.

Interestingly, while *R. ondatrae* did not establish in *B. glabrata* successfully or increase mortality, *B. glabrata* did show an initial reduction in growth with increased exposure to *R. ondatrae*. This finding suggests that juvenile *B. glabrata* may be allocating energy toward preventing, or responding to, attempted infections, which resulted in reduced growth. Previous snail–trematode studies have found that *B. glabrata* and *Lymnaea elodes* experience reduced growth when exposed to *Echinostoma* spp. (Kuris, 1980; Sandland and Minchella, 2003). However, in these studies, the snails were suitable hosts for the trematode, as opposed to our findings in which even non-host species were affected by trematode exposure. The increase in growth of exposed compared with unexposed *B. glabrata* during the second half of the study (weeks 4–7) is likely due to compensatory growth leading to no final size difference among treatments. The initial stunted growth of exposed *B. glabrata* snails highlights the fact that while species may not be a host of a parasite, they can still be affected by exposure.

The high susceptibility of *H. trivolvis* to *R. ondatrae*, as well as the concave growth response of *H. trivolvis*, has potential implications for the effects of *R. ondatrae* on snail populations in the wild. Given that *R. ondatrae* causes complete castration in its snail hosts (Harry, 1965; Huizinga, 1973), the high susceptibility of *H. trivolvis* to *R. ondatrae* infection when exposed to ≥50 eggs indicates the potential of *R. ondatrae* to influence population dynamics when comparable amounts of *R. ondatrae* miracidia are introduced to the system. Similarly, Nassi et al. (1979) found the addition of 7–9 million *R. marini* eggs resulted in a temporary collapse in a *B. glabrata* population. However, little is known about the number of *R. ondatrae* eggs and miracidia present in natural systems, and we, therefore, have no information on how our experimentally administered egg levels compare to what snails experience in nature. Our findings of *H. trivolvis* having a concave growth response, with mid-exposure treatment levels having the maximum growth rate, suggest that exposure, especially the quantity of exposure, could have important effects in natural systems. In natural populations, snail size is an important determinant of susceptibility to predators, both vertebrate and invertebrate (Osenberg and Mittelbach, 1989; Chase, 2003). Thus, parasitism and predation could interact in important ways to influence snail population indirect effects (Bernot and Lamberti, 2008).

Our results underscore the importance of using experimental infections to better understand the effects of parasites on potential host species. Through experimental exposure, these findings demonstrate the susceptibility of *H. trivolvis* collected from 2 different locations to *R. ondatrae* infection. In contrast, *B. glabrata* was not a suitable host for *R. ondatrae*, despite reports of susceptibility to similar trematode species ( *R. marini* and 1 account of *R. ondatrae* infection in a congener ( *B. obstricta*). Furthermore, we found that high parasite exposure reduced growth in juvenile snails in both species, decreased time-to-cercariae release in *H. trivolvis*, and may increase *H. trivolvis* mortality. Additional exposure studies that include smaller exposure levels (<10 eggs) are needed to determine whether infection patterns are linearly related to exposure or display thresholds. Such data will provide a more comprehensive understanding of host–parasite interactions within natural communities in which exposure is highly heterogeneous. Furthermore, studies that examine the effects of *R. ondatrae* exposure and infection on snail reproduction would aid in understanding the effects of *R. ondatrae* on snail populations. While laboratory infection experiments such as this one provide a good foundation in understanding parasite life cycles and the effects of infection on hosts, it is important to follow up these experiments with field studies.

ACKNOWLEDGMENTS

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The effect of trematode infection on amphibian limb development


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SCANNING ELECTRON MICROSCOPY AND MOLECULAR CHARACTERIZATION OF A NEW HAPLOSPORIDIUM SPECIES (HAPLOSPORIDIA), A PARASITE OF THE MARINE GASTROPOD SIPHONARIA PECTINATA (MOLLUSCA: GASTROPODA: SIPHONARIIDAE) IN THE GULF OF MEXICO

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ABSTRACT: Based on scanning electron microscopy and the small subunit ribosomal RNA (SSU rRNA), Haplosporidium tuxtlensis n. sp. (Haplosporidia), a parasite found in the visceral tissues of the false limpet Siphonaria pectinata (Linnaeus, 1758), is described. The spores are ellipsoidal (3.61 ± 0.15 μm × 2.69 ± 0.19 μm), with a circular lid (2.94 ± 0.5 μm) representing the operculum. The spore wall bears filaments occurring singly, or in clusters, of 2 to 8, fusing distally. Phylogenetic relationships of H. tuxtlensis n. sp. were assessed with other described species using the SSU rRNA sequence. Haplosporidium tuxtlensis n. sp. is sister taxon to Haplosporidium pickfordi Barrow, 1961. The morphological characteristics (spore wall structure, shape, size, and filament structure) and the unique host identity corroborate it as a new species. Additionally, this is the first record of Haplosporidia infecting striped false limpets in the Gulf of Mexico.

The phylum Haplosporidia comprises parasitic protozoa mostly marine invertebrates (Perkins, 2000) that produce spore formations, although no spores have been detected in Bonamia spp. (except Bonamia perspora) (Carnegie et al., 2006). The phylum counts about 40 named species and at least 20 unnamed putative species (Hine et al., 2009) included in 4 genera, i.e., Bonamia Pichot et al., 1980, Haplosporidium Caullery and Mesnil 1899, Minchinia Labbé 1896, and Urosporidium Caullery and Mesnil 1903. Although the monophyly of the phylum is well supported (Siddall et al., 1995; Reece et al., 2004), studies based on molecular data have consistently recovered the paraphyly of the largest genus, Haplosporidium (Burreson and Ford, 2004; Reece et al., 2004). Within the phylum, several species have been extensively studied because of their impact on commercially valued molluscs. For instance, Haplosporidium nelsoni (Haskin et al., 1966) and Bonamia ostreae Pichot et al., 1879 have been thoroughly studied as causative agents of disease in oysters (Ford and Tripp, 1996; Engelsma et al., 2010), and Bonamia exitiosa Hine et al., 2001 causes high mortality in oysters in Australia, New Zealand, and Europe (Kroeck and Montes, 2005; Corbeil et al., 2006; Abollo et al., 2008). A few species of haplosporidians have been reported in marine gastropods, i.e., Haplosporidium ludianicum Azevedo, 1984 from a true limpet Heliac pellucidus Linnaeus, 1758, and Haplosporidium montforti Azevedo et al., 2006 from the European abalone Haliotis tuberculata Linnaeus, 1758. The striped false limpet, Siphonaria pectinata (Linnaeus, 1758) (Gastropoda: Siphonariidae) is a limpet-shaped opisthobranch species with an Amphipod distribution (Kawaiuchi and Gribet, 2011). Here, we describe a new Haplosporidium species from the striped false limpet based on the spore ultrastructure and ornamentation. The present study brings the number of haplosporidian parasites of aquatic gastropods to 4 and places the new species in a molecular phylogeny of the phylum.

MATERIALS AND METHODS

Host sampling and examination

Keyhole limpets, Fissurella rosea (Gmelin, 1791) and striped false limpets, Siphonaria pectinata, were collected on rocks from 2 sites near the Estación Biología “Los Tuxtlas” Instituto de Biología, Universidad Nacional Autónoma de México, Mexico; included were 69 S. pectinata and 28 F. rosea from Barra de Sontecomapan (18°33'09"N, 94°59'37"W), collected on 20 January 2011, as well as 95 S. pectinata and 28 F. rosea from Montepio (18°38'44"N, 95°05'45"W) collected on 23 January 2011. The gastropods were examined on site by slicing open the ventral foot to uncover the viscera. The digestive glands were squashed and fresh slide preparations were made in order to look for spores using a compound microscope.

<table>
<thead>
<tr>
<th>Haplosporidian species</th>
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<tr>
<td>Bonamia ex. Crassostrea ariakensis</td>
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<tr>
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<td>Cercomonas longicauda</td>
<td>AF411270</td>
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<td>ex. Haitios iris</td>
<td>AF492442</td>
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<tr>
<td>ex. Pandalus platyceros</td>
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<tr>
<td>ex. Rudipetes decussatus</td>
<td>AY435093</td>
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<tr>
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<td>Minchinia teredivis</td>
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<tr>
<td>Urosporidium cresens</td>
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<tr>
<td>Urosporidium sp. ex. Stictodora lari</td>
<td>AY449714</td>
</tr>
</tbody>
</table>
Spore isolation and fixation

The infected tissue from specimens from Barra de Sontecomapan was fixed on site using 2 methods, i.e., part of the tissue was fixed by adding 0.1-0.2 ml of 25% glutaraldehyde to tissue in 1 ml seawater, and the rest was fixed by adding 100% ethanol to tissue in a 1.5-ml microfuge tube to use for subsequent molecular sequencing. Infected tissue from specimens from Montepio was left to degrade in seawater for 14 days, changing the water every 2 days by pipetting off 1/3 the volume and replacing it with artificial sea water. Following the degradation of host tissue, and microscopic confirmation of the remaining integrity of spores, aliquots were then fixed in 2.5% glutaraldehyde in 0.2 M Sørenson's buffer, at 4 C overnight, or fixed in 100% ethanol.

Scanning electron microscopy

For each of the isolates from 4 infected specimens of striped false limpet, duplicate preparations were made of glutaraldehyde-fixed spores pipetted onto poly-L-lysine coated coverslips and left to settle at room temperature in a moist chamber for 1 hr. Coverslips were rinsed in 0.2 M Sørenson's buffer, placed in a coverslip rack, dehydrated in graded ethanol series (50% for 15 min, 75% for 15 min, and 3 times at 100% for 15 min), critical-point dried in liquid CO₂, mounted on SEM stubs with double-sided carbon tape, and sputter coated with gold–palladium. Images were obtained at the Microscopy and Imaging Facility of the American Museum of Natural History, using a Hitachi S-4700 field emission scanning electron microscope (FE-SEM) (Hitachi Ltd., Tokyo, Japan).

Molecular phylogenetic analysis

The DNeasy® Tissue Kit (Qiagen Valencia, California) was used for extraction. Amplification reactions were conducted in an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany) and employed Taq Gold and 50 cycles of 94°C (45 sec), 47°C (30 sec), and 68°C (90 sec) following a 10 min pre-melt at 94°C with primers A and B, as described in Phillips et al. (2010). PCR amplification products were purified with AMPure (Agencourt Bioscience Corporation, Beverly, Massachusetts). Cycle sequencing reactions were performed with an Eppendorf Mastercycler using 1 μl Big Dye, 1 μl of 1 μM (A, L, C, Y, O, and B described in Phillips et al. [2010]), and 3 μl of cleaned PCR template (13 μl total volume) and analyzed with an ABI PRISM 3730 sequencer (Applied Biosystems). CodonCode Aligner (CodonCode Corporation, Dedham, Massachusetts) was used to edit and reconcile sequences. Obtained sequence was deposited in GenBank and sequences employed for comparative purposes were downloaded from National Center for Biotechnology Information (see Table I for accession numbers). Alignments were accomplished using the European Bioinformatics Institute server for MUSCLE v. 3.7 (Edgar, 2004). Parsimony analyses were conducted in TNT v 1.1 (Goloboff et al., 2000) using 10 replicates of random taxon addition, sectorial searching, the Ratchet (Nixon, 1999), and tree-fusing algorithms with a requirement that the minimum length be found at least 3 times. Trees resulting from these new technology searches were submitted to tree-bisection-reconnection branch swapping retaining up to 10,000 trees. Resampling in TNT employed the parsimony jackknife (Farris et al., 1996) with the same parameters as the initial search.

RESULTS

Of 28 specimens of Fissurella rosea, none was infected. Of 164 specimens of Siphonaria pectinata, 4 were infected, 2 from each locality. SSU rRNA sequences from the new species, extracted from the 4 infected hosts from the 2 localities, did not show variation.

DESCRIPTION

Haplosporidium tuxtlensis n. sp.

(Figs. 1, 2)

Diagnosis: All measurements are taken from the SEM images and values are indicated with standard deviation. Spores ellipsoid, length 3.61 ± 0.15 μm (n = 38); width 2.69 ± 0.19 μm; ratio of width to length 0.74 ±
FIGURE 2. Scanning electron micrographs of *Haplosporidium tuxilensis* n. sp. (A) Lateral view of a spore. (B) Basal view of a spore showing emerging filaments from the wall. (C) Spore with extended filaments. (D) Younger spore with shorter filaments. (E) Detail of the operculum. (F) Detail of filament emerging from the wall and fusing together. Abbreviations: op = operculum; f = filament. All scale bars = 1 μm.
**Taxonomic summary**

**Host:** Siphonaria pectinata (Linnaeus, 1758) (Mollusca: Gastropoda: Siphonaridae)

**Site of infection:** Digestive gland epithelium.

**Locality:** Barra de Sontecomapan, Catemaco, México (18°33'09"N, 94°59'37"W).

**Material deposited:** Hepanotype SEM stubs at the American Museum of Natural History, Accession number: AMNH Protozoa 528.

**Etymology:** The epithet refers to the location where the marine gastropods were collected at the Estación Biología Los Tuxtlas, México during the first field parasitology course taught for the Richard Gilder Graduate School of the American Museum of Natural History.

**Remarks**

Haplosporidium tuxtlensis n. sp. is most similar in spore dimension to Haplosporidium hineti Bearham et al., 2008 from Australian pearl oysters in that the latter was reported with a length of 3.5-4 μm and width of 2.5-3.0 μm. The next closest species in terms of spore dimensions are Haplosporidium costale Wood and Andrews, 1962 (~3.1 × 2.6 μm), Crassostrea virginica (Burreson and Reece, 2006), and Haplosporidium edule Azevedo et al., 2003 (3.2 × 2.2 μm) from cockles (Azevedo et al., 2003). None of these 3 haplosporidians has spore ornamentation resembling that exhibited by H. tuxtlensis. The spore filaments of H. hineti are arrayed in parallel bundles tightly adhered to the spore, while those of H. costale are much more variable in thickness and form, more of a meshwork and branching ribbons at the spore surface (Burreson and Reece, 2006). Though H. edule exhibits lateral filaments as well as the spore-adhering meshwork, those filaments are short, uniformly solitary, non-fusing, and have a terminal knob (Azevedo et al., 2003). The freshwater Haplosporidium pickfordi is similar to H. tuxtlensis in having an operculum that is wider than the maximum width of the spore (Burreson, 2001), but H. pickfordi is more than twice the size of H. tuxtlensis, and the former’s spore filaments originate only posteriorly and do not fuse.

**Phylogenetic relationships**

The phylogenetic analysis based on SSU rRNA retrieved 75 most-parsimonious trees (L = 4638; RI = 0.58; CI = 0.50). Jackknife support values above branch.

Although the paraphyly of the genus was demonstrated in recent molecular studies (Reece et al., 2004; Burreson and Reece, 2006; Siddall and Aguado, 2006), it has been recommended to assign to Haplosporidium any new species of Haplosporida possessing wall-derived ornamentation and in which molecular
phyllogenetics do not group it with *Bonamia* spp. (Burreson and Reece, 2006). The rationale for this recommendation derives from the fact that no one has managed to rediscover fresh material of the type species, *Haplosporidium scolopi* Cauley and Mesnil, 1899, from a polychaete that would permit its inclusion in phylogenetic work. The phylogenetic analysis of the SSU rRNA places *H. tuxtlensis* n. sp. among other *Haplosporidium* species without ambiguity (sister group to *H. pickfordi*). Creating a monogeneric phylum under the genus *Minchinia* Labbé 1896 would understate known diversity. Notably, *H. tuxtlensis* groups with the largest clade of species in this genus, one that includes the only sequence from a parasite of a polychaete, and does not group with *H. nelsoni* nor *Haplosporidium louisiana*, the 2 species that violate monophyly of the genus.

*Haplosporidium tuxtlensis* is closely related to *H. pickfordi*, a parasite infecting freshwater snails as discussed in the only published report of a haplosporian in a freshwater host (Barrow, 1961). *Haplosporidia* infecting freshwater hosts were reported in the United States and Europe (Burreson and Ford, 2004) even though not specifically identified. The striped false limpet belongs to Heterobranchia and, therefore, is more closely related to freshwater gastropods such as freshwater snails than it is to true marine limpets. Similarly, our phylogenetic analysis retrieves *H. tuxtlensis* as more closely related to the parasite of freshwater snails than to the parasite of a true limpet, i.e., *H. hastianum*. *Haplosporidium* spp. infecting gastropods only form a monophyletic group (*H. pickfordi, H. tuxtlensis, H. montfortii, and H. hastianum*) as opposed to the rest of the *Haplosporida* occurring in Bivalvia.

Ulrich et al. (2007) reported *H. nelsoni* in the Gulf of Mexico, but subsequent studies have cast doubt on the methods used for detection of parasites (Burreson, 2008), and no evidence from further surveys detected *H. nelsoni* in this area (Ford et al., 2011). *Haplosporidium tuxtlensis* is, therefore, the first confirmed record of a haplosporian parasite species in the Gulf. The origin for *Siphonaria pectinata* on the Western Hemisphere coasts is unclear, but it seems to be an ancient and nonanthropogenic invasion from European waters (Kawauchi and Giribet, 2011). Additional investigation of *Haplosporida* infecting striped false limpets in the Mediterranean Sea could provide more insights on the historical dispersal patterns of the host.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


ABSTRACT: The microphallid *Maritrema eroliae* parasitizes shore birds in marine ecosystems while its larval stages infect mud snails and crustacean hosts. Because it is difficult to morphologically distinguish between larvae of *M. eroliae* and other microphallids co-occurring in the same habitat, partial nucleotide sequences of the ribosomal DNA (rDNA), including the 28S and 18S in addition to complete sequences of ITS1 and ITS2, were scrutinized. This analysis was used to establish the snail-crab link in the life cycle of *M. cf. eroliae*. The rDNA 28S, 18S, and ITS sequences of metacercariae from the crab *Xantho exaratus* and sporocysts from the snail *Nanosesarma minutum* were compared. Sequence alignment demonstrated that the sporocyst and metacercaria may belong to *M. eroliae* and suggested a new second intermediate host for *M. eroliae*, the crab *X. exaratus*. The phylogenetic positions of the larval stages were determined by comparing the 28S, 18S, and ITS sequences with those of other trematodes available in GenBank. The phylogenetic trees confirmed the position of *M. cf. eroliae* within the Microphallidae and found it to be closely related to *Maritrema heardi* and *Maritrema neom*. The present study represents the first molecular study correlating the larval stages in the life cycle of *M. cf. eroliae* using partial sequences of 28S and 18S in addition to complete ITS1 and ITS2 sequences. Furthermore, the sequences elucidated the evolutionary relationship of *M. cf. eroliae* to other microphallids.

Species of *Maritrema* Nicoll, 1907 are minute flukes that parasitize the intestine of shore birds. Their life cycle involves gastropods as the first intermediate host and crustaceans as second intermediate hosts (Diaz and Cremonte, 2010). A large number of *Maritrema* species have been reported throughout the world. The wide geographic distribution of *Maritrema* species is attributed to the migratory habits of the birds that serve as their definitive hosts. About 22 species have been reported in different hosts (Yamaguti, 1975; McDonald, 1981) including *Maritrema nicollii* and *Maritrema pulcherrima* from Brazil (Deblock, 1971), *Maritrema bravoae* from Peru (Caballero and Ibanez, 1970), *Maritrema bonaerensis* and *Maritrema orensensis* from Argentina (Etchegoin and Martorelli, 1997; Cremonte and Martorelli, 1998), *Maritrema novazealandensis* from New Zealand (Martorelli et al., 2004), *Maritrema arenaria* from Ireland (Irwin and Irwin, 1980), *Maritrema medium* from the United States (Sheldon, 1938), and *M. eroliae* from South Africa (Deblock and Canaris, 1992) and Japan (Ogata, 1947). Due to morphological similarities between *Maritrema* species, Deblock (1971) considered *M. eroliae* to be a synonym of both *Maritrema magnicirrus* and *Maritrema echino­cirrata*. Kinsella and Deblock (1994) considered *Floridatrema heardi* as a separate genus from *Maritrema* based on the morphological features of the anterior pre-cecal extensions of the uterine loops. Later, this morphological feature was considered an insufficient tool for differentiating microphallid genera; thus, Tkach et al. (2003) transferred *F. heardi* to *M. heardi*. This taxonomic confusion clearly indicates that morphological characters alone cannot resolve systematic problems of the *Maritrema* species.

In Kuwait, a single *Maritrema* species has been reported, i.e., *M. eroliae* (Al-Kandari et al., 2007). Its identification was based on morphological features of the adult fluke, which was recovered from in vitro excystment experiments. The cysts used in these experiments were isolated in the crab *Nanosesarma minutum*. However, its definitive identification remains questionable for several reasons. First, the crab *N. minutum* is involved in the life cycle of another microphallid, *Probolocoryphe uca* (Al-Kandari and Al-Bustan, 2010). Second, the larval stages of both microphallids are similar morphologically. Third, both microphallids infect snails that live in the same habitat and geographic area. Therefore, more reliable and accurate techniques are required for the identification of *M. eroliae* in order to overcome the limitations raised above.

Moreover, the accurate identification of *M. eroliae* is essential for understanding the life cycle, especially in relation to the first and second intermediate hosts. The snail *Clypeomorus bifasciata* was suggested by Al-Kandari et al. (2007) as a possible first intermediate host for *M. eroliae* and the crab *N. minutum* as its second intermediate host. However, it is possible that other crabs such as *Xantho exaratus*, living in the same habitat, may serve as the second intermediate host. The crab *X. exaratus* has already been suggested to be the second intermediate host at Kuwait Bay for species of 2 other microphallids, *Longiductoterma* spp. and *Microphallus* spp. (Abdul-Salam et al., 1997).

Recent molecular biology techniques allow discrimination between closely related species by comparing ribosomal DNA (rDNA) sequences. The rDNA cluster is particularly useful for platyhelminth systematics because it is highly repeated and contains variable regions flanked by more conserved regions (Adlard et al., 1993). The 18S and 28S rDNA genes are highly conserved, relatively slow-evolving, and are used as the basis for revealing interrelationships within, and between, families of platyhelminths. However, the ITS regions in rDNA are fast evolving and highly variable. They are used as diagnostic markers for species recognition as well as for larval form identification (Nolan and Cribb, 2005; Littlewood, 2008). Hence, sequences of the 28S, 18S, and rDNA ITS together can be employed for reliable and accurate identification of the larval stages of *M. cf. eroliae*.

The present work describes, for the first time, the molecular identification and characterization of the larval stages of *M. cf. eroliae* using rDNA 28S, 18S, ITS1, and ITS2 sequences. Moreover, it adds new information that may assist in resolving the life cycle of *M. cf. eroliae* based on sequences obtained from the larval stages (position sporocysts and metacercariae). The sequence data clarify the phylogenetic relationship of *M. cf. eroliae* with respect to other microphallids.

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Table I. Primers used in this study for PCR amplification and sequencing of the 28S, 18S, ITS1, and ITS2 regions of *Maritrema cf. eroliae*.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Amplified region</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>U178</td>
<td>28S</td>
<td>5'GCACCCCGCTGAAYTAAG3'</td>
</tr>
<tr>
<td>L1642</td>
<td>28S</td>
<td>5'CCAGGGCCATCCACATTTC3'</td>
</tr>
<tr>
<td>U1148</td>
<td>28S</td>
<td>5'GACCCGAAGAGGTGTA3'</td>
</tr>
<tr>
<td>L2450</td>
<td>28S</td>
<td>5'GCCTTTCATTATAACGACTCGGA3'</td>
</tr>
<tr>
<td>U1846</td>
<td>28S</td>
<td>5'AGGCGGCAATGGGAAGGG3'</td>
</tr>
<tr>
<td>L3449</td>
<td>28S</td>
<td>5'ATTCGTACATTGGACGGTCAA3'</td>
</tr>
<tr>
<td>Primer 1</td>
<td>18S</td>
<td>5'CACACCTGGTTGATCCTGCA3'</td>
</tr>
<tr>
<td>Primer 2</td>
<td>18S</td>
<td>5'ACACGGCCATGCACACCC3'</td>
</tr>
<tr>
<td>S20T2</td>
<td>ITS1</td>
<td>5'GTAAAGTCGAAGTCATAACGC3'</td>
</tr>
<tr>
<td>5.8S1</td>
<td>ITS1</td>
<td>5'TGTCGATGAAGAGCGCAGC3'</td>
</tr>
<tr>
<td>HC2</td>
<td>ITS2</td>
<td>5'CCCGCTGAATCAGCTAT3'</td>
</tr>
<tr>
<td>LC1</td>
<td>ITS2</td>
<td>5'GACATCATGGAAGACCC3'</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Sample description and collection**

The gastropod *C. bifasciata* (Gastropoda: Prosobranchia) and the crab *X. exaratus* (Milne Edwards, 1834) were collected at low tide from Shuwaikh Bay, which is located along the northern coast of Kuwait, between latitudes 29°21'N and 47°57'E. In total, 771 snails and 136 crabs were hand picked from mud and rocks covering the shore between the period of April 2009 and September 2009. Within 24 hr of being transferred to the laboratory, snails and crabs were maintained in aerated seawater at about 20°C until further processing that took place within 72 hr. Snails were gently crushed with a hammer and their tissues were examined for the presence of sporocysts using a dissecting microscope. The tissue of each crab was macerated and examined for the metacercariae using a dissecting microscope. The cyst wall was broken for each metacercaria cyst.

The identification of larval digeneans was made on vital stained (0.5% neutral red) specimens according to Cable (1956) and Schell (1985). The identification of the larval stages of *M. cf. eroliae* was based on morphological characters as described by Abdul-Salam and Sreelatha (1998) and Al-Kandari et al. (2007). The number of infected and uninfected snails and crabs, as well as the type of infection, was recorded. For each *M. cf. eroliae* infection, the sporocysts and metacercariae were cleaned from snail tissues and stored in 95% ethanol for subsequent DNA extraction.

**DNA extraction and PCR amplification**

Individual parasite sporocysts and metacercariae were washed with mill-Q filtered water (mQ,H2O) to remove traces of ethanol. The gDNA was extracted using proteinase K digestion and organic-aqueous phase extraction (Barber et al., 2000).

The 28S partial DNA sequence was amplified in 3 overlapping sections using the primer combinations U178 + L1642, U1148 + L2450, and U1846 + L3449 (Table I) (Lockyer et al., 2003). The PCR reactions were performed in a total volume of 25 µl (2.5 µl of 10X PCR buffer, 2.5 mM MgCl2, 10 µM of each dNTP, 1.5 µM of each primer, and 1 U of amplitaq DNA polymerase). The PCR conditions used were an initial denaturation step at 94°C for 5 min followed by 40 cycles of 30 sec at 94°C, 30 sec at 52°C, and 2 min at 72°C, followed by a final extension of 7 min at 72°C and maintenance at 4°C.

The 18S partial DNA sequence was amplified using the primers described by Barker et al. (1993) (Table I). The PCR reactions were performed in a total volume of 25 µl (2.5 µl of 10X PCR buffer, 2.5 mM MgCl2, 10 µM of each dNTP, 1.5 µM of each primer, and 1 U of amplitaq DNA polymerase). The PCR conditions used were an initial denaturation step at 95°C for 3 min followed by 35 cycles of 30 sec at 95°C, 30 sec at 64°C, and 2 min at 72°C, followed by a final extension of 7 min at 72°C and maintenance at 4°C.

The ITS1 and ITS2 regions of the nuclear rDNA were amplified by PCR using 4 different primers (Eurogentec, Ougree, Belgium). The ITS1 region was amplified using the primer combination S20T2 + 5.8S1 (Table I) (Bartoli et al., 2000). The ITS2 region was amplified using the primer combination HC2 + L1 (Table I) (Després et al., 1992). The PCR reactions were performed as described by Al-Kandari and Al-Bustan (2010). A 50-base pair (bp) and 123-bp DNA ladder marker (Sigma, St. Louis, Missouri) were used to estimate the size of the amplified products. PCR products were purified using the Nucleospin Extract II kit (Macherey Nagel, Duren, Germany) according to the manufacturer's protocol.

**DNA sequencing**

The purified PCR products were then sequenced in the Gene Analyzer 3130XL (Applied Biosystems, Foster City, California) using the Big Dye Terminator kit (Version 3.1, Applied Biosystems), according to the manufacturer's instructions. The same amplification primers mentioned above were used for the forward and reverse sequencing in 2 separate reactions. The sequences were first analyzed using EMBOSs (Rice et al., 2002) and aligned using ClustalW2 (Thompson et al., 1994), then subjected to further analysis by using various bioinformatics tools. The sequences obtained in this study were deposited in the GenBank under the accession numbers JF826246, JF826247, HQ650132, and HQ650133 for 18S, 28S, ITS1, and ITS2, respectively.

**Molecular phylogenetic analysis**

The similarity search of the sequences was performed using BLAST (http://www.ncbi.nlm.nih.gov/blast). The alignment of the sequences with other digenean sequences published in GenBank was carried out using ClustalW2 (Thompson et al., 1994). Phylogenetic analysis was performed based on distance, discrete character, and maximum likelihood methods. *Ochetosoma kansense*, *Gorgoderpa sp.*, and *Fasciola hepatica* were used as outgroups in constructing phylogenetic trees based on the 28S, 18S, and ITS sequences. Maximum parsimony calculations were performed using a closest-neighbor-interchange algorithm with Mega 4.0 (Tamura et al., 2007) for 1,000 replicates. All positions containing gaps and missing data were eliminated from the dataset. Maximum likelihood calculations were obtained using PHYML of PHYLLIP (Guindon and Gascuel, 2003). The general time reversible nucleotide substitution model was used with an instantaneous rate matrix for the frequency of nucleotide proportion. The initial trees were constructed with a BIONJ algorithm (Gascuel, 1997) based on 3,000 replicates. Bayesian inference analyses were run using MrBayes (Heuelsenbeck and Ronquist, 2001) employing GTR + I + Γ model across the sites. Posterior probabilities were estimated for 1,000,000 generations, sampling every 10 generations. The first 250 trees were discarded as a burn-in.

**RESULTS**

**Percentage of infection**

Of the 771 examined snails, 8.3% were infected with sporocysts of *M. cf. eroliae* and 13.2% of the examined crabs (n = 136) were infected with the metacercariae of *M. cf. eroliae*.

**PCR amplification and analysis**

The rDNA 28S and 18S partial regions of *M. cf. eroliae* were successfully amplified using the primer combinations described above. The amplification of the 28S region using the 3 primer combinations yielded 3 fragments with molecular sizes of 1,353 bp, 1,599 bp, and 1,845 bp. The 18S region was represented by a single 1,087-bp fragment. The analysis and assembly of the PCR products revealed that the amplified 28S and 18S regions represent partial sequences with molecular sizes of 3,781 bp and 1,087 bp, respectively. The 28S and 18S partial sequences of all examined samples (including sporocysts and metacercariae) were identical in composition and length.

The rDNA ITS1 and ITS2 regions of *M. cf. eroliae* sporocysts and metacercariae yielded the single expected product for each, with a molecular size of 909 bp and 440 bp, respectively. After analysis of the PCR product sequences, the 18S, 5.8S, and 28S regions were
identifying through their comparison with identical regions of other digenean sequences available in GenBank. These regions did not overlap with the sequences of the 28S and 18S rDNA fragments amplified above. After the sequences of 28S, 5.8S, and 18S were identified and removed from the overall sequence, it was possible to identify the complete ITS1 and ITS2 sequences, which showed molecular sizes of 426 bp and 295 bp, respectively. The ITS2 sequences of all examined samples were identical in composition and length while their ITS1 sequences were found to be 99.8% similar with only 2 nucleotide differences. In addition, the ITS2 sequences of the sporocyst samples were found to be identical to the sequences obtained from the metacercariae while their ITS1 sequences were found to be 99.8% similar.

For comparative purposes, the 28S and 18S partial sequences, as well as the ITS1 and ITS2 complete sequences of *M. cf. eroliae*, were compared with those of other digeneans obtained from GenBank (Table II). No identical sequences were found in GenBank. The BLAST search result revealed that the query 28S was closely similar to *M. heardi* (synonym for *F. heardi*), *Maritrema oocysta*, and *Maritrema neomi* (Table III). However, the 18S sequence was very similar to *M. oocysta*, which is the only 18S sequence of *Maritrema* species available in GenBank. At the species level, there were 174 nucleotide differences (13.6% variation, 92 parsimony informative sites) in the 28S sequence between all *Maritrema* species; *M. cf. eroliae* and *M. heardi* differed at 30 nucleotide sites (2.3% variation) while *M.*
Phylogenetic analysis

Phylogenetic trees based on the 28S partial sequences were obtained by comparing the sequences of *M. cf. eroliae* and available sequences for trematodes in GenBank (Table II). Phylogenetic analysis, using various distance and discrete methods such as maximum parsimony and Bayesian inference, showed that the topology is similar among trees obtained (Fig. 1). Species of *Maritrema* assembled in a well-supported monophyletic clade. Bootstrap values revealed significant support for the subclade containing *M. cf. eroliae*, *M. heardi*, and *M. neomi*. The values of 70% and above in the bootstrap test of phylogenetic accuracy indicate reliable grouping among different species of microphallids. The phylogenetic analysis based on the 28S and 18S partial sequences confirmed the position of *M. cf. eroliae* within the Microphallidae, particularly within the *Maritrema* group. The constructed phylogenetic trees revealed a close relationship of *M. cf. eroliae* with *M. heardi* and *M. neomi*. However, the phylogenetic trees constructed based on the 18S partial sequences also suggested a close relationship of *M. cf. eroliae* with *M. oocysta*, although it is the only *Maritrema* species with an 18S sequence available in GenBank (Fig. 2).

The phylogenetic trees constructed based upon ITS1 and ITS2 sequences placed *M. cf. eroliae* within the well-supported Microphallidae clade (Figs. 3, 4). Moreover, *M. cf. eroliae* clearly groups with *Maritrema* sp. SP-2011 in the phylogenetic tree constructed based on the ITS1 sequence, as *Maritrema* sp. SP-2011 is the only *Maritrema* species with an ITS1 sequence deposited in GenBank. The relationship of the 2 microphallids was further supported by the high bootstrap values (70–80%). However, in the ITS2 phylogenetic tree, *M. cf. eroliae* groups with *P. uca* within the Microphallidae clade, showing a lower nucleotide substitution than *P. uca* based on the Bayesian inference analysis. The monophyletic microphallid clade based on ITS1 complete sequence included *Microphallus primas*, *Gynaeotyla longintestinata*, *Cercaria sevillana*, microphallid sp., *P. uca*, *Maritrema* sp. SP-2011, and *M. cf. eroliae* with 70–88% support values (Fig. 3). However, the monophyletic Microphallidae clade based on the ITS2 complete sequence comprised only microphallid sp., *P. uca*, and *M. cf. eroliae* with 98–100% bootstrap values (Fig. 4). The discrepancies in characterizing the microphallid clade between ITS1 and ITS2 sequences appear to be greatly influenced by the availability of the sequences in GenBank.

**DISCUSSION**

Species of *Maritrema* have ecological importance in their environment because they castrate the first intermediate host and
cause mortality in the second intermediate host (Fredensborg et al., 2005; Fredensborg and Poulin, 2006). Therefore, several studies have focused on the identification of the adult *Maritrema* species using morphological characteristics (Rankin, 1940; Irwin, 1983; Cremonte and Martorelli, 1998; Martorelli et al., 2004; Al-Kandari et al., 2007; Diaz and Cremonte, 2010). Recently, Al-Kandari et al. (2007) described the adult stage of *M. eroliae* from an in vitro excystment experiment using metacercariae naturally infecting the crab *N. minutum* from Kuwait Bay. Because of the lack of molecular data and the limitations of the light microscopy study, it was difficult to identify the larval stages of *M. eroliae* and to study its life cycle. The marked morphological difference between the adult and metacercariae stages made it impossible to establish a direct link, especially because the crab *N. minutum* is also involved in the life cycle of another microphallid, *P. uca*. Because morphological identification of *Maritrema* species caused systematic confusion, Tkach et al. (2000, 2003) used sequences of rDNA to identify and study the phylogenetic interrelationships of *M. heardi*, *M. arenaria*, *M. oocysta*, *Maritrema subdolum*, *M. neomi*, and *Maritrema proshometra*. In the present study, we clarified part of the life cycle hosts of *M. cf. eroliae* based on rDNA sequences.

Sequence analysis of the rDNA 28S, 18S, and ITS2 analyzed in the present study revealed a complete match of the sequences of sporocysts isolated from the snail *C. bifasciata* and metacercariae isolated from the crab *X. exaratus*, indicating that they belonged to the same species, *M. eroliae*. Adult parasites from definitive hosts were not identified in the present study for rDNA sequence analysis. Therefore, the xiphidocercariae parasitizing the snails with its corresponding sporocysts could be the larval stages of *M. eroliae*. Moreover, the snail *C. bifasciata* appeared to serve as the first intermediate host while the crab *X. exaratus* is considered the second intermediate host. This finding disagrees with Al-Kandari et al. (2007), who asserted that the crab *N. minutum* is the second intermediate host for *M. eroliae*. This discrepancy highlights the shortcoming of relying on just morphological characteristics for the identification of *M. eroliae*. It is also possible, however, that the
The results of the phylogenetic analysis based on the 28S sequences confirmed the position of *M. cf. eroliae* within the Microphallidae, particularly within the *Maritrema* group. Similar microphallid *M. eroliae* uses a wide range of crabs as second intermediate hosts to assure successful transmission to the definitive host and life cycle completion. This assumption supports the need to examine these microphallids at the molecular level.

The conserved 28S, 18S, and ITS2 sequences among all the examined samples revealed the presence of only 1 species of *Maritrema*. The ITS1 sequence is more variable than the ITS2, yielding low similarity values (0.2% variation) among sporocyst samples as well as for sporocyst and metacercariae sequences. This is attributed to the intraspecific variation of the ITS1 sequences where the homogenization mechanisms of ITS1 copies to a uniform repeat type are still incomplete (Kane et al., 1996; Van Herwerden et al., 1998, 1999). Some differences in the ITS1 sequences may be due to natural variation within the trematode population (Hust et al., 2004). Intraspecific variation in ITS sequences has been reported in trematodes (Adlard et al., 1993) and monogeneans (Desdevises et al., 2000).

The results of the phylogenetic analysis based on the 28S sequences confirmed the position of *M. cf. eroliae* within the Microphallidae, particularly within the *Maritrema* group. Similar to the findings of Tkach et al. (2003), *Maritrema* species formed a well-supported clade, where *Maritrema* species, i.e., *M. neomi* and *M. heardi*, which parasitize small mammals such as shrews and marsh rice rats, cluster together forming a subclade. It is interesting that *M. cf. eroliae* clustered with *M. neomi* and *M. heardi* in the same subclade. In contrast, phylogenetic analysis based on the 18S sequences grouped *M. cf. eroliae* with *M. oocysta* which may be the consequence of *M. oocysta* being the only *Maritrema* species with an 18S sequence available for analysis.

Phylogenetic analysis based on ITS1 and ITS2 sequences also confirmed the position of *M. cf. eroliae* within the Microphallidae, where it is closely related to *P. uca*. The close relationship between *M. cf. eroliae* and *P. uca* was further supported by the high bootstrap values. This close relationship may be related to the fact that these microphallids infect 2 different gastropods that occur in the same habitat and same geographic area and have possibly evolved from the same ancestors. The lack of rDNA ITS2 sequences of other *Maritrema* species in GenBank made it difficult to study their interspecific variation and phylogenetic relation based on the ITS2 sequence. The ITS sequences of other *Maritrema* species should be included in future studies in order to resolve the interspecific relationships and phylogeny of *Maritrema* spp. and to validate the use of rDNA ITS sequences in species identification among members of *Maritrema*. Data based on ITS1 and ITS2 suggest the usefulness of the ITS1 region in detecting intraspecific variations among populations, while ITS2 can be used to differentiate between closely related species and genera.

The data reported have shown that the rate of *M. cf. eroliae* infection in *C. bifasciata* at Kuwait Bay was high during the study.
period (April–September 2009). This is consistent with the previous findings of Abdul-Salam et al. (1997) who suggested that the snail C. bifasciata harbors microphallid infections throughout the year with 2 infection peaks, 1 in the summer and another in winter. It appears that snails infected with M. cf. eroliae generate a pool of infective cercariae in the summer and winter that encyst in crabs, which are then the source of infections for migratory shore birds during autumn and spring (Abdul-Salam et al., 1997). The summer peak of M. cf. eroliae infection shown in the present study is probably related to the behavior of the definitive hosts as well as to the acceleration in the development of intra-molluscan larval stages due to favorable environmental conditions such as temperature (Abdul-Salam et al., 1994). Several studies have reported that sea gulls (Larus spp.), including Larus dominicanus, Larus atlanticus, Larus maculipennis, Larus novaehollandiae, and Larus argentatus (Rankin, 1940; Deblock, 1975; Etcheogin and Martorelli, 1997; Cremon and Martorelli, 1998; Martorelli et al., 2004; La Sala et al., 2009), serve as definitive hosts for several Maritrema species. The European herring gull, L. argentatus, is one of the birds in Kuwait that migrate from Europe and Russia, passing across Kuwait Bay during the short months of autumn and spring (Haynes, 1979). Because L. argentatus has been reported to be a definitive host for M. eroliae (Rankin, 1940; Deblock, 1975), it is possible that the herring gull deposits the infective eggs at Kuwait Bay. Based on the prevalence of M. cf. eroliae in the first intermediate host, and on the migratory behavior of the definitive host, it is suggested that M. cf. eroliae is an allogenic species introduced to Kuwait Bay during the periods of herring gull migration. Further studies are required to resolve the proposed crab–bird link in the life cycle of M. cf. eroliae and whether L. argentatus is the definitive host in their life cycle.

In conclusion, the present paper represents the first molecular identification of the larval stages of M. eroliae and contributes to its life cycle characterization. The first intermediate host, C. bifasciata, is parasitized by sporocysts containing cercariae that develop into metacercariae. Encysted metacercariae were isolated from the crab X. exaratus, which constitutes a second intermediate host. Moreover, the results demonstrate the utility of ITS sequences in connecting trematode life cycle stages from different hosts as well as in distinguishing morphologically similar trematode species such as M. cf. eroliae and P. uca. Future studies relying on trematode ITS sequences may provide valuable tools for tracing the migration path of migratory birds and for understanding the relationship between different trematode species in different geographical areas.

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LITERATURE CITED


Molecular prevalence of different genotypes of Theileria orientalis detected from cattle and water buffaloes in Thailand

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ABSTRACT: Here we report on an epidemiological study regarding the molecular prevalence of different genotypes of Theileria orientalis present among domestic cattle and water buffalo populations bred in Thailand. A phylogenetic analysis based on the parasitic gene encoding a major piroplasm surface protein revealed the presence of 5 genotypes (Types 1, 3, 5, 7, and N-3) in cattle and 7 genotypes (Types 1, 3, 4, 5, 7, N-2, and N-3) in water buffaloes. Types 4, 7, and N-3 of T. orientalis were reported for the first time in water buffaloes. The previously reported C and Thai types from Thailand clustered as types 7 and 6, respectively, in the present analysis. Great similarities were observed among nucleotide sequences of isolates of the same genotype from cattle and water buffaloes, and, therefore, water buffaloes were considered to serve as a reservoir for these genotypes of T. orientalis in Thailand. In conclusion, T. orientalis parasites circulating in Thailand are more diverse in their genetic characters than previously anticipated.

Theileria parasites that infect different livestock species are relegated into 2 groups. Theileria parva and Theileria annulata form the first group that causes malignant lymphoproliferative theileriosis in cattle (Onuma et al., 1997), while another group of species whose taxonomic classifications are still under debate causes a non-lymphoproliferative bovine theileriosis (Minami et al., 1980; Kim et al., 1998). This second group includes Theileria sergenti/Theileria buffeli/Theileria orientalis (Uilenberg et al., 1985; Fujisaki et al., 1994). The disease caused by the benign group (we used “T. orientalis” as the common name in the present study) has been often described as a subclinical condition, although the clinical picture may include anemia and other nonspecific signs (Kawazu et al., 1992). Nonetheless, the disease may eventually lead to severe economic losses in endemic areas (Minami et al., 1980; Baek et al., 1990; Kim et al., 2004). Studies conducted on T. orientalis in relation to diagnosis and prevention have generated valuable information regarding survival strategies adopted by these parasites against host immunity (Katzer et al., 2010).

Many bovine hemoprotozoan parasites are capable of developing an antigenic polymorphism to escape from host immune responses, which ensures their long-term survival (Katzer et al., 1994). Similar observations have also been made with T. orientalis; the antigenic polymorphism defined by a major piroplasm surface protein (MPSP) has been extensively investigated (Zuang et al., 1994; Kubato et al., 1996).

MPSP gene sequences derived from different isolates of T. orientalis form a number of clusters in phylogenetic analyses, and, importantly, these clusters have shown different antigenic properties (Jeong et al., 2010). These findings have led researchers to conclude that the T. orientalis group can be classified with regard to any number of different genotypes (Kim et al., 1998; Ota et al., 2009; Yokoyama et al., 2011). Initially, 4 genotypes (I, C, B, and Thai types) were described for the parasites (Kakuda et al., 1998; Sarataphan et al., 1999, 2003). Kim et al. (1998) suggested that the benign Theileria species possessed 6 genotypes (Types 1-6). In addition, Types 7 and 8 were subsequently identified (Kim et al., 2004; Ota et al., 2009; Jeong et al., 2010). Interestingly, analyses of MPSP gene sequences of Vietnamese isolates indicated the presence of additional genotypes that included types N-1, N-2, and N-3 from sheep, water buffalo, and cattle, respectively (Kawasu et al., 1999; Khukhhuu et al., 2011). Our recent study on Mongolian bovine isolates also generated a range of MPSP gene sequences, with many of them resembling the novel type N-3 of T. orientalis (Altangerel et al., 2011).

In contrast to the above classification, Bai et al. (2002) and Liu et al. (2011) have described type 6 as T. sinensis, and argued that it may be a different species based on the molecular characters and transmission vectors. Similar results were obtained in a recent study on T. orientalis conducted in China, where the type 6 was not detected among the sampled populations (Liu et al., 2011).

Previously, Kakuda et al. (1998) indicated that the T. orientalis isolates collected in Thailand were phylogenetically separated from those of China and the United States. In addition, the presence of Thai, B, and C types in the Thailand cattle population was reported by Sarataphan et al. (1999). Another study using the DNA samples extracted from blood of 214 cattle and 33 water buffaloes concluded that the C and Thai types of T. orientalis were circulating among these animal populations in Thailand (Sarataphan et al., 2003). Because the C type detected in Thailand was indeed closer to Indonesian isolates than the Chitose type of Japanese isolates, the authors suggested that the C type in Thailand must belong to a different genotype of T. orientalis. Since the latter study was based on an allele-specific PCR method and few gene sequences, the findings and descriptions were limited to the existing genotypes. Therefore, we conducted the present investigation, focusing on the genetic diversity of T. orientalis in Thai isolates using greater numbers of the newly determined MPSP gene sequences based on the current criteria for genotyping.

MATERIALS AND METHODS

Sample collections

Blood samples of cattle were collected from 3 locations, including Chiang Rai, Chiang Mai, and Lampang located in the northwest region of Thailand, in September 2009. Five locations, including Roi Et, Ubon-Ratchathani, Si Sa Ket, Surin, and Buri Ram, were selected from the northeast region for sample collection from water buffaloes in January 2010 (Fig. 1) (Terkawi et al., 2011). Approximately 1.5 ml of whole blood was collected from each animal by venipuncture using 21-gauge needles. Blood samples were placed into 2 ml tubes containing 1 ml of ethylene diamine tetra-acetic acid (EDTA) and stored at -80°C until analysis.
was collected from each animal and transferred into sterile tubes containing EDTA (NIPRO, Osaka, Japan). The blood samples were collected from 200 cattle and 255 water buffaloes from the selected locations.

DNA extraction

Two hundred microliters of the whole blood were subjected to a DNA extraction protocol. A Qiagen blood DNA extraction kit (Qiagen, Hilden, Germany) was employed, according to the manufacturer’s instructions. Finally, the DNA sample was prepared in 100 μl of the elution buffer that was provided with the kit. Extracted DNA samples were stored at −30 C until use (Ota et al., 2009).

Polymerase chain reaction, cloning, and sequencing

A previously described set of forward (5’-CTTTGCTTAGATA­CTTCT-3’) and reverse (5’-ACGGCAAGTGTTGAGAACT-3’) primers was used for the PCR detection of T. orientalis MPSP genes (Ota et al., 2009). The reaction mixture of 10 μl, which contained 0.1 μl of each primer from 10 μM stocks, 5 μl of 2 x Ampdirect plus (Shimadzu Biotech., Kyoto, Japan), 0.1 μl of Extaq DNA polymerase (Takara, Tokyo, Japan), 3.7 μl of double distilled water (DDW), and 1 μl of the template DNA, was amplified under previously described thermal conditions (Ota et al., 2009). Briefly, an activation step at 94 C for 10 min was followed by 35 cycles that each consisted of a denaturation step at 94 C for 1 min, an annealing step at 58 C for 1 min, and an extension at 72 C for 1 min. After the final extension at 72 C for 4 min, positive reactions were identified by detecting a 776-bp specific band on agarose gels after electrophoresis.

Selected PCR-positive samples from each location were then amplified under conditions similar to the screening PCR, except for the composition of the reaction mixture. The total volume for each reaction was increased to 50 μl, which included 1 μl of the DNA sample, 10 μl of Expand HiFi Plus reaction buffer, 1 μl of each 10 μM primer, 0.5 μl of Taq DNA polymerase-Expand HiFi Plus; Roche Applied Science, Basel, Switzerland), 1 μl of 10 mM Nucleotide Mix (Roche Applied Science), and 35.5 μl DDW. The amplified PCR products were gel-extracted and then ligated into a TA-cloning plasmid vector (PCR 2.1-TOPO; Invitrogen, Carlsbad, California). Plasmids with the inserted DNA fragment were then transformed into E. coli competent cells (TOP 10; Invitrogen) and cloned. After overnight incubation, clones were isolated and cultured at 37 C. The plasmids were then extracted from the cultures using a commercial QIAprep Spin Miniprep kit (Qiagen), and the presence of the inserted DNA fragment was confirmed by the PCR method as described above. Finally, the nucleotide sequences of the DNA fragments were determined according to a previously described protocol (Yokoyama et al., 2011).

Phylogenetic analysis

The nucleotide sequences obtained in the present study, together with the previously registered MPSP gene sequences in GenBank, were used to construct a phylogenetic tree as described by Khukhuu et al. (2011). In brief, the GENTX 7.0 software package was used to analyze the nucleotide sequences (GENTX, Tokyo, Japan). Furthermore, a ClustalW program (Thompson et al., 1994) was used to construct the guide tree based on the multiple alignment and neighbor joining methods (Perrière et al., 1996). The confidence of branching pattern of the constructed tree was estimated by a Bootstrap test (Felsenstein, 1985).

RESULTS

The results of screening MPSP-PCR assay showed the presence of T. orientalis parasites circulating in the blood of cattle and water buffaloes bred in the 8 regions of Thailand (Fig. 1). Approximately 25% of the cattle populations in Chiang Rai and Chiang Mai regions were positive for T. orientalis infections, while relatively high numbers of positive animals (50%) were found in the cattle population of the Lampang area (Table I). However, none of the water buffaloes from the Ubon-Ratchathani region were positive in the MPSP-PCR assay, whereas buffaloes in the Roi Et region exhibited the highest positive percentages (25.6%) (Table I). The average percentages of positive animals in the studied areas were 31.5 and 9.4% for the cattle and water buffaloes, respectively.

The MPSP gene sequences of T. orientalis isolates derived from the cattle (n = 28) and water buffaloes (n = 24) were phylogenetically analyzed to identify the genotypes of T. orientalis circulating in the domestic animals of Thailand (Fig. 2). Five genotypes (Types 1, 3, 5, 7, and N-3) and 7 genotypes (Types 1, 3,
4, 5, 7, N-2, and N-3) were identified from cattle and water buffalo populations, respectively (Table I). Among the 5 types detected from the cattle population, type 3 was found in all 3 regions surveyed in the present study, while types 1 and 7 were detected from 2 locations (Chiang Rai and Lampang). Type 5 was found only in Lampang and the N-3 was detected only from Chiang Rai (Table I). The cattle population in the Chiang Rai region harbored 4 different genotypes (Types 1, 3, 7, and N-3), while the cattle in the Chiang Mai region were infected with only 2 different genotypes (Types 1 and 3) (Table I).

Among the water buffalo populations bred in the regions of Thailand selected in this study (Fig. 1), 7 genotypes (Types 1, 3, 4, 5, 7, N-2, and N-3) of *T. orientalis* were identified (Table I). Type 7 was predominantly detected in all areas where *T. orientalis*-positive...
buffaloes were found. The highest genotypic variation was found among T. orientalis isolates in the population of the Roi Et region, where all 7 genotypes identified in the current study were present (Table 1). The previously reported C type (AB081329) and Thai type (AB010703) from Thailand were clustered as types 7 and 6, respectively, in the phylogenetic analysis (Fig. 2).

DISCUSSION

The MPSP gene has been well recognized as an epidemiological molecular marker for understanding the genetic diversity of T. orientalis (Kawazu et al., 1992, 1999; Kakuda et al., 1998; Sako et al., 1999; Sarataphan et al., 1999; Kô et al., 2008; Ota et al., 2009). The multiple alignments of MPSP genes derived from different isolates indicate the presence of several clusters with different antigenic characters, and thus researchers have described them as different genotypes (Kim et al., 2004; Jeong et al., 2010; Yokoyama et al., 2011). Previously, the identification of T. orientalis genotypes has been based on the geographical locations of the isolates. However, further studies have proved the lack of relationship between the genotypes and the geographical locations (Jeong et al., 2010). Therefore, it would be more appropriate to adopt the numerical classification method proposed by Kim et al. (1998).

Initially, there were 6 genotypes (Types 1–6) of T. orientalis (Kim et al., 1998), and, subsequently, 2 additional genotypes (Types 7 and 8) were added (Jeong et al., 2010). In our recent study, we described 3 more genotypes (Types N-1, N-2, and N-3), based on phylogeny with previously reported MPSP gene sequences and those recorded in Vietnamese T. orientalis (Khukhhu et al., 2011).

The present study revealed the presence in Thailand of at least 5 and 7 genotypes in cattle and water buffaloes, respectively, and that type 3 was detected in cattle populations from all the selected locations. One of the recently added genotypes, type N-3, was also detected in cattle from the Chiang Rai region. This finding is in agreement with our recent investigation on the molecular epidemiology of T. orientalis from Mongolian cattle (Altangerel et al., 2011). In water buffaloes, type 7 of T. orientalis, which was isolated from 4 of 5 locations, was found to be the dominant genotype in Thailand. In addition, types N-2 and N-3 were also identified in the water buffaloes from Roi Et region. The existence of types 4, 7, and N-3 reported here is the first report of these forms in water buffaloes.

In the present study, the previously described C and Thai types were classified as types 7 and 6, respectively, using the present criteria. The phylogenetic analysis revealed that the Indonesian isolates (AF102500) also belong to type 7, together with Thai isolates originally described as C type (AB081329), whereas the Japanese C types (D12689) are clustered with type 1 (Fig. 2). Although the cattle and water buffalo samples were collected from different locations, the sequences of MPSP genes derived from both animals showed great similarities within particular genotypes. This finding appear to support the previous suggestion that water buffaloes may serve as a reservoir for many hemoprotozoan parasites (Oura et al., 2010).

Although a previous study detected the B type (type 3) among cattle population in Thailand (Sarataphan et al., 1999), a subsequent epidemiological survey by the same investigator failed to demonstrate the presence of B type (Sarataphan et al. 2003). However, in the present study, type 3 was identified in cattle populations from all locations. In addition, type 2 (I type) of T. orientalis, which has been reported in Japan, the Republic of Korea, and China (Kakuda et al., 1998; Kim et al., 1998; Jeong et al., 2009; Ota et al., 2009; Yokoyama et al., 2011), was not detected in the current study. This finding is in agreement with the previous observation made by Sarataphan et al. (1999, 2003).

A previous study had identified that the Thai type, which is clustered under type 6 in the present study, was the dominant genotype in Thailand (Kakuda et al., 1998; Sarataphan et al., 1999). However, none of the sequences was classified as type 6 in the present study. This finding may support the previous assumption that described type 6 as T. sinensis (Bai et al., 2002; Liu et al., 2010). The results of the pairwise comparisons revealed very low homologies between Thai type (AB010703) and other reported sequences clustered within type 6 (less than 90%). Therefore, the Thai type may form a separate branch from other registered sequences within this group (Fig. 2). Sarataphan et al. (2003) found that about 86 and 60% of cattle and water buffaloes, respectively, were positive for benign Theileria parasites in Thailand. In contrast, the present study indicates that only 31.5 and 9.4% were positive for parasite infections in the cattle and water buffalo populations, respectively. The observed differences may be due to the presence of Thai type, which could be considered as T. sinensis.

Although we were unable to detect the genotypes 2, 6, 8, and N-1 among cattle and water buffalo populations in Thailand, these findings may not be conclusive, as the blood samples were collected only from selected locations. In addition, the level of parasitemia might be below the detection limit of the PCR technique. Therefore, a large-scale epidemiological study is essential to confirm and expand the findings of the current investigation.

Consequently, we were able to identify several new genotypes of T. orientalis, which had not been described previously in Thailand. This indicates that T. orientalis parasites circulating in Thai cattle and water buffaloes have a much higher genetic diversity than previously expected.

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LITERATURE CITED


OCYST INGESTION AS AN IMPORTANT TRANSMISSION ROUTE OF TOXOPLASMA GONDII IN BRAZILIAN URBAN CHILDREN

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ABSTRACT: Toxoplasmosis is a cosmopolitan protozoan infection. Data regarding risk factors for the post-natal acquisition of Toxoplasma gondii infection in childhood are limited. We conducted a serological survey for T. gondii IgG antibodies and associated risk factors in 1,217 children 4-11-yr-old from Salvador, Brazil, using a commercial ELISA kit; antibodies were found in 17.5% of the children. Age (OR = 2.18; 95% CI: 1.50-3.17) and maternal schooling level (OR = 0.62; 95% CI: 0.42-0.92) were negatively associated with infection. A greater number of siblings (OR = 1.53; 95% CI: 1.12-2.09), cat at home (OR = 1.54; 95% CI: 1.06-2.24), house with non-treated piped water (OR = 2.54; 95% CI: 1.22-5.31), and the absence of a flush toilet at home (OR = 1.45; 95% CI: 1.04-2.01) were positively associated with T. gondii infection. Our data suggest that low socioeconomic levels and poor hygiene habits are important factors in favoring T. gondii infection.

Toxoplasma gondii infects most species of warm-blooded animals, and its prevalence in humans in Brazil is one of the highest worldwide (Dubey, 2009). As an example, 75% of blood donors from northeastern cities in Brazil were seropositive (Coelho et al., 2003). The ingestion of water or food contaminated with oocysts is considered the most important mode of transmission, at least in people of low socioeconomic status (Bahia-Oliveira et al., 2003). Most of the epidemiological information on toxoplasmosis is based on serological studies in adults, especially women of child bearing age (Pappas et al., 2009). There are many studies on post-natal infections in children (Dubey, 2009). In a study of 500 children 3-10-yr-old in Guatemala, antibodies were found in 37.8%; seroprevalence increased from 25% in 3-yr-old to 45% in 5-yr-old children (Jones et al., 2005). Souza et al. (1987) found marked differences in seropositivity among 608 school children from 2 neighborhoods, Bonsucesso (166 children) and Jacarepaguá (442 children) and 2 age groups (6-8-yr-old and 12-14-yr-old) within the city of Rio de Janeiro, Brazil. Overall, 68.4% of 608 children were seropositive (IFA, 1:16), but none had IgM antibodies; 36.5% of 81 children 6-8-yr-old and 70.4% of 81 children 12-14-yr-old in Bonsucesso, and 64% of 222 children 6-8-yr-old, and 84.5% of 220 children 12-14-yr-old in Jacarepaguá had anti-T. gondii antibodies. Results indicate a very high rate of post-natal transmission of T. gondii during childhood. Recently, using the same test (IFA, cut-off 1:16) Lopes et al. (2008) found that 46.4% of 4-11-yr-old children from Paraná, Brazil had T. gondii antibodies. Frenkel et al. (1995) found that 72 of 571 (12.6%) children in Panama City seroconverted between 1 and 6 yr of age.

In the present study, we determined T. gondii infection seroprevalence and its risk factors in children living in Salvador, a large urban center in northeastern Brazil.

MATERIALS AND METHODS

Study design

This study was conducted in the city of Salvador, northeastern Brazil, with a population of over 2.5 million. The children (n=1,445) were born between 1994 and 2001 and enrolled in a cohort recruited from 1997 and 2003 for evaluating the impact of a sanitation program on the incidence of childhood diarrhea (Barreto et al., 2007). Demographic and social data were collected using a validated questionnaire. In 2005, these children were resurveyed to collect data on asthma symptoms and risk factors for asthma; in addition, information regarding T. gondii and social demographic data were obtained. Informed written consent was obtained from the children’s parents or guardians, and ethical approval was granted by the Instituto de Saúde Coletiva at the Universidade Federal da Bahia and the National Commission on Ethics in Research (CONEP, Brazil). Among the original 1,445 children included in the 2005 survey, 1,217 with complete data set were included in the present study.

Potential risk factors for Toxoplasma gondii infection

The legal guardian of each child filled out 2 questionnaires. Between 1997 and 2003, information on socioeconomic and environmental factors was collected as part of the previous study to evaluate the impact of a sanitation program on childhood diarrhea (Strina et al., 2003). In 2005, an International Study of Asthma and Allergy in Childhood (ISAAC) Phase II-based questionnaire (ISAAC, 1998) was implemented in the same group of children. Although this questionnaire is a standardized tool for asthma and allergies, it contains several questions regarding environmental characteristics, which were used in the current work to study risk factors for T. gondii infection.

Toxoplasma gondii infection was studied as outcome and the following variables collected between 1997 and 2003 were analyzed as potential risk factors for T. gondii infection, i.e., having treated piped water at home, living in a house that flooded during the rainy season, having a flush toilet, living in a house served by a paved road, the presence of open sewage near the house, and rubbish collection frequency. Variables from the 2005 survey were also investigated as potential risk factors, i.e., maternal schooling; meat consumption (how often the child has eaten meat, chicken, beef, and pork); vegetable intake (how often the child eats vegetables); presence of older siblings; number of siblings; whether the child attended nursery; presence of rodents, flies, dogs, and cats at home; having treated piped water at home; living in a house served by a paved road; and presence of a waste system. The sex and age of the children as collected in 2005 were treated as a priori confounders.

Serological detection of anti-Toxoplasma gondii IgG antibodies

Blood collection was performed in 2005. Serum levels of anti-T. gondii IgG antibodies were determined by indirect enzyme immunoassay (ELISA), following the directions for commercially available kits (Diamedix, Miami, Florida). The assay cut-off was determined by an index value obtained by the following ratio: absorbance of sample/absorbance of a calibrator (a solution containing human serum or plasma, antibodies weakly reactive for anti-T. gondii IgG and 0.1% sodium azide). A ratio value greater than 1.00 was considered positive.

Statistical analysis

Only children with complete data were included in the analysis. We first performed a bivariable analysis between each potential risk factor and

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Table I. Sociodemographic characteristics frequencies and associations with *Toxoplasma gondii* infection of 1,217 studied children. Abbreviations: OR, odds ratio; CI, confidence interval.

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>(%)</th>
<th>OR (95% C.I.)†</th>
<th>Final model OR (95% C.I.)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (n = 564)</td>
<td>99</td>
<td>17.6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Male (n = 653)</td>
<td>114</td>
<td>17.5</td>
<td>0.99 (0.74; 1.34)</td>
<td>0.90 (0.66; 1.22)</td>
</tr>
<tr>
<td><strong>Age group (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;6 (n = 459)</td>
<td>63</td>
<td>13.7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>6 to 7 (n = 419)</td>
<td>65</td>
<td>15.5</td>
<td>1.15 (0.79; 1.68)*</td>
<td>1.10 (0.74; 1.61)</td>
</tr>
<tr>
<td>≥8 (n = 339)</td>
<td>85</td>
<td>25.1</td>
<td>2.10 (1.47; 3.02)*</td>
<td>2.18 (1.50; 3.17)*</td>
</tr>
<tr>
<td><strong>Mother’s schooling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Illiterate or primary school complete (n = 578)</td>
<td>129</td>
<td>22.3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Secondary education complete (n = 271)</td>
<td>40</td>
<td>14.8</td>
<td>0.60 (0.41; 0.89)*</td>
<td>0.73 (0.49; 1.09)</td>
</tr>
<tr>
<td>High school complete (n = 368)</td>
<td>44</td>
<td>12</td>
<td>0.47 (0.33; 0.69)*</td>
<td>0.62 (0.42; 0.92)*</td>
</tr>
<tr>
<td><strong>Number of siblings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 (n = 738)</td>
<td>106</td>
<td>14.4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>≥2 (n = 479)</td>
<td>107</td>
<td>22.3</td>
<td>1.72 (1.27; 2.31)*</td>
<td>1.53 (1.12; 2.09)*</td>
</tr>
<tr>
<td><strong>Older siblings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (n = 464)</td>
<td>69</td>
<td>14.9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes (n = 753)</td>
<td>144</td>
<td>19.1</td>
<td>1.35 (0.99; 1.85)</td>
<td></td>
</tr>
<tr>
<td><strong>Nursery attendance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (n = 1020)</td>
<td>168</td>
<td>16.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes (n = 197)</td>
<td>45</td>
<td>22.8</td>
<td>1.50 (1.04; 2.18)*</td>
<td></td>
</tr>
<tr>
<td><strong>Beef intake§</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rare (n = 67)</td>
<td>10</td>
<td>14.9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>≥once weekly (n = 1150)</td>
<td>203</td>
<td>17.7</td>
<td>1.22 (0.61; 2.43)</td>
<td></td>
</tr>
<tr>
<td><strong>Vegetable intake¶</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥once weekly (n = 956)</td>
<td>167</td>
<td>17.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Rare (n = 261)</td>
<td>46</td>
<td>17.6</td>
<td>1.01 (0.71; 1.45)</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05.
† Odds ratio and 95% confidence interval from crude analysis.
‡ Adjusted for all variables which has kept the significance in the multivariable analyses, including sex and age as a priori confounders.
§ How often has the child eaten meat (chicken, beef, or pork)?
¶ How often has the child eaten vegetables?

outcome. Second, we built a multivariable model with standard logistic regression, including only variables that had been found to be significant with the bivariate analysis including sex and age as a priori confounders. Then, we assessed all non-significant variables once more by including them in the model one at a time. If the variable remained non-significant, it was removed from the analysis permanently. Each time a variable became significant, this variable was included in the model, and all remaining non-significant variables were assessed again, one at a time. We conducted these steps in the analysis until there were no variables left to assess. The final model was then adjusted with all variables found statistically significant in the bivariate analysis and which kept the statistical significance in the multivariate model. Although the final model was split in Tables I and II, socioeconomic and environmental variables were included in the same analysis to compound the final model. The association between outcome and risk factors was estimated with odds ratio and 95% confidence interval.

**RESULTS**

**Socioeconomic and environmental risk factors for *T. gondii* infection**

Anti-*T. gondii* IgG antibodies were present in 17.5% (213/1,217) of the children (Table I). The frequency of socioeconomic and environmental variables and their association with *T. gondii* infection in both bivariate and multivariate (final model) analyses have been shown in Tables I and II, respectively. The following study variables were associated with an increased risk of infection. Children with increased age and increased number of siblings were more likely to be infected. Children with cats at home were more likely to be infected. Children living in a house without piped water facilities, in both study periods (1997–2003 and 2005) were 2.5 times more likely to be infected than those with piped water in their houses in both periods. Children living in a house without a flush toilet were more likely to be infected. The level of schooling of the children’s mothers was associated with a lower risk of infection. Children whose mothers had a higher level of schooling had less risk of being infected. Meat and vegetable intake showed no significant influence on *T. gondii* infection in this population. Nursery attendance, living in a house served by a paved road, the presence of open sewage nearby, and the frequency of rubbish collection were removed from the model due to a lack of significance in the multivariate analysis (data not shown).

**DISCUSSION**

The seroprevalence of anti-*T. gondii* IgG observed in this study was 17.5%; this is lower than the prevalence found by Rey and
Table II. Environmental characteristics frequencies and associations with *Toxoplasma gondii* infection of 1,217 studied children. Abbreviations: OR, odds ratio; CI, confidence interval.

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>%</th>
<th>OR (95% C.I.)†</th>
<th>Final model OR (95% C.I.)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flies at home</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No or rare (n = 603)</td>
<td>101</td>
<td>16.7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes (n = 614)</td>
<td>112</td>
<td>18.2</td>
<td>1.11 (0.83; 1.49)</td>
<td></td>
</tr>
<tr>
<td>Rodents at home</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>No (n = 525)</td>
<td>85</td>
<td>16.2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes (n = 692)</td>
<td>128</td>
<td>18.5</td>
<td>1.18 (0.87; 1.59)</td>
<td></td>
</tr>
<tr>
<td>Cat at home</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No (n = 1002)</td>
<td>163</td>
<td>16.3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes (n = 215)</td>
<td>50</td>
<td>23.3</td>
<td>1.56 (1.09; 2.23)*</td>
<td>1.54 (1.06; 2.24)*</td>
</tr>
<tr>
<td>Dog at home</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (n = 735)</td>
<td>116</td>
<td>15.8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes (n = 482)</td>
<td>97</td>
<td>20.1</td>
<td>1.34 (1.00; 1.81)</td>
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</tr>
<tr>
<td>Both study periods (n = 335)</td>
<td>72</td>
<td>21.5</td>
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<td></td>
</tr>
<tr>
<td>One study period (n = 395)</td>
<td>65</td>
<td>16.5</td>
<td>0.72 (0.50; 1.04)*</td>
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<tr>
<td>Never (n = 487)</td>
<td>76</td>
<td>15.6</td>
<td>0.68 (0.47; 0.97)*</td>
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</tr>
<tr>
<td>Both study periods (n = 974)</td>
<td>151</td>
<td>15.5</td>
<td>1</td>
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<tr>
<td>One study period (n = 207)</td>
<td>49</td>
<td>23.7</td>
<td>1.69 (1.17; 2.43)*</td>
<td>1.38 (0.93; 2.05)</td>
</tr>
<tr>
<td>Never (n = 36)</td>
<td>13</td>
<td>36.1</td>
<td>3.08 (1.53; 6.22)*</td>
<td>2.54 (1.22; 5.31)*</td>
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<tr>
<td>Flush toilet</td>
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</tr>
<tr>
<td>Yes (n = 761)</td>
<td>106</td>
<td>13.9</td>
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<td></td>
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<tr>
<td>No (n = 456)</td>
<td>107</td>
<td>23.5</td>
<td>1.89 (1.41; 2.55)*</td>
<td>1.45 (1.04; 2.01)*</td>
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<td>Sewage system</td>
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<td></td>
</tr>
<tr>
<td>Yes (n = 1016)</td>
<td>179</td>
<td>17.6</td>
<td>1.00 (0.64; 1.42)</td>
<td></td>
</tr>
<tr>
<td>No (n = 201)</td>
<td>34</td>
<td>16.9</td>
<td>1</td>
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<td>Open sewage nearby</td>
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<td></td>
</tr>
<tr>
<td>No (n = 646)</td>
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<td>14.4</td>
<td>1</td>
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<tr>
<td>Yes (n = 571)</td>
<td>120</td>
<td>21</td>
<td>1.58 (1.17; 2.13)*</td>
<td></td>
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<tr>
<td>Rubbish collection</td>
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<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>≥once per week (n = 924)</td>
<td>149</td>
<td>16.1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>&lt;once per week (n = 293)</td>
<td>64</td>
<td>21.8</td>
<td>1.45 (1.05; 2.02)*</td>
<td></td>
</tr>
<tr>
<td>Flooded house</td>
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<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>No (n = 842)</td>
<td>145</td>
<td>17.2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes (n = 375)</td>
<td>68</td>
<td>18.1</td>
<td>1.07 (0.78; 1.46)</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05.
† Odds ratio and 95% confidence interval from crude analysis.
‡ Adjusted for all variables which has kept the significance in the multivariable analyses, including sex and age as a priori confounders.

Ramalho (1999), who reported 40.0% seroprevalence among schoolchildren from northeast Brazil aged 2–9-yr-old, as well as other reports in the literature for similar age groups (Etheredge and Frenkel, 1995; del Castillo and Herruzo, 1998). The lower prevalence found in this study may be attributed to differences in socioeconomic level and hygiene conditions between the study populations.

In our study, approximately 30% of the infected children were under 6 yr of age, suggesting that the transmission of this infection started during their early childhood (Mohan et al., 2002). The relatively gradual increase in seroprevalence associated with age lends further support to the characteristic finding that *T. gondii* infection is generally asymptomatic (Bóia et al., 2008).

The statistical significance of variables such as mother’s level of schooling and number of siblings found in this study reinforces the contributing role of socioeconomic status in determining populations under high risk to be infected by this parasite, which preferentially affects people living in crowded conditions (Alvarado-Esquivel et al., 2008) and in households lacking basic facilities (Jones et al., 2001). The absence of treated piped water at home, for instance, showed a strong association with *T. gondii* infection; the odds of infection were greater when the absence of piped water was reported in both study periods (1997–2003 and 2005) than when it was reported in only 1 of the 2 periods. This finding supports previous reports that this parasite is water transmitted (Bahia-Oliveira et al., 2003; Dubey, 2004) and that it
may cause waterborne outbreaks of toxoplasmosis (Bowie et al., 1997).

The presence of a cat at home was shown to be a risk factor for toxoplasmosis in our study, which is in agreement with previous studies that showed that the oocysts shed by this animal and ingested by human beings are likely an important route of infection (Cook et al., 2000; Alvarado-Esquível et al., 2007).

In this work, the studied variables that showed significant associations with *T. gondii* infection, such as having a mother with a low level of schooling, having many siblings and the absence of piped water, are related to low socioeconomic status and consequently bad hygiene habits; they are directly (as absence of piped water) or indirectly (mother with low level of schooling) involved in the transmission of *T. gondii* infection. Variables related to low hygiene, as well as presence of cat at home found associated with *T. gondii*, indicate that oocyst ingestion is an important route for transmission of this parasite.

Although the current work did not evaluate the consumption of raw or undercooked meat, this route of infection has been more often reported in adult populations (Roghmann et al., 1999; Cook et al., 2000). These results are likely due to different habits between children and adults. Children in general have more contact with soil and eat less raw or undercooked meat compared with adults, although the presence of anti-*T. gondii* IgG in children from a Brazilian rural area has been associated with raw meat consumption (Souza et al., 1987). In contrast to what was expected, other variables associated with poverty and crowded living conditions, as well as the presence of animals potentially capable of spreading this infection, such as flies and rodents (Dubey, 2004), did not show any significant influence in the current study. These findings are in agreement with those of Galisteu et al. (2007), who did not find any association of these animals with toxoplasmosis in pregnant women.

The present study suggests that the presence of *T. gondii* infection in children from Salvador-Bahia, Brazil, is highly related to bad sanitation, crowded living conditions, the absence of treated water, and the presence of cats, suggesting the parasite to be mostly transmitted by oocyst ingestion. Simple improvements in education and basic sanitary conditions could help decrease childhood toxoplasmosis in this city and in other cities with populations living in similar environments.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


SURVEY OF PARASITES IN THREATENED STOCKS OF COHO SALMON (ONCORHYNCHUS KISUTCH) IN OREGON BY EXAMINATION OF WET TISSUES AND HISTOLOGY

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Department of Microbiology, Oregon State University, 220 Nash Hall, Corvallis, Oregon 97331. e-mail: jayde.ferguson@alaska.gov

ABSTRACT: We are conducting studies on the impacts of parasites on Oregon coastal coho salmon (Oncorhynchus kisutch). An essential first step is documenting the geographic distribution of infections, which may be accomplished by using different methods for parasite detection. Thus, the objectives of the current study were to (1) identify parasite species infecting these stocks of coho salmon and document their prevalence, density, and geographic distribution; (2) assess the pathology of these infections; and (3) for the first time, determine the sensitivity and specificity of histology for detecting parasites compared with examining wet preparations for muscle and gill infections. We examined 576 fry, parr, and smolt coho salmon in total by histology. The muscle and gills of 219 of these fish also were examined by wet preparation. Fish were collected from 10 different locations in 2006–2007. We identified 21 different species of parasites in these fish. Some parasites, such as Nanophyetus salmincola and Myxobolus insidiosus, were common across all fish life stages from most basins. Other parasites, such as Apophallus sp., were more common in underyearling fish than smolts and had a more restricted geographic distribution. Additional parasites commonly observed were as follows: Sanguinicolga sp., Trichodina truttae, Epistyli sp., Capriniana piscium, and unidentified metacercariae in gills; Myxobolus sp. in brain; Myxidium salvelini and Chloromyxum majoni in kidney; Pseudocapillaria salvelini and adult digenean spp. n the intestine. Only a few parasites, such as the unidentified gill metacercariae, elicited overt pathologic changes. Histology had generally poor sensitivity for detecting parasites; however, it had relatively good specificity. We recommend using both methods for studies or monitoring programs requiring a comprehensive assessment of parasite identification, enumeration, and parasite-related pathology.

Over the last half century, populations of wild salmonids have been declining at alarming rates (Lackey et al., 2006). Currently, Oregon coastal coho salmon (Oncorhynchus kisutch), an evolutionarily significant unit set by the National Oceanic and Atmospheric Administration Fisheries, are listed as threatened under the U.S. Endangered Species Act. Therefore, we are conducting studies to elucidate the impacts of parasites on these threatened populations of coho salmon. As a first step, we sought to identify the parasite species infecting these stocks of coho salmon and to document their prevalence, density, and geographic distributions. Furthermore, we conducted a pathology assessment, which provides data on impacts of parasites at the tissue and organism level (Feist and Longshaw, 2008).

The methods used for surveying parasite infections in wild populations include gross examination, microscopic evaluation of tissues in wet mounts, histology, serology, culture, and polymerase chain reaction (PCR)-based techniques. However, currently there are no specific PCR or serologic tests for most parasites of wild fishes. Thus, most fish surveys rely on identification of parasites from wet tissues, because whole fresh parasites usually provide the best morphologic information for identification (Hoffman, 1999; Ferguson, 2006) and permit enumeration of parasites (Hoffman, 1999; Jacobson et al., 2008, Ferguson et al., 2010). Examples of such surveys with salmonid fishes include Kent et al. (1998) and Arkoosh et al. (2004). Histology, however, remains useful as a general tool for conducting initial fish health surveys because it provides the best representation of pathologic changes and allows for detection of a wide variety of pathogens, including viral and bacterial agents (Kent et al., 1998; Feist and Longshaw, 2008). Indeed, most well recognized or common parasites can be readily identified by histology (Gardiner et al., 1998; Gardiner and Poynton, 1999; Bruno et al., 2006). However, aside from a few of our studies (Eaton et al., 1991; Moran and Kent, 1999; Kent et al., 2005), we are not aware of surveys that have evaluated fish by both histology and examination of wet material. Furthermore, we could not find any studies that have empirically compared these 2 methods for detecting parasites.

The Oregon Department of Fish and Wildlife (ODFW) manages life-cycle monitoring (LCM) sites for estimating fish survival as part of the state’s plan for assessing the status of threatened coastal stocks (Solazzi et al., 2000). We have shown previously that parr coho salmon from 1 of these LCM sites, West Fork Smith River, have remarkably high digenean and myxozoan infections by histology (Rodnick et al., 2008). Here, we examined coho salmon from several LCM sites in western Oregon by using histology, wet preparations, or both, from fry, parr, and smolt stages. The objectives of our study were to (1) identify parasite burdens and distributions in juvenile coho salmon; (2) assess the pathology of these infections; and (3) for the first time, determine the sensitivity and specificity of histology for detecting parasites in axial skeletal muscle and gills compared with examining wet preparations. Here, we report our results from this survey, summarize histopathologic findings, and compare the different methods used for detecting parasite infections.

MATERIALS AND METHODS

Sampling fish

Coho salmon, because of their threatened status, were opportunistically sampled by ODFW from their LCM sites (for map, see Suring et al., 2009). Fry and parr were caught by electrofishing pools during ODFW snorkeling surveys, and smolts were captured in screw traps during out-migration. In May and June 2006, fry were sampled from the following 8 ODFW LCM sites: lower main stem of West Fork (WF) Smith River (R) (43°48'54.11"N, 123°46'12.99"W), Mill Creek (Ck) Siletz R (44°44'44.89"N, 123°47'35.72"W), East Fork (EF) Trask R (45°24'55.93"N, 123°36'7.30"W), Cascade Ck of Alsea R (44°19'11.15"N, 123°50'50.73"W), Upper North Fork (UNF) (45°48'54.34"N, 123°41'34.50"W) and Lower North Fork (LNF) (45°48'43.13"N, 123°44'45.55"W) of Nehalem R, and the Upper Main Stem (UMS) (44°14'43.26"N, 123°38'28.38"W) and EF (44°14'57.05"N, 123°37'58.55"W) in the Lobster Ck sub-basin of Alsea R. In September of
2006, parr (same year class) were sampled from the same locations, excluding the Lobster Ck sites and including an additional site of Winchester Ck of the main stem of WF Smith R. South Slough of Coos Bay (43°16’55.84"N, 124°19’11.23"W). In April and May 2007, smolts (same year class) were sampled from all of the locations that parr and fry were sampled from previously, plus the additional site Mill Ck of Yaquina R (44°35’5.95"N, 123°54’11.75"W), was included. Finally, in September of 2007, parr (different year class) were sampled from the lower main stem of WF Smith R (43°51’38.96"N, 123°44’59.59"W). All fish were taken by ODFW under their permits.

**Histology**

Samples for histologic analysis were fixed in Dietrich’s solution (30 ml of 95% ethanol, 10 ml of formaldehyde, 2 ml of glacial acetic acid, and 58 ml of distilled water). Samples were processed using standard histology techniques and stained with hematoxylin and eosin (H&E), or Giemsa. Sagittal sections were made from intact fry. Parr and smolts required necropsy and removal of the following organs: liver, spleen, mesonephros, kidney, heart, intestine, pyloric caeca, brain, gonad, gill, and axial skeletal muscle (henceforth referred to simply as muscle). These organ sections were trimmed into 1–2-cm² pieces, embedded, and sectioned. One section from each fish was evaluated for parasites. Density of infection (parasites/histologic section of infected fish) was determined by counting all parasites in a section by using standard light microscopy. Myxozoans were enumerated by counting pseudocysts or spore aggregates rather than individual spores, because these all arose from a single progenitor. Only prevalence was recorded for capillarid nematodes in the gut because cross sections of single worms were possible; thus, we could not determine whether counts represented more than 1 worm.

**Infection comparisons**

Statistical comparisons were made among all 3 fish life stages across all samples sites. Comparison between sample sites was limited to descriptive assessments due to lack of replication of sampling for most watersheds. Only data from histologic evaluations were used because wet material was not available for comparisons. Mean densities of parasites in fry, parr, and smolts were compared with a non-parametric bootstrap t-test with 100,000 replications, because data were not normally distributed. Fisher’s exact tests were used to test differences in prevalence of parasites among fry, parr, and smolts. All statistical procedures were performed with quantitative parasitology (Róza et al., 2000), and significance was set at $P < 0.05$. $P$ values are 2-tailed.

**Wet preparations**

We were able to obtain fresh or frozen material from smolts collected in April 2007 (brood year 2006) from all sites and parr in October 2007 (brood year 2007) from WF Smith R. Gill were evaluated as warranted by standard practices, and muscle also was included because we were particularly interested in the density of muscle parasites given the severity of infections seen in our previous study (Rodnick et al., 2008). All wet preparations were evaluated at ×100 magnification by using a compound light microscope.

For gill tissue, 2 to 3 gill arches (including gill rakers) were collected from each fish at field sites or the laboratory and examined in wet mounts for parasites. Photomicrographs of representative parasites were obtained from gill preparations from fish that were examined in the laboratory. A few metacercariae were excysted in the laboratory as described by Irwin (1983) to better study morphology. Fish from sampling sites that were processed in the laboratory included smolts from Cascade Ck and Mill Ck Yaquina and parr (brood year 2007) from WF Smith R.

For muscle tissue, samples were prepared and evaluated in accordance with previously published methods by Ferguson et al. (2010). In short, parasites were enumerated from 1 freshly thawed and squashed fillet per fish and recorded in terms of parasites/fillet. Also, a gross examination of all fresh or frozen fresh fish was performed to enumerate neascus (black spot trematode) infecting skin and recorded in terms of parasites/fish.

Prevalence (number of infected animals/total animals), intensity (number of parasites/infected tissue) of infections are reported in accordance with the definitions provided by Bush et al. (1997). Gill wet mounts and muscle squashes are both referred to as wet preparations henceforth.

**Parasite identifications**

Parasites were identified as specifically as possible by recording morphologic characters, measurements, and tissue distribution and referring to reviews and keys including Love and Moser (1983), Lom and Dyková (1992), McDonald and Margolis (1995), Gibson (1996), Hoffman (1999), and Moles (2007).

**Diagnostic method comparison**

We calculated the sensitivity and specificity of histology by using results from wet tissue preparation evaluations as our selected reference test, because this is the most common method used by parasitologists to enumerate and identify parasites in tissues (Hoffman, 1999; Ferguson, 2006). Sensitivity was calculated by dividing the number of fish found positive by both wet preparation and histology by the total number of positive fish by wet preparation. Specificity was calculated by dividing the number of fish found negative by both wet preparation and histology by the total number of wet mount-negative fish. The 95% confidence intervals for the sensitivity and specificity of histology were determined for each parasite by using Clinical Calculator 1 (Lowry, 2010). Only smolts and parr from WF Smith R (brood year 2007) could be included in our comparison analysis of the diagnostic test methods because these were the only samples that were assessed by both techniques.

We also calculated the sensitivity of histology for each muscle parasite when only densely infected fish (top 10% of infections) and negative fish were included in the study to assess the accuracy of this diagnostic technique for detecting densely parasitized fish. This was not performed on gill infections, as there were too few fish with heavy infections.

**RESULTS**

**Total fish evaluated**

We evaluated 576 coho salmon (254 fry, 118 parr, and 204 smolts) from 10 coastal rivers in Oregon from 2006 to 2007. All groups were evaluated by histology, but wet preparations of gill and muscle also were evaluated for all smolt groups and 1 parr group. Most fish were infected with several protistan, myxozoan, and helminth parasites (Tables I-III) as we described below.

**Muscle parasites**

Using both methods, *N. salmincola* was observed in muscle of fish from at least 1 life stage at 8 sites. Based on histology, prevalence and mean density of *N. salmincola* in muscle were similar in fry, parr, and smolts (Table I). High prevalence and infection densities occurred in fry and parr from most locations (Table I). With wet preparations, prevalence of *N. salmincola* in muscle was 100% from smolts from Mill Ck. Siletz, WF Smith R, and LNF Nehalem, with a high density occurring in smolts from WF Smith R. We did not detect *N. salmincola* in any stage of fish from the EF Trask by either method, and it was only detected at a low prevalence and density with wet preparations for smolts from EF Lobster Ck. In histologic sections, *N. salmincola* was observed throughout muscle, multiple visceral organs, and even gills of smolts (Fig. 1B). The kidney was particularly densely infected. Metacercariae were usually surrounded by mild, focal, chronic inflammation.

Using both methods, *Apophallus* sp. was observed in muscle of fish from at least 1 life stage at 7 sites. In contrast to *N. salmincola*, prevalence of *Apophallus* sp. significantly declined throughout all three fish life stages ($P < 0.05$; Fisher’s exact test), with smolts having almost 20 times less prevalence than fry (Table I). Hence, using histology, this parasite was only detected in smolts at LNF Nehalem and Cascade Ck (Table I). Almost all
Table I. Prevalence and mean density (in parentheses) of parasites in axial skeletal muscle of coho salmon by 2 different methods. Only histology was available for fry and parr (brood year 2006), whereas histology and wet tissues were evaluated for smolts (brood year 2006) and parr (brood year 2007). Histo = histology, Wet = wet preparations, EF = East Fork, R = River, Ck = Creek, WF = West Fork, UNF = Upper North Fork, LNF = Lower North Fork, UMS = Upper Main Stem. Mean density data from histology refer to mean number of parasites/histologic section (1 section of muscle/fish), and mean density data from wet preparations refer to mean number of parasites/fillet. For neascus, Gross = gross examination, and mean intensity (in parentheses) refers to mean number of parasites/infected fish.

<table>
<thead>
<tr>
<th>Location and fish stage</th>
<th>Nanophyetus salmincola</th>
<th>Apophallus sp.</th>
<th>Myxobolus insidiosus</th>
<th>Myxobolus fryeri</th>
<th>Neascus (in skin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Histo</td>
<td>Wet</td>
<td>Histo</td>
<td>Wet</td>
<td>Histo</td>
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<tr>
<td><strong>EF Trask R</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fry (n = 35)</td>
<td>0</td>
<td>NA†</td>
<td>0</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Parr (n = 20)</td>
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<td>NA</td>
<td>0</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Smolts (n = 20)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Mill Ck (Siletz)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fry (n = 30)</td>
<td>93 (3)</td>
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<td>80 (1)</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Parr (n = 20)</td>
<td>85 (6)</td>
<td>NA</td>
<td>20 (3)</td>
<td>NA</td>
<td>5 (1)</td>
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<td>Smolts (n = 21)</td>
<td>5 (1)</td>
<td>100 (17)</td>
<td>0</td>
<td>86 (4)</td>
<td>19 (7)</td>
</tr>
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<td><strong>WF Smith R</strong></td>
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<td></td>
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<tr>
<td>Fry (n = 29)</td>
<td>59 (3)</td>
<td>NA</td>
<td>79 (3)</td>
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<tr>
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<td>40 (2)</td>
<td>NA</td>
<td>60 (26)</td>
</tr>
<tr>
<td>Smolts (n = 24)</td>
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* Parasite prevalence and mean density (in parentheses) in fish life stages with different lowercase superscript letters are significantly different (P < 0.05).
† NA = not available.

of the metacercariae of this species were found in myosepta and were not associated with a tissue reaction (Fig. 1A). Using wet preparations, fish from WF Smith R and LNF Nehalem had a high prevalence and density of infection, with the former harboring more than 500 worms/fillet in parr from the lower mainstem section of this river.

The 2 myxobolids infecting muscle were *M. insidiosus* within myocytes, and *M. fryeri* in the peripheral nerves. Using histology,
Table II. Prevalence and mean density (in parentheses) of gill parasites of coho salmon by 2 different methods. Only histology was available for fry and parr (brood year 2006), whereas histology and wet tissues were evaluated for smolts (brood year 2006) and parr (brood year 2007). Histo = histology, Wet = wet preparations, EF = East Fork, R = River, Ck = Creek, WF = West Fork, UNF = Upper North Fork, LNF = Lower North Fork, UMS = Upper Main Stem. Mean density data from histology refer to mean number of parasites/histologic section (1 section of 3 gills/fish), and mean density data from wet preparations refer mean number of parasites/sampled gill tissue (3 gills/fish).*

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<th>Location and fish stage</th>
<th>Nanophyetus salmincola</th>
<th>Unidentified gill metacercariae</th>
<th>Sanguinicola sp.</th>
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<th>Epistyris sp.</th>
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* Parasite prevalence and mean density (in parentheses) in fish life stages with different lowercase superscript letters are significantly different (P < 0.05).
† NA = not applicable.

no fry were infected with either species, but parr and smolts from about half the sample sites were infected by both (Table I). The overall prevalence and mean density of infections was comparable in smolts and parr, which was significantly higher than that of uninfected fry (P < 0.05; Fisher’s exact test and bootstrap t-test). Parr from WF Smith R had a high prevalence and density of M. insidiosus infections, whereas those from Mill Ck. Siletz had a high prevalence and density of M. fryeri infections (Table I). Both
Table III. Prevalence and mean density (in parentheses) of viscera or brain parasites of coho salmon by histology for fry, parr, and smolts from brood year 2006 and parr from brood year 2007. EF = East Fork, R = River, Ck = Creek, WF = West Fork, UNF = Upper North Fork, LNF = Lower North Fork, UMS = Upper Main Stem. Mean density data refer to mean number of parasites/histologic section (1 section/fish).

<table>
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<th>Myxidium salvelini</th>
<th>Chloromyxum majori</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fry (n = 10)</td>
<td>50 (3)</td>
<td>56 (13)</td>
<td>30 (3)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Parr (n = 10)</td>
<td>100 (9)</td>
<td>80 (3)</td>
<td>10 (3)</td>
<td>30</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Smolts (n = 20)</td>
<td>90 (8)</td>
<td>35 (2)</td>
<td>30 (5)</td>
<td>10 (4)</td>
<td>75 (3)</td>
<td></td>
</tr>
<tr>
<td>EF Lobster Ck</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Fry (n = 30)</td>
<td>0</td>
<td>43 (4)</td>
<td>0</td>
<td>0</td>
<td>23 (5)</td>
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</tr>
<tr>
<td>Smolts (n = 20)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>15 (1)</td>
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<td>UMS Lobster Ck</td>
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<tr>
<td>Fry (n = 30)</td>
<td>63 (4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>37 (4)</td>
</tr>
<tr>
<td>Smolts (n = 19)</td>
<td>95 (6)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>11 (1)</td>
</tr>
<tr>
<td>Winchester Ck</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Parr (n = 20)</td>
<td>30 (2)</td>
<td>0</td>
<td>20 (4)</td>
<td>25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Smolts (n = 20)</td>
<td>65 (2)</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mill Ck (Yaquina)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smolts (n = 20)</td>
<td>80 (4)</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>25 (3)</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fry (n = 254)</td>
<td>43a (3a)</td>
<td>2a (11a)</td>
<td>38a (4a)</td>
<td>6a (8a)</td>
<td>0a</td>
<td>48a (3a)</td>
</tr>
<tr>
<td>Parr (n = 118)</td>
<td>61b (6b)</td>
<td>38b (6b)</td>
<td>0b</td>
<td>21b (9b)</td>
<td>11b</td>
<td>18b (1b)</td>
</tr>
<tr>
<td>Smolts (n = 204)</td>
<td>71b (10b)</td>
<td>18b (11b)</td>
<td>3b (4b)</td>
<td>1b (3b)</td>
<td>23b</td>
<td>32b (3b)</td>
</tr>
</tbody>
</table>

* For *P. salvelini* infections, only prevalence was recorded because cross sections could represent either single or multiple worms. Parasite prevalence and mean density (in parentheses) in fish life stages with different lowercase superscript letters are significantly different (P < 0.05).

Parasites were generally common in smolts from these rivers (Table I). These parasites did not elicit any inflammatory changes when plasmodia were intact; however, after plasmodia ruptured, a mild inflammatory response was noted. The size of *M. fryeri* pseudocysts (approximately 30 μm) are approximately 6 times smaller than those of *M. insidiosus* (Ferguson et al., 2008) and hence were too small to detect in wet preparation at ×100 magnification. *Myxobolus insidiosus* was detected in smolts from every site in wet material. Mill Ck Siletz smolts and WF Smith R parr had particularly dense infections, with means of nearly 200 and 150 pseudocysts/fillet, respectively.

The presence of neascus in the skin was only evaluated from smolts and the 1 parr group (Table I) because these were the only samples that were available for fresh gross examination. Fish from most rivers were infected, and smolts from Mill Ck Yaquina had nearly 75% prevalence, with a mean intensity of approximately 15/fish (Table I).
Gill parasites

*Nanophyetus salmincola* was observed in the gills of fish from 8 sample sites by histology (Table II). The prevalence of gill infections increased with fish life stages, with smolts having almost 10 times higher prevalence than fry ($P < 0.05$; Fisher’s exact test). Smolts also had a significantly higher mean infection density than fry and parr ($P < 0.05$; bootstrap $t$-test). *Nanophyetus salmincola* within the gill filaments was easily recognized by wet preparations by the presence of its opaque posterior excretory bladder (Fig. 2A). However, this structure was not always clearly visible in metacercariae deep in tissues within the base of the gills or gill rakers.

Metacercariae of *Apophallus* sp. and *Echinochasmus milvi* were identified in wet preparations of the gills (Fig. 2C–E). One type exhibited prominent collar spines, consistent with species of Echinostomidae, whereas the other type was a small species (approximately 200 µm long) with a long pharynx, and exhibited features consistent with *Apophallus* sp., including tandem to oblique testes, a pharynx close to the oral sucker, and a long esophagus. However, many of these 2 metacercariae were indistinguishable from each other in histologic sections; hence, we classified them as “unidentified gill metacercariae.” Fish from 6 sample sites were infected with these parasites by using this method (Table II), and prevalence and mean density of infections were higher in fry than parr stage by approximately 7 and 4 times, respectfully ($P < 0.05$; Fisher’s exact test and bootstrap $t$-test). However, infections were less in parr than smolt stage by approximately half ($P < 0.05$; Fisher’s exact test and bootstrap $t$-test). A moderate to high prevalence of infection occurred in both fry and parr from Cascade Ck and a moderate to high prevalence in smolts occurred in Mill Ck Yaquina samples. These unidentified metacercariae elicited the most prominent tissue changes. Located within the base and mid-level of filaments, individual and clustered (2–4 metacercariae) cysts often caused profound intrafilamental fibrosis and concurrent chondroid hyperplasia and metaplasia (Fig. 3A–C). Cysts were surrounded by irregular, pleomorphic, and occasionally binucleated nascent chondrocytes, not directly associated or contiguous with pre-existing cartilage, within a background of dense fibrovascular tissue. The expanded intrafilamental stroma was infiltrated by chronic inflammatory cells, mostly lymphocytes, but also histiocytes and eosinophilic granular cells to a lesser extent.

Embryonated eggs of *Sanguinicola* sp. were detected in histologic sections of fish from 6 sample sites (Table II). Prevalence increased
Viscera and brain parasites by histology

Additional parasite species were detected when visceral organs and brain were examined by histology. *Nomaphytes salmioncola* (common in the muscle) was found in all visceral organs, even the brain and ovary in 1 fish. Including histologic examination of these organs often resulted in doubling the prevalence or mean density of infection compared with examination of gills or muscle only (Tables I–III). Parr and smolts had a higher prevalence and mean density of infections from tissues other than gill and muscle than fry (P < 0.05; Fisher’s exact test and bootstrap t-test).

Aggregates of *Myxobolus* sp. spores were found in the brain of fish (Fig. 3O) from 5 sample sites. Prevalence of these infections was higher in parr than fry stages but then declined in smolts (P < 0.05; Fisher’s exact test). Fry from Cascade Ck had a high prevalence (Table III). A high prevalence of this parasite also occurred in parr, especially from fish sampled from UNF Nehalem (Table III).

Two kidney myxozoans were observed, i.e., *Myxidium salvelini* and *Chloromyxum majori*, in kidney tubules and glomeruli, respectively. The prevalence of *M. salvelini* was much higher in fry than parr stage (P < 0.05; Fisher’s exact test) but then increased slightly in smolts (Table III). Conversely with *C. majori*, prevalence was higher in parr than fry stage and then much lower in smolts (P < 0.05; Fisher’s exact test). *Myxidium salvelini* was confined to the lumen of renal tubules and was not associated with tissue damage. Plasmidia had prominent, retractive granules in the cytoplasm and also contained developing and mature spores (Fig. 3K, L). Myxospores of *M. salvelini* were readily visualized with Giemsa stain (Fig. 3L, inset). Parr at 5 sites and smolts at 1 site (Cascade Ck) were infected with *C. majori* (Table III). This myxozoan was observed in the glomeruli and was identified by the presence of spherical spores with 4 polar capsules (Fig. 3M, N). Affected glomeruli were mostly effaced and expanded by the spores, with significant disruption of normal histoarchitectural features and a narrowing to near-complete obliteration of the urinary space associated with attenuated parietal epithelium. In some instances, there was mild periglomerular fibrosis surrounding the affected glomeruli.

Four helminth species were found in the gastrointestinal tract by histology. *Pseudocapillaria salvelini* (nematode) was clearly identified as a capillarid due to the presence of stichosomes and eggs with bipolar plugs (Fig. 3G). Prevalence increased with host age (P < 0.05; Fisher’s exact test); smolts from Mill Ck Siletz and both forks of Nehalem had high infection prevalences (Table III). For parr, fish from WF Smith R (broad year 2007) had a high prevalence of almost 90%. This nematode occurred within the intestinal epithelium, but was not associated with obvious tissue damage or inflammation.

Unidentified adult digenews were observed in the lumen of the pyloric caeca of a few fish (Table III) and were not associated with significant histopathologic changes (Fig. 3J). Prevalence of these worms decreased between underyearling stages but increased in smolts (P < 0.05; Fisher’s exact test), and parr had a significantly lower mean density of infection than fry and smolts (P < 0.05; bootstrap t-test). Fry from Mill Ck Siletz and WF Smith R had a high prevalence and density of infection, whereas smolts from UNF Nehalem and Cascade Ck had high levels of these indices. An adult cestode was observed in the intestine (Fig. 1C) of 1 fish from EF Lobster Ck and an adult acanthocephalan (Fig. 1D) was observed in the intestine of 4 fish from Winchester Ck. Numerous larval nematodes of *Philonema* sp. were observed in the coelomic cavity and ovary of 1 fish from LNF Nehalem (Fig. 3H, I), which was the same fish we detected larval worms in gill wet preparations. Typically, larvae were...
surrounded by many intermixed inflammatory cells, comprised of lymphocytes, histiocytes, and eosinophilic granular cells, with fewer plasma cells being present. Serosal surfaces were lined by hypertrophied reactive serosal cells overlying modest fibroplasia, indicating a low grade and chronic serositis.

Diagnostic method comparison

Using wet preparations as our reference test, the sensitivity of histology was low, i.e., 10–20%, for all the muscle parasites, and some gill parasites were not detected at all (sensitivity test; Table IV). The sensitivity was increased when we excluded muscle tissues with a low mean density of infection (Table IV), but it was still never above 45%. Three gill ciliates, *T. truttae*, *Epiptilys* sp., and *I. multifilis*, were all detected by wet preparation, but they were not found by histology (Table IV). However, histology was good for detecting *C. piscium* infections, with approximately 95% sensitivity (Table IV). Similarly, histology was fairly sensitive (approximately 45%) at detecting gill metacercariae, even though the reference test (wet preparation) consistently missed these infections across many fish populations (compare values in Table II). With the exception of the gill metacercariae, the specificity for histology was high for all other parasites, ranging from 91 to 100% (Table IV). *Sanguinicola* sp. was detected by wet preparation in only 1 group, but it was found in 4 groups in which both histology and wet preparation data were collected (Table II).

**DISCUSSION**

Study background

Diseases, including those caused by parasites, may be significant contributors to mortality in wild fish populations (Möller and Anders, 1986; Adlard and Lester, 1994; Bakke and Harris, 1998; Jacobson et al., 2008). One of the first steps for assessing impacts of parasites on wild fish populations is to identify the parasite species and to document their prevalence, density, and geographic distribution. We had previously reported heavy helminth and myxozoan infections in coho salmon parr from WF Smith R (Rodnick et al., 2008), consistent with the current survey on fry, parr, and smolt coho salmon from 10 coastal rivers. The parasites found in the present study have been described previously from salmonids in the Nearctic, mostly in parasite surveys or taxonomic descriptions.

**Nanophyetus salmincola**

One of the most common parasites observed was metacercariae of *N. salmincola*, which were found in all life stages of fish and in
TABLE IV. Sensitivity and specificity of histology, by using evaluations of wet preparations as a standard, for infections in smolts from 10 rivers and parr from 1 river; 95% confidence intervals are in parentheses. Heavy = highest 10% density based on wet preparation results for parasites in axial skeletal muscle. The mean density and (range) of these heavy infections were as follows: Apophallus sp. = 309/fillet (16–1,034/fillet), Nanophyetus salmincola = 39/fillet (22–61/fillet), and Myxobolus insidiosus = 262/fillet (116–570/fillet). Specificity for heavy infections is not presented because they were the same as those that included all data.

<table>
<thead>
<tr>
<th>Muscle parasites</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apophallus</em> sp.</td>
<td>0.15 (0.08–0.25)</td>
<td>0.99 (0.95–1.0)</td>
</tr>
<tr>
<td><em>Apophallus</em> sp., heavy</td>
<td>0.45 (0.25–0.67)</td>
<td>—</td>
</tr>
<tr>
<td><em>N. salmincola</em></td>
<td>0.26 (0.19–0.34)</td>
<td>0.91 (0.80–0.96)</td>
</tr>
<tr>
<td><em>N. salmincola</em>, heavy</td>
<td>0.32 (0.15–0.55)</td>
<td>—</td>
</tr>
<tr>
<td><em>M. insidiosus</em></td>
<td>0.03 (0.09–0.19)</td>
<td>1.0 (0.83–1.0)</td>
</tr>
<tr>
<td><em>M. insidiosus</em>, heavy</td>
<td>0.41 (0.21–0.63)</td>
<td>—</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gill parasites</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill metacercariae</td>
<td>0.31 (0.12–0.59)</td>
<td>0.80 (0.74–0.86)</td>
</tr>
<tr>
<td><em>N. salmincola</em></td>
<td>0.44 (0.21–0.69)</td>
<td>0.77 (0.71–0.83)</td>
</tr>
<tr>
<td>Sanguinicolida sp.</td>
<td>0 (0–0.37)</td>
<td>0.91 (0.86–0.95)</td>
</tr>
<tr>
<td><em>Trichodina</em> truttae</td>
<td>0 (0–0.54)</td>
<td>1 (0.98–1.0)</td>
</tr>
<tr>
<td><em>Epistylis</em> sp.</td>
<td>0 (0–0.48)</td>
<td>1 (0.98–1.0)</td>
</tr>
<tr>
<td><em>Ichthyophthirius</em> multifilis</td>
<td>0 (0–0.60)</td>
<td>1 (0.98–1.0)</td>
</tr>
<tr>
<td><em>Capriniana piscium</em></td>
<td>0.95 (0.72–0.99)</td>
<td>0.98 (0.95–0.99)</td>
</tr>
</tbody>
</table>

All but 1 of the rivers. The intermediate host for this digenean is *Juga silicula*, which is widely spread in coastal streams from northern California through Washington. This snail prefers slower moving water, with coarse substrata (Furnish, 1990), which is consistent with many locations in our study. Prominent infections in the muscle were seen in wet preparations, and this worm was detected in every organ by histology. As reported previously (Wood and Yasutake, 1956; Ferguson et al., 2010), the metacercariae were associated with localized, chronic inflammation.

**Apophallus sp.**

Metacercariae of *Apophallus* sp. were found in fish from nearly half the sites and were most dense and prevalent in fish from locations with little riparian canopy, because the surrounding lands have been either logged, e.g., WF Smith R, LNF Nehalem, and Cascade Ck, or are used for agriculture, e.g., Mill Ck Siletz (Solazzi et al., 2000). These activities are associated with increased water temperatures in the associated rivers (Beschta and Taylor, 1988), and perhaps this digenean species thrives better under these conditions. For example, summer water temperatures in the lower mainstem of the WF Smith R has often exceeded 20 C (Ebersole et al., 2006), and Rodnick et al. (2008) reported that *Apophallus* sp. metacercariae were more common in coho salmon parr in this stretch of the river compared with cooler, upstream tributaries. The first intermediate hosts for *Apophallus* species infecting different fishes in Oregon are snails of *Fluminicola* sp. (Niemi and Macy, 1974; Kent et al., 2004). Snails (Malek, 1980) and digeneans (Poulin, 2006) increase their reproduction with warmer temperatures. Hawkins et al. (1982) also reported that rivers in Oregon with little riparian canopy, presumably resulting in higher temperatures, had more snails. The abundance of definitive hosts nearby also could be an important factor. For example, the William L. Finley National Wildlife Refuge is approximately 90 km away from the WF Smith R and may allow for many piscivorous birds to frequent the area.

In contrast to *N. salmincola*, we did not observe *Apophallus* sp. in visceral organs. Indeed, the infections in muscle occurred at a specific location, i.e., within the connective tissue of the myospeta. Other reports showed that metacercariae of *Apophallus* species target skin of salmonids (Niemi and Macy, 1974), muscle of percids (Taylor et al., 1994), or bone of cyprinids (Kent et al., 2004). The latter 2 reports involved osseous hyperplasia and metaplasia associated with metacercariae. This was consistent with gill infections in our study, where metacercariae in the gills were often directly associated with chondroid hyperplasia and metaplasia.

**Unidentified gill metacercariae**

Identification of the metacercariae, other than *N. salmincola*, in the gills was difficult by histology. Occasionally, we observed a distinct spiny collar in histologic sections, a feature consistent with echinostomids. Some excyted worms from wet preparations closely resembled *Echinococbus multiv*, a common species found in gills of Oregon salmonids associated with similar lamellar damage (Hoffman, 1999). Olson and Pierce (1997) described metacercariae from the gills of Oregon steelhead trout (*Oncorhyncus mykiss*) that caused cartilage proliferation, and concluded that they were probably members of the Heterophyidae. Worms from both families have been noted to induce lamellar hyperplasia (Uzmann and Hayduk, 1964). In our study, chondrocyte hyperplasia and metaplasia associated with metacercariae were similar to that described with gill metacercariae in freshwater tropical fishes (Blazer and Gratzek, 1985). Disease produced by such infections can be serious. For example, gill-infesting *Centrocestus formosanus* (Heterophyidae) causes massive mortalities in cultured warm water fishes (Paperna, 1995).

**Sanguinicolida sp.**

Another digenean found in the gills of juvenile coho salmon was the blood fluke *Sanguinicolida* sp. It was not possible to identify this worm to the species level because only eggs and miracidia were observed; however, 2 species infect salmonids in Oregon, *Sanguinicolida klamathensis* and *Sanguinicolida alsea* (Hoffman, 1999). Embryonated eggs of *Sanguinicolida* spp. are found in blood vessels of well-vascularized organs, particularly the gills and kidney. Typical of blood fluke eggs, they elicit local inflammatory changes, and severe infections are associated with morbidity when miracidia escape from gills causing loss of blood and decreasing respiratory function (Schäperslaus, 1991; Paperna, 1995).

**Gastrointestinal helminths**

A capitlarid nematode in the lower intestine of many parr and smolts was found at nearly half the sites. We are confident that worms are *Pseudocapillaria salvelini* (syn. *Capillaria salvelini*, *Ichthyocapillaria salvelini*), because it has been reported from numerous salmonid fishes, including coho salmon from Canada (Bell and Beverly-Burton, 1981). The only other record of a different species in salmonids was *Capillaria catenata* (Fritts, 1959), which typically infects percids and centrarchids. This single
report has been regarded as suspect by Bell and Beverley-Burton (1981), due to its rarity and because the worm was found only in cutthroat trout (Oncorhynchus clarkii) and not in perids and centrarchids from the same location. Capillarids are recognized as pathogens in fishes (Dick and Choudhury, 1995), because they penetrate gut tissue and are confined with severe damage and inflammation. For example, Pseudocapillaria tomentosa causes severe inflammation and is associated with emaciation and ultimately death in zebrafish (Danio rerio; Kent et al., 2002). In contrast, all worms seen in our study were confined to the epithelium and were associated with little, or no, tissue damage.

We did not determine the identities of the adult digeneans observed in the gastrointestinal tract, because there are numerous genera and species infecting salmonids in the Pacific Northwest (Love and Moser, 1983; Hoffman, 1999), and we only observed them by histology. Adult intestinal trematodes are considered to be less pathogenic than metacercariae, because most are confined to the lumen of the gastrointestinal tract (Paperna, 1995). Numerous genera and species of acanthocephalans and cestodes infect salmon and trout in the Pacific Northwest as adult stages (Love and Moser, 1983; Hoffman, 1999; McDonald and Margolis, 1995). We were not able to identify our specimens to the genus level based on the available material.

A severe Philonema sp. infection was observed in 1 smolt, with massive numbers of larvae in the viscera and gills. Unfortunately, we were unable to collect adult worms and thus were not able to determine the precise species of this worm. Two genera of philometrids are described from salmonids in the Pacific Northwest, i.e., Philonema and Philometra (Hoffman, 1999). We identified our worms as Philonema sp. based on the tapered posterior end. There are numerous reports of Philonema oncorynchi in salmonids from the Pacific Northwest (Hoffman, 1999; Moravec and Nagasawa, 1999), and thus this may be the species seen here. This parasite is recognized to cause disease in salmonids, including coho salmon (Brocklebank et al., 1996), and the intense inflammatory response seen in the one severely infected fish certainly would have compromised this individual.

**Myxozoa**

Myxozoa are commonly observed in wild salmonids, and we found 5 species in our survey. Chloromyxum majori in the kidney and Myxobolus sp. in the brain were common in fish from rivers with high levels of digenean infections. The life cycles of these myxozoans have not been resolved, but based on other myxozoans they probably involve an oligochaete host. Many species of these worms thrive in relatively warm, oligotrophic, or eutrophic environments and this could account for their high prevalence and similar density in all rivers, suggesting that the putative oligochaete host for this parasite is widespread. These are general observations, and further studies are required to connect these parasite distributions with ecologic parameters and land use practices.

We observed renal infections by *M. salvelini* in all life stages of coho salmon in 7 of the rivers. Two Myxidium species infect the kidneys of salmonids in the Pacific Northwest, i.e., *M. salvelini* and *Myxidium minteri*. The former species has been recorded from urinary bladder, ureter (Shul’man, 1988), and kidney (Shul’man, 1988; Kent et al., 1994; McDonald and Margolis, 1995), whereas the latter has only been found in renal tubules (Yasutake and Wood, 1957; Sanders and Fryer, 1970). We identified the species seen here as *M. salvelini* based on the presence of more elongated spores and pyriform polar capsules. In contrast, the spores of *M. minteri* are oval and polar capsules are subcircular. In addition, one of us (M.K.) has observed *M. salvelini* infections in many salmonids in Canada, and, as seen here, the occurrence of refractile, brown-to-black granules in the cytoplasm of the trophozoites is a consistent finding for this species. Renal tubule degeneration can occur and spores are liberated from tubules and enter into the renal interstitium with heavy infections of *M. minteri* (Yasutake and Wood, 1957), whereas *M. salvelini* is not recognized to be pathogenic (Kent et al., 1994). There was no observable damage to the infected tubules.

Chloromyxum majori infected kidney of fry, parr, or smolts from 5 of the rivers. This species is the only species in this genus that infects glomeruli of salmonids in this geographic region (Yasutake and Wood, 1957; Hoffman, 1999). We found this parasite to predominantly infect the glomerular visceral epithelium and occlude the uniferious space, with large numbers completely effacing glomeruli. However, it also can cause degeneration of intra-renal hematopoietic tissue in heavy infections that has been associated with salmon losses in hatcheries (Yasutake and Wood, 1957). The glomerular lesions observed here were consistent with previous descriptions, and infected glomeruli were essentially obliterated by the parasite and associated inflammation.

Several neurotropic Myxobolus species infect salmonids (for review, see Ferguson et al., 2008). The spores seen here in brains from juvenile coho salmon were probably either *Myxobolus kisutchi* or *Myxobolus neurotropus* because both form oval spores in the brain and spinal cord of salmonids in this region. It is unlikely that these infections were *Myxobolus arcticus*, because this species has a more northerly range and has not been reported in parr or smolts in freshwater south of Washington state (Ferguson et al., 2008). There was minimal tissue reaction to the brain myxobolid, and infections by *M. kisutchi* and *M. neurotropus* have not been associated with disease. However, Moles and Heifetz (1998) found that *M. arcticus*, which causes similar infections, is associated with reduced swimming ability of sockeye salmon (Oncorhynchus nerka).

**Gill protists**

Several protists were observed in, or on, the gills of juvenile coho salmon, including *L. salmonae*, *T. truttae*, Epistyliis sp., Capriniana piscium (syn. Trichophyra piscium), and *I. multifilis*. Trichodina truttae was the most prevalent ectoparasitic protist, detected in fish from half of the rivers. This species has been described from salmonids in freshwater in Oregon (Mueller, 1937) and other regions in the Pacific Northwest (Arthur and Margolis, 1984), based on size and morphology. It is pathogenic when it occurs in high numbers, causing epithelial hyperplasia, decreased osmoregulatory ability, and even death (Lom, 1995). We did not observe tissue damage associated with this ciliate.

Both Epistyliis sp. (Esch et al., 1976) and *C. piscium* (Hofer et al., 2005) have been associated with fish held in water with high organic loads, and certain rivers where these were prevalent, e.g., Siletz R and Yaquinaia R, predominantly have agriculture activity
above the sites where fish were collected. The proper identification of epitylids in fishes from North America is lacking (Hoffman, 1999); thus, we refer to the organism seen here as *Epistyli* sp., which is considered more of an ectocommensal than a parasite of both skin and gills. However, high numbers of this organism have been implicated in "red sore disease" with other possible synergistic etiologies (Lom, 1995). Although *Capriniana* infections were very heavy in some fish, with every filament infected with numerous parasites, they were not associated with histologic changes. This is consistent with other reports, where *Capriniana* spp. on salmonid gills and other fishes are considered commensal, rather than parasitic (Lom, 1995; Hofer et al., 2005).

*Ichthyophthirius multifilis* is recognized as a serious pathogen (Traxler et al., 1998), but we detected this parasite in just 1 fish. Two fish were infected with the microsporidium, *L. salmonae*, which is a well-known pathogen in net pen aquaculture (Kent and Speare, 2005). Severity of tissue damage is more related to the reaction associated with destruction of xenomas rather than density of infection (Speare et al., 1998). With infections in freshwater, intact xenomas, as seen here, are associated with little damage to host tissues (Magor, 1987).

**Evidence for parasite associated mortality**

In general, fry and parr were more intensely infected by most parasites than smolts, but a few parasites had a higher prevalence in older fish. There are many examples of temporal declines in parasite burdens of host populations being associated with parasite associated mortality, often involving fishes (for reviews, see Anderson and Gordon, 1982; Lester, 1984; Hudson and Dobson, 1995; Rouset et al., 1996). Recently, Jacobson et al. (2008) reported this to occur with *N. salmincola* infections in coho salmon off the coast of Oregon. Temporal declines in parasite burdens also can be explained by recovery from infections or from non-representative samples of the population from different time points, i.e., smolts are a mixed population of many parr sub-populations. We recently showed that *N. salmincola*, *Apophallus* sp., and *M. insidiosus* persist in coho salmon from parr to smolts (Ferguson et al., 2010); thus, parasite death between parr and smolt stages would not explain the differences seen between these fish life stages in the present study. Although it is possible that the differences in parasite burden was because of the potential non-random sampling inherent with studying fish populations, it is unlikely because we observed the same trends in fish sampled across 10 different rivers. High mortality often occurs in coho salmon during the winter preceding smoltification (Ebersole et al., 2006; Hurst, 2007) and pre-overwintering fish from rivers such as WF Smith R have substantially high infections (Rodnick et al., 2008). Therefore, it is most probable that the dramatic declines in parasite burdens in fish from parr to smolt stages, as seen with *Apophallus* sp., are related to parasite associated mortality.

**Method comparison**

Diagnostic methods for identifying parasites include serology, non-lethal blood and fecal exams, molecular methods, biopsies, and necropsy with examination of tissues by wet mount or histology (Hendrix and Robinson, 2006). With fish, serologic or molecular tests have been developed for some pathogens (Cunningham, 2002; AFS-FHS, 2007), yet there are few that have been developed or verified for helminth parasites. Therefore, the most practical methods for examining fish for a variety of parasites were by histology and wet preparations. Surveys for parasites of wild fishes have been most often based on necropsies and examination of wet tissues (Arthur et al., 1976; Blaylock et al., 1998; Arkoosh et al., 2004; Butorina et al., 2008; Jacobson et al., 2008). However, some studies have relied on, or included, data obtained by histology (Kent et al., 1998; Longshaw et al., 2004; Stentiford and Feist, 2005).

We used 2 diagnostic methods, histology and wet tissue examinations, and compared the sensitivity and specificity of histology by using the latter method as a reference test. Although we have used both methods together in parasites surveys (Eaton et al., 1991; Moran and Kent, 1999; Kent et al., 2005), we are unaware of any studies in which both methods were compared empirically with sensitivity and specificity tests. Wet preparations consistently detected more parasites. On average, only 15% of *Apophallus* sp. were correctly detected by histology (Table IV). When histology was applied to determine prevalence of gill and muscle parasites in population surveys, it had an effect on the prevalence of infection. For example, when using histologic evaluation, *Apophallus* sp. was only detected in 2 smolt populations, but it was found in 6 populations by using wet preparations. Likewise, the use of only histology would have resulted in missed *M. insidiosus* infections in smolts from 4 sites, and the prevalence of infection would have been greatly underestimated for most parasite infections except infections with gill trematodes (Tables II, III).

The sensitivity of histology improved when we excluded lightly infected fish (Table IV). However, this increase was still below 50% for all parasites examined, so less than 50% of intensely parasitized fish would be detected by histologic evaluation alone. Clearly, using wet preparations was better for detecting parasites than histology, probably because more tissue is examined. Our calculations suggest that wet tissue examination, using 1 g of muscle, represents almost 2,400 times more volume than the average amount of muscle examined in one histologic section. The estimated difference in material evaluated by both methods is similar to that reported by Kocan et al. (2011). An alternative histlogic assessment, such as use of more than 1 section, or more than 1 location, within a given organ, would probably improve sensitivity.

Histology is also relatively poor for evaluating external gill parasites (Table IV) such as ciliates, because they often detach when gills are placed in fixatives; thus, data obtained by histology for these parasites are generally underestimated. For example, the reason why *C. piscium* was the only external ciliate that was detected by histology was probably because of the severity of infection by this parasite. In contrast, although *L. salmonae* infections were relatively light, this parasite was detected by both methods as this parasite is intracellular and thus would not be removed from the tissues during processing. Wet tissues also provide another advantage in that whole parasites are examined and thus important morphologic features are better visualized.

Histology, however, provides certain advantages. With the exception of the gill metacercariae, the specificity for histology was high for all other parasites (91–100%); thus, histology was equally accurate as wet preparations for identification of these parasites. Embryonated eggs of the *Sanguinieola* sp. were more often detected in histology compared with wet tissue examination (Table II; note low specificity Table IV). These eggs are deep
within blood vessels, are small and not motile, and thus are probably easier to detect by histology. Although histology had poor specificity for gill metacercaire in our analysis, this was actually due to the choice of wet preparations for the reference test. Furthermore, detection of *N. salmincola* in gills seemed to be easier by histology, which could have been due to the difficulty of detecting this worm deep in thicker wet preparations of gill.

In conclusion, we detected a wide variety of parasites in fish from some sites, and few were associated with prominent histologic changes. We found that parasites were more often detected in wet preparations. However, histology provides more information on pathology and may be more amendable for detecting parasites in certain tissues, e.g., brain of very small fish and thick tissues such as cardiac muscle. Finally, histology is a broad-based diagnostic method, allowing for detection of a variety of etiologic agents beyond parasites. We propose the use of both methods when possible to provide the most accurate identification, enumeration, and determination of impact of parasites. This is particularly important when sample availability is limited.

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**LITERATURE CITED**


Parasite survey comparing methodologies

FERGUSON ET AL.

Parasite survey comparing methodologies


A DESCRIPTION OF MATURE ONICCOLA VENEZUELENsis (ACANTHOCEPHALA: Oligacanthorhynchidae) FROM A FERAL HOUSE CAT IN THE U. S. VIRGIN ISLANDS

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ABSTRACT: A road-killed feral house cat from the U.S. Virgin Islands was parasitized by 87 acanthocephalans of the species Oniccola venezuelesis Marteau, 1977. The finding allowed for the documentation of a suitable definitive host for the species in the Virgin Islands and permits a more comprehensive description of the species, including the first of fully mature adults and completely formed eggs. Sexually mature males from the cat were 6.5-8.4 (8.0) mm long; gravid females were 13.2-18.3 (15.5) mm long. Fully formed eggs dissected from the trunk of females were 67-72 (69) μm long by 43-50 (47) μm wide. The life cycle of O. venezuelesis in the Virgin Islands is now apparent, i.e., termites serve as intermediate hosts, lizards and birds as paratenic hosts, and domestic cats as definitive hosts. Extra-intestinal infections in mongooses are likely incidental.

The acanthocephalan species Oniccola venezuelesis Marteau, 1977, was originally described from 4 specimens (2 males, 2 females) found in an ocelot, Leopardus pardalis (Linnaeus, 1758), collected in Venezuela. Although the worms were in the intestine of the ocelot, none was completely mature, hindering description of some structures and precluding description of fully formed eggs (Marteau, 1977). Subsequently, O. venezuelesis was not reported again until Nickol et al. (2006) described cystacanths of this species obtained from the hemocoels of termites, the subcutaneous tissues of lizards, the greater omentum of mongooses, and the mesenteries of birds from the U. S. Virgin Islands. Studies of infected termites demonstrated parasite-induced changes in behavior, color, and risk of predation (Fuller et al., 2003) that had implications for reproductive success in termite colonies (Fuller and Jeyasingh, 2004). Because the only mammals native to the Virgin Islands are a few kinds of bats, there are not many species to serve as possible definitive hosts in these islands. Nickol et al. (2006) speculated that domestic and feral house cats were the most likely definitive hosts in the U. S. Virgin Islands.

Recently, a road-killed feral cat (Feliis catus Linneaus, 1758) was fortuitously found on St. John, U. S. Virgin Islands. Necropsy of the cat revealed 87 specimens of O. venezuelesis, including sexually mature males and gravid females, in the intestine. These specimens demonstrate a definitive host for O. venezuelesis in the Lesser Antilles Islands and permit a more comprehensive description of the species, including the first description of gravid females and fully formed eggs.

MATERIALS AND METHODS

A freshly killed cat (F. catus), retrieved from the roadside on St. John Island, U. S. Virgin Islands, was examined for acanthocephalans. The intestine contained 87 specimens that were later identified as O. venezuelesis. After the worms were held in tap water overnight to promote evacuation of proboscides, 68 of them were fixed in a solution of alcohol, formalin, and acetic acid (AFA) and the remaining 19 were transferred to 95% ethanol to permit future DNA analysis. After fixation, 36 specimens were stored in 70% ethanol until being prepared for microscopic study. These were stained in Mayer’s carmalum, dehydrated in a series of ethanol concentrations, cleared in xylene, and mounted in Canada balsam. All of the specimens were examined, but the following description is of fully mature (9 male and 15 female) worms only, males with a secretion in efferent ducts and females with fully developed eggs. Marteau (1977) described younger forms taken from the intestine of an ocelot, and Nickol et al. (2006) described cystacanths of this species from intermediate and paratenic hosts. Unless stated otherwise, measurements are in micrometers and widths were taken at the widest point of the structures. All measurements for proboscis hooks were made in full lateral profile and lengths are the straight-line distance from the tip of the thorn to the junction with the root, i.e., hook length does not include the anteriorly directed root. Ranges for counts and measurements are followed by the mean in parentheses.

Voucher specimens were deposited in Harold W. Manter Laboratory, University of Nebraska State Museum (HWML).

DESCRIPTION

Oniccola venezuelesis Marteau, 1977

(Figs. 1–4)

Sexually mature adults: Trunk elongated, widest in anterior 1/3, tapering to posterior extremity; males 6.5–8.4 (8.0) mm long by 1.2–1.3 (1.2) wide, females 13.2–18.3 (15.5) mm long by 1.8–2.0 (2.2) mm wide. Proboscis globular; males 336–348 (344) long by 476–480 (479) wide; females 432–480 (458) long by 528–566 (547) wide; with 36 hooks in 6 circles of 6 each. Hooks robust, chisel-shaped at tip. Hooks in distal 4 circles with roots much elongated anteriorly, less elongated in fourth circle; hook roots in proximal 2 circles flat, plate-like. Hooks of similar size in both sexes; 115–139 (127), 120–139 (131), 98–110 (102), 91–96 (92), 77–86 (82), 74–82 (77) long in circles from anterior to posterior. Neck 250–302 (289) long by 384–422 (396) wide, with pair of lateral sensory pits 48–55 (51) by 29–39 (34) just posterior to root of last proboscis hook.

Proboscis receptacle 1.01–1.39 (1.21) mm long; single walled, tightly surrounded by protrusor muscles; with ventral cleft through which passes aperistaltic muscles and retinacula. Cerebral ganglion along ventral wall of receptacle at cleft; 110–149 (131) long by 62–86 (73) wide. Lemnisci very long, looped; ribbonlike, about 10 mm long, extending into posterior 1/4 of females and often to the level of cement glands in males. Protopnephridia 2; dendritic type; fan-shaped, 192–202 (200) long by 94–96 (95) wide; attached to anterior wall of Saefltigen’s pouch in males, dorsal wall of uterine bell in females.

Males: Reproductive tract extends from posterior end to slightly beyond mid-trunk; 2 tandem, elliptical testes, neither consistently larger than the other, 950–1,507 (1,136) long by 342–442 (381) wide. Cement glands 8; arranged in pairs with anterior pair contiguous with posterior testes; each gland elliptical with a single large nucleus; 528–672 (595) long by 269–288 (281) wide. Saefltigen’s pouch, alongside vas deferens, 1,056–1,392 (1.264) mm long by 288–480 (384) wide; duct accompaniments vas deferens to bursa.

Females: Reproductive system in posterior 10% of trunk. Uterine bell, from anterior margin to back of selector apparatus, 499–538 (520) long by 259–336 (301) wide; uterus 672–816 (752) long; vagina almost entirely surrounded by well-developed sphincter, 264–384 (330) long. Genital pore slightly subterminal. Fully developed eggs, dissected from AFA-fixed female, 67–72 (69) long by 43–50 (47) wide.

Taxonomic summary

Host: Feral domestic cat, Feliis catus Linneaus, 1758.
Locality: 18°19′8.04″N, 64°42′19.5″W, on St. John Island, U. S. Virgin Islands.

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**DISCUSSION**

The cat from the U.S. Virgin Islands contained specimens of *O. venezuelensis* in stages of growth that ranged from that found in lizards and birds presumed to be paratenic hosts (Nickol et al., 2006) through fully mature worms including gravid females with completely developed eggs. The range in stages of growth, along with the large number of worms present, suggests that the cat acquired acanthocephalans during a prolonged period. Male acanthocephalans die and are lost from the host shortly after copulation (Crompton, 1970); for this reason, the dynamics of the infrapopulation might explain why males in our study are shorter than those described by Marteau (1977). The 2 males present in Marteau's ocelot were likely the same age as, and had copulated with, the 2 females present, which did not contain fully formed eggs. Males that had copulated with the females containing fully formed eggs from our cat had probably been lost, and those remaining were likely younger and would mate with females present that were not yet gravid.

*Oncicola venezuelensis* clearly is established in the U. S. Virgin Islands where termites harbor developing cystacanths and cystacanths and lizards, birds, and mongooses harbor extra-intestinal cystacanths (Nickol et al., 2006). In the present report, we show that a feral house cat is a suitable definitive host. Although infection of lizards with cystacanths from termites is the only part of the life cycle that has been completed in the laboratory (Nickol et al., 2006), the life cycle in the Virgin Islands seems obvious, i.e., termites are intermediate hosts, likely ingesting eggs from cat feces, and lizards and birds are paratenic hosts that transfer the parasite to domestic cats, which serve as definitive hosts. Mongooses probably have no predator in the Virgin Islands, and they are likely incidental hosts for cystacanths.

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**LITERATURE CITED**


DISPERSAL IN THE ACANTHOCEPHALAN ACANTHOCHEPHALUS DIRUS

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ABSTRACT: In acanthocephalans, dispersal typically occurs when eggs that have been released in the intestines of definitive hosts are expelled with the feces. We examined whether the acanthocephalan _Acanthocephalus dirus_ adopts a strategy of dispersal in which eggs are carried into the environment by gravid females. Using a combination of field surveys and lab-based experiments, we showed that the _A. dirus_ female retained eggs as they passed out of the intestines and that these eggs could develop in intermediate hosts (sediment-dwelling isopods). Lab-based behavioral experiments revealed that the bodies of gravid females were attractive to foraging isopods. We propose that a strategy of egg dispersal could occur in _A. dirus_ in which eggs are carried into the environment by females. This strategy could increase transmission success by dispersing eggs closer to the sediment, rather than in the water column, and by directing the feeding behavior of target hosts.

Dispersal strategies of helminths can be relatively diverse, with factors such as the properties of the habitat and the feeding behavior of hosts influencing the optimal strategy (Combes et al., 1994; Bush et al., 2001; Moore, 2002). Contrary to this general pattern, acanthocephalans appear to have a relatively conserved strategy of dispersal (Kennedy, 2006). Typically, eggs are released into the intestines of definitive hosts and dispersed with the feces. Transmission to the intermediate hosts then occurs when these eggs are consumed (Kennedy, 2006). Although this strategy is associated with many acanthocephalans, some authors have suggested that some species may adopt an alternative strategy of dispersal in which eggs are carried into the environment by gravid females, i.e., _Polymorphus minutus_, _Polymorphus marilis_, and _Acanthocephalus jacksoni_, (Nicholas and Hynes, 1958; Denny, 1968; Muzzall and Rabalais, 1975).

Here, we have examined the potential occurrence of this strategy in the acanthocephalan _Acanthocephalus dirus_ (= _A. jacksoni_ = _Acanthocephalus parksidei_). Populations of _A. dirus_ are common in streams throughout the midwestern United States (Amin, 1985; Crompton and Nickol, 1985), where they infect sediment-dwelling isopods ( _Caecidotea spp._, _Lirceus spp._) as intermediate hosts and creek chub ( _Semotilus atromaculatus_ ) as definitive hosts (Seidenberg, 1973; Muzzall and Rabalais 1975; Camp and Huizinga, 1980; Amin, 1985; Sparkes et al., 2004). Development in _A. dirus_ is relatively synchronous, with immature acanthellae dominating infections of intermediate hosts during the summer and mature cystacanths dominating infections of definitive hosts during late spring-early summer (Seidenberg, 1973; Amin, 1980; Camp and Huizinga, 1980). Similar patterns of development have been identified in European acanthocephalans (Brattey, 1988). Inside the creek chub, _A. dirus_ females produce eggs which are dispersed in the stream during the spring and summer (Seidenberg, 1973; Çamp and Huizinga, 1980). Muzzall and Rabalais (1975) proposed that eggs are carried into the stream by female _A. dirus_ because eggs were not present in the feces and females were expelled from definitive hosts intact. We tested this hypothesis using a combination of field surveys and lab-based experiments. Field surveys were used to determine whether eggs were released into the intestines of definitive hosts or retained by females; lab-based experiments were used to determine whether eggs contained in expelled females could develop in intermediate hosts. We then used a lab-based experiment to examine whether the bodies of gravid females were attractive to target hosts and, hence, could facilitate transmission.

MATERIALS AND METHODS

Site of study

All organisms were collected from Buffalo Creek, located 60 km northwest of Chicago in Lake County, Illinois. At this site, juvenile isopods ( _Caecidotea intermedia_ ) are infected by _A. dirus_ eggs during the summer and creek chub ( _S. atromaculatus_ ) are infected by _A. dirus_ cystacanths during the autumn, winter, and spring (Sparkes et al., 2004). Gravid female _A. dirus_ are present in the creek chub during late spring and early summer.

Intestinal contents of _S. atromaculatus_

To determine whether eggs were released into the intestines of definitive hosts, we examined the intestinal contents of creek chub. Twenty-one creek chub were collected during the spring of 2008 and 2009 using a combination of seine and hand nets (n = 12, 22 May 2008; n = 9, 21 May 2009). Immediately following capture, each fish was killed (MS222), fixed in formalin (10%, 24 hr), and stored in ethanol (70%) prior to necropsy. The intestinal parasites present were removed and identified and the contents of the intestines examined for eggs using a dissecting microscope. In each case, the contents were washed into a Petri dish and both the transferred contents and the remaining intestines were examined for eggs. If a fish contained a female _A. dirus_ whose body was broken, it was excluded from the analysis because eggs present in the intestines may have been liberated during necropsy.

To determine whether eggs were present in female acanthocephalans, we dissected the females recovered from the creek chub and recorded the presence or absence of eggs. For a sub-sample of these females (n = 10), we estimated total egg content to allow the determination of the relationship between intestinal egg counts and female egg counts. For this analysis, each female was placed into a small Petri dish (35 × 10 mm), measured (length, width), cut into small sections, and the eggs and ovarian balls (if present) removed. Female size was estimated by calculating total volume (volume = (π × length × width) / 6; Dezfuli et al., 2001). To obtain egg counts, the eggs and ovarian balls recovered from each female were transferred into a glass tube containing distilled water (13 × 100 mm; total volume = 5 ml). Prior to each count, the tube was gently vortexed for 15 sec and a 4-μl sub-sample was transferred to a Cell-VU (Millennium Sciences, Inc., New York, New York) slide (n = 30). Each sub-sample was placed into the middle of a marked circle that had been divided into 8 sections of equal size. One drop of Lugol’s solution was then added to stain the eggs. The number of eggs that contained acanthors was counted, the average number of eggs present per sub-sample calculated, and the total egg content per parasite estimated (based on the initial volume of liquid). The same procedure was used to estimate the total number of ovarian balls present. We used a second sub-sample of the females recovered from the creek chub (n = 13) to estimate the level of egg maturity. For each female, we removed a random sample of 30 eggs and determined egg maturity using the number of membranes present. Eggs that possessed 4 membranes were considered mature (West, 1964). The length of the eggs was also measured.

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Experimental infection of *C. intermedius*

To determine whether eggs contained in expelled female *A. dirus* could develop in intermediate hosts, we experimentally infected *C. intermedius* in the laboratory. Juvenile *C. intermedius* were collected and transported to the laboratory at DePaul University (25 June 2007). The isopods were then exposed to either eggs recovered from a female or the bodies of gravid females.

To obtain the female *A. dirus*, creek chub were collected (8 May 2007) and transported to the laboratory (n = 10). Each fish was housed individually (38-L aquarium, filled with a mixture of stream water and conditioned tap water), and fed fish pellets ad libitum. A plastic grate exposed to either eggs recovered from a female or the bodies of gravid females was positioned approximately 4 cm from the bottom of the tank was used to isolate expelled parasites. The tanks were monitored daily for 23 days (8 May–30 May 2007) and expelled females were removed and stored in conditioned tap water at 4 °C (n = 13, mean number of days prior to expulsion = 18). Five of these females were used for the experimental infections.

Experimental infections using free eggs were carried out between 30 July and 29 August 2007. Eggs used for the infections were removed from the body of a female *A. dirus* and stored in a Petri dish (35 × 10 mm) in conditioned tap water at 4 °C. Body size measurements were used to standardize isopod size between infection groups (n = 54 per group). Maple leaves were pre-soaked, cut into squares (100 × 100 mm), and placed into individual Petri dishes (35 × 10 mm). Using a fine pin attached to a probe, either 5 or 10 eggs were placed onto each leaf square. An individual isopod was then allowed to feed in a dish containing 1 leaf square for 24 hr. Each juvenile was then transferred into a cup (80 mm, 300 ml) containing leaf material that was partially filled with conditioned tap water and aerated. Isopods were monitored daily for 30 days, after which the surviving isopods were preserved (70% ethanol), measured, dissected, and infection status and parasite size (length) was recorded. Body size measurements of the parasites were used to distinguish between field-based (pre-existing) and lab-based (experimental) infections.

Experimental infections with gravid females were carried out between June and September 2007. Juvenile *C. intermedius* (n = 1,000) were collected (25 June 2007), transferred to the lab, and housed in 2 holding arenas (55 × 45 × 25 cm, n = 500 per group). Each arena contained a mixture of stream and conditioned tap water, maple leaves obtained from the DePaul campus, and algal material obtained from the field site. For the experimental infections, 4 gravid female *A. dirus* were introduced into an arena (8 August 2007, ‘experimental’ group). The other arena was left undisturbed (‘control’ group). After 30 days, 50 juveniles were captured at random from each arena and preserved (70% ethanol). Isopods were then measured, necropsied, and infection status and parasite length recorded.

To obtain measures of the typical infection dynamics in nature, we examined juveniles collected from Buffalo Creek during the first month of infection (June). These juveniles were captured from vegetation and from the underside of rocks using hand nets and were preserved in 70% ethanol (n = 140). In the lab, each isopod was measured (body length), dissected, and infection status and parasite size (length) recorded.

**Feeding behavior of *C. intermedius***

To determine whether juvenile isopods were attracted to the bodies of female *A. dirus*, we exposed isopods to either gravid females and leaf material or mature males and leaf material. Juvenile isopods were collected from Buffalo Creek (25 June 2007), transported to DePaul University, and placed into a holding arena (55 × 45 × 25 cm) containing aerated stream water and detritus. Behavioral assays were conducted in a feeding arena (8.5-cm diameter Petri dish) that was partially filled with conditioned tap water. For each trial, individual isopods were placed in an arena with a leaf square (1 × 1 cm) and a parasite (gravid female or mature male) for 20 min. The leaf and parasite were attached to mesh screens (2 × 2 cm) that were placed equidistant from the middle of the Petri dish. After an isopod was released in the center of the dish, encounters and feeding attempts on both the leaf material and parasite were recorded. An encounter was counted if the isopod made contact with the food item. A feeding attempt was counted if the head of the isopod remained in contact with the item for more than 15 sec. For each parasite, we collected behavioral data for 10 isopods. These data were converted to single values for each parasite by calculating percent response values, i.e., percent of isopods that encountered the food item and percent of isopods that attempted to feed on the item. To determine whether isopods preferred to feed on the parasite or the leaf material, we compared the feeding responses between food types using paired t-tests (paired because the isopods were exposed to the leaves and parasites simultaneously). To determine whether isopods preferred to feed on gravid females or mature males, we compared the feeding responses of isopods between parasite types using unpaired t-tests. Following the trials, the isopods were dissected and infection status determined. To resolve whether body size or prior infection status differed between groups, we compared these variables using an unpaired t-test (body size) and a G-test (prior infection status). For analysis that required parametric tests, normality of the data was determined using Systat 10 (Systat, Chicago, Illinois).

**Results**

**Intestinal contents of *S. atromaculatus***

A total of 163 *A. dirus* individuals were recovered from the 21 creek chub collected from Buffalo Creek (prevalence = 95%, median intensity = 5, range = 1–31). Sixty-six percent of these parasites were female (n = 108), 93% of the females contained eggs, and the average egg to ovarian ball ratio per female was 92.8. Estimates of fecundity on a sub-set of these individuals (n = 10) revealed that the females contained an average of 40,000 eggs (SE = 6,800, mean female volume = 2.4 mm³, SE = 0.35). Six of these parasites contained eggs and no ovarian balls and 4 contained a mixture of eggs and ovarian balls. For the 4 females that contained a mixture, the average ratio of eggs to ovarian balls was 98.2, indicating that most of the eggs that would most likely be produced were present. Estimates of egg maturity, based on 30 eggs per female, revealed that, on average, 67% of the eggs were mature (SE = 6.1, n = 13). Mature eggs were larger than immature eggs (mature: mean length = 114 μm, SE = 1.8, n = 13; immature: mean length = 74 μm, SE = 1.2, n = 13).

Seventeen of the original 21 creek chub contained female *A. dirus*. Five of these fish were excluded from the analysis of intestinal contents because the bodies of 1, or more, females were broken during dissection. The results obtained for the remaining 12 fish are shown in Table I. Eight of these fish contained no eggs in their intestines and 4 contained a small number of eggs (1, 2, 5, and 13, respectively). To determine the expected number of
mature eggs present per fish, we used values obtained from the parasites present in the focal fish (number of gravid females per fish, proportion of eggs to ovarian balls per gravid female = 0.92:0.08) and values obtained from the other sub-groups of females (proportion of mature eggs per gravid female = 0.67, egg content per gravid female = 40,000). Based on these values, the estimated number of eggs present per creek chub was approximately 75,000. Given this estimate, the highest number of *A. dirus* eggs found in the intestines would represent less than 0.02% of the estimated number of mature eggs present per fish.

**Experimental infection of *C. intermedius***

To distinguish between pre-existing and experimental infections, we examined variation in body size among the parasites

**FIGURE 1.** Size distributions of *Acanthocephalus dirus* recovered from juvenile *Caecidotea intermedius* in both experimental infections and natural infections. Isopods in the control group (A) were not exposed to *A. dirus* eggs. Isopods in the experimental group (B) were exposed to free eggs or the bodies of gravid females. Parasites recovered from isopods (C) collected in June represent the natural infection levels during the first month of infection (Fig. 1).

For the isopods exposed to free eggs, 24% acquired new infections (Table II). Prevalence of new infections was not influenced by pre-existing infection (5 eggs: \( G = 0.4, df = 1, P > 0.05 \); 10 eggs: \( G = 0.01, df = 1, P > 0.05 \)) and there was no effect of egg density (5, 10) on establishment success (\( G = 0.5, df = 1, P > 0.05 \)). For the isopods exposed to gravid females, 88% acquired new infections (Table II). For these isopods, there was no effect of pre-existing infections on prevalence of new infections (\( G = 2.0, df = 1, P > 0.05 \)).

**Feeding behavior of *C. intermedius***

The results obtained from the behavioral experiment are shown in Figure 2. There was a significant difference in encounter rates between leaf material and parasite within each experimental group (gravid female, \( t = 5.6, df = 5, P = 0.003 \); male, \( t = 12.9, df = 5, P < 0.001 \)), but there was no difference in encounter rates with the different parasites types (\( t = 1.5, df = 10, P = 0.2 \)). Isopods that encountered gravid females exhibited feeding responses that were comparable to responses to leaf material (\( t = 0.2, df = 5, P = 0.8 \)). In contrast, isopods that encountered males fed more on the leaf material (\( t = 6.2, df = 5, P = 0.002 \)). Comparison of the feeding responses between groups showed that isopods were more likely to feed on females than on males (\( t = 3.7, df = 10, P = 0.004 \)). There was no difference in either body size of isopods (\( t = 0.02, df = 118, P = 0.9 \)) or prevalence of pre-existing infections (\( G = 1.4, df = 1, P > 0.05 \)) between the experimental groups.

**DISCUSSION**

In most acanthocephalans, dispersal occurs when eggs released in the intestines of definitive hosts are expelled with the feces (Kennedy, 2006). Contrary to this pattern, Muzzall and Rabalais (1975) found that eggs of the acanthocephalan *A. dirus* were not present in the feces and proposed that gravid females carried the eggs into the environment prior to dispersal. Similar mechanisms have been proposed for the aquatic acanthocephalans *P. minutus* and *P. marilis* (Nicholas and Hynes, 1958; Denny, 1968). Consistent with this type of strategy, we found that eggs did not appear to be released into the intestines of definitive hosts by female *A. dirus*, but instead were retained by gravid females when they were expelled from the definitive host intact. Experimental infections revealed that the eggs contained in these females could

**TABLE II.** Experimental infection of *Caecidotea intermedius* by *Acanthocephalus dirus*. Isopods were exposed to free eggs (n = 5 or 10) or gravid females (n = 4). Shown are mean (length) or median (intensity) values and sample sizes for isopods and parasites. Juvenile isopods collected in June from Buffalo Creek represent the natural infection levels during the first month of infection.

<table>
<thead>
<tr>
<th>Source of infection</th>
<th>Infection location</th>
<th>No. isopods</th>
<th>Isopod length, mm (SE)</th>
<th>Prevalence %</th>
<th>Intensity (range)</th>
<th>No. parasites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free eggs</td>
<td>Lab</td>
<td>55</td>
<td>3.6 (0.10)</td>
<td>24</td>
<td>1 (1–4)</td>
<td>21</td>
</tr>
<tr>
<td>Gravid females</td>
<td>Lab</td>
<td>50</td>
<td>4.3 (0.10)</td>
<td>88</td>
<td>5 (1–25)</td>
<td>257</td>
</tr>
<tr>
<td>Natural infection</td>
<td>Field</td>
<td>140</td>
<td>4.1 (0.07)</td>
<td>61</td>
<td>1 (1–16)</td>
<td>119</td>
</tr>
</tbody>
</table>
develop in intermediate hosts. These results are consistent with the hypothesis that an alternative strategy of egg dispersal occurs for *A. dirus*. However, given that acanthocephalan eggs are often released into the intestines of definitive hosts in spurts (Crompton and Whitfield, 1968), we cannot exclude the possibility that eggs were also released with the feces.

Several factors associated with both the properties of the stream habitat and host biology could potentially favor a strategy of egg retention in which eggs are carried into the stream by female *A. dirus*. In streams, dispersing eggs in the feces could be problematic because acanthocephalan eggs are buoyant (e.g., George and Nadakal, 1973). Thus, eggs released in the water column could be carried downstream and deposited in microhabitats that are not occupied by target hosts, e.g., surface algae. In contrast, if eggs are retained by females, they could be carried into the microhabitat of the target hosts when the females sink through the water column. Egg dispersal would then occur within the microhabitat of the sediment-dwelling hosts when the bodies of the females degrade. Given that water velocity is reduced close to the sediment in streams (Gordon et al., 1992), dispersal within this microhabitat could be relatively localized. In addition, because *A. dirus* eggs possess fibrils (West, 1964; Oettinger and Nickol, 1974; Nikishin, 2001), eggs dispersed within the microhabitat could potentially retain their position by attaching to structures on the sediment, e.g., leaf material. Thus, egg retention by female *A. dirus* could facilitate transmission by increasing the likelihood that eggs are dispersed within the microhabitat of the target hosts.

Another factor that could favor egg retention by females is that the bodies of gravid females attract foraging isopods. In the lab-based behavioral experiment, the feeding response of isopods to the bodies of gravid females was greater than the response to males and comparable to the response to leaf material. Thus, the presence of gravid females in the microhabitat of the target hosts could potentially facilitate transmission by directing the feeding behavior of the hosts. This type of host attraction is somewhat consistent with other parasites that appear to attract hosts during the free-living stage, e.g., cestodes and trematodes (Evans et al., 1992; Beuret and Pearson, 1994).

Several authors have proposed that factors such as habitat properties and host feeding behavior could play a significant role in determining strategies of dispersal in acanthocephalans (Nickol, 1985; Nikishin, 2001; Kennedy, 2006). For example, eggs of the acanthocephalans *Pallisenis nagpurensis* and *Pallisenis rex* expand and migrate into the water column where they can potentially encounter copepod hosts (George and Nadakal, 1973; Wongkham and Whitfield, 2004). Similarly, eggs of the acanthocephalan *Leptorhynchoides thecatus* attach to algae suspended in the water column, where they are potentially able to encounter foraging amphipods (Uznanski and Nickol, 1976; Barger and Nickol, 1998). The results presented here are consistent with the interpretation that eggs are carried into the microhabitat of target hosts by female *A. dirus*. This strategy could be adaptive in response to both habitat properties (stream current) and host biology (microhabitat use, feeding behavior). However, future studies are required to determine the importance of this strategy in nature.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


**FIGURE 2.** Behavior of juvenile *Cucidotea intermedia* in response to gravid females, mature males, and leaf material. Isopods were exposed to *Acanthocephalus dirus* parasites (female or male) and leaf material simultaneously. Shown are mean percent response values (±1 SE) for (A) encounter rate and (B) feeding response.


IDENTIFICATION AND CHARACTERIZATION OF A MITOCHONDRIAL MANGANESE SUPEROXIDE DISMUTASE OF SPIROMETRA ERINACEI

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ABSTRACT: A gene encoding the manganese superoxide dismutase (Mn-SOD) of Spiorometra erinacei was identified, and the biochemical properties of the recombinant enzyme were partially characterized. The S. erinacei Mn-SOD gene consisted of 669 bp, which encoded 222 amino acids. A sequence analysis of the gene showed that it had typical molecular structures, including characteristic metal-binding residues and motifs that were conserved in Mn-SODs. An analysis of the N-terminal presequence of S. erinacei Mn-SOD revealed that it had physiochemical characteristics commonly found in mitochondria-targeting sequences and predicted that the enzyme is located in the mitochondria. A biochemical analysis also revealed that the enzyme is a typical Mn-SOD. The enzyme was consistently expressed in both S. erinacei plerocercoid larvae and adult worms. Our results collectively suggested that S. erinacei Mn-SOD is a typical mitochondrial Mn-SOD and may play an important role in parasite physiology, detoxifying excess superoxide radicals generated in the mitochondria.

Spiorometra erinacei is a pseudophyllidean tapeworm that inhabits the intestines of cats and dogs. The infections are usually asymptomatic, but in more severe cases, the infected animals may exhibit weight loss, irritability, and changes in appetite. The most important clinical manifestation, sparganosis, is developed when the plerocercoid larvae of the parasite (spargana) infect other intermediate vertebrate hosts, including humans. Human infections are mainly acquired by ingesting water containing Cyclops spp. in which tapeworm eggs mature into procercoid larvae, by ingesting uncooked mammalian, reptilian, or amphibian flesh, or by placing frog poultices on open lesions (Sarma and Weilbacher, 1986). Spargana can invade muscle, subcutaneous tissues, visceral and urogenital organs, orbital tissue, and occasionally the central nervous system, causing inflammation and fibrosis (Chang et al., 1992). Symptoms differ depending on the particular tissues or organs involved.

Highly reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, are generated through numerous pathways in aerobic organisms (Fridovich, 1995). ROS induce peroxidation of membrane lipids, DNA damage, and inactivation of proteins (Imlay and Linn, 1988). Oxidative stress resulting from ROS is much greater in parasitic organisms because they must detoxify ROS produced endogenously as well as those produced by host immune effector cells. Activated host immune effector cells undergo an oxidative burst and produce ROS as a part of their defense against parasitic infections (Butterworth, 1984; Callahan et al., 1988; Brophy and Pritchard, 1992; Maizels et al., 1993). Therefore, effective detoxification of host-generated ROS is important for parasitic organisms in evading host immune responses and for surviving inside the host (Callahan et al., 1988; Mkoji et al., 1988; Nare et al., 1990; Batra et al., 1993; Maizels et al., 1993; Vaca-Paniagua et al., 2008; Mourão Mde et al., 2009).

Superoxide dismutases (SODs; EC 1.15.1.1) are a group of ubiquitous metalloenzymes that constitute a major component of antioxidant systems in most aerobic organisms (Fridovich, 1995). They play an essential role in protecting organisms from oxidative damages from superoxide anions, which are produced by both enzymatic and non-enzymatic metabolic processes (Fridovich, 1998). SODs have been identified and characterized in various helminth parasites, and their critical roles in parasite survival in hosts have been well addressed (Henkle et al., 1991; Hong et al., 1992; Tang et al., 1994; Henkle-Dührens et al., 1995; Kim et al., 2000; Tawe et al., 2000; Castellanos-González et al., 2002; Lee et al., 2005; Li, Kong et al., 2005; Li, Na et al., 2005; Wu et al., 2006, 2008; Li et al., 2010). In a previous study, we characterized a copper-zinc SOD (Cu/Zn-SOD) of S. erinacei (Li et al., 2010). The enzyme is functionally expressed in both S. erinacei plerocercoid larvae and adult worms, and its expression is significantly increased under exogenous oxidative stress conditions, indicating its essential role in parasite physiology. In the present study, we identified a novel manganese SOD (Mn-SOD) of S. erinacei and partially characterized its biochemical properties to expand our knowledge of the antioxidant defense system of the parasite.

MATERIALS AND METHODS

Parasite

Spargana of S. erinacei were collected from the subcutaneous tissues of naturally infected snakes, Elaphe rydodorsata. Dogs were experimentally infected with 20 spargana by oral administration. Two months after infection, the dogs were killed, and S. erinacei adult worms were collected from their small intestines. The worms were washed with cold physiological saline several times to remove any host contaminants and used immediately for RNA preparation or stored at −70°C until use.

mRNA isolation and cDNA synthesis

mRNA was isolated from S. erinacei adult worms using an Oligotex mRNA purification kit (QIAGEN, Valencia, California) according to the manufacturer’s instructions. Single-stranded cDNA was synthesized using a BD SMART™ RACE cDNA amplification kit (BD Biosciences, Palo Alto, California) following the manufacturer’s instructions.
Cloning of a gene encoding Mn-SOD

Two degenerate oligonucleotide primers were designed based on the conserved amino acids of Mn-SODs from various eukaryotic organisms (Li, Kong et al., 2005). Polymerase chain reaction (PCR) was performed using the 2 primers, with S. erinacei cDNA as a template. The amplified PCR product was analyzed on 1.5% agarose gel, purified from the gel, and cloned into a pCR2.1 vector (Invitrogen, Carlsbad, California). The ligation mixture was transformed into Escherichia coli DH5α competent cells (Invitrogen), and the nucleotide sequence of the cloned PCR product was analyzed with automatic sequencing. Sequence analysis and a Blast search of the National Center for Biotechnology Information (NCBI) for a cloned gene fragment revealed that it contained a partial sequence that was well conserved in Mn-SODs. To obtain the sequences of the 5' - and 3' -regions for S. erinacei Mn-SOD, rapid amplification of cDNA ends (RACE) was performed using gene-specific primers designed based on the nucleotide sequence of the cloned pREP4 (Novagen, La Jolla, California), which was predigested with the same restriction enzymes. The resulting plasmid was transformed into E. coli M15 (pREP4) cells (QIAGEN), and the positive clones were selected. Expression of the recombinant protein was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37 °C for 3 hr. The bacterial cells were harvested by centrifugation, suspended in a native lysis buffer (50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole, pH 8.0), and disrupted by French cell press. Then, the supernatant was collected by centrifuging at 10,000 g for 20 min at 4 °C. The recombinant protein was purified with nickel-nitritotriacetic acid chromatography (QIAGEN), and the purity and purification of the recombinant protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A specific antibody to recombinant S. erinacei Mn-SOD was produced by immunizing BALB/c mice with the purified recombinant protein (50 μg) 3 times at 2-wk intervals, as described in a previous study (Kang et al., 2008).

Enzyme assay

The SOD activity of the recombinant enzyme was assayed in a microplate format with a neotetrazolium chloride (NTC) reaction assay (Kang et al., 2008). The reaction mixtures (100 μl) contained 10 μl of 0.5 M sodium phosphate (pH 7.5), 5 μl of 16% Triton X-100, 0.5 μl of 10 mM ethylenediamine tetraacetic acid, 15 μl of 1.2 mM NTC, 0.5 μl of xanthine oxidase (1 U), the sample, 5 μl of 2 mM hypoxanthine, and distilled water. Absorbance was monitored at 540 nm with a Multiscan FC microplate photometer (Thermo Scientific, Vantaa, Finland) after the addition of 100 μl of stop solution containing 1 M formate buffer (pH 3.5), 10% Triton X-100, and 40% formaldehyde. One unit of enzyme activity was defined as the amount of enzyme required for 50% inhibition of NTC reduction under the assay condition. For in-gel activity staining of SOD activity, the recombinant enzyme was separated by SDS-PAGE, and the gel was stained with the riboflavin-nitroblue tetrazolium method (Beauchamp and Fridovich, 1971). The gel was soaked simultaneously in a solution of 0.2% nitroblue tetrazolium, 0.028 M N,N,N',N'-tetramethylethylenediamine, and 2.8 × 10−3 M riboflavin in 50 mM potassium phosphate buffer (pH 7.8) for 30 min.

Expression and purification of recombinant S. erinacei Mn-SOD

The entire coding region of S. erinacei Mn-SOD gene was amplified by PCR with the primers 5' -GATGGTTGGGTCTCTGTCCTGT-3' for 5' RACE and 5' -GAATTCATGCTTCGCTGCT-3' for 3' RACE. The RACE procedures were performed with a BD SMART RACE cDNA amplification kit (BD Biosciences), according to the manufacturer's instructions. The PCR products were gel purified, ligated, transformed into E. coli, and sequenced as described above. A primary structure analysis of the deduced amino acid sequence was performed with DNA STAR (DNASTAR, Madison, Wisconsin) and Signal P (http://www.cbs.dtu.dk/services/SignalP). TargetP1.1 (http://www.cbs.dtu.dk/services/TargetP) was used to predict protein localization. Secondary structures were predicted using GOR4 (http://npsa-pbil.ibcp.fr) and Protean Program (DNASTAR).

![Multiple sequence alignment of the deduced amino acid sequence of S. erinacei Mn-SOD with those of other helminth parasites and humans.](image)

**Figure 1.** Multiple sequence alignment of the deduced amino acid sequence of S. erinacei Mn-SOD with those of other helminth parasites and humans. Gaps are introduced to maximize alignment. The putative amino acid residues required for coordinating manganese are marked with asterisks. The (Base-F-N-G-G)-G-(H)-X-(N)-H-(L-F)-W, which is one of the critical characteristics that discriminate Mn-SOD from Fe-SOD, is labeled with a dotted line on the sequence. Predicted mitochondria targeting signals are marked with a box. The G-S-G-W-X-L consensus sequence is marked with a bold line on the sequence. C. sinensis (AY563102), P. westermani (AY675508), S. japonicum (AY814748), T. pseudospiralis (AF521909), Onchocerca volvulus (X82172), and Human (AY049787).
Characterization of biochemical properties of \textit{S. erinacei} Mn-SOD

The optimal pH for enzyme activity was assessed with a standard assay method, using 0.5 M sodium phosphate (pH 7.0–7.5), 0.5 M Tris-HCl (pH 8.0–8.5), and 0.5 M glycine-NaOH (pH 9.0–9.5) buffers instead of standard buffer. For the inhibition assay, each enzyme was mixed with different concentrations of potassium cyanide (KCN), sodium azide (NaN$_3$), or hydrogen peroxide (H$_2$O$_2$) and incubated at room temperature for 20 min. The remaining enzyme activity was assayed and compared with a control without any inhibitor. To determine the thermal stability of the enzyme, it was incubated at various temperatures (37, 50, and 70 °C) for indicated time, and the residual enzyme activity was measured. The pH stability of the enzyme also was examined for a pH range of 5.0 to 9.0 by incubating the enzyme at 37 °C in the appropriate buffers. The remaining enzyme activity of each sample was measured as described above.

Expression profile of \textit{S. erinacei} Mn-SOD in plerocercoid larvae and adult worms

The expression profile of Mn-SOD in \textit{S. erinacei} plerocercoid larvae and adult worms was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and immunoblot analysis. The total RNA was isolated from each developmental stage of the parasite using TRlzol reagent (Invitrogen) and was treated with RNase-free DNase (Invitrogen) to remove any contaminating DNA. Reverse transcription and the subsequent amplification of \textit{S. erinacei} Mn-SOD was carried out with the same amounts of total RNA (1 µg each) and primers (5'-ATGCTTTTGCG-TTGCTGCTTCCTTGGACT-3' and 5'-CTAGTGCTTTTGAAGAGC-ACAAAGCG-5') using a Protoscript® II RT-PCR kit (Invitrogen), according to the manufacturer's instructions. The amplified PCR products were analyzed on 1% agarose gel. For the immunoblot analysis, \textit{S. erinacei} plerocercoid larvae and adult worms were homogenized in physiological saline containing Complete protease inhibitor cocktails (Roche Diagnostics, Mannheim, Germany) on ice and then centrifuged at 15,000 g for 20 min at 4 °C. The supernatant was collected, and each worm extract (100 µg) was separated with 10% SDS-PAGE. The resulting proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California). The membrane was blocked with phosphate-buffered saline supplemented with 0.05% Tween 20 (PBST; pH 7.4) and 5% skim milk for 1 hr. The membrane was then incubated with an antibody raised to the recombinant \textit{S. erinacei} Mn-SOD and was diluted 1:1,000 in PBST at room temperature for 2 hr. After several washes with PBST, the membrane was incubated with 1:1,000 diluted horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Sigma, St. Louis, Missouri). The blot was visualized with 4-chloro-1-naphthol (Sigma), and the reaction was stopped by washing the membrane with distilled water.

RESULTS

Cloning and molecular characterization of a gene encoding \textit{S. erinacei} Mn-SOD

After PCR with the degenerate primers for Mn-SOD, we obtained a 153-bp gene fragment that putatively encoded the
Mn-SOD of *S. erinacei*. Sequence analysis and a Blast search of the NCBI for the gene fragment revealed that it contained metal-binding residues and a motif that were characteristic for Mn-SODs. The full-length gene sequence of the gene was obtained with RACE procedures. The open reading frame of the *S. erinacei* Mn-SOD gene consisted of 669 bp encoding 222 amino acids. The predicted molecular mass and theoretical isoelectric point of the protein were 24.8 kDa and 8.55, respectively. The amino acid residues responsible for binding to Mn, His49, His97, Asp182, and His186, were present in the sequence. The (Base-F-N-G-G)-G-(H)-X-(N)-H-X-(L)-F-W motif, which is 1 of the critical characteristics that distinguishes Mn-binding residues and a motif that were characteristic for Mn-SODs.

The open reading frame of the *S. erinacei* Mn-SOD and related enzymes of other helminth parasites was 48.4–67.1%. No putative signal peptide or transmembrane domain was identified in the sequence, but a putative mitochondria-targeting sequence was found in the N-terminal portion of the protein. Targeting and translocation of mitochondrial proteins is usually mediated by N-terminal extensions known to mitochondrial targeting sequences or presequences (Schatz and Butow, 1983; Roise and Schatz, 1988; von Heijne et al., 1989). The N-terminal presequences, usually 15–40 amino acid residues, do not show sequence identities that are common to each other, but they do share characteristic physicochemical properties, such as high content of positively charged, hydroxylated (mostly serine), and hydrophobic amino acid residues, as well as the potential to form an amphipathic α-helical structure (von Heijne, 1986; Roise and Schatz, 1988).

The ability to form the α-helices is also important for recognition by the translocation machinery in the outer and inner mitochondrial membranes (Roise and Schatz, 1988; Abe et al., 2000). Analysis of the 27-amino acid N-terminal sequence of *S. erinacei* Mn-SOD revealed that it had a high content of hydrophobic and basic amino acid residues (Fig. 2A). It also contained a typical R-2 motif, 10FARV ↓ FSV16 (signature amino acids in bold), a putative cleavage site which is recognized by mitochondrial processing peptidase (Gakh et al., 2002). A secondary structure prediction analysis of *S. erinacei* Mn-SOD suggested that the presequence might form an α-helix between amino acid residues 10 and 18 (Fig. 2B). A hydrophilicity plot and amphipathic region prediction analysis also revealed that the amino acid residues had hydrophobic properties, suggesting the probability of a bifacial amphipathic α-helix between these amino acids (Fig. 2B). A helical wheel plot of the *S. erinacei* Mn-SOD presequence also indicated that the region may form an amphipathic α-helix, with positively charged residues localized to 1 side of the helix, whereas uncharged and hydrophobic residues located on the opposite side of the structure provide additional support for the notion that the N-terminal presequence of *S. erinacei* Mn-SOD is a mitochondria-targeting signal (Fig. 2C).

An in silico analysis of the *S. erinacei* Mn-SOD sequence using TargetP programs also suggested that its location is in the mitochondria (Table I).

### Expression and characterization of biochemical properties of *S. erinacei* Mn-SOD

The recombinant protein of *S. erinacei* Mn-SOD was produced in *E. coli* in a soluble form. The molecular mass of the purified recombinant protein was approximately 26 kDa, which was close to the size estimated, deduced from the 24.8-kDa amino acid sequence of *S. erinacei* Mn-SOD combined with the approximate 1 kDa of His tag (Fig. 3A). The recombinant protein had SOD activity when analyzed with native gel electrophoresis followed by in-gel SOD activity staining (Fig. 3B). The recombinant *S. erinacei* Mn-SOD showed optimum activity over a broad range of pHs (7.0–9.5), with a maximum activity level between pH 7.5 and 8.0 (data not shown). The enzyme was relatively stable at 37°C, but it was rapidly inactivated at 50 and 70°C (data not shown). It was highly stable in neutral and alkaline pHs (pH 7.0–9.0) and fairly unstable in acidic conditions (pH 6; data not shown). The enzyme's activity was strongly inhibited by NaN₃ but not by H₂O₂ or KCN (Fig. 3C).

### Expression profile of *S. erinacei* Mn-SOD

The expression profile of *S. erinacei* Mn-SOD in plerocercoid larvae and adult stages of *S. erinacei* was analyzed by RT-PCR and immunoblot. RT-PCR analysis suggested that the gene was consistently expressed in both plerocercoid larvae and adult stages (Fig. 4A). An immunoblot analysis also showed that the protein was present in both developmental stages of the parasite (Fig. 4B).

### DISCUSSION

In the present study, we identified and characterized an Mn-SOD of *S. erinacei*. The enzyme is a typical Mn-SOD that shares similar structural and biochemical characteristics with other Mn-SODs. The primary and the secondary structure analysis of N-terminal presequence of *S. erinacei* Mn-SOD implied its

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**Table I. Summary of in-silico predicted protein localizations.***

<table>
<thead>
<tr>
<th></th>
<th>mTP</th>
<th>SP</th>
<th>Other</th>
<th>Loc</th>
<th>RC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. erinacei</em> Mn-SOD</td>
<td>0.820</td>
<td>0.028</td>
<td>0.173</td>
<td>M</td>
<td>2</td>
</tr>
<tr>
<td><em>C. sinensis</em> Mn-SOD</td>
<td>0.890</td>
<td>0.015</td>
<td>0.197</td>
<td>M</td>
<td>2</td>
</tr>
<tr>
<td><em>P. westermani</em> Mn-SOD</td>
<td>0.577</td>
<td>0.051</td>
<td>0.311</td>
<td>M</td>
<td>4</td>
</tr>
<tr>
<td><em>S. japonicum</em> Mn-SOD</td>
<td>0.883</td>
<td>0.024</td>
<td>0.103</td>
<td>M</td>
<td>2</td>
</tr>
<tr>
<td><em>T. pseudospiralis</em> Mn-SOD</td>
<td>0.407</td>
<td>0.119</td>
<td>0.366</td>
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<td>5</td>
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<tr>
<td><em>S. cerevisiae</em> Mn-SOD</td>
<td>0.695</td>
<td>0.048</td>
<td>0.327</td>
<td>M</td>
<td>4</td>
</tr>
<tr>
<td>Human Mn-SOD</td>
<td>0.757</td>
<td>0.041</td>
<td>0.222</td>
<td>M</td>
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<tr>
<td>Rat Mn-SOD</td>
<td>0.826</td>
<td>0.024</td>
<td>0.217</td>
<td>M</td>
<td>2</td>
</tr>
<tr>
<td>Mouse Mn-SOD</td>
<td>0.823</td>
<td>0.034</td>
<td>0.173</td>
<td>M</td>
<td>2</td>
</tr>
</tbody>
</table>

* Values were calculated with TargetP 1.1 using nonplant algorithms. mTP, mitochondrial target peptide; SP, signal peptide; Other, cytosolic localization or undetermined; Loc, localization based on “winner takes all” (M, mitochondrion); RC, confidence index (1, excellent; >5, poor). *Chlororhis sinensis* Mn-SOD (AY363102), *Paragonimus westermani* Mn-SOD (AY675508), *Schistosoma japonicum* Mn-SOD (AY814748), *Trichinella pseudospiralis* Mn-SOD (AF521909), *Saccharomyces cerevisiae* Mn-SOD (X02156), human Mn-SOD (AY649787), Rat Mn-SOD (NP_008747), and mouse Mn-SOD (X04972).
targeting for mitochondria. The mitochondrion is an organelle that produces large amounts of superoxide radicals as by-products of cellular respiration. Because oxidative stress within the mitochondrion is the starting point for the programmed cascade of apoptosis, the effective detoxification of superoxide radicals in the organelle is important for maintaining cell health (Beyer et al., 1991; Alzate et al., 2007; Piacenza et al., 2007). The mitochondrion is also known to play an essential role in the synthesis of heme, a cofactor of the antioxidant enzymes catalase and ascorbate peroxidase (Ryter and Tyrrell, 2000). Escherichia coli and Saccharomyces cerevisiae mutants lacking mitochondrial SOD showed increased sensitivity to oxidative stress (Carlioz and Touati, 1986; van Loon et al., 1986; Balzan et al., 1995). When mitochondrial SOD of Trypanosoma brucei (TbSODA) was down-regulated, the sensitivity of the parasite against paraquat significantly increased (Wilkinson et al., 2006). These studies collectively suggest the critical roles that mitochondrial SODs play in the maintenance of cellular physiology. It is well known that the extracellular and cytosolic SODs (mostly Cu/Zn-SODs) of helminth parasites play essential roles in parasite survival in the host by detoxifying superoxide anions produced endogenously by the parasite as well as the host immune effector cells (Henkle et al., 1991; Hong et al., 1992; Tang et al., 1994; Kim et al., 2000; Piedrafita et al., 2000; Tawe et al., 2000; Castellanos-González et al., 2002; Li, Na et al., 2005; Li, Kong et al., 2005; Lee et al., 2005; Wu et al., 2006).

Our previous study of the Cu/Zn-SOD of S. erinacei suggested that its primary role in parasite survival was in protecting the parasite from endogenous oxidative stress as well as detoxifying exogenous superoxide anions (Li et al., 2010). Compared with the extensive work that has been performed on the Cu/Zn-SODs of helminth parasites, only a few helminth Mn-SODs have been identified (Henkle-Dührsen et al., 1995; Li, Kong et al., 2005; Wu et al., 2008), and information on their biochemical and biological properties is limited. Considering the mitochondrial

**FIGURE 3.** Expression and biochemical characterization of S. erinacei Mn-SOD. (A) Expression and purification of recombinant protein. Proteins were analyzed by SDS-PAGE and stained with Coomassie Blue. Lane M, molecular weight size markers; lane 1, uninduced E. coli lysate; lane 2, IPTG-induced E. coli lysate; and lane 3, purified recombinant S. erinacei Mn-SOD. (B) In-gel SOD activity staining. Lane 1, purified recombinant S. erinacei Mn-SOD; and lane 2, negative control without protein. (C) Inhibition test. S. erinacei Mn-SOD was incubated with different concentrations (2 or 5 mM) of KCN, H2O2, or NaN3 at room temperature for 20 min, and the residual enzyme activity was assayed and compared with that of a control without any inhibitor.

**FIGURE 4.** Expression profile of S. erinacei Mn-SOD. (A) Transcriptional profile of Mn-SOD gene in plerocercoid larvae and adults of S. erinacei. RT-PCR was performed as described in Materials and Methods. A reaction also was conducted in the absence of RT enzyme (RTase−) to verify DNA contamination. PCR products were analyzed on 1% agarose gel with ethidium bromide staining. PL, plerocercoid larvae; and A, adults. (B) Immunoblot analysis. Each worm extract (100 µg) was separated on 10% SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane. Immunoblot analysis was performed using an antibody for S. erinacei Mn-SOD. M, molecular weight size markers; PL, plerocercoid larvae; A, adults; and R, purified recombinant S. erinacei Mn-SOD (5 µg).
localization and consistent expression of Mn-SOD in different developmental stages of *S. erinacei*, the enzyme is likely to play a primary housekeeping role in parasite physiology, protecting the parasite’s cells from excess superoxide radicals generated in the mitochondria.

In conclusion, we identified and characterized a novel mitochondrial Mn-SOD of *S. erinacei*. This characterization provides important insight into the antioxidant system of the parasite and will contribute to subsequent studies of its biology. Many questions remain unanswered regarding the physiological significance of the enzyme in the parasite. Therefore, further studies will be necessary to understand the in-depth physiological roles of the enzyme in the biology and pathology of the parasite.

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**LITERATURE CITED**


CRYPTOSPORIDIUM-MALNUTRITION INTERACTIONS: MUCOSAL DISRUPTION, CYTOKINES, AND TLR SIGNALING IN A WEANED MURINE MODEL


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ABSTRACT: Cryptosporidiosis is a leading cause of persistent diarrhea in children in impoverished and developing countries and has both a short- and long-term impact on the growth and development of affected children. An animal model of cryptosporidial infection that mirrors closely the complex interaction between nutritional status and infection in children, particularly in vulnerable settings such as post-weaning and malnutrition, is needed to permit exploration of the pathogenic mechanisms involved. Weaned C57BL/6 mice received a protein-deficient (2%) diet for 3–12 days, then were infected with 5 × 10⁷ excysted C. parvum oocysts, and followed for rate of growth, parasite stool shedding, and intestinal invasion/morphometry. Mice had about 20% reduction in weight gain over 12 days of malnutrition and an additional 20% weight loss after C. parvum challenge. Further, a significantly higher fecal C. parvum shedding was detected in malnourished infected mice compared to the nourished infected mice. Also, higher oocyst counts were found in ileum and colon tissue samples from malnourished infected mice, as well as a significant reduction in the villous height–crypt depth ratio in the ileum. Tissue Th1 cytokine concentrations in the ileum were significantly diminished by malnutrition and infection. mRNA for toll-like receptors 2 and 4 were diminished in malnourished infected mice. Treatment with nitazoxanide did not prevent weight loss or parasite stool shedding. These findings indicate that, in the weaned animal, malnutrition intensifies cryptosporidial infection, while cryptosporidial infection further impairs normal growth. Depressed TLR2 and 4 signaling and Th1 cytokine response may be important in the mechanisms underlying the vicious cycle of malnutrition and enteric infection.

Cryptosporidium parvum is an obligate intracellular protozoan parasite that invades and resides in the epithelial cells of the small intestine (Tzipori and Ward, 2002; Sasahara et al., 2003). Discovered by Tyzzer in 1907, it is now well recognized as a major cause of diarrheal illness and gastroenteritis in children and adults worldwide (Dillingham et al., 2002; Huang et al., 2004; Tzipori and Widmer, 2008). Cryptosporidiosis is associated with severe life-threatening illness among immunocompromised individuals, most notably those with AIDS (Pozio et al., 1997). Different from immunocompetent adults, in whom the disease is usually a self-limited diarrhea, people with AIDS are susceptible to a devastating form manifested by chronic, voluminous diarrhea and increased mortality (Pantenburg et al., 2008; Borad and Ward, 2010). Furthermore, in patients with AIDS, infections may result in malabsorption of antiretroviral drugs and, possibly, in increased drug resistance (Brantley et al., 2003). In resource-limited countries, the disease probably exerts most of its impact on pediatric health, particularly as an important cause of morbidity and mortality in young children (Agnew et al., 1998; Newman et al., 1999). In these areas, successive or persistent infections of Cryptosporidium spp. intensified by malnutrition have been considered responsible for substantial long-term consequences for the later physical and cognitive development of these children and even leading to death (Guerrant et al., 1999; Gendrel et al., 2003). Malnutrition itself can increase susceptibility to infection, which, in turn, contributes to further malnutrition, resulting in a vicious cycle, with potentially serious health consequences for the host (Guerrant et al., 2000; Katona and Katona-Apte, 2008). Attempts to treat cryptosporidial infections in humans have included antimicrobial drugs, as well as other pharmacologic approaches, but have yielded limited or only partial success (Armson et al., 2003; Gargala, 2008; Rossignol, 2010). Nitazoxanide (NTZ) was effective in treating diarrhea in immunocompetent children and adults but is not effective in the absence of an appropriate immune response; in AIDS patients, restoration of the immune system, along with antimicrobial treatment, is necessary (Maggi et al., 2000). The development of effective intervention is hampered by the still incomplete knowledge about the human immune response toward C. parvum, how the parasite causes disease, and the contribution of immunocompromising conditions, e.g., AIDS or malnutrition.

Our goal is to study the interaction of malnutrition and C. parvum infection in weaned mice, a model that is more easily executed than previous neonatal models and correlates more closely to the complex interaction between the immune response and infectious agents in children transitioning from breast feeding to solid foods. We observed the weight loss caused by malnutrition, as well as infection, quantified fecal parasite shedding, and intestinal tissue parasite burden, and analyzed morphometric and histopathologic characteristics of crypts and villi from the ileal mucosa. In addition, we examined the effectiveness of the treatment with NTZ in this model and assessed the innate and adaptive immune responses by measuring toll-like receptors (TLRs) and Th1/Th2 cytokines production in the intestinal tissue.

MATERIALS AND METHODS

Animal studies

Animals and malnutrition protocol: Twenty-one-day-old female C57BL/6 mice were purchased from Charles River Laboratories, Inc. (Wilmington, Massachusetts). Mice were acclimated, fed a regular diet for 3 days, and then assigned to experimental groups matched for body weight. On day 24 of life, mice assigned to the nourished groups received chow containing 20% protein (Harland Labs, Madison, Wisconsin), and mice assigned to the malnourished groups received isocaloric chow with 2% protein (Harland Labs). The animals remained on their diets for 3–12 days before oocyst challenge and continued their diet until the end of the experiment. In some experiments, mice were killed by cervical dislocation on the peak of infection (day 3 or 4 post-infection [PI]), at which time ileal and colonic specimens were collected and processed. The protocol described here was approved and is in accordance with the Institutional Animal Care and Use Committee policies of the University of Virginia.

Preparation and administration of C. parvum: The C. parvum oocysts were obtained from experimentally infected calves (Iowa isolate;
Waterborne, New Orleans, Louisiana). Oocysts of C. parvum were stored in phosphate-buffered saline (PBS) at 4 C and used within 8 wk of their receipt from Waterborne. The concentration of oocysts in the stock solution was measured using a hemocytometer by adding 1 part sodium hypochlorite to 4 parts of the oocysts solution to make a final concentration of 20% sodium hypochlorite solution. The tube was briefly vortexed and incubated in room temperature for 10 min. The number of oocysts was then counted before and after hypochlorite to compute for the excystation rate (30-35% in these experiments). Each infected mouse received 75 µl of PBS containing freshly prepared excysted oocysts or a recently vortexed solution (5-10 oocysts per mouse) by oral gavage directly into the stomach.

Administration of NTZ: Nitazoxanide was purchased as a powder, formulated for oral administration to children (Almina™, Romark Pharmaceuticals, Tampa, Florida). It was prepared as a suspension, containing 2 mg per 50 µl of water, a concentration allowing a 50-µl dose to be equivalent to 100-150 mg/kg depending on the weight of each animal. For mice treated with NTZ, each received 50 µl daily for 3 days via gavage, beginning 24 hr after challenge with excysted oocysts.

Stool collection and weight measurements: Mice were weighed daily, and stools were collected daily from the infected and non-infected groups in pre-weighted tubes and stored at −20 C until DNA or protein was extracted.

Analysis of samples

DNA extraction: The DNA was extracted from the frozen stool samples using Qiagen QIAamp DNA Stool Kit (Qiagen, Inc., Germantown, Maryland) with minor modifications as follows. First 400 µl Buffer ASL (warmed to 60 C) was added to each sample, and each sample was vortexed at 1,500 rpm overnight until homogenized. The samples were incubated at 82.5 C for 5 min and then vortexed for 1 min at full speed. The supernatant was pipetted into a new tube with 500 µl Buffer AL and incubated at 70 C for 10 min; 400 µl of ethanol (100%) was added and mixed by vortexing. The “Protocol for Isolation of DNA from Stool for Pathogen Detection” was then followed to completion, and the DNA was eluted in 200 µl Elution Buffer and stored at −20 C. DNA from tissue samples was extracted from frozen tissue samples using the QIAamp DNA Tissue Kit (Qiagen), following the protocol exactly.

Real-time PCR for C. parvum quantification: Extracted DNA (5 µl) was added to the master mix (20 µl) to give a total reaction volume of 25 µl per sample. Master mix was prepared by mixing 12.5 µl of Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, California), 5.5 µl of DEPC-treated nucleic acid free sterile water (Fisher Scientific, Pittsburgh, Pennsylvania), and 1.0 µl (6.2 µM) each of both forward and reverse primers (Invitrogen, Carlsbad, California). The primers target the 18s rRNA gene of the parasite (forward: 5'-CGCGAATTGCTCATTA- TAATCCGTATTTAAGAATG-3' and reverse: 5'-GGCCAATACCTACCGCTT-3') (GenBank no. AF164102). The reaction was performed in a Bio-Rad iCycler iQ multicolor PCR Detection System using iCycler software (version 3.0). Amplification consisted of 15 min at 95 C followed by 40 cycles of 15 sec at 95 C, 15 sec at 52 C, and 20 sec at 72 C, followed by 0.5-degree increments for 10 sec starting at 75 C and ending with 95 C for the Melt Curve. Fluorescence was measured during the annealing step of each cycle. Ct values of each run were compared to standards with known amounts of rRNA gene of the parasite (forward: 5'-CTGCGAATGGCTCATTAA- TAATCCGTATTTAAGAATG-3' and reverse: 5'-GGCCAATACCTACCGCTT-3') (GenBank no. NM_011905), TLR4 (forward: 5'-AGCGGAAATTGGTTGTTTAC-3', reverse: 5'-TTGTCGCTTTTTCCTCTCTTT-3'), GenBank no. NM_021297.2 and TLR9 (forward: 5'-GCAATGGAAGGACTGTCACCATCTTTGAGG-3', reverse: 5'-ATGCCTTTGTCCGTTTACGAGG-3') (GenBank no. AY649791) were purchased from Invitrogen. Amplification consisted of 10 min at 95 C, followed by 40 cycles of 25 sec at 95 C, 25 sec at the respective annealing temperature to each pair of primers (60 C for TLR2 and 4, 45 C for TLR9, and 54 C for GAPDH), and 20 sec at 72 C, followed by 40 cycles of 10 sec starting at 75 C with 0.5 C increment for the Melt Curve. Fluorescence was measured during the annealing step of each cycle. Reagents and equipments were the same as what were used in C. parvum quantification above. The relative gene expression was determined using the 2^(-ΔΔCt) method (Pfaffl, 2001).

Cytokine concentrations in ileal homogenates: Ileal tissue was homogenized using cold lysis buffer (50 mM HEPES, 1% Triton x100 and 1:100 protease inhibitor), centrifuged at 4 C at full speed for 10 min, then supernatant was collected and stored at −80 C. Levels of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN-γ, and TNF-α were measured using a Bio-Plex Pro Mouse Cytokine Assay (Pro Mo Cyto TH1/TH2 1× 96, M60000037; Bio-Rad) according to the manufacturer’s instructions. A dual-laser, flow-based microplate reader system was used to report the level of target protein in each sample, employing a Bio-Plex 200 reader system (Bio-Rad).

Histopathology analyses: Digital micrographs of H&E sections were taken using a high-resolution microscope Aperio ScanScope Slide Scanner and analyzed with ImageScope software (Aperio Technologies, Vista, California). For villus height and crypt depth, well-oriented sections of ileum from 4 mice at day 3 or 4 post-challenge were analyzed.

Statistical analyses: Analyses of weight loss/increase were expressed as a percent change in baseline body weight. The analyses of stool shedding (number of parasites per mg of stool) and tissue burden (number of parasites per mg of tissue) were performed using SPSS 17.0 software (Chicago, Illinois). Statistical analyses were performed using ANOVA with Bonferroni post-hoc correction. A P value less than 0.05 was considered significant. Data were presented as mean ± SEM.

RESULTS

Malnutrition is critical for generating a murine model of excysted C. parvum infection

Protein deprivation and C. parvum challenge influence growth: The inability to attain an expected rate of growth has been proposed as a biological marker of malnutrition, or enteric infection, or both, in humans and in small animal models (Coutinho et al., 2008; Mondal et al., 2009). Following up to 12 days of a special diet containing 2% protein, 2 of 4 groups of 36-day-old C57BL/6 mice had a profound failure to gain weight (day 26–36; P < 0.01) (Fig. 1). Challenge with excysted C. parvum oocysts (5 × 10^7/mouse) was associated with a rapid impairment in the rate of weight gain of malnourished mice, compared to iso-calorically nourished mice, which steadily gained weight despite infection (P < 0.001). Malnourished infected mice had about 20% reduction in weight gain over 14 days caused by protein deprivation, and an additional 20% weight loss caused by C. parvum challenge. Similar results were observed in a second independent study. These findings suggest that malnutrition in association with C. parvum infection in weaned C57BL/6 mouse results in disease of substantial severity in which there is profound weight loss.

Stool shedding of parasites: We reasoned that the intensity of enteric infection in mice might be best reflected in the number of and duration over which challenge organisms are shed into the stool. To test this hypothesis, we collected stool from each oocyst-challenged mouse for 14 days and quantified the number of organisms per mg of stool by quantitative PCR (Fig. 2). Malnourished mice shed more organisms than nourished mice.
FIGURE 1. Growth of mice as altered by nutritional status and by challenge with \( C. \) parvum. Female C57BL/6 mice at 24 days of life were assigned to either a 2% (malnourished) or 20% (nourished) protein diet. Groups to be infected were then challenged 12 days later (day 36 of life) with \( 5 \times 10^7 \) excysted oocysts/mouse. Shown is the body weight change (percentage of initial body weight) from each group starting at day 24 until day 50 of life; *\( P < 0.01 \) for both malnourished groups compared with both nourished groups; **\( P < 0.01 \) for malnourished infected mice compared with malnourished uninfected mice. Results are shown as mean ± SEM.

(by 1–3 logs on days 1–7 PI), with shedding gradually abating over time. This result suggested that malnourished and nourished mice can both be infected orally, but that the intensity of infection, as mirrored by shedding of organisms into the stool, is markedly increased in those that are malnourished. Findings were similar in a second independent experiment with a shorter duration of observation.

Tissue burden of organisms: While stool shedding may reflect the rate of generation of new intra-luminal parasites, the ability of the organism to infect the epithelium may be more indicative of disease activity. Therefore, stool-free colon and distal ileum were taken from C57BL/6 mice 3 days after challenge with \( 5 \times 10^7 \) excysted oocysts/mouse and analyzed for \( C. \) parvum, by quantifying the amount of \( C. \) parvum-specific DNA. Normalized by wet weight of tissue, malnourished mice were observed to have 3.5-fold more \( C. \) parvum organisms per mg of tissue, in both the ileum and the colon, than nourished mice (\( P < 0.05 \)) (Fig. 3).

Duration of protein deprivation: While the malnourished \( C. \) parvum-challenged weaned mouse does manifest enhanced infection as assessed by prolonged stool shedding and a substantial tissue burden (Figs. 2, 3), the duration and extent of protein deprivation needed to enable parasite colonization and stool shedding in these mice is not clear. Therefore, we subjected the 2% protein diet identical groups of mice for 3, 7, or 10 days, then challenged them with \( 5 \times 10^7 \) excysted oocysts per mouse

FIGURE 2. Malnutrition increases stool shedding of oocyst-challenged mice. Parasite shedding was increased in malnourished infected relative to the nourished infected mice over the first 9 days PI. Shown is the number of parasites per mg of stool determined by quantitative real-time PCR. *\( P < 0.05 \) at day 3 PI (n = 11). Results are shown in log scale as mean ± SEM.

FIGURE 3. Tissue burden of \( C. \) parvum organisms in the ileum and colon of challenged mice, as altered by nutritional status. Female C57BL/6 mice, nourished or malnourished (12 days), were challenged with \( 5 \times 10^7 \) excysted oocysts/mouse and then killed 3 days later in order to determine the level of ileal and colonic tissue burden of organisms. The number of parasites per mg of tissue was determined by quantitative PCR. Data are shown as mean ± SEM. n = 5 mice per group. For ileum and colon, *\( P < 0.05 \) for malnourished infected vs. nourished infected mice.

FIGURE 4. Stool shedding as affected by the duration of protein deprivation. Mice were malnourished for 3, 7, or 10 days (A, B, C, respectively), then challenged with \( 5 \times 10^7 \) excysted oocysts per mouse. Shown is the number of parasites per mg of stool, determined by qPCR over time after \( C. \) parvum challenge. Results are shown in log scale as mean ± SEM. n = 4 per group.
Impact of malnutrition and infection on ileal morphology. Ileal sections were harvested from C57BL/6 mice 3 days after *C. parvum* challenge with $5 \times 10^7$ oocysts/mouse. (A) Villus height was diminished in malnourished mice but was not statistically different from other groups. (B) Crypt depth was increased in malnourished infected mice compared with any other group ($P < 0.05$). (C) Villus height:crypt depth was lower in malnourished infected vs. any other group ($P < 0.01$). $n = 3–5$ per group. Bars show mean ± SEM.

**Cryptosporidial infection causes intestinal crypt changes in malnourished mice**

**Histopathological changes:** *Cryptosporidium parvum* infection in humans is associated with villus blunting and crypt hyperplasia (Orenstein, 1997; Alcantara et al., 2008); hence, we examined whether there would be similar changes in the excysted oocyst-challenged mouse. On day 3 following oocyst challenge, ileal segments were examined for villous height and crypt depth. A reduction in villous height was noted in the malnourished mice (Fig. 5A), and crypt depth was observed to be significantly greater in the ileum of malnourished infected animals compared with any other group ($P < 0.05$) (Fig. 5B). Nourished infected mice did not show significant differences in villous height or crypt depth compared with nourished and malnourished uninfected animals. The ratio of villous height to crypt depth was significantly lower in malnourished infected animals compared with any other group ($P < 0.01$) (Fig. 5C). Representative ileal histopathology from infected and uninfected nourished and malnourished mice is shown in Figure 6.

**TLR mRNA expression:** Previous studies have shown that TLR2 and TLR4, through activation of NFκB, mediate defense against cryptosporidiosis in human cholangiocytes (Chen et al., 2005, 2007) while in *C. parvum*-infected neonatal mice, activation of TLR9 by CpG-ODN decreased intestinal parasite load by 80–95% (Barrier et al., 2006). To begin to explore innate immune events underlying the effects of infection and malnutrition, ileal tissues were harvested from C57BL/6 mice 3 days after challenge with $5 \times 10^7$ excysted oocysts/mouse and analyzed for mRNA expression for TLR2, 4, and 9. Malnourished uninfected mice showed higher TLR2 and TLR4 expression compared with malnourished controls (Fig. 7; $P < 0.05$ for TLR2). Malnourished infected mice had lower TLR2 and TLR4 expression compared with malnourished uninfected controls ($P < 0.05$ for TLR2). No difference was observed on TLR9 expression. These data suggest that infection with *C. parvum* is associated a marked reduction in expression of TLR2 mRNA from that induced by malnutrition alone. Similar, but not statistically significant, effects were seen with TLR4.

Stool shedding through days 7–11 was observed, with a tendency to prolonged shedding with longer periods of protein deprivation. Similarly, challenge of mice with half as many excysted oocysts ($2.5 \times 10^7$) was associated with modestly (but not significantly) fewer shed oocysts than in mice given the higher inoculum challenge (data not shown). Thus, while short periods of protein deprivation and challenge with as few as $2.5 \times 10^7$ excysted oocysts/mouse appear sufficient for enabling infection in a weaned C57BL/6 mice model, longer duration (10 days) was associated with more prolonged stool shedding through day 11.

Taken together, these data (Figs. 1–4) suggest that *C. parvum* challenge of C57BL/6 mice that are made protein deficient results in a loss of growth velocity, more intense infection as reflected in stool shedding of organisms, and heavier epithelial infection compared to nourished mice.
Cryptosporidium parvum generation of cytokines: Given studies above showing that C. parvum elicits morphological changes and an increased tissue burden of organisms in the ileum of malnourished mice, we examined whether local (mucosal) generation of pro-inflammatory cytokines might play a role. Therefore, ileal tissues from nourished (n = 4) and malnourished (n = 4) weaned mice challenged with $5 \times 10^7$ excysted oocysts/mouse, were examined for cytokine concentrations 4 days post-challenge. IFN-γ, shown previously to be important for resistance to C. parvum infection (Coutinho et al., 2008), was $292 \pm 72$ pg/ml in normal uninfected ileum, but was reduced with malnutrition ($37 \pm 32$ pg/ml), with infection ($103 \pm 13$ pg/ml), as well as in the setting of malnutrition and infection ($107 \pm 14$ pg/ml) ($P < 0.02$, compared to normal uninfected tissue). The TNF-α concentrations determined in ileum followed a similar pattern ($929 \pm 413$ pg/ml dropping to 693, 236, and 584 pg/ml, respectively) ($P < 0.03$, comparing nourished control and nourished infected tissue). IL-2 was also highest in concentration in nourished, uninfected challenge.

**Figure 6.** Ileal morphology in malnourished mice. Tissue from uninfected control (A) and infected (B) mice were examined at 4 days PI, demonstrating diminished villous height (*) and increased crypt depth (**) in malnourished, infected mice. Brackets mark the length of villi and crypts in representative specimens.

**Figure 7.** Relative mRNA expression for TLR2, TLR4, and TLR9 measured with RT-qPCR. C57BL/6 mice were malnourished for 10 days, challenged with $5 \times 10^7$ excysted C. parvum oocysts, and killed 3 days later for ileal tissue. Age-matched nourished untreated mice were studied as controls. Malnourished uninfected mice showed significantly higher TLR2 and TLR4 expressions compared with nourished controls (*). Malnourished infected mice have significantly lower TLR2 and TLR4 expressions compared to malnourished control (**). Ct values were normalized for GAPDH (used as reference gene) in the same sample. Expression ratio calculated using the $2^{-\Delta\Delta C_t}$ method. n = 4 per group. Bars show mean ± SEM.

**Figure 8.** Effect of three consecutive days of treatment with NTZ (days 1 to 3 PI) on body weight change in malnourished female C57BL/6 mice challenged with $5 \times 10^7$ excysted oocysts/mouse at day 36 of life. Results are shown as mean ± SEM. $P < 0.05$ for malnourished uninfected compared to any infected group. NTZ had no significant effects on weight change.
control tissue (5.1 ± 3.9 pg/ml), while IL-4 and IL-5 were essentially unchanged. These findings suggest that malnutrition, as well as infection with *C. parvum*, is associated with marked reductions in at least 2 important Th1 cytokines in affected tissue, compared to control, and may contribute to inability of the host to combat the parasite after challenge.

**Nitazoxanide is not effective against cryptosporidiosis in malnourished mice:** Prior reports suggested that nitazoxanide may be therapeutic for *C. parvum* infection in pediatric populations (Diaz et al., 2003). Therefore, nitazoxanide was given by gavage (2 mg/mouse/day) on days 1, 2, and 3 after challenge with 5 × 10⁵ excysted *C. parvum* oocysts, to C57BL/6 mice malnourished for 10–12 days with a 2% protein diet. Nitazoxanide did not ameliorate weight loss in malnourished infected mice, relative to malnourished non-infected mice, studied over 14 days post-challenge (Fig. 8). Furthermore, nitazoxanide did not diminish the intensity of stool shedding of parasites relative to controls (malnourished infected but non-treated mice; Fig. 9). Nourished challenged mice continued to shed the fewest oocysts. These findings suggest that, at oral doses similar on a weight basis to those used in human pediatric populations, nitazoxanide had no effect on preserving weight gain or on reducing the intensity of infection measured by stool shedding in *C. parvum*–challenged malnourished mice.

**DISCUSSION**

Cryptosporidiosis is a diarrheal illness that is self-limited for many healthy travelers but is frequently devastating and persistent for young malnourished children living in many areas, as well as immune-compromised patients such as those with AIDS (Dillingham et al., 2002; Huang et al., 2004; Tzipori and Widmer, 2008). Several animal models of *C. parvum* infection have been published, primarily in the neonatal host or in the immune-suppressed adult mouse (Ungar et al., 1990). Here we report a new model in the weaned malnourished mouse, unique in its ability to be easily generated (change in diet), require minimal manipulation, be free of the need for inborn genetic defects in protective cytokines, and have robust quantifiable end points (growth rate, stool shedding) that can be followed longitudinally, associated with tissue invasion and morphometric changes, and whose size allows a wealth of tissue to be available for study in vitro. This new malnourished weaned mouse model should provide an excellent tool to elucidate disease mechanisms contributing to the infection-malnutrition cycle, as well as putative therapies, with several distinct advantages compared to other models. The time interval before disease onset is one such area, particularly in comparison with genetically manipulated models. SCID mice require a minimum of 2–3 wk before shedding is detected (Mead et al., 1991; Tzipori et al., 1995). In the interferon gamma knockout adult mouse infected with small doses of oocysts, body weight change does not occur for 7 days, while high doses of oocysts (~10⁹/mouse) are needed to observe profound weight loss (Griffiths et al., 1998). Models dependent on steroid administration require continuous immune suppression, as well as a high challenge dose of oocysts in the adult mouse (Rasmussen and Healey, 1992; Cheng et al., 1996; Surl and Kim, 2006). In contrast, the current model we report demonstrated weight loss and robust shedding of oocysts on the first day post-challenge, with a time course that extended for at least 11 days. Our finding of prolonged stool shedding, detected by PCR, is consistent with that clinically observed in humans and strongly supports the notion that nutritional status affects host susceptibility to cryptosporidiosis (Agnew et al., 1998; Bushen et al., 2007). The absence of genetic manipulation of the immune system/response in the current model enables it to be used to explore the naturally occurring innate and acquired immune response to cryptosporidiosis. While parasite attachment/invasion of the ileal and colonic mucosa was marked in the malnourished challenged animal, atrophy of villi and elongation of crypts were seen in the ileum when the host was both malnourished and infected with *C. parvum*.

The new malnourished weaned mouse model can be useful to study already established chemotherapies and new potential therapeutics against *C. parvum*. NTZ is the drug of choice to treat cryptosporidiosis (Rossignol, 2010). The effect of NTZ has been studied in immunocompetent and immunodeficient people, including young children and patients with AIDS and highly active antiretroviral therapy (HAART) (Rossignol et al., 2001; Smith and Corcoran, 2004; Anderson and Curran, 2007), in many animal models as well as in vitro (Gargala et al., 2000). In the present study, we analyzed for the first time the effect of the treatment with NTZ on malnutrition and *C. parvum* infection using a weaned mouse model. We observed that malnourished infected mice treated with NTZ did not show significant differences in stool shedding of parasites or body weight loss compared to malnourished infected mice that did not receive the treatment. These findings contrast with previous studies showing that NTZ can reduce *C. parvum* infection in neonatal mice (Blagburn et al., 1998) and SCID adult mice (Theodos et al., 1998). This discrepancy with the current study likely reflects the effects of malnutrition that intensifies cryptosporidiosis as was previously observed in neonatal mice (Coutinho et al., 2008) and, potentially, of wasting among HIV-infected pediatric populations in developing countries, contributing to a lack of efficacy of NTZ (Zulu et al., 2005; Abubakar et al., 2007; Amadi et al., 2009), indicating the need for improved chemotherapy and alternative approaches to treatment.

Intestinal epithelial cells play an important role in the activation of immune responses to *C. parvum* infection. Epithelial
cells can trigger innate and adaptive immune responses via production of cytokines and activation of TLRs, for example, TLR2, 4, and 9 (Abreu et al., 2005). We analyzed the mRNA expressions of these TLRs from ileal tissue of nourished and malnourished mice that were infected and uninfected. Compared with control (nourished, uninfected), malnourished uninfected mice exhibited up-regulation of TLR2 and TLR4 mRNA expression. However, in the presence of infection at day 4 post-challenge, both were diminished to near control levels. TLR9 message expression did not change with malnutrition or with infection (Fig. 7). Prior in vitro studies using epithelial cells showed that TLR2 and TLR4 mediated defense to C. parvum infection (Chen et al., 2005, 2007; O'Hara et al., 2009) and that MyD88 (signaling adaptor of most TLRs) mediates protective responses against cryptosporidiosis in adult mice (Rogers et al., 2006). Moreover, malnutrition and infection in weaned mice resulted to inadequate local humoral response as evident in the reduced ileal TNFα and IFNγ levels. Our findings suggest that the intensified and prolonged shedding in the current model may be due to the inability to sustain protective innate immune responses when the malnourished host is challenged with C. parvum in vitro.

Our results have several limitations. Inherent to the nature of animal studies, it is uncertain whether our findings would hold true in other mouse strains or in humans. Although weight loss and intestinal histopathology were noted, infected mice, unlike in human disease, were not observed to develop diarrhea. Only selected TLRs and cytokines were examined, and no time course experiments were performed, possibly missing changes that occur at different time points after infection and during recovery of the epithelium. Besides malnutrition, there may be other host, parasite, or environmental factors, not examined by us, that could have resulted in poor response to nitazoxanide.

In conclusion, we described cryptosporidiosis in a novel weaned mouse model with biological end points (weight loss, stool worsened by malnutrition. In this model, NTZ did not change the useful in elucidating mechanisms that intersect malnutrition and potentially helping to explain the increased infection and infection (Fig. 7). Prior in vitro studies using epithelial cells could have resulted in poor response to nitazoxanide. 

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LITERATURE CITED


COSTA ET AL.—CRYPTOSPORIDIOsis AND MALNUTRITION 1119


NEOSPORA CANINUM IS ASSOCIATED WITH ABORTION IN ALGERIAN CATTLE

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ABSTRACT: Neospora caninum is a major cause of abortion in cattle worldwide. However, little information is available for Algeria. Accordingly, 799 cattle from 87 farms in the north and northeast of Algeria were enrolled in a seroepidemiological survey. An indirect fluorescence antibody test (IFAT) revealed a seroprevalence of 19.6%. The animals were divided into 3 groups according to their breed: imported European cattle, local breeds, and crossed animals (European × local). Seroprevalences were 16.0%, 34.3%, and 18.6% in groups 1, 2, and 3, respectively. A case control study was performed to investigate the link between global seropositivity to N. caninum and abortion risk in those cattle farms. There was a significant (P < 0.01) association between the seroprevalence against N. caninum and the occurrence of abortion in those farms (odds ratio [OR] = 12.03). This was also observed at the individual level (OR = 2.79). The analysis of results according to the breed revealed a significant association between seroprevalence and abortion in groups 1 and 3, but not for group 2, despite the fact that the highest seroprevalence was observed in group 2. Cerebral tissues from 5 aborted fetuses were available for histology and polymerase chain reaction (PCR). One sample was found positive both by histology and by PCR, 2 samples were positive by PCR only, and 2 samples were negative in both tests.

Neospora caninum is an apicomplexan protozoon responsible for abortion and neonatal mortality in cattle worldwide (Dubey et al., 2007). This parasite has a heterogenous life cycle, with the dog as definitive host and cattle as the main intermediate host (McAllister et al., 1999; Dubey et al., 2006). In dogs, N. caninum can cause neuromuscular disorders and death (Lindsay and Dubey, 2000), but, most of the time, the infection is asymptomatic (Dubey and Lindsay, 1993). Dogs produce fecal oocysts, which are infective for cattle. After ingestion and release of tachyzoites from oocysts, the parasites enter epithelial cells in the gut wall; some may eventually enter the placenta, leading to fetal infection. After the induction of a specific immune response, cysts are formed in the neural and muscular tissues. These cysts are packed with infective cysts, the parasites enter epithelial cells in the gut wall; some may eventually enter the placenta, leading to fetal infection.

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Serology is widely used to diagnose the infection, but also for the conduct of wide-scale epidemiological studies. Several serologic techniques are used, including indirect immunofluorescence antibody test (IFAT) and different immunoenzymatic assays (ELISA) (Dubey et al., 1988; Wouda et al., 1998; Ghalmi et al., 2009a). Immunobots were also used as a confirmation method with the advantage of being able to determine the molecular weight of the reactive antigens (Dubey and Scharies, 2006). Serology was used to evaluate the prevalence of N. caninum in bovines worldwide (Dubey et al., 2007). Depending on the study, prevalence varied considerably (Dubey et al., 2007; Ghalmi et al., 2007).

More recently, classic (Müller et al., 1996; Payne and Ellis, 1996; Yamage et al., 1996), nested (Baszler et al., 1999; Paula et al., 2004), or real-time polymerase chain reaction (PCR) (Müller et al., 2002; Ghalmi et al., 2008) methods have been developed to detect N. caninum in dogs and cattle. Case control studies are efficient epidemiological tools used to investigate the relationship between a disease and a particular factor. Some case control studies were performed to investigate the role of N. caninum in cattle abortion (Davison et al., 1999; De Meerschman et al., 2000; Sager et al., 2001; Vaclavek et al., 2003). All studies indicated a clear association between seroprevalence and abortion in cattle. In aborted fetuses, the association of brain lesions (nonsuppurative encephalomyelitis and necrosis) and the presence of specific DNA are usually considered as conclusive evidence (Dubey and Scharies, 2006).

In the present study, the seroprevalence against N. caninum was investigated in cattle farms in Algeria and a case control study was performed to determine the role of N. caninum in cattle abortion in these farms. Finally, a few aborted fetuses were examined for the presence of N. caninum.

MATERIALS AND METHODS

Animals

The owners of 87 farms were willing to participate. From these farms, 799 cattle were enrolled in this study. The farms were located in the northern (Algiers County and Blida) or the eastern regions of Algeria (Bejaia and Setif). These areas represented approximately 25,000 km². The sampling took place from November 2006 to January 2009. The farms were classified into 3 groups according to the breed of cattle: group 1 (European imported cattle such as Holstein, Primholstein, Frisean, Montbeliarde, or Fleckvie, 30 farms; n = 324); group 2 (local breeds such as the Brown of Atlas, 14 farms; n = 105); and group 3 (crosses between local and European breeds, 43 farms; n = 370).

Case control study

A farm was considered as a case when at least 1 abortion event was recorded during the previous 5 yr, whereas a farm was considered as a control if no abortion event occurred during the same period of time. Among the 87 farms, 30 were considered as case and 57 as controls. The farm size was slightly larger (P = 0.03) for case (24 ± 28.9) than for controls (14 ± 12.8).

Serology

Ten milliliters of whole blood was collected from the tail vein of each animal. The tubes were centrifuged for 10 min at 2,700 g and the sera stored at −20 C until further use. An IFAT was performed as previously described (Ghalmi et al., 2009b). To determine the cutoff for the test, a validation step was performed on 100 sera by comparing IFAT results to a

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Aborted fetuses

The brains of aborted fetuses were collected. A small piece (10 g) was kept for PCR and several small fragments were stored in 10% formaldehyde for histological examination (hematoxylin and eosin-stained preparations). For PCR, DNA was extracted with the use of a Chemagen DNA tissue kit (Chemagen, Achen, Germany). Two PCR systems were used, i.e., classic PCR targeting the ITS1 DNA region (Payne and Ellis, 1996) and a previously described real-time PCR targeting the NC5 DNA region (Ghalmi et al., 2008). For ITS1 PCR (Payne and Ellis, 1996), an internal PCR control has been developed by amplifying invA gene of Salmonella typhimurium with the use of CI-ITS1F (5’-gctgataatgaaagtgtg-3’) and CI-TSIR (5’-aataaatggtggaaaa ctetctatggtgtaaagac-3’) primers. The resulting amplicon (240 base pairs [bp]) has been reamplified by N. caninum-specific primers NS1 (5’-gctgataatgaaagtgtg-3’) and SR1 (5’-aataaatggtggaaaa ctetctatggtgtaaagac-3’). The resulting amplicon was diluted and the lowest amplifiable amount was determined and used as internal PCR control. For ITS1 PCR, the PCR mixture included 50 ng of target DNA, 1 U of Taq polymerase (New England Biolabs, Ipswich, Massachusetts), 2 μl of 10× thermopolbuffer (200 mM Tris-HCl, 100 mM [NaH]SO₄, 100 mM KCl, 20 mM MgSO₄, 1% Triton X-100), pH 8.8 at 25°C, 400 nM of each primer, 1 μl of internal PCR control, 200 μM dNTP, NAT water up to 20 μl. The amplification cycles were as follows: 1 cycle for 5 min at 95°C, 40 cycles at 95°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, 1 cycle at 72°C for 5 min. The PCR products were analyzed by 2% gel agarose electrophoresis. The target DNA was 137 bp and the IPC was 240 bp.

RESULTS

Statistics

Specificity, sensitivity, Kappa, odds ratios, and confidence intervals were calculated with the use of Winepiscose 2.0 and Stat A 9.1 software.

Seroprevalence

Overall, 52.9% of herds (46/87) had at least 1 seropositive animal. Seroprevalences within herds varied from 0 to 100%. Of 799 serum samples, 157 (19.6%) were positive (confidence interval 95%: 16.8–22.5). When the different cattle groups are considered, the seroprevalences were 16.0% (11.9–20.1), 34.3% (25.0–43.5), and 18.6% (14.6–22.6) in groups 1, 2, and 3, respectively. The seroprevalence in cattle of the local breeds (group 2) was significantly (P < 0.01) higher than in the 2 other populations. All positive samples were confirmed as positive by immunoblot.

Case control study

Among the 87 studied farms, 30 (34.5%) were considered as case and 57 (65.5%) as control with respect to the presence or absence of abortion events during the previous 5 yr. Among the 799 sampled cattle, 369 (46.2%) belonged to case farms and 430 (53.8%) to control farms. Among the 57 control farms, 20 (35.1%) were seropositive for N. caninum and 37 (64.9%) were negative, whereas among the 30 case farms, 26 (86.7%) were seropositive and 4 (13.3%) seronegative. The seroprevalence within herds varied from 0 to 100% in case farms, and from 0 to 57.1% in control farms. The association between seroprevalence and abortion was calculated with the use of odds and risk ratios (Table I). The risk and odds ratios were significantly greater than 1, indicating an association between seroprevalence to N. caninum and the presence of abortion events on the farms. Furthermore, when individual seroprevalence was considered with respect to the origin of a given animal (case versus control), the calculated OR and RR were still significant (Table II). Finally, when the data were analyzed according to the breed, a significant association (P < 0.01) was observed in groups 1 and 3, but not in group 2 (P > 0.05) (Table III).

Dog involvement

Previously (Ghalmi, Dramchinini et al., 2009), we have shown that there was an association between the presence of dogs and abortion in cattle farms in Algeria. Here, we investigated this association more thoroughly. When the serological status of dogs was compared with the presence of abortions in farms (Table IV), a strong association was observed (P < 0.01) between the serological status of dogs and the probability of abortion.

Other abortions causes

The sera of cattle from case farms (n = 320) were tested for the presence of antibodies against BHV1, BHV4, and BVD viruses, which are the major viral causes of abortion in cattle. Twenty-one (6.5%), 35 (10.9%), and 71 (22.2%) sera were positive for BHV1, BVD, and BHV4, respectively. The overall seroprevalence of N. caninum in the same serum samples was 27.2%. Among the tested sera, 54 belonged were from aborting cows. Among these 54 sera, 26.8, 13, and 1 were positive for N. caninum, BVDV, BHV4, and BHV1, respectively. Therefore, it was possible to calculate risk for each pathogen, i.e., 17.9, 6.7, 1.8, and 0 for N. caninum, BVDV, BHV1, and BHV4, respectively.
TABLE III. Case control study at the cattle population level.

<table>
<thead>
<tr>
<th>Group</th>
<th>Seropositive</th>
<th>Seronegative</th>
<th>Seropositive</th>
<th>Seronegative</th>
<th>Total</th>
<th>Odds ratio (OR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>8</td>
<td>0</td>
<td>8</td>
<td>14</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>11</td>
<td>3</td>
<td>8</td>
<td>21</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>7</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>4</td>
<td>20</td>
<td>37</td>
<td>87</td>
<td></td>
</tr>
</tbody>
</table>

* OR = ∞, the $\chi^2$ has been calculated and was significant ($P < 0.01$).

BHV4, and BHV1, respectively. The probability of causing abortion was also calculated for each relevant pathogen and was found to be 59.8%, 29.6%, and 9.9% for N. caninum, BVDV, and BHV4, respectively. An increased risk of abortion (44.4%) with concurrent infections (N. caninum, BVDV, and BHV4) was observed.

Aborted fetuses

To confirm a direct link between N. caninum infection and abortion, 5 fetuses were analyzed. The serological status of the mothers was examined by IFAT. Four cows were positive and 1 was negative. The brains of fetuses were analyzed both by histology and PCR. For PCR, we used NC-5 real-time PCR and ITS1 classical PCR. Histological examination revealed the presence of compatible lesions in 1 fetus. This sample was also positive in PCR. Two additional samples were positive by PCR (NC-5 and ITS1), whereas the latter 2 were negative with both techniques.

DISCUSSION

The seroprevalence of N. caninum in bovine populations can vary according to country, region, methodology, and cutoff value (Dubey et al., 2007; Ghalmi et al., 2007). Our observation that 52.9% of the herds studied had at least 1 animal seropositive to N. caninum suggests that neosporosis is widespread among dairy herds in at least some parts of Algeria. In the present study, the sera of 799 Algerian cattle were examined and a global seroprevalence of 19.6% was recorded (16.8–22.5). This seroprevalence is comparable with those found in other studies using the same technique (IFAT) and the same cut off (1/200), as that performed in Argentina (Moore et al., 2002, 2003), Brazil (Corbellini et al., 2006; Minervino et al., 2008), Japan (Koiwai et al., 1999), Korea (Hur et al., 1998), Australia (Atkinson et al., 2000), and the United States (Dyer et al., 2000).

Several studies indicated that N. caninum seropositive cows have a higher risk for abortion. Only a few real case control studies have been performed to evaluate the association between the presence of specific antibodies and the occurrence of abortion in cattle farms (De Meerschman et al., 2000; Vaclavek et al., 2003). In these studies, the OR ranged from 3.1 (De Meerschman et al., 2000) to 22.1 (Sager et al., 2001) and the results were significantly different from 1. The present data are in agreement with the previous studies with an OR of 12.0 at the farm level and 2.8 at the individual level. Thurmond and Hietala (1995) considered that an OR of 2 indicated a state of endemic abortion, whereas a higher value was indicative of an epidemic situation.

Because dogs are the definitive host, the presence of dogs on a cattle farm is a major risk factor for the propagation of the parasite and for cattle abortion. The association between cattle abortion and the presence of dogs was previously investigated (Ghalmi, Dramchini et al., 2009). Here, we extended the analysis by determining the presence of antibodies against N. caninum in farm dogs and cattle abortion. There was a very clear association (OR = 48.2) between the presence of seropositive dogs in farms and the occurrence of abortion. A cross infection between dogs and cattle is possible, because dogs infect cattle via contaminated food or water and cattle infect dogs via contaminated aborted fetuses. A similar observation was reported from Netherlands (Wouda et al., 1999) and Brazil (Guimaraes et al., 2004), showing a correlation between seropositivity to N. caninum in farm dogs and a high seroprevalence of neosporosis in cattle.

Additional tests were carried out on seropositive animals from case farms. These indicated that viral pathogens like BHV1, BHV4, and BVDV also may be responsible for abortions. However, N. caninum clearly appears to be the most important agent for abortion in cattle.

The present study also demonstrated an increased risk of abortion when infection is concurrent among N. caninum, BHV4, and BVDV (44.4% versus 28.6%). However, in another study, Davison et al. (1999) was unable to demonstrate the existence of an association between N. caninum, BVD, IBRV, and Leptospira hardjo-bovis.

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A NEW CHOLEOEIMERIA SPECIES (APICOMPLEXA: EIMERIIDAE) INFECTING THE GALL BLADDER OF SCINCUS MITRANUS (REPTILIA: SCINCIDAE) IN SAUDI ARABIA

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ABSTRACT: Choleoeimeria mitranusensis n. sp. is described from the gall bladder of the lizard Scincus mitranus in Saudi Arabia. The prevalence of the infection was 20% (6/30). Oocysts were ellipsoidal and measured 29 μm × 20 μm. Sporocysts were dicyoic and elliptical in shape. The endogenous development was confined to the gall bladder epithelium. Meronts, gamonts, and young oocysts were detected.

The descriptions of many Eimeria species from reptiles are based solely on the morphology of their mature oocysts and sporocysts, following Levine (1973), with no consideration for any differences that might exist in the rest of the parasites’ life cycles (Lainson, 2002). Paperna and Landsberg (1989) assigned 2 new generic names for eimeriid parasites of reptiles that undergo a peculiar mode of development in biliary or intestinal epithelia of their host (Lainson and Paperna, 1999). Choleoeimeria occupies the distal part of the biliary epithelial cells, which become hypertrophied and displaced to the surface of the bile duct, while Acroeimeria develops immediately beneath the brush border of the intestinal epithelial cells and, within the displaced host cell, bulges out above the surface of the intestinal mucosa (Abdel-Baki et al., 2008, 2009; Lainson et al., 2008). The oocysts of both genera possess 4 sporocysts, which, like species of reptilian Eimeria, are devoid of a Stieda body and contain 2 sporozoites (Lainson and Paperna, 1999).

The status of Choleoeimeria as a sister clade to the Eimeriidae was recently confirmed (Jirků et al., 2002). Modrý and Jirků (2006) and Sloboda and Modrý (2006) provide a taxonomic revision for Eimeria-like species from Scincidae and Chamaeleonidae, respectively, and place several of them into 2 new genera, Choleoeimeria and Acroeimeria. They stress the necessity of revising the taxonomic status of Eimeria-like species from other lizards through study of the endogenous stages. Using this prospective, here we describe a new species of the genus Choleoeimeria based on the exogenous and endogenous stages from the gall bladder of the sandfish lizard, Scincus mitranus, collected from Riyadh, Saudi Arabia.

MATERIALS AND METHODS

During May 2010, 30 adult sandfish lizards, Scincus mitranus Anderson, 1871, were captured by hand from Riyadh, Saudi Arabia. These animals were caged separately. Their fecal pellets were placed individually in a thin layer of 2.5% aqueous potassium dichromate solution and subsequently examined microscopically for the presence of oocysts after concentration by the flotation technique.

To determine the site of infection, tissue samples from the intestine and gall bladder were fixed in 10% buffered formalin. Fixed tissues were processed for histological examination; they were sectioned and stained with hematoxylin and eosin (H & E). All the developmental stages were observed and photographed using an Olympus BX51 microscope with an Olympus DP71 camera. Measurements were made with an eyepiece micrometer and the oil-immersion lens; they are given in micrometers (μm); mean ± standard deviation, followed by the range in parentheses and shape index (ratio of length/width).

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RESULTS

The prevalence of infection was 20% (6/30). The entire infected lizard appeared healthy. However, the gall bladder was a deep blue color, and the bile contained a huge number of oocysts in different stages of maturation.

DESCRIPTION

Choleoeimeria mitranusensis n. sp. (Figs. 1–12)

Diagnosis: Nonsporulated oocysts characterized by presence of granulated sporont without nucleus filling entire oocyst volume (Fig. 1). Sporulation process started with condensation of sporont to an elliptical mass, leaving space between sporont and oocyst wall (Fig. 1), which developed into 4 sporocysts (Fig. 2). Oocysts fully sporulated when each sporocyst contains 2 banana-shaped sporozoites (Fig. 3). Sporulated oocysts tetrapsorocystic and elliptoidal in shape (Fig. 12), measuring 29 ± 0.8 (28–31) × 20 ± 0.9 (19–21), L/W 1.4 ± 0.05 (1.3–1.5). Oocyst wall smooth, bilayered, and colorless, 1.0 thick. Micropyle, polar granule, oocyst residuum, and polar body or other inclusions absent. Sporocysts elliptical, measuring 11 ± 0.9 (9–12) × 8 ± 0.7 (7–9), L/W 1.3 ± 0.13 (1.2–1.5). Sporocyst wall unlayered, smooth, and colorless. Stieda body absent. Free sporozoites in oocysts (Fig. 4); crescent-shaped, with slightly tapering ends.

Histological examination revealed all endogenous stages, including trophozoites, meronts, macrogamonts, and microgamonts in gall bladder epithelium of infected lizards (Figs. 5–11). No endogenous stages observed in intestine. Infected epithelial cells displaced from mucosa toward lumen, usually in contact with basal membrane only by thin pedicle (Fig. 5). Uninucleated meronts subsporoblastic (Fig. 6) measuring 8.2 ± 0.4 (7.9–9) × 5.3 ± 0.3 (5–7). Mature meronts round (Fig. 7), 12 ± 0.2 (11–13) and estimated to produce 10–15 merozoites. Microgamonts elliptical to spherical in shape (Figs. 8, 9), approximately 11 ± 1.1 (10–13) × 9 ± 1.8 (8–11). Macrogamonts mostly spherical (Fig. 10), 13 ± 0.3 (11–14) in diameter. During maturation, zygote or young oocysts (Fig. 11) elongated; mature oocysts released in large numbers in bile secretion.

Taxonomic summary

Type host: Scincus mitranus Anderson, 1871.
Type locality: Riyadh City, Saudi Arabia.
Prevalence: Six of 30 (20%).
Site of infection: Gall bladder.
Sporulation: Sporulation endogenous; both sporulated and unsporulated oocysts were found in the gall bladder lumen and intestinal contents prior to being voided in the feces.
Type material: Phototypes are deposited at Zoology Department, College of Science, King Saud University, Riyadh City, Saudi Arabia.
Etymology: The specific epithet is derived from the host name Scincus mitranus.

Remarks

Four species of Choleoeimeria have been described so far from scincid hosts (Table I), including Choleoeimeria sciici Phisalix 1923 (Modrý and Jirků, 2006), Choleoeimeria hengrichii Alyousif & Al-Shawa, 2005.
Figures 1–11. Photomicrographs (1–4) of freshly collected oocysts of *Choleoeimeria mitranusensis* n. sp. at different stages of development from the gall bladder of *Scincus mitranus*. (1) Non-sporulated oocysts with granulated cytoplasm. (2) Immature oocyst containing 4 sporoblasts (S). (3) Mature oocysts with 4 sporocysts (S). (4) Mature oocyst surrounded with outer-layer (OL) and inner-layer (IL) membrane and containing ruptured sporocysts and free sporozoites (Sp). (5–11) Endogenous stages of *Choleoeimeria mitranusensis* n. sp. at different stages of development from the gall bladder of *Scincus mitranus*. (5) Low-power view showing intense infection with hypertrophy of the infected cells toward the lumen (L). (6) Uninucleated meront (M). (7) Mature meront (M). (8, 9) Microgamont (Mi). (10) Macrogamont (Ma). (11) Zygote (Zy). All scale bars = 10 μm, with exception of Figure 5, which is 40 μm.

(Abdel-Baki et al., 2008), *Choleoeimeria saqanqouri* Abdel-Baki et al., 2008, and *Choleoeimeria riyadhae* (Alyousif and Al-Shawa, 2010). The oocysts of *C. scincis* n. sp. have cylindrical, often longer, oocysts, and markedly larger sporocysts. *Choleoeimeria hemprichti*, *C. saqanqouri*, and *C. riyadhae* have much longer oocysts; *C. riyadhae* has markedly larger sporocysts.

**DISCUSSION**

The generic taxonomy of eimerid coccidia of reptilian hosts has traditionally been based solely on the size and structural characters of the oocysts and sporocysts (Abdel-Baki et al., 2009). In general, oocyst morphology both between and within host species can be quite diverse, with the only constant feature being their 4 sporocysts, each with 2 sporozoites (Asmundsson et al., 2001). Therefore, it is impossible to be sure of the generic status of many parasites without reference to the endogenous stages (Lainson and Paperna, 1999). The majority of *Eimeria* species from reptiles has been, and still is, however, described based on oocyst morphology alone. Considering the combination of oocyst and sporocyst morphology and site and characters of the endogenous development, Paperna and Landsberg (1989)
epithelial cells and undergo a similar mode of development on the surface of the epithelium. Species of the second genus, designated as Acroeimeria, develop immediately beneath the brush-border of the gut epithelium and undergo a similar mode of development on the surface of the epithelium.

In the present work, we demonstrated that the gall bladder was the only site for the endogenous development of the new species, and no endogenous stages were detected in intestine. Therefore, we follow Paperna and Landsberg (1989) and assign the biliary coccidium recorded in this study as a species of Choleoeimeria.

**ACKNOWLEDGMENTS**

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through research group project number RGP-VPP-004.

**LITERATURE CITED**


**Table 1. Comparative descriptive measurements (in μm) of Choleoeimeria mitranusensis n. sp. with morphologically similar species from genus Scincus spp.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Oocyst shape (SI)</th>
<th>Oocyst size</th>
<th>Sporocyst shape (SI)</th>
<th>Sporocyst size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Choleoeimeria scincii</em></td>
<td>Scincus</td>
<td>Cylindrical (?)</td>
<td>25–36 long</td>
<td>??</td>
<td>14 × 10</td>
</tr>
<tr>
<td><em>(Phisalix 1923)</em></td>
<td><em>scincus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cited from Modry and Jirků, 2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Choleoeimeria henprichi</em></td>
<td>Scincus</td>
<td>Ellipsoidal (?)</td>
<td>34.6 (32.5–36.3)</td>
<td>Ellipsoidal (?)</td>
<td>11.6 (11.2–12.6)</td>
</tr>
<tr>
<td><em>(Alyousif &amp; Al-Shawa, 2005)</em></td>
<td><em>henprichi</em></td>
<td>× 21.4 (20.7–22.5)</td>
<td></td>
<td>× 8.0 (7.5–8.4)</td>
<td></td>
</tr>
<tr>
<td>(cited from Abdel-Baki et al., 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Choleoeimeria saqanqouri</em></td>
<td>Scincus</td>
<td>Ellipsoidal 1.5</td>
<td>34.8 (33.5–37)</td>
<td>Ellipsoidal 1.3</td>
<td>11.5 (10.5–12)</td>
</tr>
<tr>
<td><em>(Abdel-Baki et al., 2008)</em></td>
<td><em>scincus</em></td>
<td>(1.4–1.6) × 23.4 (22–25)</td>
<td></td>
<td>(1.1–1.6) × 8.9 (7.5–10)</td>
<td></td>
</tr>
<tr>
<td><em>Choleoeimeria riyadhae</em></td>
<td>Scincus</td>
<td>Ellipsoidal 1.6</td>
<td>36.8 (33.4–39.1)</td>
<td>Ellipsoidal 1.63</td>
<td>14.8 (13.7–15.5)</td>
</tr>
<tr>
<td><em>(Alyousif &amp; Al-Shawa, 2010)</em></td>
<td><em>riyardhae</em></td>
<td>1.21 (1.19–1.23) × 30.5 (28.7–32.5)</td>
<td></td>
<td>(1.52–1.74) × 9.1 (8.1–10.4)</td>
<td></td>
</tr>
<tr>
<td><em>Choleoeimeria mitranusensis n. sp.</em></td>
<td>Scincus</td>
<td>Ellipsoidal 1.4</td>
<td>29 (28–31) × 20 (19–21)</td>
<td>Ellipsoidal 1.3</td>
<td>11 (9–12) × 8 (7–9)</td>
</tr>
<tr>
<td><em>(the present study)</em></td>
<td><em>mitranus</em></td>
<td>(1.3–1.5)</td>
<td></td>
<td>(1.2–1.5)</td>
<td></td>
</tr>
</tbody>
</table>

? = no data, SI = length/width ratio.


A NEW EIMERIA SPECIES PARASITIC IN ISOODON OBESULUS (MARSUPIALIA: PERAMELIDAE) IN WESTERN AUSTRALIA

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ABSTRACT: Feces from southern brown bandicoots, Isoodon obesulus, inhabiting the Perth metropolitan area were examined using fecal flotation and light microscopy, and were frequently found to contain oocysts (10/24; 42%). To enable formal description of the proposed new Eimeria species, i.e., Eimeria quenda n. sp., fecal oocysts from 1 juvenile male I. obesulus were allowed to sporulate in 2% potassium dichromate solution at room temperature. Sporulated oocysts are spheroidal to subspheroideal, 24.5 × 23.6 (22.5–26.0 × 22.5–24.8) μm, with L/W ratio of 1.04 (1.00–1.13), lack a micropyle and oocyst residuum, and are contained within a smooth trilaminar oocyst wall 1.8 (1.6–2.0) μm thick. Sporocysts are ovoid, 12.6 × 9.2 (12.0–13.8 × 8.5–10.0) μm, with L/W ratio of 1.37 (1.20–1.53), have a sporocyst residuum, and 2 comma-shaped sporozoites, each containing 2 spheroidal refractile bodies. Sporulation takes 1–3 days at room temperature. This is the second formal description of an Eimeria species parasitic in the order Peramelemorphia.

The southern brown bandicoot, Isoodon obesulus (Shaw, 1797), is an Australian peramelid marsupial species that usually inhabits southern mainland Australia, Tasmania, and several islands off the southern coast of Australia (Braithwaite, 1997). In Western Australia, the southern brown bandicoot is commonly known as the ‘quenda’, from the name for I. obesulus in the local Nyoongar language (Braithwaite, 2002).

Eimeria kanyana Bennett, Woolford, O’Hara, Nicholls, Warren, and Hobbs, 2006, has previously been reported from the feces of western barred bandicoots (Perameles bougainville) in Western Australia (Bennett et al., 2006). Two Eimeria species that have not yet been formally described have been noted in other peramelid species, including the eastern barred bandicoot Perameles gunnii (Obendorf and Munday, 1990) and the northern brown bandicoot Isoodon macrourus (Mackerras, 1958; Mackerras and Mackerras, 1960). No measurements or description of sporulated oocysts were given for either of these Eimeria species, making comparison to Eimeria quenda n. sp. impossible.

MATERIALS AND METHODS

To estimate the local prevalence of coccidian oocysts in I. obesulus feces, 24 fecal samples were collected from wild I. obesulus brought to Kanyana Wildlife Rehabilitation Centre (KWRC) in late 2007 and early 2008. These individuals were admitted to KWRC for a variety of reasons, including suspected predation, suspected poisoning, habitat destruction, and motor vehicle trauma. Their feces were collected within 2 days of admission and stored at 4°C until further laboratory analysis could be conducted. Feces were mixed in a 10 ml-conical tube with fecal flotation solution (saturated sodium chloride and 30% sucrose [w/v]) to produce a homogenous slurry; then further fecal flotation solution was added to achieve a total volume of 10 ml, and the mixture was centrifuged at 720 g for 5 min. Oocysts were transferred from the liquid at the meniscus of the conical tube onto glass microscope slides using a flamed wire loop and mounted using a cover slip.

Fresh feces from a juvenile male I. obesulus kept by a wildlife care-giver associated with KWRC were collected directly from that individual’s enclosure and placed in 2% potassium dichromate solution (K₂Cr₂O₇), mixed thoroughly, then poured into large Petri dishes to a depth of <1 cm and kept at room temperature in the dark to facilitate oocyst sporulation. Sporulated oocysts were concentrated by fecal flotation as described above. Slides were again produced using a flamed wire loop to transfer oocysts; however, these slides were mounted using a cover slip edged with petroleum jelly. Sporulated oocysts were observed using a ×100 oil immersion objective of an Olympus BX50 microscope and measured by Olympus Advanced digital micro-imaging DP71 technology. Bright field and Nomarski differential interference microscopy techniques were used to measure and photograph sporulated oocysts. Sporulation time in potassium dichromate was determined in a concurrent experiment in which feces from the juvenile male I. obesulus were collected as described above and distributed into 6-well plates. The content of each well was concentrated and prepared as described above and then examined for the relative proportions of sporulated, unsporulated, and degenerate oocysts at 8 time points throughout the experiment, which lasted 9 days. The number of oocysts examined at each time point was between 348 and 512 (mean ± SD) ± 465. Measurements of 50 sporulated oocysts are given in micrometers (mean ± SD) with the observed range in parentheses.

DESCRIPTION

Eimeria quenda n. sp. (Figs. 1–4)

Diagnosis: Sporulated oocysts spheroidal to subspheroideal, with trilaminar oocyst wall, 1.8 ± 0.1 (1.6–2.0) μm thick. Outermost wall smooth and yellow, encircling middle brown layer and inner black layer. Oocysts with 4 ovoid sporocysts. Oocyst length, 24.5 ± 0.8 (22.5–26.0); oocyst width, 23.6 ± 0.6 (22.5–24.8); oocyst L/W ratio, 1.04 ± 0.03 (1.00–1.13). Micropyle and oocyst residuum absent. Sporocysts with 2 comma-shaped sporozoites. Sporocyst length, 12.6 ± 0.5 (12.0–13.8); sporocyst width, 9.2 ± 0.4 (8.5–10.0); sporocyst L/W ratio, 1.37 ± 0.06 (1.2–1.53). Stieda, parasidetia, and substiedetia bodies absent. Each sporozoite with 2 refractile bodies; diffuse, granular sporocyst residuum surrounding sporozoites.

Taxonomic summary

Type host: Isodon obesulus (Shaw, 1797), southern brown bandicoot.
Type locality: Forrestfield, Western Australia (31°59′S, 116°10′E).
Prevalence: Unknown. Ten of 24 (42%) fecal samples from I. obesulus contained coccidian oocysts; however, only 3 of the 10 oocyst-containing samples were sporulated. All 3 of these fecal samples contained E. quenda n. sp. oocysts.
Sporulation time: One to 3 days at 25°C in 2% (w/v) potassium dichromate (see Fig. 5).
Material deposited: Photomicrographs of sporulated oocysts have been deposited in the U.S. National Parasite Collection, Beltsville, Maryland, USNPC No. 104679 and the Australian Registry of Wildlife Health, Taronga Zoo, Mosman, New South Wales, Australia, ARWH No. 8015.1.
Etymology: The specific epithet quenda is the Nyoongar (local Australian aboriginal) word for I. obesulus.

Remarks

Compared with Eimeria kanyana, the only other species of Eimeria formally described from a peramelid host, E. quenda n. sp. is approximately 30% larger in its dimensions. In contrast to E. kanyana,
**Eimeria quenda** n. sp. oocysts lack a polar granule, and their sporocysts lack a Stieda body.

Nearly 50 species of *Eimeria* have been described from members of other families of marsupials in Australia, mostly from the Macropodidae (O’Donoghue and Adlard, 2000; O’Callagan and O’Donoghue, 2001). Only 3 of these species have oocysts that are spheroidal or subspheroidal in shape, and the sporocysts of all 3 have a Stieda body.

**DISCUSSION**

It is worth noting that the juvenile male *I. obesulus* whose feces were used in this formal description of *E. quenda* n. sp. had a male pouch-mate, whose feces also contained *E. quenda* n. sp. oocysts, though the concentration of oocysts in his feces was much lower. Furthermore, their mother’s feces also contained a low concentration of *E. quenda* n. sp. oocysts.

Adult *I. obesulus* were more likely to have fecal oocysts (5/8 = 62.5%) compared with juveniles (5/16 = 31.25%). Of the 16/24 individuals for which a sex was recorded, males were slightly more likely to have fecal oocysts (5/8 = 62.5%) than females (3/8 = 37.5%). However, owing to the low number of samples in this epidemiological analysis, no clear conclusions regarding any possible age or sex predilection can be drawn.

In 1 fecal sample from an adult female *I. obesulus* victim of motor vehicle trauma, a heavy fecal oocyst burden was detected. While the majority of these unsporulated oocysts were morphologically consistent with *E. quenda* n. sp., there were also other coccidian oocysts with a distinctly oval, rather than spheroidal to subspheroidal morphology. Furthermore, there are 2 other morphologically distinct sporulated oocyst types recovered from *I. obesulus* feces (both in 1999), recorded in the archives of the Parasitology Department, Murdoch University School of Veterinary and Biomedical Sciences. These findings indicate that further coccidian species parasitic in the bandicoots of Western Australia are awaiting description.

**FIGURE 4.** Composite line drawing of *Eimeria quenda* n. sp. sporulated oocyst. Bar = 10 μm.

**FIGURE 5.** Graph illustrating the percentage of unsporulated, sporulated, and degenerate oocysts of *Eimeria quenda* n. sp. over 9 days of incubation in 2% (w/v) potassium dichromate solution at room temperature.
ACKNOWLEDGMENTS
Thanks to June Butcher, Ruth Haight, and Margaret Robinson from Kanyana Wildlife Rehabilitation Centre for their kind assistance.

LITERATURE CITED


SPHAERIDIO Trema GLOBULUS AND SPHAERIDITREMA PSEUDOGLOBULUS (DIGENEA): SPECIES DIFFERENTIATION BASED ON mtDNA (BARCODE) AND PARTIAL LSU–rDNA SEQUENCES

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ABSTRACT: Flukes belonging to Sphaeridiotrema are important parasites of waterfowl, and 2 morphologically similar species Sphaeridiotrema globulus and Sphaeridiotrema pseudoglobulus, have been implicated in waterfowl mortality in North America. Cytochrome oxidase I (barcode region) and partial LSU-rDNA sequences from specimens of S. globulus and S. pseudoglobulus, obtained from naturally and experimentally infected hosts from New Jersey and Quebec, respectively, confirmed that these species were distinct. Barcode sequences of the 2 species differed at 92 of 590 nucleotide positions (15.6%) and the translated sequences differed by 13 amino acid residues. Partial LSU-rDNA sequences differed at 29 of 1,208 nucleotide positions (2.4%). Additional barcode sequences from specimens collected from waterfowl in Wisconsin and Minnesota and morphometric data obtained from specimens acquired along the north shore of Lake Superior revealed the presence of S. pseudoglobulus in these areas. Although morphometric data suggested the presence of S. globulus in the Lake Superior sample, it was not found among the specimens sequenced from Wisconsin or Minnesota.

Trematodes belonging to Sphaeridiotrema Odhner, 1913 are important parasites of waterfowl. Historically, specimens found in waterfowl in eastern North America have been identified as a single species, Sphaeridiotrema globulus (Rudolphi, 1814) (see Price, 1934; Gower, 1938; Cornwall and Cowan, 1963; Trainer and Fischer, 1963; Speckmann et al., 1972; Roscoe and Huffman, 1982, 1983; Hoeve and Scott, 1988; Huffman and Roscoe, 1989; Sauer et al., 2007). In most cases, reports of the parasite are accompanied by reports of hemorrhagic enteritis and mortality. Exceptions to this include studies by Gower (1938) and Hoeve and Scott (1988). Thus, Gower (1938) found no effects of S. globulus in lightly infected goldeneyes (presumably Bucephala clangula) or in lesser scaup (Aythya affinis) infected by “literally hundreds” of flukes in Michigan. Hoeve and Scott (1988) found mean intensities ranging from 4,699 to 12,419 flukes in blue-winged teal (Anas discors), black ducks (Anas rubripes), and mallards (Anas platyrhynchos) found dead in Quebec in 1984 and 1985, but there was no report of hemorrhagic enteritis in these birds or in blue-winged teal that acquired 4–2,757 flukes when exposed for 24 hr in sentinel cages. Similar pathological inconsistencies have been reported elsewhere (see McLaughlin et al., 1993; Huffman, 2008), suggesting the possibility that other species may have been mistakenly identified as S. globulus.

The pathological effects of S. globulus in naturally and experimentally infected waterfowl in New Jersey have been well described with hemorrhagic enteritis and mortality being the common endpoints (Roscoe and Huffman, 1983, 1984; Huffman and Roscoe, 1989; Mucha and Huffman, 1991). However, neither blue-winged teal nor domestic ducklings experimentally infected with metacercariae from Bithynia tentaculata in Quebec, Canada, displayed the symptoms or mortality characteristic of S. globulus reported in New Jersey (Gagnon, 1990). Subsequent morphological study of adult flukes from ducks experimentally infected with metacercariae from New Jersey and Quebec revealed that the specimens were different species (McLaughlin et al., 1993). Specimens from New Jersey, which cause severe and frequently fatal hemorrhagic enteritis in waterfowl, were similar to the type specimens of S. globulus and were referred to that species. The specimens from Quebec were designated as a new species, Sphaeridiotrema pseudoglobulus McLaughlin, Scott, and Huffman, 1993. These flukes do not cause the acute hemorrhagic enteritis in experimentally infected ducks, but nonetheless may be pathogenic to waterfowl when present in large numbers (Hoeve and Scott, 1988).

Morphologically, S. globulus and S. pseudoglobulus are readily identifiable at the generic level, but are difficult to distinguish at the species level. These difficulties are exacerbated by specimen deterioration caused by delays in necropsy common in hosts examined during die-offs, and by freezing of the carcass. In view of the difficulties encountered in identifying such specimens, alternative methods for resolving species within Sphaeridiotrema are required.

Ribosomal DNA sequences provide an alternative to morphologically based taxonomic methods within the platyhelminthes and, more specifically, the digenea (Nolan and Cribb, 2005; Olson and Tkach, 2005). They are particularly useful when specimens are unsuitable for morphological assessment. Mitochondrial gene sequences are less commonly used (Olson and Tkach, 2005). However, recent work by Moszczynska et al. (2009), Locke, McLaughlin, and Marcogliese (2010), Locke et al. (2011) has shown that DNA barcode sequences are also an effective means of distinguishing species within an array of digenean genera. The DNA barcode region near the 5′ end of the cytochrome c oxidase gene has been used as a marker for species identification across a wide range of animal taxa (e.g., Hebert et al., 2003; Cywinska et al., 2006; Hajibabaei et al., 2006). The LSU region is the largest of the conserved regions in the rDNA gene. Although used primarily in investigations of higher-level phylogenies, it has also been used successfully for species-level discrimination of species within the digenea (see Olson and Tkach, 2005). In the present study, we assess the effectiveness of DNA barcode sequences and partial sequences of the large subunit region of ribosomal DNA (LSU-rDNA) as a means of distinguishing between these species.

MATERIALS AND METHODS

The specimens examined were obtained from naturally and experimentally infected waterfowl and from naturally infected snails collected from

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various localities over a period of several years. *Sphaeridiotrema globulus* were obtained from domestic ducks experimentally infected with metacercariae from *Elminia virginica* (Lake Musconetcong, New Jersey [McLaughlin et al., 1993]), from infected mute swans (*Cygnus olor*) (Lake Musconetcong), and from *C. olor* and *E. virginica* from Furnace Lake, New Jersey. Additional samples of adult flukes, provisionally identified as *S. globulus*, were obtained from lesser scaup (*Aythya affinis*) found dead at the South Shore Yacht Club in Port Washington near Milwaukee, Wisconsin in 2002 and on Lake Winnibigoshish, Minnesota in 2008. Larval stages were obtained from *B. tentaculata* from Lake Onalaska, Wisconsin in 2007. Specimens of *S. pseudoglobulus* were obtained from domestic ducks experimentally infected with metacercariae from *B. tentaculata* from Rivière du Sud, Quebec, Canada (McLaughlin et al., 1993), from 2 greater scaup (*Aythya marilis*) found dead on Lake St. Louis, which borders the Island of Montreal, Canada, in 2007 and 2008, and from *B. tentaculata* collected from Lac St. Louis in 2007. The flukes retained from the study by McLaughlin et al. (1993) had been frozen at ~80 °C in a small drop of water. The remaining adult specimens were taken from waterfowl that were found dead and had been frozen until they were examined. Samples from these birds and samples of larval stages from freshly killed snails were preserved in either 95% ethanol or absolute ethanol.

We also examined specimens of formalin-fixed flukes identified as *S. globulus* collected from 10 greater scaup found dead on the north shore of Lake Superior near Black Bay, Ontario, Canada, in the spring of 1990. These were stained in acetocarmine, dehydrated, cleared, and mounted in Canada balsam, and the largest mature egg in each specimen was measured. *Sphaeridiotrema globulus* has smaller eggs than *S. pseudoglobulus* (although the upper range of the former overlaps slightly with the lower range of the latter) and measurements were taken as a means of potentially distinguishing the species should the molecular evidence support previous morphometric work (McLaughlin et al., 1993).

DNA was extracted from individual flukes, rediae, and cercariae with the use of DNeasy™ tissue extraction kits (Qiagen, Valencia, California) in accordance with the manufacturer's protocols. PCR reactions had a total volume of 25 μl that included 17.5 μl ultrapure water, 2.5 μl 10x PCR buffer, 1.25 μl 50 mM MgCl2 (both provided with the Taq), 0.125 μl 10 mM dNTP, 1.25 μl 10 pmol of each primer, 0.125 μl Platinum Taq Polymerase (Invitrogen, Carlsbad, California), and 3 μl of template. The COI primers used were MplatCOXldF and MplatCOXldR developed by Moszczynska et al. (2009). These primers include M13 tails (M13 [-21]F and M13 [-27]R) at the 5’ ends. The original cycling parameters reported in Moszczynska et al. (2009) have been modified as follows: 94 °C min 1, 5 cycles (94 °C 40 sec, 45 °C 40 sec, 72 °C 1 min) followed by 35 cycles (94 °C 40 sec, 51 °C 40 sec, 72 °C 1 min) with a final extension at 72 C for 1 min. These primers were also used for sequencing.

A region at the 5’ end of the 28S ribosomal DNA gene including variable domain D1-D3 was amplified by PCR according to the protocol described by Tkach et al. (2003). Forward primer dig2 (5’-AGAACATACCTAGCGG-3’) and reverse primer 1500R (5’-GCTATCTGAGGGAAACTCTCG-3’) were used for amplification. PCR primers as well as several internal primers were used in sequencing reactions. These included internal forward primers: 300F (5’-CAAGTACCTGAGGGAAACTCTCG-3’), 900F (5’-CGTCTGGAAAACGACCGGAACTCG-3’), and internal reverse primers: 300R (5’-CAGCTCTCCCTACGGTACTCTCG-3’), ECD2 (5’-TTGGTCTCGGTTCATAGGCGG-3’).

PCR products were visualized under UV light on 1% agarose gels containing ethidium bromide, purified using Qiagen Qiapquick™ columns, and cycle-sequenced directly using ABI BigDye™ (Foster City, California) chemistry. Sequencing of approximately half of the barcode and LSU sequences was done at the Genome Quebec Centre, McGill University, Montreal, Quebec, Canada. The remaining barcode samples were sequenced at the Canadian Centre for DNA Barcoding at the University of Guelph, Guelph, Ontario, Canada; the remaining LSU samples were sequenced at the University of North Dakota. Nucleotide and amino acid sequences (translated with the use of trematode mitochondrial codons) were assembled, edited, and aligned with the use of Invitrogen Vector 10 and Sequencher™ (GeneCodes Corp., ver. 4.1.4) software. We took a conservative approach to sequence analysis by using only data from the regions covered by the forward and reverse strands. Barcode sequences and partial LSU-rDNA sequences have been deposited in Genbank under accession numbers GQ890328-GQ890331. Additional barcode sequences and their trace files are available in project SPHAE at www.barcodinglife.org.

**RESULTS**

Identification of the Quebec and New Jersey samples was based on specimens from the populations from experimentally infected hosts obtained during morphological studies on *S. globulus* and *S. pseudoglobulus* (McLaughlin et al., 1993).

We obtained 65 partial barcode sequences from Quebec (*N* = 21), Wisconsin/Minnesota (*N* = 19) and New Jersey (*N* = 25) and 14 partial LSU sequences; Quebec (*N* = 5), Wisconsin/Minnesota (*N* = 5) and New Jersey (*N* = 4). The majority of the barcode sequences were from adult flukes, but several sequences in each group were from larval stages. All of the LSU sequences were from adult specimens.

All specimens of *S. pseudoglobulus* (Quebec and Wisconsin/Minnesota) had identical barcode and LSU sequences. The LSU sequences and 22 of 25 barcode sequences from the New Jersey specimens (*S. globulus*) were identical; 3 barcode sequences had a single C/G substitution at position 360 in the alignment shown in Figure 1.

Comparison of the barcode sequences of *S. pseudoglobulus* and *S. globulus* revealed differences in 92 of the nucleotide positions (15.6%) over the length of the aligned region (590 nucleotides) (Fig. 1). The translated sequences differed by 13 amino acid residues (6.5%) (Fig. 2). The single substitution observed in the 3 samples of *S. globulus* did not affect the amino acid sequence. All of the LSU sequences from *S. pseudoglobulus* were identical, as were those from *S. globulus*. The LSU sequences differed at 29 nucleotide positions (2.4%) over the length of the alignment (1,208 nucleotides) (data not shown).

One hundred forty-three specimens obtained from waterfowl in the die-off on Lake Superior in 1990 had measurable eggs in the uterus. Seven specimens had eggs 96–100 μm long (*S. globulus*) range, 102 specimens had eggs 111–125 μm long (*S. pseudoglobulus*) range, and 34 had eggs in the overlapping range 101–110 μm. Within the overlapping group, most clustered at the ends of the range; 10 were ≤102 μm long and 18 were ≥108 μm, or longer.

**DISCUSSION**

Differences in the barcode and LSU sequences between the 2 species are consistent with differences reported in morphology (McLaughlin et al., 1993), pathology (Roscoe and Huffman 1982, 1983; Huffman and Roscoe, 1989; Gagnon, 1990; Mucha and Huffman, 1991; McLaughlin et al., 1993), egg hatching times (McKinsey and McLaughlin, 1994), and species of first intermediate host (*E. virginica* vs. *B. tentaculata*) (Huffman and Fried, 1983; Hoeve and Scott, 1988). The DNA results indicate that the lower and upper ranges in egg size reported by McLaughlin et al. (1990; 1993) are reliable diagnostic features for the separation of these species; however, the overlapping portion remains problematic. Overall, we obtained the most consistent results with the COI primers. These were part of primer set developed specifically for the barcode region of parasitic flatworms. Virtually all of the specimens amplified successfully with them. The magnitude of the differences of the barcode sequences found between the species falls within the range of those found between other congeneric digeneans (Moszczynska et al., 2009). The LSU specimens were more difficult to sequence, likely due to deterioration of the samples. The sequences obtained were less variable than those of
the barcode sequences. However, the number of differences observed was comparable to those found between LSU sequences of congeneric flukes in other studies (e.g., Razo-Mendivil et al., 2004; León-Rézagoon et al., 2005) and support results obtained with the DNA barcode data.

*Sphaeridiotrema pseudoglobulus* was the only species detected in the Wisconsin/Minnesota samples; we did not detect *S. globulus*. The first intermediate host of *S. pseudoglobulus*, *B. tentaculata*, is an invasive species that was first detected in Lake Michigan in the 1870s (see Mills et al., 1993). It now occurs widely throughout the Great Lakes Basin (Burch, 1989) and the St. Lawrence River drainage system (Clarke, 1981) and is present in the upper Mississippi River, including areas adjacent to Minnesota, Iowa, and Wisconsin (Sauer et al., 2007; Herrmann and Sorensen, 2009).

**Figure 1.** Nucleotide alignment of partial barcode sequences of *Sphaeridiotrema pseudoglobulus* and *Sphaeridiotrema globulus*.

**Figure 2.** Amino acid alignment of the translated barcode sequences of *Sphaeridiotrema pseudoglobulus* and *Sphaeridiotrema globulus*.
and Lake Winnibigoshish, Minnesota (NWHC, unpubl. obs.). The spread of this snail has also led to the subsequent establishment of *S. pseudoglobulus* throughout these regions.

The presence of *S. pseudoglobulus* in the Wisconsin/Minnesota samples, however, is not consistent with the reports of hemorrhagic enteritis in waterfowl in the region. *Sphaeridiotrema pseudoglobulus* has been associated with waterfowl mortality and is a potential threat to waterfowl when present in large numbers (Hoeve and Scott, 1988), but there is no evidence from natural or experimental infections that this species causes the hemorrhagic enteritis characteristic of *S. globulus* infections (Hoeve and Scott, 1988; Gagnon, 1990; McLaughlin et al., 1993). However, these studies were limited to observations on surface feeding ducks. There are no experimental data regarding the effects of *S. pseudoglobulus* on diving species such as scaup, which seem to be more severely affected by the parasite. Moreover, these studies involved parasite intensities that were far less than those found in lesser scaup (see Sauer et al., 2007). Vascular leakage (as demonstrated by Evans blue injection prior to necropsy) occurs in comparatively light infections, i.e., <90 (Burch, 1989) although its distribution is not well documented.

In recent years, our research has focused on experimental infections of dabbling ducks with large numbers of *S. pseudoglobulus* and *S. globulus*. The few flukes from this sample in New Jersey (Huffman and Fried, 1983) also occurs in the Great Lakes basin (Burch, 1989) although its distribution is not well documented. Our Ontario data indicate that *S. globulus* is present in Lake Superior and its presence in states bordering Lake Superior and Lake Michigan would not be surprising.

**ACKNOWLEDGMENTS**

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HEMATOZOA AND A NEW HAEMOPROTEID SPECIES FROM CATHARTIDAE (NEW WORLD VULTURE) IN SOUTH CAROLINA

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ABSTRACT: A survey of turkey vultures (Cathartes aura) in South Carolina revealed the presence of a novel haemoproteid, Haemoproteus catharti n. sp. is described from the slides developed from the blood of this vulture. The new species is a thick, halteridial form with complete margins, and is considered distinct from the other recognized haemoproteids from the diurnal raptors in shape, pigment number, parasite outline, and host-family specificity. While reviewing blood films, a series of unusual immature schizonts of Plasmodium sp. were also observed, and these are illustrated, along with another distinct haemoproteid from old slides of poor quality produced from turkey vulture blood.

The taxonomy of Haemoproteus is based on morphology and host specificity at the avian host family level (Pierce, 2005). Pierce et al. (1990) reviewed the known species of Haemoproteus in the Falconiformes and his summary was used for comparison with our proposed new species. Genetic sequences do not always support this tradition, as sometimes the same nucleic acid sequences of haemoproteids are found in birds from different families (Fallon et al., 2003; Krizanaukiene et al., 2006; Svensson and Ricklefs, 2009). Until experimental infections are completed to determine the true extent of host specificity, we should use the conservative approach that discovery of a haemoproteid in an avian family with no prior species names is justification for proposing a new species name. This is a better option than only providing a generic name because it provides more information for future work. If synonyms are discovered, the science will be corrected. If no species name is provided, useful information may be lost.

Webb et al. (2005) examined 22 black vultures (Coragyps atratus) and 11 turkey vultures (Cathartes aura) and determined that 1 was infected with a species of Haemoproteus. They reviewed the literature of blood parasites from new world vultures and found that Haemoproteus spp. prevalence varied in turkey vultures from 14/79 (18%) (Wetmore, 1941) in Washington, D.C., 29/12% in Maryland and New Jersey (Williams and Bennett, 1978), 34/75% in Panama (Galindo and Sousa, 1966), Georgia (Love et al., 1953), and Panama (Darling, 1912). Black vultures were rarely found with patent hematooza infections. The primary purpose of the present article is to name the previously undescribed taxon from the turkey vulture.

MATERIALS AND METHODS

Vultures were trapped in baited walk-in traps and by rocket nets at the Savannah River Site, a United States Department of Energy nuclear production and research facility near Aiken, South Carolina (DeVault et al., 2004, 2005). While the birds were being handled for placement of radio transmitters, blood samples were collected from the brachial vein. Blood smears were made on site and the slides were methanol fixed and stained with Diff-Quik (Webb et al., 2005). Positive smears were sent to the University of Florida for identification of the parasite. Gametocytes were photographed with an Olympus DP25 Digital Camera on a Zeiss compound microscope under oil immersion; images were printed, and then measured by superimposing a grid of known size to match the print magnifications. Standard deviations and means of measurements were made with Sigma Stat 2.03. All measurements are in microns.

DESCRIPTION

Haemoproteus catharti n. sp.

(Figs. 1–4)

Immature gametocytes: Elongate; immature gametocytes develop lateral to erythrocyte nucleus (Fig. 1).

Macrogametocytes (n = 20): Thick, halteridial gametocytes with complete margins infect mature erythrocytes; usually longer than length of RBC (Figs. 2, 3). Gametocytes partially displace host cell nucleus (nuclear displacement ratio [NDR] = 0.59 ± 0.2 [0.2–1.0]); gametocyte length 15.4 ± 1.1 (14–18), width 3.4 ± 0.5 (2.5–5), and area 56.0 ± 8.3 (43–74). Nucleus centrally located; gametocytes not usually in contact with red-blood-cell nucleus. Normal staining differentiation with macrogametocytes a darker blue and microgametocytes faintly staining. Infected red blood cell slightly hypertrophied, with length 15.4 ± 1.0 (14–17), width 7.7 ± 0.7 (7–9), and area 96.8 ± 10.3 (82–115). Pigment granule number 24.4 ± 7.0 (19–33), highly refractive, moderately sized, and scattered throughout gametocytes.

Microgametocytes (n = 3): Thick, halteridial gametocytes with entire margins infecting mature erythrocytes (Fig. 4), usually bending around ends of red-blood-cell nucleus; usually not in contact with red-blood-cell nucleus. Gametocytes partially displace erythrocyte nucleus (NDR = 0.5 ± 0.2 [0.3–0.7]). Gametocyte length 14.5 ± 0.6 (14–15), width 3.7 ± 0.6 (3–4), and area 55.0 ± 7.0 (48–62). Infected erythrocytes length 15.2 ± 1.3 (14–16.5), width 7.8 ± 0.8 (7–8.5), and area 93.3 ± 16.3 (75–106); slightly hypertrophied. Pigment granule number 18.0 ± 7.0 (11–25), with similar traits of macrogametocyte.

Uninfected erythrocytes (n = 10): Length 14.2 ± 0.8 (13–16), width 7.9 ± 0.5 (7–9), and area 88.6 ± 10.9 (71–119).

Taxonomic summary

Type host: Cathartes aura turkey vulture.

Type locality: Savannah River Site, Aiken, South Carolina (33°16′N, 81°34′W).

Basis for description: Parasites are described from 2 blood smears taken from a turkey vulture collected at the Savannah River Site by T. L. DeVault on 28 June 2001.

Specimens deposited: The hepantotype and the parahepantotype are deposited in the International Reference Centre for Avian Haematozoa (IRCAH) in Queensland Museum, South Brisbane, Queensland, Australia G465453 and G465454, respectively.

Distribution: It is presumed that this taxon is present throughout the range of the Cathartidae.

Etymology: The parasite-specific epithet is a reflection of the family of birds it infects.

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FIGURES 1–9. *Haemoproteus catharti* gametocyte. (1) Immature macrogametocyte (size bar = 10 μm, same for all figures). (2) Macrogametocyte in contact with RBC nucleus. (3) Macrogametocyte not in contact with RBC nucleus. (4) Thicker variety of microgametocyte. (5) Microgametocyte. (6,7) *Plasmodium* sp. immature schizont. (8,9) *Haemoproteus* sp. or *Plasmodium* sp. slender, less halteridial macrogametocyte.

Remarks

This is the first species named from vultures in the Cathartidae. The new species resembles *Haemoproteus tinnunculi* (Wasielewski and Wulker, 1918) Wingstrand, 1947 (see Figs. 2 and 3 of Pierce et al., 1990), which is a parasite of the Falconidae and *Haemoproteus elani* de Mello, 1937 (see Figs. 6 and 7 of Pierce et al., 1990) of the Accipitridae, although we observed no volutin granules in the gametocytes of the blood smears. The new species has complete margins, unlike both *Haemoproteus nisi* Pierce and Marquiss, 1983 of the Accipitridae and *Haemoproteus brachiatius* Valkiūnas and Ezhova, 1989 of Falconidae, which have thin gametocytes with amoeboid or incomplete margins. The new species does not have circumnuclear, or broadly ovate, gametocytes as does, *Haemoproteus janovyi* Greiner and Mandal, 1979 of the Accipitridae.

Three slides from turkey vultures originating from Maryland (C.M. Herman collected in 1940), were borrowed from IRCAH, although the quality of these slides was poor. One smear (53216), however, contained a taxon with thinner gametocytes (Figs. 8, 9), with fewer pigment granules, entire margins, and usually straighter gametocytes than *H. catharti* n. sp.; some were halteridial and the NDR was about 1.0. This parasite does not fit the morphology of any of the known species from diurnal raptors (Pierce et al., 1990), but the limited material available for study precluded us naming it. Whether this is a second species of *Haemoproteus* or possibly represents a second species of *Plasmodium*, possibly *Plasmodium*.
elongatum Huff, 1930 (see Valkiunas, 2005 Figs. 273:16-17), in which no schizonts were detected, is not clear. The other 2 slides could not be used because of the faint stain of one and no parasites were seen on the other. We do not have molecular data on any of these species and thus feel justified in naming *H. catharti* based mainly on host specificity at the avian-host family level and its morphology.

In resampling the slides trying to find specimens of immature forms to photograph, we discovered that there was also a series of unusually thick, elongate immature schizonts lateral to the red-blood-cell nucleus (Figs. 6, 7). These immature schizonts were most similar to *Plasmodium circumflexum* (Kikuth, 1931) (see Valkiunas, 2005 Figs. 240--247) and *Plasmodium gabaldoni* Garnham, 1977 (see Valkiunas, 2005, Figs. 254--256). Species identifications are not based on immature schizonts, and thus the species could not be determined.

**DISCUSSION**

The new world vultures are infected with blood parasites. No species of *Haemoproteus* has, however, been named from this family of birds, and until it is determined that this parasite is a synonym of a preexisting species, we propose the name *H. catharti* n. sp. for the broad halteridial species that infects cathartids. There may be a second species as suggested by the morphology of gametocytes on one of the early smears from Maryland, but until better-quality slides are available, it will not be named.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


A NEW EIMERIAN SPECIES (APICOMPLEXA: EIMERIIDAE) FROM THE BLUE-FRONTED AMAZON PARROT AMAZONA AESTIVA L. (AVES: PSITTACIDAE) IN BRAZIL

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ABSTRACT: The Neotropical psittacine species Amazona aestiva, commonly known as the blue-fronted Amazon, is one of the most common and best-known psittacine birds kept as a pet worldwide. However, very little is known about the diseases or parasites of these birds. In this study, we describe a new species, Eimeria aestivae, associated with these parrots. The new species is characterized by: ovoid smooth oocysts (n = 60), 36.8 (33.2-41.5) × 23.7 (21.7-25.7) μm, length/width ratio = 1.55; polar granule present; ellipsoidal sporocysts (n = 25), 19.8 (17.5-21.6) × 9.3 (8.3-9.9) μm; Stieda, sub-Stieda body, and sporocyst residuum present. Sporozoites (n = 20), 2 per sporocyst, elongate and curved, 17.6 (15.8-19.2) × 3.8 (3.2-4.8) μm; each with 2 refractile bodies. The oocysts of the other 2 eimerian species described for Amazona are larger than those of the present species, but they all seem to be closely related because of some similarities among them.

The Neotropical psittacine species Amazona aestiva L., the blue-fronted Amazon parrot, occurs in Brazil, Bolivia, Paraguay, and Argentina, with 2 subspecies currently recognized, Amazona aestiva aestiva and Amazona aestiva xanthopteryx. These birds have always attracted much attention because of their beauty and ability to imitate human speech and sounds from domestic animals. These characteristics have resulted in their over-exploitation in nature and extensive trade worldwide. From 1980 to 1992, approximately 400,000 birds were “legally” exported from Argentina to Europe and the United States (Juniper and Parr, 1998). The intense exploitation of these birds by legal and illegal dealers is increased by the difficulty in breeding this species in captivity (Beissinger and Snyder, 1992).

In this report, we describe a new species of Eimeria from blue-fronted Amazon parrots. This parasite was discovered during a parasitological survey of fecal material from several parrot species maintained in zoos in the State of São Paulo, southeastern Brazil.

MATERIAL AND METHODS

Fecal samples were collected in August 2009 from several specimens of A. a. aestiva kept in the municipal zoo at Americana and were stored in 2.5% potassium dichromate solution (K₂Cr₂O₇). In the laboratory, the fecal material was filtered through a 154-μm sieve with distilled water and concentrated by centrifugation at 1,200 g for 5 min. Parasite oocysts were obtained by flotation in sucrose solution (d = 1.2 g/ml) using the Sheather (1923) method. Unsporulated oocysts obtained in this way were allowed to sporulate on a dish containing 2.5% potassium dichromate solution at room temperature for a few days. The oocysts, sporocysts, and sporozoites were photographed with a Zeiss light photomicroscope and then measured with Image Manager IMS® 4 software (Leica IMS 4.0 Imagie Bildverarbeitung AG, Leica Microsystems Imaging Solutions Ltd., Cambridg, U.K.). Measurements were taken from 60 oocysts; sizes are expressed in μm.

Williams et al. (2010) demonstrated that a 2.5% (w/v) aqueous solution of potassium dichromate at 4°C can preserve the morphology and DNA of eimerian oocysts from chickens for at least 25 yr, so we also chose to retain the oocysts of the present material in potassium dichromate rather than employing alcohol or formalin as a preservative.

DESCRIPTION

Eimeria aestivae sp. n.
(Figs. 1, 2)

Diagnosis: Oocysts (n = 60) ovoid, bilayered, 36.8 (33.2-41.5) × 23.7 (21.7-25.7), length/width ratio = 1.55; outer layer: smooth and colorless.

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FIGURE 1. Photomicrograph of Eimeria aestivae sp. n., sporulated oocyst. Scale bar = 10 μm.
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Table I. Eimeria species formally described from Amazona.

<table>
<thead>
<tr>
<th>Species</th>
<th>Type hosts</th>
<th>Length (µm)</th>
<th>Width (µm)</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eimeria amazonae</em></td>
<td><em>Amazona ochrocephala</em></td>
<td>48.9</td>
<td>36.2</td>
<td>Hofstatter and Kawazoe (2011)</td>
</tr>
<tr>
<td><em>Eimeria ochrocephalae</em></td>
<td><em>Amazona ochrocephala</em></td>
<td>43.8</td>
<td>27.7</td>
<td>Hofstatter and Kawazoe (2011)</td>
</tr>
<tr>
<td><em>Eimeria aestivae</em> n. sp.</td>
<td><em>Amazona aestiva</em></td>
<td>36.8</td>
<td>23.7</td>
<td>This study</td>
</tr>
</tbody>
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LITERATURE CITED


REDESCRIPTION OF *CORALLOBOTHRIUM SOLIDUM* (CESTODA: PROTEOCEPHALIDEA) AND ERECTION OF A NEW GENUS, *ESXEIIELLA*, FOR TAPEWORMS FROM CHANNEL CATFISHES (ICTALURIDAE)

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ABSTRACT: The proteocephalidean tapeworm, *Corallobothrium solidum*, type species of the genus, is redescribed on the basis of the examination of its type specimens and extensive material recently collected from *Malapterurus electricus* (type host). Some morphological characteristics of taxonomic importance are reported for the first time, such as the presence of semilunar (U-shaped) sphincters on the external (outer) margin of the suckers, a vaginal sphincter, a well-developed seminal receptacle, and a unique morphology of the eggs. *Corallobothrium solidum* differs from the 2 remaining species of the genus, both parasitic in channel catfishes (*Ictalurus*), in its scolex shape, morphology of its suckers, presence of longitudinal and transverse grooves on the body surface, dense network of excretory canals in the apical part of the scolex, morphology of the eggs, and uterine development. The non-monophyletic nature of *Corallobothrium* is further supported by molecular data (partial sequences of the 28S rRNA gene) because *C. solidum* and the 2 remaining species from ictalurids do not form a monophyletic assemblage. Therefore, *Esxeiella* n. gen. is proposed to accommodate *Esxeiella fimbriatum* new comb. (type and only species; syn. *Corallobothrium fimbriatum*) from channel catfish. *Esxeiella* n. gen. differs from *Corallobothrium*, *Megathylacoides*, and *Megathylacus* by the absence of a sphincter in the suckers, from *Corallobothria* by the shape of the scolex and the number and shape of proglottids, and from *Paraproteocephalus* by the structure of the uterus. The diagnosis of *Corallobothrium*, which becomes monotypic and restricted to electric catfishes in Africa, is emended. The remaining species of *Corallobothrium*, *Corallobothrium parafimbriatum*, is tentatively transferred to *Corallobothria* as *Corallobothria parafimbriata* n. comb., based on molecular data, small size of the strobila, and shape of the scolex.

The cestode fauna of freshwater fishes in Africa is relatively species poor and includes, besides species of other groups, 19 species of proteocephalideans (Khalil and Polling, 1997; de Chambrier et al., 2009). Two species, *Electrotaenia malopteruri* (Fritsch, 1886) and *Corallobothrium solidum* Fritsch, 1886, parasitize the electric catfish, *Malapterurus electricus* (Gmelin) (Khalil, 1971; Khalil and Polling, 1997; de Chambrier, Scholz, and Ibraheem, 2004). Brujniščak et al. (2004, 2005) provided data on spermiogenesis and the ultrastructure of spermatozoa of the latter species, which is the type species of *Corallobothrium* Fritsch, 1886 (Proteocephalidae: Corallobothriinae), but only limited information exists on its general morphology.

The original description of *C. solidum*, which is based on poorly preserved and fragmented material, is incomplete, and almost no additional morphological data (illustrations, measurements, photomicrographs) have been provided by subsequent authors (Fuhrmann, 1916; Janicki, 1928; Khalil, 1963; Freze, 1965; Ibraheem, 1998). A study of specimens of *C. solidum* collected from the type host, the electric catfish, in Egypt (type locality) and 4 additional African countries made it possible to provide previously unreported morphological data and to discuss the species composition of the genus. Scanning electron microscopical (SEM) observations also enabled us to provide the first detailed information on scolex morphology and surface structures.

Besides *C. solidum*, *Corallobothrium* currently includes 2 additional species, namely, *Corallobothrium fimbriatum* Essex, 1928, and *Corallobothrium parafimbriatum* Befus and Freeman, 1973, both from channel catfishes (Ictaluridae) in North America (Schmidt, 1986). The Corallobothriinae Freze, 1965 is composed of 5 genera (*Corallobothrium; Corallotria* Freze, 1965; *Megathylacoides* Jones, Kerly, and Sneed, 1956; *Megathylacus* Woodland, 1934; and *Paraproteocephalus* Chen, 1962), but molecular data indicate that the subfamily may be paraphyletic or polyphyletic with *Megathylacus*, which includes species parasitic in catfishes in South America, being apparently unrelated to the remaining genera (de Chambrier, Zehnder et al., 2004; Hýpá et al., 2005).

MATERIALS AND METHODS

The following material of corallobothrine cestodes was examined: (1) *C. solidum* Fritsch, 1886, all from *M. electricus*—syntypes (5 whole-mounts with fragments of mature specimens, including 2 scolices; 3 slides with longitudinal sections, and 3 slides with cross sections) from the Nile River in Egypt (exact locality not specified), deposited in the Natural History Museum in Geneva (MHNG INVE 36747); vouchers (whole-mount of 1 mature specimen) from the Nile River in Khartoum, Sudan, collected by L. Khalil (MHNG INVE 34803; see Khalil, 1963); vouchers (whole-mounts of 7 mature specimens, 8 slides with cross sections of proglottids, and 4 slides with longitudinal sections of the scolex) from the Nile River in Luxor, Egypt (25°41'N; 39°39'W), collected by A. de Chambrier in 2001 (MHNG INVE 31550, 32761-32767); vouchers (whole-mounts of 3 mature and 5 juvenile specimens, and 1 slide with cross sections) from the Nile River in El Minia, Egypt, collected by M. Ibraheem in 2002 (MHNG INVE 33997 and 36043); vouchers (whole-mounts of 8 specimens) from the Nile River in Khartoum and Omdurman, White Nile River in Kosti, Sudan, all collected by A. de Chambrier and T. Scholz in 2006 and M. Jirků in 2010, and from Al Kawa, Sudan, collected by Z. N. Mahmoud in 2008 (MHNG INVE 63109-63111, 63197; helminthological collection of the Institute of Parasitology, České Budějovice, Czech Republic, IPCAS C-507; The Natural History Museum, London, U.K., BMNH 2011.2.10.1; U.S. National Parasite Collection, Beltsville, Maryland, USNPC 104286); vouchers (whole-mounts of 2 mature specimens) from Turkana Lake, Kenya, collected by M. Jirků in 2009 (MHNG INVE 75469); vouchers (whole-mounts of 8 mature specimens) from the Senegal River in Richard Toll, Dagana, Senegal (16°29′N; 15°36′W), collected by A. Sène (date not given; MHNG INVE 37857-37859); vouchers (whole-mounts of 3 mature specimens) from the Kwilu River in Bagata, Zaïre (now Democratic Republic of the Congo; 4°49′S; 18°46′E), collected by A. Fain (date not given; MHNG INVE 55307); (2) *Corallobothrium cf. solidum* Fritsch, 1886—vouchers (whole-mounts of 6 mature specimens and 10 slides with cross sections) from *Malapterurus gossei* Norris, lower Congo River at Pioka, Democratic Republic of the Congo, collected by M. Jirků in 2008 (MHNG
Redescription of *Corallobothrium floridanum* Fritsch, 1886 (Figs. 1–16, 19–27)

Redescription (based on 16 newly collected specimens from *M. electricus* from Egypt and the Sudan; type specimens are strongly contracted and thus their measurements could not be taken); Protocecalphalaeae, *Corallobothriinae*. Testes, ovary, vitelline follicles, and uterus mediulary (Figs. 12–14). Strobila 38–57 mm long and 1.7–2.6 mm wide, massive, acraspedote, covered with papilliform filitriches, consisting of 92–116 proglottids; 61–71 immature, 11–17 mature, and 20–28 pregravid and gravid. Body surface with deep longitudinal and transverse grooves (wrinkles) forming rectangular network (Fig. 25).

Internal longitudinal musculature weakly developed, formed by narrow band of isolated muscle fibers, more dense on lateral sides of strobila (Figs. 12, 13). Osmoregulatory canals well developed, thin-walled, forming dense network of thin canals in scolex (Figs. 4–6). Foramina secundaria described by Janicki (1928) not observed in cross sections (Figs. 12–14). Ventral canals lateroventral to testes; dorsal canals dorsal to vitelline follicles and dorsolateral to testes. Dorsal canals situated more laterally than ventral ones (Figs. 8, 12, 13). Scolex large, 1,060–1,660 (x = 1345; n = 8) long by 2,260–3,230 (x = 2,665, n = 8) wide, with well-developed metacoelex, much wider than proliferative zone (neck) (Figs. 1, 2, 19–21). Scolex umbrella-shaped, with widely pyramidal apex and well-developed metacoelex, which forms folded collar surrounding suckers (Figs. 1, 2, 19–21). Scolex wider laterally than dorsosventrally (Figs. 20, 23). Suckers large, uniloculate, deeply embedded, 450–595 (x = 510, n = 20) long by 325–455 wide (x = 395, n = 20) (Figs. 4–6, 19–23); external (outer) margins of suckers with semispherical (U-shaped, i.e., interrupted anteriorly) musculature serving as sphincter (Figs. 1–3, 5, 6). Sucker cavity covered with thin valve-like structure (velum) with slit-like opening (Figs. 1, 3, 19, 22, 23). Proliferation zone 895–1,365 wide.

Testes mediulary, spherical to oval, 40–70 in diameter, 224–281 in number (x = 244, n = 5, CV = 11%), in 2 irregular (incomplete) layers (Figs. 8, 11–13), in 2 lateral fields connected anteriorly (Fig. 8). External sperm duct (vas deferens) winding, reaching to median line of body (Figs. 8, 10). Cirrus-sac gradually narrowing to distal end, thick-walled proximally (Fig. 15), 240–450 long by 95–200 wide, representing about 16–24% of proglottid width (x = 20%, n = 22, CV = 11%). Internal sperm duct thick-walled, forming several loops in proximal third or half of cirrus-sac. Cirrus straight, long, about 2/3 of cirrus-sac length. Genital pores irregularly alternating, close to anterior margin of proglottids, situated at 19–36% (n = 25; CV = 17%) of proglottid length from anterior end. Genital atrium deep (Fig. 15), 55–250 in depth.

Ovary mediulary, compact, with small follicles on surface (Figs. 8, 11), bilobed, with short and wide lateral lobes connected by ventrally situated isthmus (Figs. 8–11). Length of ovary 215–440; total width of ovary 545–830, representing 40–52% (n = 25; CV = 8%) of proglottid width. MEHLS’ glands slightly posterior to ovarian isthmus, about 70–110 in diameter, representing 4–6% of proglottid width (Figs. 8–10).

Vaginal canal narrow, almost straight or slightly sinuous, thick-walled and lined with chromophilic cells, usually posterior to cirrus-sac (80%, n = 257). Terminal (distal) part of vaginal canal with small vaginal sphincter near genital atrium, formed by diffuse muscle fibers (Fig. 15). Seminal receptacle small, dorsal to ovarian isthmus (Fig. 11), observed also in type material.

Vitelline follicles mediulary, small, arranged in 2 narrow lateral bands starting at distance from anterior margin of proglottid (Figs. 8–10), widened posteriorly and bent inward near posterior margin of proglottid (parallel to posterior edge of proglottid), usually reaching mediadly to ovarian lobes (Figs. 8–10). Total length of bands of follicles represents 66–86% (oral) and 75–95% (aporal) of proglottid length. Poral band interrupted ventrally (Fig. 8), with follicles dorsal to cirrus-sac (Figs. 9, 15).

Uterus mediulary, type 1 development (de Chambrier, Zehnder et al., 2004): uterine stem with numerous intensively-staining cells concentrated along its wall in immature proglottids. Uterine lumen appears in last immature proglottids simultaneously with dorsolateral outgrowths (diverticula); lateral branches (diverticula) asymmetrical, occupying up to 71% of width of gravid proglottids (Fig. 9).
FIGURES 1–11. *Corallobothrium solidum* Fritsch, 1886, from *Malapterurus electricus*. (1, 2) Dorsoventral view of scolex (1, MHNG INVE 31550; 2, MHNG INVE 31554), Egypt. (3) Detail of sucker showing the sphincter (MHNG INVE 63109), Egypt. (4–6) Sagittal sections of scolex (MHNG INVE 32761), Egypt. Note the dense network of osmoregulatory canals in the apical region. (7) Scolex of juvenile specimen (MHNG INVE 33997), Egypt. (8) Mature proglottid, ventral view (MHNG INVE 32762), Egypt. (9) Gravid proglottid, testes omitted, dorsal view (MHNG INVE 63197), Sudan. (10) Pre gravid proglottid, with cirrus everted, dorsal view (MHNG INVE 31554). (11) Detail of the posterior region of a pre gravid proglottid, dorsal view (MHNG INVE 31554), Egypt. Abbreviations: ad, additional opening; ao, apical organ; mg, Mehlis' gland; ms, metascolex; oc, osmoregulatory canals; ov, ovary; sp, circular musculature serving as sphincter; sr, seminal receptacle. Scale bars: 1, 11 = 200 μm; 2 = 1,000 μm; 3, 7 = 100 μm; 4–6, 9–10 = 500 μm; 8 = 300 μm.
Figures 12–18. (12-16) *Corallobothrium solidum* Fritsch, 1886 from *Malapterurus electricus*. (12-14) Cross sections of pregravid proglottid at level of anterior, middle, and posterior part, respectively (MHNG INVE 32762 and 32763), Egypt. (15) Terminal genitalia, dorsal view (MHNG INVE 63197), Sudan. (16) Eggs in distilled water (MHNG INVE 48053), Sudan. (17) *Megathylacoides giganteum* (Essex, 1928) from *Ictalurus punctatus*. Eggs in distilled water, with collapsed outer hyaline envelope (MHNG INVE 75439). (18) *Corallobothrium fimbriatum* Essex, 1928 (= *Essexiella fimbriata*), from *I. punctatus*. Eggs in distilled water, with collapsed outer hyaline envelope (MHNG INVE 75464). Abbreviations: cs, cirrus-sac; doc, dorsal osmoregulatory canal; em, bilayered embryophore; ga, genital atrium; ilm, longitudinal internal musculature; oe, outer envelope; on, oncospheres; ov, ovary; te, testes; tp, tubercle-like projections; ut, uterus; va, vagina; vd, vas deferens; vf, vitelline follicles; voc, ventral osmoregulatory canal; vs, vaginal sphincter. Scale bars: 12–14 = 500 μm; 15 = 100 μm; 16–18 = 20 μm.

Eggs spherical, with hyaline outer membrane 95–100 in diameter (collapsed in permanent mounts) and spherical, bilayered embryophore, with outer layer 23–27 in diameter, denser than inner nuclei-containing layer. Tubercle-like projections (outgrowths) on 1 pole of embryophore (Fig. 16). Oncospheres spherical to oval, 13–15 long by 12–14 wide, with 3 pairs of embryonic hooks 5–6 long.

**Taxonomic summary**

*Type host:* *Malapterurus electricus* (Gmelin, 1789) (Siluriformes: Malapteruridae).

*Type locality:* Nile River in Egypt (exact locality not specified).

*Type specimens:* MHNG INVE 36374.

*Additional host:* *Malapterurus gossei* Norris, 2002 (but see Discussion).

*Site of infection:* Intestine.

*Prevalence:* In Luxor, Egypt, prevalence = 100% (n = 2), mean intensity of infection 38 (range 11–64); in Khartoum, Sudan, 75% (n = 12), 3.7 (range 1–10); Omdurman, Sudan, 67% (n = 6), 3.3 (1–8); Kosti, Sudan, 67% (n = 3), 4.0 (4).

*Distribution:* Basins of the Nile (Egypt, Sudan), Congo (Democratic Republic of the Congo), and Omo (Turkana Lake, Kenya) rivers, Senegal (last 3 localities represent new zoogeographical records).
Paraproteocephalus parasili (AJ388604)

Corallobothrium cf. solidum (Congo)
- 100
- 100

- Corallobothrium solidum (AJ583450)

- Megathyacoides giganteum (AY307117)

- Megathyacoides lamothei (AY307119)

- Megathyacoides lamothei (AY548165)

- Megathyacoides giganteum (AY307118)

- Corallobothrium fimбриatum (AY548160)

- Corallobothrium fimбриatum (AY548161)

- Corallobothrium fimбриatum (AY548162)

- Corallobothrium parafimbriatum (AY548163)

- Corallotaenia minutia (AY548164)

- Corallotaenia intermedia (AJ275232)

0.01 subst/site

**Figure 28.** Phylogram (maximum likelihood—ML) showing the relationships of 13 corallobothriine tapeworms inferred from partial sequences of the 28S rRNA gene. The same topology is obtained from a parsimony analysis. Parsimony bootstrap values are above the nodes and ML ones below the nodes.

**Remarks**

The original description of *C. solidum* by Fritsch (1886) was very brief; only 2 measurements (total length and maximum width of the body), a sketch of the whole worm, and figures of sections of an apparently contracted specimen were provided (Fritsch, 1886). Fuhrmann (1916), who studied Fritsch’s original material (currently deposited in MHNG, but of very poor quality due to strong contraction of the worms), provided additional data on the scolex, proglottids, and genital organs, including the number of testes (140–180). However, he erroneously reported as many as 50-60 lateral branches of the uterus (10-12 branches in 3-5 layers), which was not confirmed by a study of type material, and mentioned the absence of a seminal receptacle in *C. solidum*, which is in fact present (Fig. 11).

Janicki (1928) described a dense network of excretory canals on the ventral side of the strobila (fig. 25 in his paper), but this network and foramina secundaria were not observed in cross sections of the new material. In contrast, we confirm the presence of numerous longitudinal and transverse grooves on the body surface (compare Fig. 25 in the present paper with figs. 22 and 24 in Janicki, 1928), and the shape of the bands of vitelline follicles, which bent inward (medially) near the posterior margin of proglottids (Figs. 8–10).

Some previously unreported morphological characteristics were observed in the present study. The most important feature is the presence of a well-developed, semi-circular (U-shaped) musculature on the anterior margin of suckers, which forms a large sphincter interrupted anteriorly (Figs. 2, 3). Such a sphincter has been found in some other proteocephalidean genera, including species of the corallobothriine *Megathyacoides* Jones, Kerly, and Sneed, 1956, from North American ictalurid catfish (Freze, 1965; Rego, 1994), but also in *Barsonella* de Chambrier, Scholz, Beletew, and Mariaux, 2009, from *Clarias* catfish in Africa (de Chambrier et al., 2009). In contrast, sphincters are absent on the suckers of both remaining species of *Corallobothrium*, *C. fimбриatum* and *C. parafimbriatum*, both from channel catfish (*Ictaluridae*) (Essex, 1928; Freze, 1965; Befus and Freeman, 1973; present study).
Another character of *C. solidum* reported here for the first time is a unique egg morphology. The embryophore of the eggs possesses tubercle-like projections (outgrowths) (Fig. 16). These projections have not been observed on the eggs of species of *Corallobothrium* and *Megathylacoides* from ictalurids (Figs. 17, 18; see also Essex, 1928; Befus and Freeman, 1973) or those of other proteocephalidean cestodes (Freze, 1965; Scholz, 1999).

Other characteristics of *C. solidum*, which were unreported previously, are the presence of a well-developed seminal receptacle (observed also in type material) and a vaginal sphincter (Figs. 11, 15). However, the former structure serving for storage of sperm is probably present in all proteocephalidean cestodes, yet difficult to observe in some species, and the presence of the sphincter surrounding the distal end of the vaginal canal is also common (Freze, 1965; de Chambrier and Vaucher, 1999).

Another feature typical of *C. solidum* is a concentration of muscle fibers of the inner longitudinal musculature on the lateral sides of the strobila (Figs. 12–14). Such a feature is uncommon in proteocephalidean cestodes and has been reported for a few species only, such as those of *Amphoteromorphus* Diesing, 1850 (see Carfora et al., 2003).

New, well-fixed material of *C. solidum* also enabled us to better describe the shape of the scolex. SEM observations have shown that it is in fact markedly different (asymmetrical in apical view, i.e., wider laterally than dorsoventrally; Fig. 20) from that described by previous authors, who apparently studied contracted specimens (see fig. 2 in Fritsch, 1886, and fig. 22 in Janicki, 1928). Juvenile specimens possess a large arcal organ, spherical to widely oval in shape (Fig. 7), 190–285 long by 150–300 wide, but their metascolex is weakly developed (Fig. 7).

Neither of the 2 species from ictalurid catfish, that is *C. fimbriatum* and *C. parafimbriatum*, possess the following characteristics observed in *C. solidum*: a sphincter on the suckers, tubercle-like projections on the embryophore of the eggs (see Figs. 17, 18), longitudinal and transverse wrinkles on the strobilar surface, and type 1 development of the uterus (Essex, 1928; Befus and Freeman, 1973; present study).

These conspicuous morphological differences cast doubts upon the current species composition of the genus *Corallobothrium* as a natural, monophyletic assemblage. The genus is also heterogeneous when considering its spectrum of definitive hosts (ictalurids vs. malapterurids) and its markedly disjunct geographical distribution (North America vs. Africa).

**Molecular analysis**

The phylogenetic relationships of all 12 available sequences of corallobothrine cestodes (11 published, 1 new; the sequence of *Megathylacus jandia* Woodland, 1934 was not included because this species was found to be unrelated with the remaining corallobothrinines—see de Chambrier, Zehnder et al., 2004) were analyzed using both parsimony and maximum likelihood methods applied to about 1 kb of the 5' end of the 28Sr RNA gene. *Paraprotocephalus parasituri* (Zmeev, 1936) was chosen as a functional outgroup. The alignment obtained was 1,022 bp long, including 49 gaps, and comprised 84 parsimony informative positions. The shortest parsimony tree (uninformative positions excluded) has a length of 123 (CI = 0.64, RI = 0.83). Treating the gaps as a fifth character did not change the tree topology. The best-fit model selected by AIC in jModeltest is TPM3uf+G with the following parameters: Lset base = (0.1939, 0.2202, 0.3400, 0.2459); nsr = 6; rmat = (0.3707, 5.9067, 1.0000, 0.3707, 5.9067, 1.0000); rates = gamma; shape = 0.1590; pinvar = 0. The best ML tree under these conditions has a −ln likelihood of 2524.9436 and the same topology as the parsimony tree (Fig. 28).

Samples of *C. solidum* and *C. cf. solidum* from Congo are sister taxa and basal. The other taxa group in 2 clades. The first is composed of the *Megathylacoides* samples (*M. giganteum* and *M. lamothei*) and shows a possibly paraphyletic *M. giganteum* (as in Rosas-Valdez et al., 2004; with addition of 2 new sequences, the same data were used in the present analysis). The second comprises *C. fimbriatum*, *C. parafimbriatum*, and 2 species of *Corallotaenia* Freze, 1965 [*Corallotaenia intermedia* (Fritts, 1959) Freze, 1965, and *Corallotaenia minuta* (Fritts, 1959) Freze, 1965], with *C. parafimbriatum* as sister taxon to both *Corallotaenia* species (Fig. 28).

**Species composition of *Corallobothrium***

Based on the above mentioned morphological, bionomical (spectrum of definitive hosts and geographical distribution), and molecular data, species of *Corallobothrium* are newly placed in 2 genera. The former, *Corallobothrium*, now includes only a single species, *C. solidum*, parasitic in African electric catfish (*Malapterurus* spp.), whereas a new genus is proposed to accommodate *C. fimbriatum* from channel catfish (*Ictaluridae*) occurring originally in North America.

**Essexiella new genus**

_Diagnosis:_ Proteocephalidea, Proteocephalidae, Corallobothriinae. Large tapeworms, with massive strobila and well-developed inner longitudinal musculature. Testes, ovary, vitelline follicles, and uterus medullary. Main osmoregulatory canals thin-walled, or evaginated. Suckers deeply embedded, oval, without sphincter. Genital pores irregularly alternating, situated pre-equatorial. Ovary bilobed, its width representing less than 50% of proglottis width. Vagina posterior or anterior to cirrus-sac. Seminal receptacle present. Vitelline follicles arranged in 2 lateral bands, with posterior follicles turning medially, reaching ovarian lobes. Uterine development of type 2 according to de Chambrier, Zehnder et al. (2004). Eggs spherical, with smooth embryophore. Parasites of channel catfish (*Ictaluridae*), originally in North America. Type and only species: _Essexiella fimbriata_ (Essex, 1928) new. comb.

_Etymology:_ The generic name commemorates the contribution of Hiram E. Essex to the systematics of fish cestodes, especially his monograph on *Corallobothrium* from North America (Essex, 1928); the name should be treated as a feminine.

**Differential diagnosis**

The new genus is placed in the Corallobothriinae sensu Rego (1994) because genital organs (testes, ovary, vitelline follicles, and uterus) are situated in the medulla and a metascolex is present (Freze, 1965; Rego, 1994). _Essexiella_ differs from the remaining 5 genera of the Corallobothriinae, i.e., *Corallobothrium*, *Corallotaenia*, *Megathylacoides*, *Megathylacus* Woodland, 1934, and *Paraprotocephalus* Chen, 1962, as follows:
(1) *Corallobothrium* possesses a U-shaped sphincter on the external (outer) margin of suckers, tubercle-like projections on the embryophore, and a network of osmoregulatory canals in the scolex (all structures absent in *Essexiella*); it also differs by the type of uterine development (type 1) and testes arranged in 2 separate fields confluent anteriorly (in 1 field in *Essexiella*).

(2) *Corallotaenia* can be distinguished from *Essexiella* by the small size of its body, which consists of only a few proglottids, with mature ones being longer than wide (much wider than long in *Essexiella*), and a weakly developed metascolex formed by a few lobes in the posterior part of otherwise globular scolex (see Rosas-Valdez et al., 2004) (Jones et al., 1956; Freze, 1965).

(3) *Megathylacoides* differs by the presence of a sphincter in suckers (absent in *Essexiella*) and by a different shape of the scolex, with a less developed metascolex (see Scholz et al., 2003; Rosas-Valdez et al., 2004).

(4) *Megathylacus* possesses sphincters in the suckers (absent in *Essexiella*), the scolex is of a markedly different shape (see Freze, 1965; Rego, 1994), and the species possesses a type 1 development of the uterus (de Chambrier, Zehnder et al., 2004).

(5) *Paraproteocephalus* is conspicuously different from *Essexiella* in its unique morphology of the uterus, which forms external branches (diverticula) in the vertical direction (see Yamaguti, 1934; Freze, 1965; Rego, 1994) (the uterus of *Essexiella* has lateral diverticula as in all other proteocephalideans); it also differs in the shape of the scolex, which is discoidal and possesses anteriorly situated suckers and an apical sucker (Rego, 1994).

**Phylogenetic relationships**

Molecular data demonstrated that *C. solidum* and the 2 remaining species previously placed in *Corallobothrium*, i.e., *C. fimbriatum* and *C. parafimbriatum*, are unrelated and should not be placed in the same genus (Fig. 28). However, this analysis has also indicated that the species composition of North American corallobothriine genera may not reflect their phylogenetic relationships, because *Paraproteocephalus* did not form a monophyletic clade with *C. fimbriatum*, but represented a sister taxon to both species of *Corallotaenia* (*C. intermedia* and *C. minuta*, the latter being synonymized by Freze [1965] with the former) (Fig. 28).

In some morphological characteristics, such as small size of the body and the shape of the scolex, *C. parafimbriatum* resembles species of *Corallotaenia* (see Freze, 1965; Befus and Freeman, 1973). Based on this morphological resemblance between *C. parafimbriatum* and *Corallotaenia* spp., and new molecular data, the former species is tentatively placed in *Corallotaenia* as *Corallotaenia parafimbriata* n. comb., but further studies should confirm this generic placement.

Molecular data also indicate that *M. giganteum*, type species of the genus, may represent a complex of cryptic species, some of them being unrelated to each other (Rosas-Valdez et al., 2004). It is evident that the whole group of proteocephalidean cestodes parasitic in ictalurids, including *Corallotaenia*, *Essexiella*, and *Megathylacoides*, is pending taxonomic revision, which should include morphological and molecular data.

**Corallobothrium Fritsch, 1886—emended diagnosis**

**Diagnosis:** Proteocephalidea, Proteocephalidae, Corallobothriinae. Testes, ovary, vitelline follicles, and uterus medullary. Large tapeworms, with massive strobila and well-developed inner longitudinal musculature with muscle fibers concentrated on lateral sides of strobila. Body surface with longitudinal and transverse grooves. Main osmoregulatory canals thin-walled, ventral canals overlapping testicular field. Scolex large, with metascolex, wider laterally than dorsoventrally. Suckers deeply embedded, oval, external (outer) margin of suckers with well-developed semi-circular (U-shaped) musculature serving as sphincter. Testes in 2 fields confluent anteriorly, in 1 complete or 2 incomplete layers. Cirrus-sac oval. Genital pores irregularly alternating, pre-equatorial. Genital atrium deep. Ovary bilobed, its width occupies more than 50% of proglottis width. Vagina usually posterior to cirrus-sac. Seminal receptacle present. Vitelline follicles arranged in 2 lateral bands, with posterior follicles turning medially, reaching ovarian lobes. Uterine development of type 1 according to de Chambrier, Zehnder et al. (2004). Eggs with tubercle-like projections on embryophore. Parasites of electric catfishes (Malapteruridae) in Africa. Type and only species: *C. solidum* Fritsch, 1886.

**DISCUSSION**

As many as 11 species of proteocephalidean cestodes possessing a metascolex have been placed in *Corallobothrium* Fritsch, 1886 (see Freze [1965] for overview of the taxonomic history of the genus). However, only 3 species, namely *C. solidum*, *C. fimbriatum*, and *C. parafimbriatum*, were retained in the genus more recently (Schmidt, 1986). Based on the present study, the genus should be considered to be monotypic, with its single species specific to electric catfishes in Africa, whereas the 2 species from ictalurids are accommodated in newly proposed genera, *Essexiella* and *Corallo­taenia*, respectively. These 2 latter species were originally described from North America (Essex, 1928; Befus and Freeman, 1973), but they may occur outside of North America as a result of the import of channel catfishes to other continents, especially to Europe (Scholz and Cappellaro, 1993; C. Vaucher, unpubl. data; MHNG INVE 32944).

A study of the type material and newly collected specimens of *C. solidum* has revealed some morphological characteristics of taxonomic importance, which were previously unreported. The most important novelty is the presence of muscular sphincters in suckers and tubercle-like projections on the outer layer of the embryophore of the eggs. The presence/absence of a sphincter in suckers is considered to be a taxonomically important character that is suitable for differentiation of genera of proteocephalidean cestodes (Jones et al., 1956; Freze, 1965; Rego, 1994; de Chambrier and Rego, 1995; de Chambrier and Vaucher, 1999, de Chambrier et al., 2009). Egg morphology is also considered to represent a taxonomic character suitable for differentiation of species (Gil de Pertierra and de Chambrier, 2000; Carfora et al., 2003; de Chambrier et al., 2007, 2009, 2010; Scholz et al., 2009).

Presence of a dense network of osmoregulatory canals within the scolex is an unusual observation for the Proteocephalidea, although it was already reported for *Proteocephalus turulosus* (Batsch, 1786), a parasite of cyprinids in the Holarctic, by Wagner (1917), for *M. lamothei* (Garcia-Prieto, 1990), a parasite of
**ACKNOWLEDGMENTS**

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Current studies on proteocephalidean cestodes (e.g., de Chambrier et al., 2007, 2009) have also demonstrated that complicated 3-dimensional structures such as the metascolex of corallobothriine and other proteocephalidean cestodes may be difficult to correctly describe without use of scanning electron microscopy. In addition, fixation may considerably influence the appearance of these complicated structures, which can then lead to incorrect taxonomic conclusions. Besides SEM observations, longitudinal sections (frontal or sagittal) of the scolex appear to be of great value for correct description of the internal morphology of the scolex. This also concerns the structure of the suckers and the apical part of the scolex, which may contain gland cells, apical structures and/or a dense network of excretory canals as observed in *C. solidum*.

In juvenile *C. solidum* tapeworms, the metascolex of which was not yet fully developed, a large apical organ with granular content was observed (Fig. 7). However, this organ, or its traces, were not observed in any of the numerous adult tapeworms studied (Figs. 1, 2, 4–6), which indicates that this organ disappears completely during the maturation of the worms within the definitive host. This organ may serve as an additional attachment organ important for firm fixation of juvenile, recently recruited tapeworms in the intestinal lumen of the fish host, but no data are available on the life-cycle and ontogenetic development of *C. solidum* to confirm this assumption, which is based on existing information about life-cycles of other proteocephalideans (for review, see Freze, 1965; Scholz, 1999; Scholz and de Chambrier, 2003).

Congeneric tapeworms with a well-developed metascolex and robust strobila were found in another electric catfish, *M. gossei* Norris, from the lower Congo River in the Democratic Republic of the Congo. They slightly differ from *C. solidum* in the shape of the scolex (Figs. 23, 24) and sequences of the 28S rRNA gene (Fig. 28). They may represent a new, yet undescribed species of the genus, but the material available is not sufficient for a reliable taxonomic description of the new taxon. Accordingly, **Corallobothrium** is tentatively considered to be a monotypic genus, specific to electric catfish (*Malapterurus* spp.) in Africa.

**LITERATURE CITED**


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ESSEX, H. E. 1928. The structure and development of *Corallobothrium* with descriptions of two new fish tapeworms. Illinois Biological Monographs 11, No. 3, 64 pp. + 5 Plates.


DISCOVERY OF OPISTHORCHIS LOBATUS (TREMATODA: OPISTHORCHIIDAE): A NEW RECORD OF SMALL LIVER FLUKES IN THE GREATER MEKONG SUB-REGION

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ABSTRACT: Metacercariae, morphologically similar to those of small liver flukes, were found to parasitize red-tailed snakehead fish, Channa limbata, collected from the city of Vientiane, Lao People’s Democratic Republic. Adult worms that were recovered from experimentally-infected hamsters showed characteristics distinctly different from Opisthorchis viverrini, but closely similar to Opisthorchis lobatus, which was first reported in poultry (Anas sp.) from Pakistan. The present study aimed to redescribe O. lobatus based on the adult worms recovered from experimentally-infected hamsters. Additionally, it aimed to document the genetic relationships among O. lobatus and other opisthorchid liver flukes using the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene and the internal transcribed spacer 2 (ITS2) region. DNA alignment of the O. lobatus and O. viverrini COI partial sequences (330 bp) showed 3.03% fixed differences (2.72% of amino acids changed) while the ITS2 region (350 bp) indicated a 0.86% difference for nucleotides. Species boundaries between the 2 parasites were determined by neighbor-joining analysis using the molecular sequence data. The phenogram confirmed that O. lobatus was distinctly different from O. viverrini, representing the first reported instance of O. lobatus in the Greater Mekong Sub-region (GMS) and the first record of C. limbata as the second intermediate host of a small liver fluke. Questions regarding human infection and the extent of the geographic distribution of these species should be investigated further.

Currently, only 2 species of small liver flukes (Opisthorchiidae Looss, 1899) are known to exist in Southeast Asia as human pathogens. Clonorchis sinensis (Cobbold, 1875) Looss, 1907 has been reported in North Vietnam (De et al., 2003; Do et al., 2007) and a few cases have been reported in central Thailand (Traub et al., 2009). Opisthorchis viverrini (Poirier, 1886) Blanchard, 1895 has been observed in Cambodia (Touch et al., 2009), Laos’ Democratic Republic (Loa PDR) (Chai et al., 2005; Sayasone et al., 2009), Thailand (Kaewpitoon et al., 2008), and central and South Vietnam (De et al., 2003). Opisthorchiasis and clonorchiasis are major parasitic infections and are significant public health problems in the Greater Mekong Sub-region (GMS) (Young et al., 2010). Indeed, opisthorchiasis has been reported as the major cause of cholangiocarcinoma in Thailand (Sripa et al., 2004).

The life cycles of these small liver flukes involve freshwater fish as second intermediate hosts. Reports from the GMS noted that many species of freshwater fish harbor metacercariae of these flukes, with most fish species being cyprinids (Cyprinidae) (Manivong et al., 2009) and the internal transcribed spacer 2 (ITS2) region. DNA alignment of the O. lobatus and O. viverrini COI partial sequences (330 bp) showed 3.03% fixed differences (2.72% of amino acids changed) while the ITS2 region (350 bp) indicated a 0.86% difference for nucleotides. Species boundaries between the 2 parasites were determined by neighbor-joining analysis using the molecular sequence data. The phenogram confirmed that O. lobatus was distinctly different from O. viverrini, representing the first reported instance of O. lobatus in the Greater Mekong Sub-region (GMS) and the first record of C. limbata as the second intermediate host of a small liver fluke. Questions regarding human infection and the extent of the geographic distribution of these species should be investigated further.

In this study, the metacercariae of a small liver fluke were found in the red-tailed snakehead fish, Channa limbata (Cuvier, 1831) collected from Naxon Village in the Pakngeum district of Vientiane, Lao PDR. Adult worms recovered from experimentally-infected hamsters had a small body and oral sucker. Morphological observations suggested that the worms present were O. lobatus because of traits that distinguished them from other congeners described in the GMS region.

The original description of this species was incomplete, lacking in several details regarding its defining characteristics. Here, O. lobatus is re-described based on the adult specimens recovered from experimentally-infected hamsters. In addition, molecular sequence data are used to determine genetic differences between O. lobatus and other opisthorchid liver flukes using the COI gene and ITS2 region.

MATERIALS AND METHODS

Several species of freshwater fish were collected from Naxon Village in the city of Vientiane, Lao PDR in October 2008. Fish were examined using the ‘compression’ method. Metacercariae that were morphologically identifiable as the larval stage of small liver flukes were found in 1 species of cyprinid fish, Barbodes gonionotus (Bleeker, 1850), and in the red-tailed snakehead fish, C. limbata. The metacercariae were separated from the muscles of the cyprinid and snakehead fish and 50 metacercariae were fed to each of the 3 hamsters. Infected hamsters were killed after fluke eggs were detected in their feces (about 4 wk post-infection). During necropsy, small liver flukes were collected from the hamsters’ bile ducts. Ten flukes were fixed in hot formalin, stained in acetic carmine, and mounted in Canada balsam for morphological analysis. Ten flukes were also fixed in 70% alcohol for DNA sequence study and kept at −20 °C until used. The use of experimental hamsters in this study was approved by the Animal Care and Use Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (FTM-ACUC 007/2008).

Genomic DNA was extracted from 3 adult worms using a Genomic DNA mini kit (issue, Geneaid, Taipei, Taiwan) according to the manufacturer’s protocol. A portion of the cytochrome c oxidase subunit 1 (COI) gene and
Table I. Sample collections and sequences used.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Developmental stage</th>
<th>Locality</th>
<th>COI sequence accession no.</th>
<th>ITS2 sequence accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ophisthorchis lobatus</td>
<td>Channa limbata</td>
<td>Metacercaria</td>
<td>Vientiane, Laos</td>
<td>HQ328539</td>
<td>HQ328545</td>
</tr>
<tr>
<td>O. lobatus</td>
<td>Channa limbata</td>
<td>Metacercaria</td>
<td>Vientiane, Laos</td>
<td>HQ328540</td>
<td>HQ328546</td>
</tr>
<tr>
<td>O. lobatis</td>
<td>Channa limbata</td>
<td>Metacercaria</td>
<td>Vientiane, Laos</td>
<td>HQ328541</td>
<td>HQ328547</td>
</tr>
<tr>
<td>Ophisthorchis viverrini</td>
<td>Barbonymus gonionotus</td>
<td>Metacercaria</td>
<td>Vientiane, Laos</td>
<td>HQ328542</td>
<td>HQ328548</td>
</tr>
<tr>
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<td>Barbonymus gonionotus</td>
<td>Metacercaria</td>
<td>Vientiane, Laos</td>
<td>HQ328543</td>
<td>HQ328549</td>
</tr>
<tr>
<td>O. viverrini</td>
<td>Barbonymus gonionotus</td>
<td>Metacercaria</td>
<td>Vientiane, Laos</td>
<td>HQ328544</td>
<td>HQ328550</td>
</tr>
<tr>
<td>Clonorchis sinensis</td>
<td>Dog</td>
<td>Adult</td>
<td>China</td>
<td>FJ965384</td>
<td>*</td>
</tr>
<tr>
<td>C. sinensis</td>
<td>Cat</td>
<td>Adult</td>
<td>China</td>
<td>FJ965391</td>
<td></td>
</tr>
<tr>
<td>C. sinensis</td>
<td>N/A†</td>
<td>N/A</td>
<td>South Korea</td>
<td>-</td>
<td>AF217094</td>
</tr>
<tr>
<td>Ophisthorchis felineus</td>
<td>N/A</td>
<td>Metacercaria</td>
<td>Russia</td>
<td>EF688123</td>
<td>-</td>
</tr>
<tr>
<td>O. felineus</td>
<td>N/A</td>
<td>Metacercaria</td>
<td>Russia</td>
<td>EF688127</td>
<td>-</td>
</tr>
<tr>
<td>O. felineus</td>
<td>N/A</td>
<td>Metacercaria</td>
<td>Russia</td>
<td>EF688128</td>
<td>-</td>
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<tr>
<td>O. felineus</td>
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<td>Metacercaria</td>
<td>Russia</td>
<td>-</td>
<td>EF688142</td>
</tr>
</tbody>
</table>

* (-) indicates unavailable data.
† N/A = not applicable.

The internal transcribed spacer subunit II (ITS2) was amplified using COI-OV-Hap-F&R (Thaenkham et al., 2007) and the 3S and BD2 primers (Bowles et al., 1993), respectively. The total volume of the PCR reaction was 50 μl and consisted of 25 μl 2× GoTaq colorless mixture (Promega, Madison, Wisconsin) with 50 pmol of each primer. About 10 ng of genomic DNA was added to each reaction. PCR amplicons were obtained using the following amplification conditions: initial denaturation at 94°C for 3 min, 30 cycles at 94°C for 30 sec, annealing at 52°C for 30 sec for the COI and at 50°C for 30 sec for ITS2 extension at 72°C for 30 sec, and a final extension at 72°C for 8 min. The PCR amplicons were electrophoresed in 1.0% agarose gel and visualized on a UV-transilluminator. Each PCR amplicon was sequenced using the 2 amplification primers on an ABI Prism 377 DNA sequencer (Macrogen Inc., Seoul, Korea). The sequences obtained were checked with BLAST to confirm the PCR target. The electropherogram of each sequence was examined for sequence accuracy by BioEdit program version 7.0 (Hall, 1999).

The DNA sequences of the COI and ITS2 markers were aligned with the Ophisthorchis felineus and Clonorchis sinensis sequences deposited in GenBank (Table I) using Clustal X (Thompson et al., 1997). All gap sites in the sequences were excluded. The numbers of fixed nucleotide differences were estimated from the DNA sequence alignment of each marker. The COI sequences of all species studied were translated to amino acid sequences and aligned to verify the position of any nucleotide change. Cluster diagrams were constructed from the COI and the ITS2 sequences by neighbor-joining (NJ) method by estimating 1,000 replications with p-distance in MEGA 4.1 (Kumar et al., 2008).

RESULTS

Small liver fluke metacercariae were found in 2 of 24 (8.3%) specimens of Barbodes gonionotus (Cyprinidae) and in 2 of 85 (2.4%) specimens of C. limbata (Channidae) (Table II).

Beginning 3 wk post-infection (PI) in the hamsters, fecal samples from infected hamsters were examined twice a week. At

Table II. Metacercariae found in freshwater fish collected at Naxon Village, Vientiane, Lao People’s Democratic Republic, 8–17 October 2008.

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Common name in Laos</th>
<th>Number of fish examined</th>
<th>Small liver fluke</th>
<th>Clinostome</th>
<th>Unknown</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anabas testudineus</td>
<td>Pia Kheng</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Barbonymus gonionotus</td>
<td>Pia Pak Na</td>
<td>24</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Channa limbata</td>
<td>Pia Kho Kang</td>
<td>85</td>
<td>(8.3)</td>
<td>(16.7)</td>
<td>(25)</td>
<td></td>
</tr>
<tr>
<td>Channa striata</td>
<td>Pia Kho</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Eumyconius metallicus</td>
<td>Pia Seaw Nuad Yao</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Hampsala dispar</td>
<td>Pia Sood Jam</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hampsala macrolepidota</td>
<td>Pia Sood Kan</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Henicorhynchus siamensis</td>
<td>Pia Soi</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Labeobarbus siamensis</td>
<td>Pia Kui Lam</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Luciosoma blekeri</td>
<td>Pia Seaw Aow</td>
<td>103</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Trichogaster trichopterus</td>
<td>Pia Ka Dird</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>16.7</td>
<td>16.7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>303</td>
<td>4</td>
<td>7</td>
<td>29</td>
<td>40</td>
</tr>
</tbody>
</table>

* (1.3) (2.3) (9.6) (13.2)
4 wk PI, small fluke eggs were found in the fecal samples. Adult *O. viverrini* were recovered from 1 hamster infected with cyprinid fish metacercariae; the adult recovery was 54% (28 worms/50 metacercariae). The adults recovered from another 2 hamsters that were infected with metacercariae from snakehead fish were not similar to *O. viverrini*; the adult recovery was 26% (13 worms/50 metacercariae) in 1 hamster and 46% in the second (23 worms/50 metacercariae). After staining, morphology of the specimen was studied. The small liver fluke from the snakehead fish was identified as *O. lobatus*. All measurements in the description are in micrometers, unless otherwise noted.

**EMENDED DESCRIPTION**

*Opisthorchis lobatus*  
(Fig. 1A, B)

**Adult:** Body spatulate, oval to elongate, thin and transparent, anterior end broad to pointed, posterior end broad, 4 (4–6) mm long and 0.9 (0.7–1.1) mm wide (Fig. 1B). Oral sucker terminal, very small, 94 (60–100) in diameter; prepharynx absent; pharynx small, 105 (80–110) long and 90 (60–100) wide; short esophagus, 89 (20–200). Narrow ceca extended to posterior end of terminating body, lateral to excretory bladder. Pharynx and cecal bifurcation densely covered with unicellular glands, with short ducts opening into lumen of covered area. Ventral sucker small, 91 (70–125) in diameter, located in mid-body of anterior third. Testes 2 in number, tandem, large, irregularly lobed, located at posterior end; posterior testis 481 (400–500) long and 364 (380–460) wide, anterior testis 474 (380–550) long and 376 (310–520) wide. Sperm duct from each testis running anteriorly and joining to form seminal vesicle immediately anterior to vitelline follicles. Vitelline follicles 50 (20–90) long and 20 (15–20) wide. Ovary with 3–4 finger-like lobes, close to and anterior to seminal receptacle, (230–380) long and 165 (100–230) wide. Oviduct runs to open into ootype; oocyte covered with numerous Mehlis’ glands; uterus runs transversely towards anterior end, forming transverse irregular folds in mid-body between ceca, with some folds overlapping ceca. Metraterm dorsal to ventral sucker, joins with ejaculatory duct, opening into genital pore. Seminal receptacle large, voluminous, pre-testicular; duct from seminal receptacle opens into ootype. Laurer’s canal branches off duct of seminal receptacle, relatively long, opening on dorsal side anterior to anterior wall of excretory bladder. Vitelline follicles relatively large, forming 7–8 groups on each lateral side in middle third of body; posterior groups do not extend posterior to ovary; vitelline ducts not always clearly visible, vitelline chamber triangular, ventral to ovary; common vitelline duct running anterior to open into ootype. Uterine eggs numerous, 24 (22–28) long and 11 (10–12) wide (20 eggs), operculate, thick-walled, distinctly shouldered, operculum large; posterior end sometimes with abopercular knob. Excretory bladder tubular, winding between testes and seminal receptacle.

*Metacercaria:* Encysted metacercariae oval, moving vigorously in thin, transparent, single-layered cyst wall, 150 (100–200) long and 80 (70–90) wide (15 cysts) (Fig. 1A). Encysted metacercariae elongated oval in shape, 432 (320–480) long and 72 (60–80) wide. Oral sucker terminate, 48 (45–50) long and 37 (35–37) wide.

**FIGURE 1.** Morphology of metacercaria and adult worm of *Opisthorchis lobatus*. (A) Excysted metacercaria from a red-tailed snakehead fish from Naxon, Vientiane, Lao People’s Democratic Republic. (B) Line drawing of adult obtained by experimental infection of a golden hamster after 2.5 mo.
throughout entire body. Excretory bladder large, oval, and located in posterior third of body.

**Taxonomic summary**

**Host:** Red-tailed snakehead fish, *Channa limbata* (Cuvier, 1831) (Channidae: Perciformes).

**Location:** Muscle of the tail fin.

**Locality:** Vientiane City, Lao PDR (18°05.311′N, 102°58.998′E).

**Prevalence:** 2.4%.

**Specimens deposited:** Natural History Museum, U.K. (BMNH 2010.9.15.1); U.S. National Parasite Collection, United States (USNPC 103739); Meguro Parasitological Museum, Japan (MPM 18912); Unit of Parasitology, Faculty of Basic Science, University of Health Science, Lao PDR (UHSPC-2010-112); Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand (MUTMHM 2553001/2).

**Remarks**

Compared to the original description (Bilgees et al., 2003), every morphological feature of the worms obtained in this study are smaller. This is true to the extent that even the smallest recorded measurement from Bilgees et al. (2003) was, in fact, larger than the largest recorded measurement found in our study. In figure 1 of Bilgees et al. (2003), the body of the worm was wide and the uterine coils were separated, suggesting that the worm was severely flattened, which may have occurred during the fixation process.

Our study provides more detailed descriptions of reproductive organs including the vitelline follicles, the seminal vesicle, the seminal receptacle, the oviduct, the ootype, and the ovary. All of these characteristics were lacking in the original description of Bilgees et al. (2003), as they cannot be seen clearly in a flattened specimen.

Several features of the co-existing species *O. lobatus* and *O. viverrini* from the same locality are distinguishable. The metacercaria cyst of *O. lobatus* is smaller than that of *O. viverrini* but the excysted metacercaria has a larger body (Table II). The adult *O. lobatus* is two-thirds the width of *O. viverrini*. The oral sucker of *O. lobatus* is about half the size of that of *O. viverrini* (Table III). The size of the oral sucker is quite similar to the size of the pharynx in *O. lobatus* (oral sucker:pharynx is 0.9:1.0), while the oral sucker is larger than the pharynx in *O. viverrini* (oral sucker:pharynx is 1.7:1.0) (Table III). However, the oral sucker in both species is smaller than the ventral sucker; the ratio of the oral sucker:ventral sucker of *O. lobatus* is 0.9:1.0 and of *O. viverrini* is 0.8:1.0. Other than the differences in body and sucker sizes, the 2 species also differ in other characteristics. For example, the esophageal glands are larger and more numerous in *O. lobatus* while they are small and scattered in *O. viverrini*. The vitelline follicles are located in the middle third of the body and do not extend beyond the ovary in *O. lobatus*, but in *O. viverrini* they are in the middle two-fifths of the body and extend beyond the ovary to the anterior testis. In *O. lobatus*, the vitelline ducts are not clearly visible, but they are in *O. viverrini*. The uterus and seminal vesicle do not extend beyond the

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**Table III.** Measurement (range) and ratios (average) of diagnostic characteristics of *Opisthorchis viverrini* and *Opisthorchis lobatus.*

<table>
<thead>
<tr>
<th>Diagnostic characters</th>
<th><em>Opisthorchis viverrini</em> (µm)</th>
<th><em>Opisthorchis lobatus</em> (µm)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metacercariae (n = 15)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Encysted metacercaria</td>
<td>L 185-210</td>
<td>145-155</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>W 150-170</td>
<td>97-110</td>
<td>1.5</td>
</tr>
<tr>
<td>Excysted metacercaria</td>
<td>L 315-325</td>
<td>420-435</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>W 55-65</td>
<td>68-75</td>
<td>0.8</td>
</tr>
<tr>
<td>Oral sucker</td>
<td>L 33-40</td>
<td>42-53</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>W 33-42</td>
<td>32-43</td>
<td>1.0</td>
</tr>
<tr>
<td>Ventral sucker</td>
<td>L 42-50</td>
<td>47-55</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>W 41-48</td>
<td>47-55</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>Adults (n = 20)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body</td>
<td>L 4,180-7,000</td>
<td>3,620-5,600</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>W 1,200-1,800</td>
<td>720-1,100</td>
<td>1.6</td>
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<tr>
<td>Oral sucker</td>
<td>L 160-240</td>
<td>70-113</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>W 100-220</td>
<td>50-100</td>
<td>2.1</td>
</tr>
<tr>
<td>Pharynx</td>
<td>L 100-200</td>
<td>80-140</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>W 60-140</td>
<td>60-100</td>
<td>1.2</td>
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<tr>
<td>Esophagus</td>
<td>L 100-400</td>
<td>62.5-200</td>
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<td>Ventral sucker</td>
<td>L 160-260</td>
<td>70-125</td>
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<td>W 160-260</td>
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<td>Posterior testis</td>
<td>L 320-480</td>
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<td>Anterior testis</td>
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<td>W 10-17.5</td>
<td>10-12.5</td>
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ventral sucker in *O. lobatus* but they sometimes do in *O. viverrini*. The lobes of the testes are irregular in shape and size in *O. lobatus* but more regular in *O. viverrini*.

**COI sequence differences**

The percentages of fixed differences in the nucleotide variations of the partial COI gene (282 bp) were determined using pairwise comparison between the nucleotide sequences of *O. lobatus* and *O. felineus*, and of *O. lobatus* and *C. sinensis*; they were 17.37% and 13.82%, respectively. Pairwise comparison between the partial COI genes (330 bp) of *O. lobatus* and *O. viverrini* indicated 3.03% fixed nucleotide differences. After COI sequence translation, the number of amino acid differences between *O. lobatus* and *O. viverrini* were the same as between *O. lobatus* and *C. sinensis* (3 amino acid changes, i.e., 2.72%) while the number of amino-acid changes between *O. lobatus* and *O. felineus* was 6 (6.38%) (Fig. 2). Using the ITS2 region as a DNA marker, the percentage of fixed nucleotide positions was smaller than the COI gene. The percentages of fixed nucleotide differences in the ITS2 sequences (264 bp) between *O. lobatus* and *O. felineus* was 3.03% while it was 2.37% between *O. lobatus* and *C. sinensis* (337 bp). A pairwise comparison between the partial ITS2 region (347 bp) of *O. lobatus* and *O. viverrini* revealed 0.86% fixed nucleotide differences (data not shown). The sequence length of each comparison was different because the indel positions were deleted after alignment.

The cluster diagram showed that *O. lobatus* has the greatest sequence similarity to *O. viverrini* when the COI gene and the ITS2 region were used as DNA markers. The cluster diagram reconstructed from the amino acids of both the COI genes and ITS2 sequences revealed a high degree of sequence similarity between *O. lobatus* and *O. viverrini* and both, in return, were more similar in sequence to *C. sinensis* than to *O. felineus* (Fig. 3A, B).

**DISCUSSION**

The results of the present study increase the number of species of small liver flukes known to exist in the GMS. An examination of fish collected in the city of Vientiane found *O. lobatus* metacercariae for the first time in Laos and the GMS. Two species of *Opisthorchis* were confirmed in Laos, i.e., *O. viverrini* and *O. lobatus*; both species were found in the same locality of Vientiane but in different groups of fish. *Opisthorchis viverrini* metacercariae had been previously reported in cyprinid fish (Manivong et al., 2009) and there was a single report of *O. viverrini* metacercariae in snakehead fish, *Channa striata*, collected in An Giang Province, Vietnam. Although the parasite species was identified provisionally, due to the small number of metacercariae found and to the inability to carry out any experimental animal infections, it has nonetheless been recorded that the snakehead fish is a second intermediate host of a small liver fluke (Thu et al., 2007). The present study reported *Opisthorchis* metacercariae in another species of snakehead fish, *C. limbata*. We were able to identify these metacercariae as *O. lobatus* by the morphology of the adult worms obtained from experimental infections. It is possible that the metacercariae found in the snakehead fish in Vietnam were not *O. viverrini* but were instead *O. lobatus*. The results obtained through the COI DNA marker revealed at least 3.03% fixed amino acid differences between *O. lobatus* and *O. viverrini*.

These molecular data indicate that *O. lobatus* is distinct from *O. viverrini*.

Metacercariae of small liver flukes from cyprinid fish collected from the same locality as the red-tailed snakehead fish developed into adult *O. viverrini* in experimentally-infected hamsters. In the present study, the small liver fluke metacercariae were not found in another species of fish apart from the snakehead fish, *C. striata*, collected from the same site. This finding suggests potential host specificity by the 2 species of *Opisthorchis*, i.e., cyprinid fish are the second intermediate host of *O. viverrini* and the red-tailed snakehead fish is the only species that is the second intermediate host for *O. lobatus*. However, further investigation is needed to confirm this secondary intermediate host specificity among the 2 species of *Opisthorchis* liver flukes.

The discovery reported here is also significant because it confirms another species of small liver fluke in the GMS. *Opisthorchis lobatus* has not been previously recognized in studies.
of small liver flukes in this area. Lacking morphological information, misidentification may easily occur because of the similar size and shape of *O. viverrini* and *O. lobatus* metacercariae and adult worms. In the future, human infection, geographical distribution, and host–parasite specificity should be investigated in order to elicit more information about this species in the GMS.

ACKNOWLEDGMENTS

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INTERrenal DISEASE in BLUEgILLS (Lepomis MacROCHIRUS) Caused By a NEW GENUS and SPECIES of MyxozoAN

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ABSTRACT: A myxozoan species not matching with any described genus was encountered in a survey of fish parasites of freshwater fishes in New York. The parasite was observed in the kidney tubules of bluegill sunfish (Lepomis macrochirus). Two polar capsules measure 9.4-12.5 μm long by 2.9-4.0 μm wide and contain a filament coiled 10-15 times. Phylogenetic analysis places A. hoffmani n gen., n. sp. as a sister to a Myxobilatus and a Hoferella species. The clustering of these 3 similar genera in the larger ‘freshwater’ clade of myxozoans does not support their inclusion in the Sphaerosporida but instead supports the distinct status of the Myxobilatidae Shulman 1953.

Myxozoan parasites infect freshwater and marine fishes the world over. The most well-known myxozoan species are those associated with severe pathology and economic loss (Kent et al., 2001), but a wealth of biodiversity exists with over 2,180 species described to date (Lom and Dyková, 2006). In an ongoing survey of fishes of New York State, high levels of myxozoan parasitism were documented in young of the year bluegill sunfish (Lepomis macrochirus). The most well-known myxozoan species are those parasitism were documented in young of the year bluegill sunfish (Lepomis macrochirus). A new genus is described to accommodate Acuda hoffmani n. gen., n. sp., which exhibits pyriform spores with longitudinal ridges and polar capsules perpendicular to the sutured plane. Spores measure 17.9-21.8 μm long and 6.9-11.0 μm wide, with spore valves each with 11-12 ridges. Two polar capsules measure 9.4-12.5 μm long by 2.9-4.0 μm wide and contain a filament coiled 10-15 times. Phylogenetic analysis places A. hoffmani n gen., n. sp. as a sister to a Myxobilatus and a Hoferella species. The clustering of these 3 similar genera in the larger ‘freshwater’ clade of myxozoans does not support their inclusion in the Sphaerosporida but instead supports the distinct status of the Myxobilatidae Shulman 1953.

MATERIALS AND METHODS

Fish were collected by seine net during the period between 10 June and 6 September, 2009 and again in July 2010 from the south shore of Cazenovia Lake, New York (Latitude 42°55′29.52″N, 75°52′10.38″W). Fish were transported to the Fish and Wildlife Disease Laboratory at SUNY-ESF, Syracuse, New York and held in static aquaria until they could be examined for parasites (approximately 1 day to 2 wk). Fish were killed with 250 mg/L of tricane methanesulfonate (Argent Laboratories, Redmond, Washington)buffered with 250 mg/L sodium bicarbonate. Fish were then weighed and fork length measured. Parasitological examinations were conducted by standard methods with the aid of a Nikon SMZ-800 Stereo-zoom microscope (Nikon Instruments Inc., Melville, New York). Giemsa stained imprints and squash preparations were examined using a Nikon 80i compound microscope with Nomarski and phase objectives. Images were captured with the 3mp IDEA digital camera and analyzed with photomicrography software (Diagnostic Instruments, Inc., Spot RT Software 4.6, Sterling Heights, Michigan).

Tissues containing myxozoan parasites were preserved in both 95% ethanol and paraformaldehyde (at least a 1:1 fixative to tissue ratio) for molecular analyses and scanning electron microscopy (SEM), respectively. For morphological analysis of spores, an infected tissue was placed in a 1.5-ml Eppendorf tube containing phosphate-buffered saline and gently suspended with a microtube pestle. An aliquot of this suspension was mixed with agarose (to a final concentration of 0.5%) and mounted on a microscope slide. At least 50 measurements were made for each relevant spore dimension following the guidelines of Lom and Arthur (1989). The number of polar filament coils was noted where possible. For SEM, spores were allowed to settle on coverslips coated in poly-L-lysine, dehydrated in a 50-70% ethanol series, fixed in OsO4 for 60 min, then dehydrated in a 80-100% ethanol series and critical point dried in CO2. Coverslips were sputter coated with gold and examined using a JEOL JSM-5800 LV (JEOL USA, Inc., Peabody, Massachusetts) at 10 kV. Approximately 25 mg of infected kidney tissue was used for DNA extraction using the Qiagen DNAeasy Blood & Tissue Kit following the manufacturer’s instructions (Qiagen Inc., Valencia, California). PCR was performed in 50-μl reaction volumes in Quick-Load Taq 2X Master Mix (New England Biolabs, Ipswich, Massachusetts), 0.25 μM of each primer, and 3 μl of template DNA. A first round amplification used primers 18E and 18R (Whipps et al., 2003) followed by a second round of PCR with MyxospF (Fiala, 2006) and 18R. Amplifications were performed on a C1000™ Thermal Cycler (BioRad Laboratories, Hercules, California) with initial denaturation at 95 °C for 3 min followed by 35 cycles of 94 °C for 30 sec, 56 °C for 45 sec, 68 °C for 90 sec, and a final extension at 72 °C for 7 min. Product amplification was evaluated by observation on a 1% agarose gel and the remainder of the sample was purified using the E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek, Norcross, Georgia). DNA was quantified using a DNA spectrophotometer (NanoDrop Technologies Wilmington, Delaware). Direct sequencing was performed using the primers MyxOF, 18R, and MyxgenF (Diamant et al., 2004). Reactions were carried out with the ABI BigDye® Terminator Cycle Sequencing Ready Reaction Kit v3.1 using the ABI3730xl Genetic Analyzer (Applied Biosystems, Foster City, California).

Sequences were assembled in BioEdit (Hall, 1999) and verified as myxozoan by GenBank BLAST search. To estimate the phylogeny of this species, 102 sequences were aligned with Clustal X v. 1.8 (Thompson et al., 1997). The alignment consisted of the top 25 BLAST search matches and representatives of neighboring clades based on earlier phylum (Fiala, 2006) and family level analyses (Liu et al., 2010). Phylogenetic analysis was carried out on this 1,763 character alignment as follows. Optimal evolutionary models for maximum likelihood (ML) and Bayesian analysis (BA) were determined using jModeltest (Posada, 2008), which identified the optimal evolutionary model using the Akaike information criteria as the general time reversible model (GTR+I+G). Nucleotide frequencies were estimated from the data (A = 0.2286, C = 0.1710, G = 0.2882, T = 0.3121). Six rates of nucleotide substitution were estimated. Bootstrap confidence values were calculated with 100 replicates. Bayesian ML analysis was performed using PhyML (Guindon and Gascuel, 2003).
analyses were conducted in Mr. Bayes (Ronquist and Huelsenbeck, 2003) using the evolutionary model as above, with 10^6 generations, tree sampling every 100 generations, and a burn-in of 100 trees. Trees were initially examined in TreeView X (Page, 1996) and edited and annotated in Adobe Illustrator (Adobe Systems Inc., San Jose, California).

**DESCRIPTION**

**Family Myxobilatidae Shulman 1953**

*Amended diagnosis:* Spores elongated, with 2 striated valves, and 2 polar capsules at 1 pole of the spore in a plane perpendicular to that of suture. Spores with or without caudal projections or filaments. Sporoplasm with iodophilic vacuole. Vegetative form, plasmodia. Parasites of uriniferous tubules of kidney and urinary bladder. Family includes 3 genera: *Hoferellus*, *Myxobilatus*, and *Acauda* n. gen.

*Acauda* n. gen.

*Diagnosis:* Myxospores with longitudinally ridged valves, pyriform or miter-like in sutural and valvular views, round to nearly round in apical view. Valvular extensions and filaments absent. Two polar capsules, each situated on either side of sutural plane. Polysporous plasmodia in coelomic spaces of kidney with asynchronous development of spores.

**Acauda hoffmani** n. gen., n. sp. (Figs. 1-4)

*Diagnosis:* Coelozoic polysporous plasmodia in kidney tubules and collecting ducts containing spores at multiple stages of development. Macroscopic plasmodia white, round, with distinct margin, approximately 250–500 μm. Microscopic plasmodia within tubules approximately 30 μm or greater. Spores pyriform in sutural and valvular view, round in apical view. Occasional spore with less-rounded posterior. Two equal shell valves, each with 11–12 ridges, bisected by straight suture running longitudinally to spore. Two polar capsules equal in length, pyriform in sutural view, slightly curved in opposite directions in valvular view. Polar filament coiled 10–15 times, with most (31/40 counted) having 11 or 12 coils. Rarely, spores contain 3 to 4 polar capsules. Sporoplasm at posterior
of spore. Mean spore dimensions in micrometers with number of measurements, standard error, and range in parentheses as follows: spore length 19.7 (n = 50, ±0.12, 17.9–21.8); spore width 8.5 (n = 50, ±0.13, 6.9–11.0); polar capsule length 11.1 (n = 63, ±0.08, 9.4–12.5); polar capsule width 3.4 (n = 60, ±0.04, 2.9–4.0).

Taxonomic summary

Type host: Lepomis macrochirus Rafinesque, 1819 (Teleostei, Centrarchidae).
Other hosts: Fundulus diaphanus (Lesueur, 1817) (Teleostei, Fundulidae).
Site of infection: Kidney, specifically the renal tubules.
Prevalence: Lepomis macrochirus 8 of 18 (44.4%); F. diaphanus 2 of 4.
Locality: Cazenovia Lake, Madison County, New York (42 ° 55.49'N, 75 ° 52.17'W).
Specimens deposited: Deposited at the Harold W. Manter Laboratory collection (HWML), University of Nebraska State Museum, Lincoln, Nebraska. Syntaxype hematoxylin and eosin-stained slide of bluegill kidney tissue containing plasmodia with spores, HWML 49552; infected kidney tissue from same fish in 95% ethanol, HWML 66691. GenBank Accession number for partial ssrDNA sequence, HQ913566.

Etymology: The specific name 'hoffmani' is given in honor of Dr. Glenn Hoffman, world renowned fish parasitologist who informally proposed the name 'Acauda' in his extensive survey "Parasites of North American Freshwater Fishes" (Hoffman, 1999).

Remarks

Acauda hoffmani n. gen., n. sp. bears spores resembling Acauda elongata (Kudo 1919) in overall form, but the 2 species may be distinguished as follows. Spores of A. elongata are shorter in length and width than those of A. hoffmani, although some reduction in myxospor size might be expected as a result of fixation prior to measurement (Parker and Warner, 1970) in the case of A. elongata. The polar capsules of A. hoffmani are much larger (11.1 × 3.4 μm) than those of A. elongata (7.5 × 2 μm), a difference well beyond what might be expected as a result of fixation. In addition, the new species and A. elongata differ in the number of valve ridges and polar capsule filament coils they possess, with 11–12 versus 14–16 ridges, and 10–15 versus 7–8 coils, respectively. The polar capsules of A. hoffmani are equal in length but, in many cases, when both capsules could be measured from a single spore, 1 capsule was slightly longer than the other. Statistically, the lengths were not different, and this subtle difference may have more to do with the curvature of the polar capsules, 1 toward, and 1 away from the examiner in sutural view (Figs. 1D, E, 2B), than with a true difference in length.

Phylogenetic analysis of partial ssrDNA sequence from A. hoffmani places this species within a cluster of species with similar myxospor body types (Fig. 3). Both Myxobilatus gasterostei and Hoferellus gilsoni bear spores with longitudinal valve striations and a suture line that bisects the spore. However, these genera are easily distinguished from Acauda in having either long tails (Myxobilatus) or filaments (Hoferellus) projecting from the posterior of the spore.

In wet mount preparations, A. hoffmani plasmodia were often observed within the renal tubules (Fig. 1F). Yet grossly, visible cysts were seen and, in histological sections, microscopic plasmodia were encapsulated within the tissue (Fig. 4). Similar observations were made for A. elongata by Kudo (1919), who noted the presence of the parasite primarily in tubules in the early summer, then the more-regular occurrence of conspicuous cysts throughout the kidney 5–6 wk later. It is unclear whether the parasites expand within the tubules and become encapsulated or invade the tissue. Other myxozoa, like Parvicapsula minibicornis, begin their development histozoically and then migrate into the lumen of the kidney tubules (Kent et al., 1997). Although it is speculative, the reverse is likely true for Acauda species because sister genera are primarily coelozoic.

EMENDED SPECIES DESCRIPTION

Acauda elongata (Kudo 1919) n. comb.

Syn. Mitraspora elongata Kudo 1919, Hoferellus elongata Lom 1986

Diagnosis: In urinary tubules and kidney tissue of green sunfish (Lepomis cyanellus), Crystal Lake, Urbana, Illinois. Spores oblong with pointed anterior and truncated posterior, nearly circular in apical view. Two shell valves each with 14–16 striations. Preserved spore dimensions: length 15–17 μm, width 5–6 μm, thickness 4.5–5.5 μm. Two polar capsules, mostly equal, pyriform, and elongate, measuring 7.5 μm by 2 μm. Polar filament coiled 7 to 8 times, 40–50 μm when extruded.

DISCUSSION

Acauda hoffmani n. gen., n. sp. occurs in the kidneys of 2 freshwater fish species in New York and possesses spores morphologically distinct from any previously described myxosporan genus. Another previously described species with similar spores from green sunfish in Illinois was classified as Mitraspora elongata by Kudo (1919). However, when Mitraspora was designated a junior synonym to Hoferellus by Lom (1986), Kudo's species became Hoferellus elongata, although Lom made no specific mention of this species. The description of Hoferellus does not

FIGURE 2. Line drawing of Acauda hoffmani n. gen., n. sp. spores. (A) Internal features of spore in sutural view. (B) Internal features of spore in valvular view. (C) Spore surface in sutural view. Bar = 5 μm.

FIGURE 3. Scanning electron micrograph of Acauda hoffmani n. gen., n. sp. showing detail of ridged valve surface. Bar = 5 μm.
In deciding whether to assign this species to a previously described genus such as Hoferellus or Myxobilatus, or to the newly proposed Acauda, several lines of evidence were considered. First, and foremost, the descriptions of Hoferellus and Myxobilatus do not accommodate the current species because it lacks posterior projections. The taxonomic value of posterior projections on spores across the myxozoans is not known. In the case of Henneguya (with tails) and Myxobolus (without tails), this character is phylogenetically uninformative, as is demonstrated by the analysis of dozens of species within the Myxobilidae (Fig. 5; Kent et al., 2001; Fiala and Bartošová, 2010; Liu et al., 2010). This does not reduce the potential value of posterior extensions in other suborders of Myxosporea, and in absence of evidence to the contrary for Acauda, the utility of these projections must be considered a real possibility. The second major point to consider is, if presence of projections were to be discounted, to what genus should the current species be accurately assigned? Acauda hoffmani is sister to Myxobilatus gasterostei and part of a phylogenetic group also containing Hoferellus gilsoni (Fig. 5). Ignoring posterior projections altogether might suggest that all species are Hoferellus by priority, or perhaps only Acauda and Myxobilatus should be synonymous. Such hypotheses were not tested here and, therefore, the most conservative approach is to describe a new genus. Acauda does not disrupt the monophyly of any other genus, and revision of either Hoferellus or Myxobilatus would be arbitrary. It may be that future research recommends synonymization of some or all of these species, but the current data cannot reject the hypothesis of 'Acauda' as a distinct taxon.

Phylogenetically, A. hoffmani is most closely related to Hoferellus and Myxobilatus species (Fig. 5). All genera share common features of longitudinally striated spore valves and a sutural plane that bisects a pair of polar capsules. The 3 species that represent these genera form a genetic cluster distinct from all other myxosporeans, supporting the monophyly of the group. Current classification schemes (Lom and Dyková, 2006) include both Hoferellus and Myxobilatus in the Sphaerosporidae Davis, 1917, creating some taxonomic complications as a result. The true sphaerosporids species form an early branching lineage in the Myxosporea (Fiala, 2006; Holzer et al., 2007; Jirků et al., 2007) that is separate from other members of the class. In addition, the description of Sphaerosporidae by Davis (1917) that includes "spores pyramidal or approximately spherical; not distinctly longer than wide; with or without lateral processes," does not readily accommodate the genera under consideration here. Thus, reassignment of these genera is warranted. The Myxobilatidae Shulman 1953 (Shulman 1966) was proposed to accommodate Myxobilatus species, and Molnár (1988) supported the inclusion of Hoferellus in this family as well, based on similar spore development observed in the 2 genera. The description of the family requires modest modification to include all genera, however. Specifically, Shulman (1966) included species with "spores with 2 caudal appendages," which has been modified here to spores that may, or may not, have caudal appendages or filaments.

Considering other potential familial relationships here, the myxobilatids are sister to a species cluster consisting of a Chloromyxum species (Chloromyxidae) and Myxidium and Zschokkeella species (Myxidiidae) (Fig. 5). Assignment of Acauda, Hoferellus, or Myxobilatus to any one of these already paraphyletic families would only further complicate myxozoan
FIGURE 5. Phylogenetic tree generated from Bayesian analysis of small subunit ribosomal RNA gene sequences. With the exception of the Myxobilatidae, none of the remaining ingroup taxa (genera, families, suborders) is monophyletic. Genbank accession numbers are listed adjacent to species names. Support values in percent units at branching points are listed as: Bayesian posterior probabilities/bootstrap values from ML analysis. Asterisks are shown where values exceeded 95%. Dashes are shown for values under 65%. Embedded line drawings show stereotypical spore morphologies for the 3 genera of the Myxobilatidae.
classification. In an earlier analysis (Fiala, 2006), these species clustered within the Myxobilatidae, but that relationship was not supported here or in other estimates of phylogeny (Holzer et al., 2007; Jirků et al., 2007). At the suborder level, the Myxobilatidae fits well into the description of the suborder Variisporina. Although the Variisporina is paraphyletic, with the Platyisporina nested within this clade as evidenced here and in previous evaluations (Kent et al., 2001; Jirků et al., 2006), reconciliation of this taxon is beyond the scope of the present study.

The classification of the Myxozoa is based heavily on the overt characteristics of myxospores. This is despite the lack of support for monophyly of some of these taxa through phylogenetic analyses (Kent et al., 2001; Fiala, 2006). Still, morphological classification is a practice of necessity, as molecular data are not always available; archival specimens of type tissues are not preserved in a manner amenable to DNA analysis, and morphological features that are consistent with molecular phylogenies have yet to be found. Within subclades, there are some tendencies toward host groupings or tissue specificity, but these cannot be applied universally (Ferguson et al., 2008). Members of the Myxobilatidae, for example, are mostly coelozoic in the urinary system. However, in their summary of myxozoans from African fishes, Fomena and Bouix (1997) listed 2 Myxobilatus species (Myxobilatus accessobranchialis and Myxobilatus synodontis) from other tissues, although the authors questioned these generic assignments. In the absence of available type material, additional collections and analyses are required to determine if there is yet another exception to the rule or inadequate morphological features that are consistent with molecular phylogenies have yet to be found. Within subclades, there are some tendencies toward host groupings or tissue specificity, but these cannot be applied universally (Ferguson et al., 2008). Members of the Myxobilatidae, for example, are mostly coelozoic in the urinary system. However, in their summary of myxozoans from African fishes, Fomena and Bouix (1997) listed 2 Myxobilatus species (Myxobilatus accessobranchialis and Myxobilatus synodontis) from other tissues, although the authors questioned these generic assignments. In the absence of available type material, additional collections and analyses are required to determine if there is yet another exception to the rule or inadequate morphological features that are consistent with molecular phylogenies have yet to be found. Within subclades, there are some tendencies toward host groupings or tissue specificity, but these cannot be applied universally (Ferguson et al., 2008). Members of the Myxobilatidae, for example, are mostly coelozoic in the urinary system. However, in their summary of myxozoans from African fishes, Fomena and Bouix (1997) listed 2 Myxobilatus species (Myxobilatus accessobranchialis and Myxobilatus synodontis) from other tissues, although the authors questioned these generic assignments. In the absence of available type material, additional collections and analyses are required to determine if there is yet another exception to the rule or inadequate morphological features that are consistent with molecular phylogenies have yet to be found. Within subclades, there are some tendencies toward host groupings or tissue specificity, but these cannot be applied universally (Ferguson et al., 2008). Members of the Myxobilatidae, for example, are mostly coelozoic in the urinary system. However, in their summary of myxozoans from African fishes, Fomena and Bouix (1997) listed 2 Myxobilatus species (Myxobilatus accessobranchialis and Myxobilatus synodontis) from other tissues, although the authors questioned these generic assignments. In the absence of available type material, additional collections and analyses are required to determine if there is yet another exception to the rule or inadequate morphological features that are consistent with molecular phylogenies have yet to be found. Within subclades, there are some tendencies toward host groupings or tissue specificity, but these cannot be applied universally (Ferguson et al., 2008). Members of the Myxobilatidae, for example, are mostly coelozoic in the urinary system. However, in their summary of myxozoans from African fishes, Fomena and Bouix (1997) listed 2 Myxobilatus species (Myxobilatus accessobranchialis and Myxobilatus synodontis) from other tissues, although the authors questioned these generic assignments.


**DEVELOPMENT AND EVALUATION OF REAL-TIME PCR ASSAY FOR THE DETECTION OF BABESIA ORIENTALIS IN WATER BUFFALO (BUBALUS BUBALIS, LINNAEUS, 1758)**


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**ABSTRACT:** *Babesia orientalis* is the causative agent of babesiosis in water buffalo (*Bubalus bubalis*, Linnaeus, 1758). In this study, a TaqMan real-time PCR assay was developed for quantitative detection of *B. orientalis* in water buffalo. Hybridization probe and oligonucleotide primers were designed based on the v4 region of 18S rRNA gene. Detection limit was determined at 2 parasites. Blood samples were collected from experimentally infected water buffalo, as well as from 180 field samples, which were collected from 4 different geographical locations to the north and south of the Yangtse River. The parasite was detected by real-time PCR on day 2 until day 39 post-infection, while reverse line blot (RLB) was on day 6 until day 36 in experimentally infected water buffalo. For the results of 180 field samples, statistical analysis showed no significant difference in relative effectiveness of real-time PCR and RLB. The analysis also indicated that there was no difference in the prevalence of *B. orientalis* between the regions of south and north of the Yangtse River by both the real-time PCR assay and RLB detection. These results indicated that the parasite infection has spread to the north of the Yangtse River.

Water buffalo babesiosis caused by *Babesia orientalis* is one of the most important diseases of water buffalo in central and south China, resulting in enormous economic losses. The disease is characterized by fever, anemia, icterus, hemoglobinuria, and high mortality (Liu et al., 1986, 1997; Liu et al., 2005). The only reported mammalian hosts for *B. orientalis* are water buffalo (Liu et al., 1997). *Rhipicephalus haemaphysaloides*, which is widely distributed in central and south China, is the only reported tick vector for *B. orientalis*. This tick transmits the parasite to water buffalo transovarially (Liu et al., 1997).

Several useful methods for detecting *B. orientalis* have been developed, but most of them have limitations. Microscopical examination of thin blood smear is a traditional diagnostic test, but it is difficult to discriminate between previous infections and current infections (Baoan et al., 2002; He et al., 2009; Zhou et al., 2009).

Molecular-based diagnostic tests have higher sensitivity and specificity than those of serological methods and can detect parasites with very low parasitemia (Bhoora et al., 2010). Recently molecular techniques based on species-specific PCR assays targeting 18S rRNA gene have been proven very useful for the detection and classification of piroplasms, such as those in the Theileria/Babesia group, including 18S rRNA-based nested PCR (Altay et al., 2005); real-time PCR, which is reported to be highly sensitive and quantitative (Kim et al., 2007; Bhoora et al., 2008); and reverse line blot (RLB), which is very powerful for the identification of novel genotypes or species and for the detection of mixed infections (Gubbels et al., 1999). Previously, quantitative real-time PCR has been widely used to detect various hemoparasites: for example, the detection of *Anaplasma marginale* in cattle targeting msplb gene (Carelli et al., 2007), *Ehrlichia ruminantium* in livestock blood and ticks from the field targeting pC520 gene (Steyn et al., 2008), *Theileria sergenti* from the blood samples of cattle based on p33 gene (Jeong et al., 2003), *Babesia bovis* and *Babesia bigemina* in bovine based on cytochrome b gene and 18S rRNA gene (Buling et al., 2007; Kim et al., 2007), and *Babesia caballi* and *Theileria equi* from equines and *Theileria parva* from Cape buffalo based on the 18S rRNA gene (Kim et al., 2008; Sibeko et al., 2008; Bhoora et al., 2010). However, using molecular approaches, a semi-nested PCR was developed for investigating the epidemiology and enzootic potential of *B. orientalis* in Hubei Province (Liu et al., 2007). Then, a loop-mediated isothermal amplification (LAMP) assay based on the v4 hypervariable region of 18S rRNA gene was established with high sensitivity and specificity (He et al., 2009). However, none of the above methods is quantitative.

In order to investigate the parasitemia in field and experimentally infected animals, a TaqMan real-time PCR assay was developed for detecting *B. orientalis* in water buffalo blood samples. The present study will provide significant scientific data to correlate the clinical symptoms of infection with parasitic load in the future.

**MATERIALS AND METHODS**

**Collection of blood samples**

Two 1-yr-old water buffaloes, free of *B. orientalis* infection as confirmed via microscopy and semi-nested PCR (Liu et al., 2007), were splenectomized 14 days prior to *B. orientalis* infection. Each buffalo was subcutaneously injected with 4 ml of *B. orientalis*-infected blood (percentage parasitized erythrocytes [PPEs] 1%). Blood samples were collected every day in the first wk and every 3 days until 39 days post-infection (PI) and subjected to RLB and real-time PCR assay, respectively. Additionally, thin blood smears were prepared, stained with Giemsa, and examined for the presence of hemoparasites. Blood samples containing *B. orientalis* Wuhan strain, with a PPE of approximately 1%, was used as the gold standard positive control. A total of 180 water buffalo blood samples were collected from south and north of the Yangtse River, including 66 from Daye located to the south and 43, 45, and 26 from Hongan, Suizhou, and Xiaogan located to the north of Yangtse River, respectively. All the samples were collected in EDTA and then were directly used for DNA extraction or stored at −20°C until use.

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TABLE I. Primers and probes used for the real-time PCR of Babesia orientalis and reverse line blot.

<table>
<thead>
<tr>
<th>Method</th>
<th>Primer and probe</th>
<th>Sequences (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>Real-time PCR</td>
<td>Bo-F</td>
<td>GTGCCGTGTGCTTTTGT</td>
</tr>
<tr>
<td></td>
<td>Bo-R</td>
<td>GCCTGCTTGAACACTCAAT</td>
</tr>
<tr>
<td>Reverse</td>
<td>Bo-P</td>
<td>FAM-CCGTCTCATTGGTTTTTATT-TAMRA</td>
</tr>
<tr>
<td>line blot</td>
<td>RLB-F2</td>
<td>GACACGGAGGTAGTGACAG</td>
</tr>
<tr>
<td></td>
<td>RLB-R2</td>
<td>Biotin-CTTACAGTTTCACCTGTACAGT</td>
</tr>
<tr>
<td></td>
<td>RLB_B. orientalis</td>
<td>CTTCTTTTGCCGGCTCTCAG</td>
</tr>
</tbody>
</table>

DNA extraction

Genomic DNA of all samples was extracted from 200 µl of blood using the QIAamp blood and tissue extraction kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. DNA was eluted in 100 µl elution buffer and stored at −20°C until further analysis.

Reverse line blot

The v4 hypervariable region of 18S rRNA gene was amplified by touchdown PCR using the Theileria and Babesia genus-specific primers RLB-F2 and RLB-R2 (Gubbels et al., 1999). The resulting PCR products were analyzed with RLB following the procedure of Gubbels et al. (1999). For the detection of B. orientalis, a species-specific oligonucleotide probe was designed and used. Table I shows primers and oligonucleotide probe sequences for RLB.

Real-time PCR assay

Real-time PCR primers and TaqMan probe: For the quantitative real-time PCR assay, a forward primer (Bo-F: 5'- GTG CGT GTG CCT TTG TTG -3'), a reverse primer (Bo-R: 5'-GCC TGC TTG AAA CAC TCT GTG CGT GTG CCT CTT TTG -3'), a reverse primer (Bo-R: 5'-GCC TGC TTG AAA CAC TCT GTG CGT GTG CCT CTT TTG -3'), and a TaqMan probe (Bo-P: 5'-FAM-CCGTCTCATTGGTTTTTATT-TAMRA) were designed and used. Table I shows primers and oligonucleotide probe sequences for RLB.

Reaction conditions: The TaqMan real-time PCR assay was performed in an Applied Biosystems 7500. The PCR reaction was prepared in a total volume of 25 µl, consisting of 1 µl of DNA template, 12.5 µl of TaqMan Master Mix (TOYOBO, Tokyo, Japan), 200 µM of each primer, and 100 µM of the TaqMan probe. The PCR reaction was performed in a 96-well optical plate (AXYGEN, Foster City, California) to specifically amplify a 75 bp fragment of the v4 hypervariable region of 18S rRNA gene of B. orientalis (AY596279).

Detection of Babesia orientalis in field samples

To evaluate applicability of the real-time PCR assay, 180 field water buffalo samples, which were collected from Hubei Province, were subjected to lO-fold serial dilutions and subjected to real-time PCR, and a typical amplification was demonstrated by the real-time PCR assay using experimentally infected water buffalo samples.

Real-time PCR was performed in a total volume of 25 µl, consisting of 1 µl of DNA template, 12.5 µl of TaqMan Master Mix (TOYOBO, Tokyo, Japan), 200 µM of each primer, and 100 µM of the TaqMan probe. The PCR reaction was performed in a 96-well optical plate (AXYGEN, Foster City, California) under the following conditions: 2 min at 50°C to achieve optimal Amplifluce uracil-N-glycosylase activity, 10 min at 95°C, and then 40 cycles of 15 sec at 95°C, and 1 min at 65°C. All reactions included a negative control, genomic DNA extracted from blood of uninfected water buffalo as negative control, and genomic DNA extracted from blood of B. orientalis–infected water buffalo (PPE 1%) as a positive control. The collected fluorescence data were analyzed using Applied Biosystems Sequence Detection Software (v. 1.3.1).

Sensitivity and specificity of real-time PCR: Babesia orientalis Wuhan strain with PPE approximately 1% (about 2.0 × 10^8 parasites/µl) was subjected to 10-fold serial dilutions (2.0 × 10^8 to 2.0 × 10^2 parasites/µl) using non-infected water buffalo RBC. Genomic DNA was extracted in triplicate from each diluted sample, subjected to real-time PCR, and a standard curve was generated. In order to evaluate the specificity of the established real-time PCR assay, genomic DNA of B. orientalis, B. gibsoni, B. bovis, T. sergenti, and T. buffeli were extracted from PPE 1% blood and subjected to amplification. Babesia orientalis and B. gibsoni are normally reported from buffaloes and dogs, respectively, while B. bovis, T. sergenti, and T. buffeli can infect both cattle and buffaloes. Control DNA from blood of an uninfected water buffalo and a PCR negative control were included.

Statistical analysis

In order to compare the parasite detection ability of the real-time PCR and RLB, and the area prevalence of B. orientalis, cross-tabulation of categorical data and a chi-square test were carried out using SAS (v. 8.0; SAS Institute, Cary, North Carolina). The test results were considered significantly different when P < 0.01.

RESULTS

Sensitivity and specificity of the Babesia orientalis real-time PCR assay

The detection limit of assay was determined from the sensitivity curve. The cycle threshold (Ct) values plotted against the log of 2 × 10^0 to 2 × 10^6 B. orientalis parasites were significantly linear (r^2 = 0.9956) and allow for the quantification of the parasite within this range. Detection limit was established as 2 × 10^0 (corresponding to 2) B. orientalis parasites. As shown in Figure 1, 2 to 200,000 parasites were detected by the real-time PCR assay. For the specificity, a typical amplification was demonstrated by B. orientalis DNA, whereas no positive signal was observed from the negative control, uninfected water buffalo blood DNA, and DNAs from B. bovis, B. gibsoni, T. sergenti, and T. buffeli DNAs (data not shown).

Evaluation of real-time PCR assay using experimentally infected water buffalo samples

Babesia orientalis was detected by real-time PCR on day 2 PI (data not shown). With the RLB and microscopy, B. orientalis was first detected on days 6 and 9, respectively. Using the real-time PCR, B. orientalis was consistently detectable up to day 39 PI in experimentally infected buffaloes, whereas RLB could only detect the parasite until day 36 PI. However, it was difficult to consistently detect the parasite by microscopic examination from day 24 PI.
in China, were tested by the real-time PCR assay and RLB. As shown in Table I, 28 and 21 samples were found positive by real-time PCR and RLB, respectively. Although the real-time PCR showed a higher proportion (15.6%) of positive samples than RLB assay (11.7%), the difference was not statistically significant ($\chi^2 = 1.6312$, $P = 0.2218$).

To compare the prevalence of *B. orientalis* to the north and south of the Yangtse River, statistic analysis was performed. As showed in Table I, the prevalence of *B. orientalis* was higher to the north of the Yangtse River (18.2%, 12/66) than that to the south (14.0%, 16/114) by real-time PCR. Similarly, RLB detected higher prevalence in the north (13.6%, 9/66) and lower to the north (10.5%, 12/114) of Yangtse River. However, statistical analysis indicated that the prevalence of *B. orientalis* by both real-time PCR and RLB was not significant ($\chi^2 = 0.5238$, $P = 0.4595$ and $\chi^2 = 0.6310$, $P = 0.5311$, respectively).

The quantity of parasites estimated in the positive samples using real-time PCR were as follows: from $2.0 \times 10^5$ to $2.0 \times 10^6$ parasites/μl in 6 samples collected from Hongan, from $2.0 \times 10^3$ to $2.0 \times 10^4$ parasites/μl in 9 samples collected from Suizhou, and from $2.0 \times 10^4$ to $2.0 \times 10^6$ parasites/μl in 12 samples collected from Daye. However, only 1 positive sample collected from Xiaogan was estimated to be from $2.0 \times 10^5$ to $2.0 \times 10^3$ parasites/μl (Table II).

**DISCUSSION**

Buffalo babesiosis is a serious problem; it was first reported in 1984 with a high prevalence in 9 provinces of China (Chen, 1984). This pathogen was originally considered as *B. bovis* and *B. bigemina* due to the similar morphology and pathogenicity. However, in 1997, it was definitively identified as *B. orientalis* (Liu et al., 1997). Many molecular diagnostic methods have been developed to detect this parasite in water buffaloes, i.e., nested-PCR, LAMP, and RLB (Liu et al., 2007; He et al., 2009), but still some molecular approaches are required to establish the identification and quantification of *B. orientalis*, such as real-time PCR. In the present study, a TaqMan real-time PCR was developed to detect and quantify the parasite. Moreover, this is the first study in which quantitative PCR was used to investigate the epizootiology of *B. orientalis* in China.

The 18S rRNA gene is highly conserved, evolutionarily stable with limited intra-species sequence variation (Criado et al., 2006). One of the important evaluation standards for the successful development of a DNA detection assay is possibly to amplify an appropriate gene target. For *B. orientalis*, the v4 region of 18S rRNA gene was successfully used as target gene in various detection methods, with high sensitivity and specificity, including semi-nested PCR and loop-mediated isothermal amplification (LAMP) methods (Liu et al., 2007; He et al., 2009). Therefore, in the present study, the v4 region of 18S rRNA was again used as a target gene in real-time PCR.

It is difficult to detect hemoparasites in the early stage of infection and in carrier animals by microscopic examination of blood smears (Calder et al., 1996; Almeria et al., 2001). However, considering the sensitivity, real-time PCR was found to be a powerful tool for the detection of this parasite in both early and later stages. Positive signals were observed on day 2 PI and until 39 days PI in the experimentally infected water buffaloes, whereas the RLB and microscopy could only detect the parasite on days 6 and 9 PI in the early stage, and until on days 21 and 36 PI in the later stages, respectively. There were 28 (15.6%) positive samples tested by real-time PCR, while only 21 (11.7%) positive samples were detected by RLB (Table III), which is consistent with our finding of the higher sensitivity of real-time PCR compared to RLB. For the specificity, only the DNA of *B. orientalis* Wuhan strain was amplified, but DNAs of other *Babesia* or *Theileria* species endemic in China, including *B. gibsoni*, *B. bovis*, *T. sergenti*, and *T. buffeli*, did not show any signal, indicating that the present developed real-time PCR is highly specific for the detection of *B. orientalis*.

In a previous report, the LAMP assay tested 31.2% *B. orientalis*-positive animals to the south of Yangtse River, which was much higher than 6.8% to the north (He et al., 2009). In the present study, the real-time PCR results showed that 14.0% (16/114) and 18.2% (12/66) samples were positive, which were collected from the north and south of the Yangtse River, respectively. Statistical analysis indicated that the prevalence difference of south and north to the Yangtse River was not significant by the real-time PCR assay. Similar results were obtained via RLB detection. These results confirmed the previous speculation, i.e., that the natural barrier of Yangtse River was breached and the epidemic areas of *B. orientalis* were expanded.

<table>
<thead>
<tr>
<th>Table II. Quantities of <em>B. orientalis</em> in field water buffalo samples.</th>
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<tbody>
<tr>
<td><strong>Quantity</strong></td>
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<tr>
<td>$2.0 \times 10^5 - 2.0 \times 10^6$</td>
</tr>
<tr>
<td>$2.0 \times 10^4 - 2.0 \times 10^5$</td>
</tr>
<tr>
<td>$2.0 \times 10^3 - 2.0 \times 10^4$</td>
</tr>
<tr>
<td>$2.0 \times 10^2 - 2.0 \times 10^3$</td>
</tr>
<tr>
<td>$2.0 \times 10^1 - 2.0 \times 10^2$</td>
</tr>
<tr>
<td>Total</td>
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</tbody>
</table>

* Quantities are expressed as the number of *B. orientalis* parasites per microliter of infected blood.

<table>
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<tr>
<th>Table III. Detection of <em>Babesia orientalis</em> from water buffalo field samples using the real-time PCR and reverse line blot (RLB).</th>
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<tbody>
<tr>
<td><strong>North of Yangtse River</strong></td>
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<tr>
<td>Real-time PCR</td>
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<td>RLB</td>
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Accordingly, it is imperative to develop a highly sensitive, quantitative diagnostic method for the early detection of B. orientalis.

RLB detected about 40 samples with different species-specific probes at a time, while real-time PCR can quantitatively assess the parasite in an individual host. The latter methodology was also useful for quickly testing the cases that microscopic findings could not differentiate, as well as for the diagnosis at early stages when the parasitemia was very low. In veterinary clinics, confirmatory diagnosis of buffalo babesiosis by PCR could achieve timely and effective treatment (Zhou et al., 2009). Generally, the TaqMan real-time PCR assay and RLB, used in combination, should improve epizootiological surveys and clinical diagnosis of B. orientalis.

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LITERATURE CITED


Human Infections with *Dicrocoelium dendriticum* in Kyrgyzstan: The Tip of the Iceberg?

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These authors contributed equally to the work reported here. †Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland; ††University of Basel, Basel, Switzerland; ‡‡Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, U.K.; ‖Department of Pathology and Animal Health, Faculty of Veterinary Medicine, University of Naples Federico II, CREMOPAR Regione Campania, Naples, Italy. †To whom correspondence should be addressed. e-mail: peter.steinmann@unibas.ch

**ABSTRACT:** *Dicrocoelium dendriticum* is the causative agent of a rare food-borne zoonosis of the human biliary tract, dicroclosis, for which few human prevalence data are available. Infection occurs through the ingestion of ants containing metacercariae, whereas pseudo-infections (presence of *D. dendriticum* eggs in stool in the absence of adult worms) are due to the consumption of infected animal liver. Here, results from a cross-sectional survey carried out among 138 children aged 2–15 yr in a peri-urban area of Kyrgyzstan are reported. Each child provided 1 stool sample that was subjected to the FLOTAC technique. Eggs of *D. dendriticum* were diagnosed in 11 children (prevalence 8.0%; 95% confidence interval 4.5–13.7%). Although no distinction could be made between true and pseudo-infections, the prevailing animal husbandry system and the diet and hygiene conditions of the study area suggest that the social-ecological system in Kyrgyzstan is conducive for human transmission of *D. dendriticum*. There is a need to investigate the epidemiology of dicroclosis in Kyrgyzstan, placing emphasis on the distinction between true and pseudo-infections.

The trematode *Dicrocoelium dendriticum* (Rudolph, 1819) is a small lancet fluke parasitizing the small bile duct and gall bladder of various mammals, such as domestic and wild ruminants (e.g., buffalos, goat, sheep, and deer), rabbits, and, occasionally, dogs, horses, pigs, and humans. The life cycle is complex, with 2 intermediate hosts, namely, land snails and ants. Human infection, like that of the other definitive hosts, occurs by ingestion of ants containing the infective stage, i.e., metacercariae, which can be found on grass, herbs, raw fruit, and vegetables, and even in drinking water (Haridy et al., 2003). Pseudo-infections (i.e., presence of a diagnostic marker, here eggs, in the absence of the etiological agent, here adult *D. dendriticum*) are due to the consumption of infected ant parts. Palaeoepidemiological studies suggest that *D. dendriticum* has a history of at least 550,000 yr in Western Europe, whereas the parasite was only recorded in the New World around the 17th century, following the colonization of Canada by Europeans (Le Bailly and Bouchet, 2010). The current geographic distribution of *D. dendriticum* covers Europe (especially the Mediterranean area), Asia (in particular the Middle East, Indonesia, Japan, Malaysia, People’s Republic of China, and the former Soviet Union), North Africa, South America, and some foci in North America.

Dicroclosis is generally considered an infection of ruminants, with prevalence of infections usually higher in sheep (up to 100% in many Mediterranean and Middle Eastern areas) than in cattle and buffalo (Cringoli et al., 2002; Manga-González and González-Lanza, 2005; Rinaldi et al., 2009). The biology, diagnosis, epidemiology, etiology, pathogenesis, and treatment of dicroclosis in ruminants have been extensively reviewed (Otranto and Traversa, 2002, 2003). The availability of reliable data on this parasite in livestock contrasts sharply with the situation in humans, where infections are considered to be rare. Indeed, there are only few reports on the prevalence and incidence of this food-borne zoonosis in humans. Among the reported cases, the subclinical form predominates, but clinical cases of human dicroclosis have also been reported from different parts of the world, including Iran (Ashrafi, 2010) and Turkey (Otranto and Traversa, 2002). Fecal egg counts have been found positive for *D. dendriticum* eggs. In 8 children, a concurrent *Ascaris lumbricoides* infection was found, and in the feces of 3 children eggs of *Hymenolepis nana* were also present. In the stools of the remaining 5 children only *D. dendriticum* eggs were detected. The presence of *D. dendriticum* eggs showed no significant association with sex (4 boys versus 7 girls), nor with age (<8 yr-old; stratified into 2-yr classes). Fecal egg counts varied between 2 eggs per g (EPG) of stool and 818 EPG, with a median of 16 EPG. Fecal egg counts were not statistically significantly associated with sex and age.

In Kyrgyzstan, and elsewhere in Central Asia, there is growing interest in the epidemiology and control of human and animal helminthiases (Torgerson et al., 2003, 2009; Jeandron et al., 2010; Steinmann et al., 2010; Zeddinov et al., 2010). However, data for *D. dendriticum* in human and livestock populations are scant.

Estimates of the at risk-population, number of infections, and global burden are being assessed by expert groups for a number of food-borne trematodes, such as the liver flukes *Clonorchis sinensis*, *Fasciola hepatica* and *Opisthorchis viverrini*, and may include chronic diarrhea, constipation, and may include chronic diarrhea, constipation, liver disease, hepatic steatosis, portal hypertension, and cholangitis. In infected humans, complications may be related to immune-suppression; cases of patients infected with the human immunodeficiency virus (HIV) and *D. dendriticum* have been reported (Drabick et al., 1988; Zali et al., 2004). *Dicrocoelium dendriticum* eggs have been recently found in the stool of a patient with Crohn’s disease (Schweiger and Kuhn, 2008). In addition, acute urticaria has been reported as a complication of the infection (Sing et al., 2008). Most reports concern autochthonous populations in Europe and the Middle East and immigrants from North Africa (Gualdieri et al., 2011), although *D. dendriticum* has also been reported in humans from Canada (Schweiger and Kuhn, 2008). At present, there are only 2 drugs available to treat liver fluke infections, namely, praziquantel and triclabendazole. The former is the drug of choice against *D. dendriticum* infections (Rana et al., 2007).

Evaluating the public health impact of dicroclosis is complicated by the pseudo-infections. Some have said that such spurious infections can be readily distinguished from true infections on the basis of egg morphology (Blajin, 1930), but no recent example of the successful application of this method is known to the authors. *Dicrocoelium dendriticum* eggs are, however, readily distinguished from those of other trematodes endemic in Central Asia, such as *F. hepatica*. They have an asymmetrical oval shape and a mean measure approximately 40 × 25 μm, are of dark brown color, and have a smooth thick shell and an indistinct operculum (Fig. 1). Here, findings from a cross-sectional parasitological survey among children of a peri-urban area of Kyrgyzstan in Central Asia are reported. In this research note, emphasis is placed on *D. dendriticum* prevalence, whereas the broader study compared the diagnostic accuracy of different methods for the detection of helminth infections as reported by Jeandron and colleagues (2010).

The study setting, ethical considerations, enrollment of participants, and field, laboratory and data management procedures have been described elsewhere (Jeandron et al., 2010). In brief, within 3 wk, up to 3 stool samples were collected from 138 children (72 girls and 66 boys), aged 2–15 yr and living close to Bishkek, the capital of the country. The first stool collected from each child was preserved with 5% formalin at a dilution ratio 1:4 and subjected to the FLOTAC dual technique (Cringoli et al., 2010). FLOTAC preparation was performed with a hand centrifuge commercially available from Hettich (Tuttlingen, Germany), using the specific FLOTAC adapter. Eggs of *D. dendriticum* were counted using flotation solution no. 7 (FST; zinc sulfate with a specific gravity of 1.35).

Of the 138 fecal samples subjected to the FLOTAC dual technique, 11 (prevalence 8.0%; 95% confidence interval CI = 4.5–13.7%) were found positive for *D. dendriticum* eggs. In 8 children, a concurrent *Ascaris lumbricoides* infection was found, and in the feces of 3 of these children eggs of *Hymenolepis nana* were also present. In the stools of the remaining 5 children only *D. dendriticum* eggs were detected. The presence of *D. dendriticum* eggs showed no significant association with sex (4 boys versus 7 girls), nor with age (<8 yr-old; stratified into 2-yr classes). Fecal egg counts varied between 2 eggs per g (EPG) of stool and 818 EPG, with a median of 16 EPG. Fecal egg counts were not statistically significantly associated with sex and age.

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Estimates of the at risk-population, number of infections, and global burden are being assessed by expert groups for a number of food-borne trematodes, such as the liver flukes *Clonorchis sinensis*, *Fasciola spp.*, and *Opisthorchis spp.*, the lung fluke *Paragonimus spp.*, and intestinal flukes (*Echinostoma spp.* and *Heterophyidae*). (Keiser and Utzinger, 2009; Keiser

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et al., 2010). The global burden of dicrocoeliasis in humans has yet to be determined. Of note, in 2007, *D. dendriticum* has been included in the list of causative agents for which burden of disease estimates should be derived by Task Force 1 of the Foodborne Disease Burden Epidemiology Reference Group (FERG) of the World Health Organization (WHO, 2007).

The results of the present study are based on stool examination using the recently developed FLOTAC technique with FS7. The FLOTAC has been successfully validated for *D. dendriticum* diagnosis both for animals and humans (Gualdieri et al., 2011; Rinaldi et al., 2011). The prevalence of *D. dendriticum* eggs reported in the present study (8%) is higher than the prevalence of 1.8% found during a parasitological survey among school children in Osh oblast in south Kyrgyzstan (Steinmann et al., 2010). A possible explanation is that the FLOTAC dual technique used in the study described here is more sensitive than duplicate Kato-Katz thick smears with consecutive days or collect 3 or more stool samples over a period of 10 days with participants specifically instructed not to eat liver, or (2) examine duodenal or biliary fluid for *D. dendriticum* eggs (Wolfe, 2007). Alternatively, the method described in the cited report, suggesting visual distinction between true and pseudo-infection based on egg morphology were possible, should be revisited (Blajin, 1930). The social-ecological conditions in Kyrgyzstan seem permissive for true infections. The Kyrgyz economy relies heavily on agriculture, mainly animal husbandry. The number of small ruminants was estimated at about 4.2 million in 2009 (FAO, 2011), scattered in small-scale husbandry, with an average of 6 to 10 animals per owner (WFP, 2010). The Soviet inherited veterinary surveillance system, developed for concentrated breeding methods, is inappropriate for this new mode of production, and many owners never received proper training in animal care and cannot afford veterinary services. About half of the urban and all rural households have access to land for growing their own crops, vegetables, and fruits (WFP, 2010). Wild berries are also traditionally collected in summer. Access to tap water and adequate sanitation is lacking in rural areas, and washing raw vegetables before consumption is reported by only half of the households (Steinmann et al., 2010). Together, these conditions are conducive to a sustained *D. dendriticum* life cycle, accidental ingestion of ants by humans and, therefore, occurrence of true infections with *D. dendriticum*.

In conclusion, the present study calls for the investigation of the real epidemiological significance of *D. dendriticum* in humans in Kyrgyzstan, with a particular emphasis on distinguishing between true and pseudo-infections, infection pathways, and risk behavior. If a significant number of true infections are detected, the clinical presentation of infections and its public health importance need to be established. Moreover, awareness for this parasite would need to be raised among laboratory technicians involved in the diagnosis of intestinal parasites and among the wider medical community.

**LITERATURE CITED**


A cross-sectional coprolological survey of liver flukes in cattle and sheep from an area of the southern Italian Apennines. Veterinary Parasitology 108: 137–143.


Examination of a Virulence Mutant Uncovers the Ribosome Biogenesis Regulatory Protein of Toxoplasma gondii

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ABSTRACT: Several insertional mutants identified in a screen for Toxoplasma gondii that were defective in establishing a chronic infection had a common site of plasmid insertion. This insertion site was determined to be 43 bp upstream of the transcription initiation site of a gene whose predicted product has homology to ribosome biogenesis regulatory protein Rrs1p, an essential protein required for ribosome biogenesis in Saccharomyces cerevisiae. Northern blot analysis of this locus, termed TgRRSI, showed that in the C3 mutant, the full-length transcript is down-regulated and at least 1 new smaller transcript is present. Restoration of the intact predicted promoter and locus to TgRRSI insertional mutant strain C3 did not restore brain cyst formation to the levels of the parent strain. Epitope-tagged TgRRSI was found to localize to the parasite nucleus seen during murine infection in an area corresponding to the granular component region of T. gondii. TgRRSI can serve as a marker for the sub-nuclear granular component region of T. gondii.

Toxoplasma gondii is an obligate intracellular pathogen capable of establishing a chronic infection in any warm-blooded animal. Acquired by consumption of undercooked contaminated meats or produce, the parasite causes mild flu-like symptoms in immunocompetent hosts. In these cases, acute infection is followed by chronic asymptomatic infection of long-lived immune-competent hosts. Strain C3, which itself is defective in establishing chronic infection in mice (Montoya and Lieniesen, 2004), was used. This technique involved creation of strains identifiable by their tagged plasmid, followed by insertional mutagenesis of these strains with a ribosome biogenesis regulatory protein TgME49_120450 (Fig. 1D). The plasmid was located within a NotI site 904 bp upstream of the translational start site of TgME49_120440, and 472 bp upstream of the translational start site of the oppositely transcribed TgME49_120450 locus. Interestingly, the insertion site of the signature-tagged plasmid in C3 is identical to that of mutagenic vector plK47 in 72F2, another strain from the same family of mutants that also displayed a cyst-depletion defect (Craver et al., 2004). Identical insertion sites likely arose because of the use of NotI restriction enzyme-mediated insertion for plasmid integration during creation of the library (Frankel et al., 2005).

To assist in determining which gene may be impacted by the plasmid insertion, the transcriptional start site of the most proximal gene, TgME49_120450, was determined by rapid amplification of cDNA ends (RACE) using RNA derived from WT T. gondii (RLM-RACE kit; Invitrogen, Carlsbad, California). The transcriptional start site of TgME49_120450 was found to be 436 bp upstream of the predicted translational start codon, and 43 bp from the plasmid insertion site, likely affecting TgME49_120440 transcription. Because plasmid insertion occurred just upstream of the transcriptional start site of TgME49_120450, we sought to determine if transcription of the locus was affected in the C3 mutant. Total RNA derived from tachyzoites of C3 was compared to WT T. gondii for steady-state levels of TgME49_120450 transcript by northern hybridization (Figs. 1B, D). A transcript of 2.3 kb was detected in mutant and WT strains but was clearly less abundant in the C3 mutant. At least 1 new approximately 1 kb transcript hybridized in the C3 mutant, but not in the WT. When compared to the levels of transcript determined for T. gondii housekeeping gene α-tubulin (TUB1) as a loading control, the combined TgME49_120450 transcript levels from the bands of mutant C3 were equivalent to those of the single transcript found in the WT. This result indicates that the plasmid insertion is likely causing addition of an alternative transcriptional start site for the TgME49_120450 locus.

The TgME49_120450 transcript was mapped by RT-PCR, using random-hexamer primed cDNA from WT T. gondii (Superscript III First-Strand Synthesis System; Invitrogen, Carlsbad, California) and sequencing. The determined open reading frame consisted of 5 exons, making up a 960 bp coding region in agreement with the predicted gene architecture indicated by ToxoDB (Fig. 1D). The 5' UTR as determined by RACE was 436 bp, 32 bases longer than predicted by ToxoDB based on expressed sequence tag evidence. The 3' UTR was unable to be determined by RACE, though expressed sequence tag evidence suggests it is at least 75 bp downstream of the stop codon (ToxoDB). The 2.3 kb transcript size and 4 kb of predicted non-coding region of TgME49_120450 and the downstream locus suggest the 3' UTR is likely to be approximately 800 bp. The TgME49_120450 locus encodes a protein product of 319 amino acids and 35.7 kDa. This predicted protein was searched for conserved domains using Pfam v24.0 (http://pfam.janelia.org; Marchler-Bauer et al., 2007), which identified a ribosome biogenesis regulatory protein RRS1 domain (Pfam 04939) from amino acid 24 to 213 (expect value = 6.13 X 10^-35). Rrs1p of Saccharomyces cerevisiae is an essential nuclear protein that regulates ribosome synthesis by aiding in maturation of the 25S rRNA and assembly of the 60S ribosomal subunits (Tsuno et al., 2000; Miyoshi et al., 2002; Morita et al., 2002) and is required for export of the 60S subunit from the nucleolus to the cytoplasm (Miyoshi et al., 2004; Zhang et al., 2007). The protein product of TgME49_120450 was compared to the non-redundant protein sequence database by protein BLAST (http://www.ncbi.nlm.nih.gov/BLAST/; Johnson et al., 2008), which identified RRS1 proteins across numerous genera. Given these data and the absence of other RRS1 homologs identified in the T. gondii genome, we have named this locus in T. gondii TgRRS1.

Although TgRRS1 is not predicted to be a structural component of the mature ribosome, its locus shares sequence similarity with ribosomal
protein loci. Two conserved sequence motifs, termed *Toxoplasma* Ribosomal Protein (TRP)-1 and TRP-2 elements, have been identified within the promoter regions of genes encoding *T. gondii* ribosomal proteins (Van Poppel et al., 2006). These 2 motifs alone, or in combination, have been identified in 95% of ribosomal proteins in this parasite and are located 10 to 303 bases upstream of predicted transcriptional start sites. A perfect consensus TRP-2 element, TGCATGCA, is located 27 bases upstream of the RACE-predicted TgRRS1 transcriptional start site. This motif matches the *Plasmodium falciparum* PF14_0633 Apetala2 (AP2) DNA-binding protein recognition sequence, suggesting possible transcriptional control by an AP2-like transcription factor (Dixon et al., 2010). While TRP-2 is found in the promoters of ribosomal protein genes, it is also found ubiquitously throughout the *T. gondii* genome (Van Poppel et al., 2006). By contrast, the TRP-1 element is enriched within the upstream of ribosomal protein-encoding loci and has the consensus sequence TCGGCTTATATCGG. A similar sequence, CCGGGTTTCTTTCTT, is found 155 bases upstream of the RACE-predicted transcriptional start site of TgRRS1. This sequence is similar to the TRP-1 consensus, and deviating bases are variants found in previously identified ribosomal protein TRP-1 elements, with the exception of positions 10 and 15 (underlined; Van Poppel et al., 2006). Though the functions of TRP-1 and TRP-2 are not yet characterized, it is interesting to note that they are conserved within the promoter of a locus whose product is not predicted to be within the mature ribosome. Sequence elements involved in transcriptional control of ribosomal protein-encoding genes may also be employed at a locus whose product impacts ribosomal assembly and maturation. This arrangement would allow TgRRS1 transcription to be coordinately regulated with transcription of structural ribosomal protein loci. In strain C3, plasmid insertion at the TgRRS1 locus occurred between the predicted TRP-1 and TRP-2 elements, effectively displacing the TRP-1 element from the transcriptional start site by several kilobases.

Inoculation with strain C3 results in a reduced number of cysts in the brains of mice during the early stages of a chronic infection. This phenotype is in contrast to that of a conditional knockdown of *T. gondii* ribosomal structural protein RPS13, which was unable to replicate in vivo because of arrest at the G1 stage of the cell cycle (Hutson et al., 2010). The plasmid insertion upstream of TgRRS1 may be responsible for cyst formation defect of C3. The shorter TgRRS1 transcript detected in strain C3 may result from transcription initiation within the coding region, possibly creating a truncated form of TgRRS1 in the C3 mutant. To determine if introduction of additional copies of TgRRS1 could restore the cyst-formation phenotype to constitutive WT levels, 2 plasmids were generated. One contained the TgRRS1 cDNA transcribed from the TUB1 promoter using a derivative of the vector pT230-TUB5CAT (Kim et al., 1993). The second was made up of the genomic TgRRS1 locus, including 1.375 kb upstream of the translational start and 1.88 kb downstream of the translational stop codon. Analysis by Southern hybridization confirmed C3 transformants. During murine infection, a comparison of C3 and WT strains indicated that addition of TgRRS1 to C3 did not restore cyst formation to WT levels (data not shown). For the other TgRRS1 mutant strain, 72F2, introduction of identical constructs also did not complement its cyst-formation defect (data not shown). Perhaps the truncated form of TgRRS1 in the mutants acts as a dominant negative.
| TgRRS1 | 1 | MASASMPGPSPFLLDSSAPGASNAMESHLNFLCC----IDFSPVHDDLSDLTRQNAQS |
| PfRRS1 | 1 | MD------------------------IDFCSHLLA--YDNSIINDENEIAKVEQNLPL |
| ChRRS1 | 1 | MEEAQLETSF---LEVCLSNLIG----IDISSINET--EISKSGANNQQL |
| ScRrslp | 1 | MSAEDYKNLPVTVEKPIPVYVYDLGNLAAFDSNVLDKNDLSNNARREKEIKSLTRDNLQ |

| TgRRS1 | 58 | MVAQLCLAP--HETTDDG-------LILALPPPKNSTFKLRPMHPAPAAKLTRWAEFAKE |
| PfRRS1 | 35 | IFSKINLQ--KEKEDE-------EDIYISVTNDN-IFNMPRSIPKEKKKTQWLFPAEN |
| ChRRS1 | 42 | MLNSIWSLDSELKNDG-------TVVSLPSKQE---ILLPRAYLPAKREKTRWEKFAEL |
| ScRrslp | 61 | LINQKLSSLPMKTSTTSVGQTQSSVMITYSLPDTDPDDLPREKPLPKAKMTKWEKFAAK |

| TgRRS1 | 111 | KGIDKKKRK-RLVWDANTKDWPRWGHKGIQQMNALAEAVIEDKDGEYVGRGDRGKK |
| PfRRS1 | 86 | KLRMKKNKS-GLIYDVSKGWVRFFQ-KKQIKLNEQKNNFHEKYN |
| ChRRS1 | 93 | KGKJKRKRS-RKYYDPITDDDFVPRGRNRSKIKQKHNEAIEIKG |
| ScRrslp | 121 | KGKPKERAGKMIYDEASGEWVPGK--YKL-------------------------- |

| TgRRS1 | 170 | LQKRSDAQKRRRTSOQVECPFEAARKDEKQLRQKRSLKRESQKRFKARLQKRELSK |
| PfRRS1 | 132 | -----KEDIYED------FPEEQEEKDIDKMKQKRNLKDFQ-------KG-ISTE |
| ChRRS1 | 138 | -----NMDEKND------PREAINKDRMMKNQRLKKNMKSNKKNSKDEFAL |
| ScRrslp | 150 | ---------------------ANKKLDQWVLEDDVKQG--------TDNELI |

| TgRRS1 | 230 | AAGGEAAAEKGGAAGLHRHRRTKEELKEVMMRATTSFQFDRALKAKNEK--KREKQKR |
| PfRRS1 | 172 | DIKypiQKCRK------------R--ENNDNLKMAIQISSSTFGRKDKKLLKEKLLQNDK |
| ChRRS1 | 186 | GIGNLKDTNV--------RSKTQVFELDPVSLSTASYGRDCAKLED-RTTNVK |
| ScRrslp | 175 | DPRTLNLRAERK--------RLVK--KNEK-- |

| TgRRS1 | 289 | TKGVSLSLDDERSKRYRHLKNILSAAESADV |
| PfRRS1 | 220 | ITNQKYEKRDLKDEVKQNNRLLAALVKS |
| ChRRS1 | 235 | KVKKILDCTNEKEYKSIYSKILKQSNL |
| ScRrslp | 194 | -------------------QQRNNMKAL |

**Figure 2.** TgRRS1 aligned with RRS1 family members. The amino acid sequence of TgRRS1 was aligned with RRS1 proteins from apicomplexan parasites *Plasmodium falciparum* (PfRRS1; accession XP_001347930.1) and *Cryptosporidium hominis* (ChRRS1; accession XP_6653581), as well as the defining family member from *Saccharomyces cerevisiae* (ScRrslp; accession NP_014937.1) using ClustalW in MacVector v9.0. Amino acid positions are shown at the left of each line. Conserved residues are indicated by asterisks below the alignment. Possible nucleolar localization sequences contained in stretches of sequence similar to monopartite nuclear localization signals, marked by consensus (R/K)(R/K)X(R/K), are boxed on the TgRRS1 sequence.

It remained possible expression from the other locus proximal to the plasmid insertion, *TgME49_120440*, may have been impacted by the virulence defect seen in strain C3. As northern hybridization did not reveal a *TgME49_120440* transcript (data not shown), quantitative real-time PCR (Q-PCR) was performed on cDNA prepared from WT and C3 tachyzoites to quantify transcripts for *TgRRS1* (using primers 5′ -CACCTCCCTTCCTCGACTCT and 5′ -TCGTTTCGTGAGGCAGAGCG), *TgME49_120440* (5′ -GAGAGACGTTCTTCTTGCCGA and 5′ -CCTGCGACTGCTTTCACTC), and *TUB1*, as previously described (Rooney et al., 2011). Normalized to transcript levels of housekeeping gene *TUB1*, steady-state levels of both *TgRRS1* and *TgME49_120440* transcripts were increased in strain C3 relative to WT tachyzoites by 4.4 ± 0.7-fold and 4.5 ± 0.5-fold, respectively. Though the region of *TgRRS1* transcript detected by Q-PCR was within exons 1, 2, and 3, whereas the probe used in northern hybridization spanned the 3′ end of exon 3 to the stop codon, this difference in detected regions does not explain the contrasting outcomes from these 2 methods of transcript assessment. However, the possibilities that TgRRS1 is overexpressed in C3, or that transcription of both genes proximal to the insertion site are affected, provide explanations as to why addition of TgRRS1 to strain C3 would not restore WT levels of in vivo cyst formation.

Several member proteins in the RRS1 superfamily were localized to the nucleolus, the site of transcription of ribosomal DNA and maturation of pre-rRNA transcripts (Tsuno et al., 2000; Andersen et al., 2002; Carnemolla et al., 2009). TgRRS1 contains basic stretches sharing similarity to monopartite nuclear localization signals, including the core sequence motif of (R/K)(R/K)X(R/K), which is found repeatedly in several known nucleolar proteins (Fig. 2; Hork et al., 2004). To determine if TgRRS1 has a similar localization pattern, a hemagglutinin (HA) tag was...
TgRRSI was used to infect human foreskin fibroblasts. Infected monolayers were fixed, permeabilized, and reacted with anti-HA polyclonal antisera (Zymed/Invitrogen, Carlsbad, California) and antifibrillarin monoclonal antibody. Reactivity was detected using fluorescently conjugated secondary antibodies. Images were captured using a Zeiss Axiosplan 2 microscope equipped with a rear-mounted excitation filter wheel (DAPI/FITC/Texas Red) emission cube, differential interference contrast (DIC) optics, and a Hamamatsu ORCA-CCD camera. Volocity version 4 (PerkinElmer) was used to deconvolve images using a constrained iterative algorithm. All images are at the same scale, indicated by a 2.5 μm bar in the upper left panel. At left are DIC panels showing 2 tachyzoites for each strain. Anti-HA reactivity identifies TgRRSI (green), and anti-fibrillarin (FIB) is used to highlight the dense fibrillar component of the nucleolus (red). Apicoplast and host and parasite nuclear DNA is shown in the merged image at right by 4',6-diamidino-2-phenylindole (DAPI) reactivity (blue).

We were unable to identify a B23 homolog within the parasite nuclear DNA is shown in the merged image at right by 4',6-diamidino-2-phenylindole (DAPI) reactivity (blue).

CITED LITERATURE


Genetic Characterization of Protostrongylus shiozawai From Japanese Serows (Capricornis crispus)

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ABSTRACT: Protostrongylus shiozawai (Nematoda: Strongylida) infection is known to occur in the lungs of wild Japanese serows (Capricornis crispus) and, to date, has been classified only by its morphological characteristics and, as well as host specificity. To characterize P. shiozawai genetically, a partial sequence of the second internal transcribed spacer (ITS-2) region was determined and compared with those of related nematodes. Our subsequent classification of P. shiozawai based on phylogenetic analysis was consistent with the current classification. Phylogenetic analysis also showed that P. shiozawai is more closely related to Protostrongylus stilesii than to Protostrongylus rufescens. PCR using generic markers in the ITS-2 region should provide a useful tool for expanded studies of P. shiozawai and other Protostrongylus species.

The lungworm Protostrongylus shiozawai was first reported in the cavities of the bronchioles, alveolar ducts, and alveoli of wild Japanese serows (Capricornis crispus) in 1974 and was placed in the Protostrongylidae (Strongylida) (Ohbayashi and Ueno, 1974). In a previous study, pulmonary lesions caused by P. shiozawai were found in >70% of the 119 wild serows examined (Suzuki et al., 1981); the parasite is apparently widespread in wild serow populations (Shiozawa et al., 1975; Kato, 1979; Oyamada et al., 2002). Protostrongylus includes many species, some of which infect wild ruminants, i.e., Protostrongylus stilesii in bighorn sheep (Ovis canadensis canadensis) (Forrester and Lankester, 1997) and Dall’s sheep (Ovis dalli dalli) (Kutz et al., 2001; Hoberg et al., 2002), Protostrongylus rufescens in moullons (Ovis musimon) (Panayotova-Penecheva, 2006), Protostrongylus rupicaprae in chamois (Rupicapra rupicapra) (Nocture et al., 1998), and Protostrongylus rushi in bighorn sheep (Forrester and Lankester, 1997). To date, the only animal reported to be infected by P. shiozawai is the Japanese serow. Although the species of Protostrongylus are identified by morphological characteristics and host specificity, the genetic relationship between P. shiozawai and other lungworms remains unclear. Here, we determined the sequence of the second internal transcribed spacer (ITS-2) of P. shiozawai and compared the findings among related nematodes. This is the first report of genetic characterization of P. shiozawai.

Lung samples that showed a brown-tanned granulomatous nodule were obtained from 2 dead Japanese serows in Gifu Prefecture in February 2007 (Fig. 1A). Impression smears of the freshly cut surfaces of the granulomatous nodule were used for cytopathological evaluation, and the remaining lung samples were stored at -80 C until DNA extraction. Impression smears were fixed in methanol and then immersed in hemacolor stain solution (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. Nematodes were detected in granuloma in lungs using light microscopy. Morphologically, the nematodes were identified as P. shiozawai, and they included first-stage larvae to adult worms, as determined according to Ohbayashi and Ueno (1974). Numerous macrophages and alveolar epithelium, plus a few neutrophils and eosinophils, were also observed (Fig. 1B).

After confirmation of the presence of the lungworms, total DNA was extracted from the granuloma lesions of 2 independent lungs using a DNAeasy Tissue kit (Qiagen, Hilden, Germany) or a QuickGene DNA tissue kit S (Fujifilm, Tokyo, Japan) according to the manufacturers’ instructions. PCR primers NCI (5’-AGCTCTGGTCAAGGTTTGTT-3’) and NCI2 (5’-TTAGTTCCTTTTCCCTCCTC-3’) were used to amplify the region of ITS-2 (Gasser et al., 1993) and for subsequent, direct sequencing. A 50-μl aliquot of PCR reaction mixture contained 0.4 μM of each primer, 800 μM dNTP mix, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California), and 2 μl of extracted DNA. PCR consisted of 9 min at 95 C, followed by 35 cycles of 94 C for 30 sec, 51 C for 30 sec, and 72 C for 30 sec, with a final extension at 72 C for 7 min. The PCR products were purified using a QIAquick PCR Purification kit (Qiagen), and nucleotide sequences were determined by direct sequencing using a DNA sequencer 310 with a BigDye Terminator Cycle Sequencing kit (Applied Biosystems). For verification, sequencing was performed in both directions. PCR of the genomic DNA from lung tissue containing the nematode produced an amplicon of approximately 440 bp. The ITS-2

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Figure 1. Granuloma associated with Protostrongylus shiozawai in the lung of a Japanese serow. (A) Cut section at necropsy. Arrow indicates granuloma. Scale bar represents 1 cm. (B) Hemacolor staining of impression smears from the lung tissue of a Japanese serow. Numerous larval stages of P. shiozawai were observed. Scale bar represents 200 μm. Inset: a larva showing a sharply pointed posterior end. Scale bar represents 50 μm.
sequences determined from the 2 independent lung samples showed no nucleotide differences. The determined sequence was submitted to DDBJ/EMBL/GenBank under accession number AB478249.

Next, the nucleotide sequence determined for *P. shiozawai* was aligned and compared with the published sequence data of *P. rufescens* and *P. stilesi*, as well as that of 6 species from 3 different protostrongylids and of 3 species from different families (Table I). A phylogenetic tree was constructed using a 315-bp sequence from the determined sequence of *P. shiozawai* (excluding the regions of the NC1 primer and the 28S rRNA gene) and an approximately 340-bp sequence from the corresponding region of related nematodes. Sequences were aligned using the Clustal W program, and phylogenetic analysis was conducted using MEGA5 software (Tamura et al., 2011) with Kimura’s 2-parameter method. A phylogenetic tree was constructed using the neighbor-joining method with bootstrap values calculated from 1,000 replicates. In the tree, *P. shiozawai* was placed into the same clade as *P. rufescens* and *P. stilesi*, which demonstrates that *P. shiozawai* is more closely related to *P. stilesi* than to *P. rufescens* (Fig. 2). This phylogenetic tree produced the same classification of *Prostomstrongylus* as that produced using traditional morphological characteristics and host specificity.

For members of the Protostrongylidae, morphological characterization has traditionally been used for analysis of evolutionary relationships (Carreno and Hoberg, 1999). Recently, phylogenetic analyses of various nematodes were conducted for the same purpose using sequences of the ITS-2 region (Jenkins et al., 2005; Kutz et al., 2007) and 18S and 28S rRNA regions (Chilton et al., 2006). *Prostomstrongylus shiozawai* had not been characterized genetically prior to the present study. To define the phylogenetic position of *P. shiozawai*, we compared a partial sequence of the ITS-2 region with the available sequence data of 2 other protostongylid species, namely, *P. rufescens* and *P. stilesi*, as well as with related nematodes. This phylogenetic analysis revealed that *P. shiozawai* is more closely related to *P. stilesi* than to *P. rufescens*. For a more detailed characterization of the phylogenetic relationship of *P. shiozawai* with the other members of the Protostrongylidae, further genetic analyses of other species in the Protostrongylidae and of other genes, such as the ITS-2 region (*Parasitology 85*: 638–648), would be required.

*Prostomstrongylus stilesi* infection has been reported in bighorn sheep (Forrester and Lankester, 1997) and Dall’s sheep (Kutz et al., 2001; Hoberg et al., 2002). In contrast, *P. rufescens* infects mainly domestic sheep. Bighorn and Dall’s sheep are distinct species from domestic sheep in the genus *Ovis*, but they are phylogenetically more closely related to each other than to domestic sheep (Groves and Shields, 1996), suggesting that *P. stilesi* has established host specificity in bighorn and Dall’s sheep during their phylogenetic evolution. Our findings and the fact that there has been no report of infections in other animals by *P. shiozawai* suggest that *P. shiozawai* infection is host specific to Japanese serows. However, the possibility and concern remain that sheep and goats, or other ruminants, may in fact be susceptible to infection by *P. shiozawai*.

The protection of Japanese serows as a natural monument by law and the extinction of the Japanese wolf have resulted in an increasing serow population and expansion of their territory. Therefore, the foraging ranges of Japanese serows appear to be highly specific, this overlapping of grazing areas could result in host switching, as previously reported for *P. stilesi* from Dall’s sheep to muskoxen (*Ovis moschatus wardi*) (Hoberg et al., 2002, 2008). Continuing and expanded studies are required for both molecular characterization and the current status of *P. shiozawai* infection in other animals.

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**LITERATURE CITED**


Internal Movement of Estuarine Digenean Trematodes Through Their Intermediate Snail Host *Cerithidea californica*

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ABSTRACT: The ability of free-swimming larval parasites to control emergence from their hosts can be critical in increasing the chances of successful infection transmission. For a group of estuarine trematodes, emergence of cercariae from their snail hosts is known to match favorable temperature, tidal activity, and light intensity. How the larvae time this behavior is not well understood, but the pathway that the larvae take through their host may play a role. Through video and histological analysis, we were able to localize the snail's anus as the emergence point and the peri-intestinal sinus dorsal to the intestines as the route by which they reach that point. By moving through this open sinus, the larvae have an energetically efficient pathway to reach their emergence point while minimizing damage to the host. Most importantly, it allows control over emergence to be maintained by the parasite, not the host, thus increasing the chances of the larva successfully reaching its intended destination.

For parasites, transmitting infection to new hosts is critical to both maintain and increase their prevalence in host populations. For those that use free-living transmission stages, emergence timing is often synchronized with host availability and favorable environmental conditions to increase the chances of encountering a suitable host (Combes et al., 1994). This is particularly important if conditions change rapidly, such as in estuarine environments where tides, light levels, salinity, and temperature can vary at scales as small as a few minutes.

Some parasites show a remarkable level of sophistication in terms of their emergence patterns, e.g., the estuarine digenean trematodes found in southern California intertidal marshes (Martin, 1972; Fingerut et al., 2003a). Their definitive hosts may be a shorebird from which either the egg or the hatched miracidia are fecalated onto the mudflat. Depending on species, miracidia actively seek out, or eggs may be ingested by, the intermediate host Cerithidea californica which serves as their first intermediate host. Once inside the host, different parasite species can localize in different organs, e.g., gonads, hepatopancreas, etc., with multiple asexually reproducing stages producing free-living forms known as cercariae. Mature cercariae leave the snail, emerging into the environment to seek out their next host, e.g., a crab, fish, or another snail.

In studying members of this group, Fingerut et al. (2003a) found that cercaria emergence was correlated with a hierarchy of abiotic factors, e.g., light, temperature, and tidal submergence of the host. The presence of light and an inundated host is necessary for properly oriented swimming towards their next host. In addition, each species has a specific temperature below which they will not emerge. The annual temporal distribution of these temperatures appears to match the occurrence in the habitat of each species' next host (Fingerut et al., 2003a). The ability of these larvae to synchronize their emergence to all 3 of these factors, the timing of which may change daily, increases their chances of successful transmission. While conditions necessary for larval emergence, as well as behavior once emerged, have previously been studied (Fingerut et al., 2003a, 2003b; Zimmer et al., 2009), how larvae are able to have such precise control is still unknown.

The goal of the present study was to determine the pathway by which cercariae move through their gastropod host from their natal location in the gonads to where emergence occurs. To determine this pathway, we used both video and histological analysis of in vivo parasite infection for 2 trematode species, *Himasthla rhigedana* and *Euhaplorchis californiensis*, that represent 2 ends of a spectrum in terms of number and size of emerging cercariae (Fingerut, 2003a).

*Cerithidea californica* snails were shipped overnight from Carpenteria Salt Marsh Reserve in Santa Barbara County, California (34°24′16″N, 119°31′30″W) to Philadelphia, Pennsylvania. Upon arrival, all snails were placed in an aerated, 37.9-L, slanted tank, filled partly with artificial seawater to provide both wet and dry habitat for these intertidal snails.

Snails were removed from the population tank at least 3 hr prior to being placed in individual small, covered compartments for shedding. Compartments were then filled with artificial seawater and illuminated for 4 hr (600W FotoLite®, Testrite Instrument Co., Newark, New Jersey) to mimic both heat and light from the sun. Cercariae from each compartment were collected and identified via microscopic analysis using a dichotomous key (Martin, 1972). Each snail was then labeled with nail polish to identify the particular infection it carried. Only snails with single-species infections were used.

To determine the cercariae emergence point, 15 snails known to have trematode infections were videotaped after being glued dorsal-side down to glass slides, with the foot and opercular opening facing upwards. Snails were placed into a large fingerbowl, illuminated as described above, and artificial seawater was added until the snail was submerged. The fingerbowl was positioned under a Hitachi KP-D20A CCD camera (Hitachi Kokusai Electric America, Ltd, Woodbury, New York) with a Nikon 52.5-mm lens with a 2× extender and +5 magnifying lens. Video was taken for 2 hr and recorded on a Panasonic DVD Recorder (DMR-ES25) (Panasonic of North America, Secaucus, New Jersey).

After the anus was identified as the point of emergence (see below), we initiated a second set of experiments on snails with known infections using histological techniques to track the cercariae pathway back toward the gonad region. To isolate the intestinal tract, the shell was removed from the body; the foot and the gonads were removed to enable the body to lie flat, and the intestinal tube from the anus to the stomach was excised. Twenty-three *C. californica* were necropsied and analyzed; 15 were infected with *H. rhigedana*, 3 with *E. californiensis*, and 5 were uninfected.

Excised intestines were fixed in 3.7% formaldehyde in 0.1 M sodium phosphate buffer at a pH of 7.4 for at least 24 hr, after which they underwent 2, 15 min washes in deionized water. The tissues were then dehydrated in increasing concentrations of ethanol. After the tissues were dehydrated in 100% ethanol for 15 min, the intestines were infiltrated with JB4 infiltration solution (JB-4 Embedding Kit™, Electron Microscopy Sciences, Hatfield, Pennsylvania) in ethanol. After 2 medium changes of 100% JB4 infiltration solution, tissues were cut into smaller pieces to allow for complete infiltration and placed into plastic embedding tubes containing JB4 embedding medium. Nitrogen was slowly streamed into the container for 24 hr to provide an anoxic environment. After an additional 24 hr, the embedded tissues were removed from the container and placed in an oven at 50 C for 24 hr and then at room temperature for a second 24 hr for further hardening.

Fixation of cercariae released from the host occurred immediately after they were shed. Cercariae were fixed by adding approximately 2 ml of 3.7% formaldehyde in phosphate buffer at a pH of 7.4. Because larvae are too small to embed following the protocol described above, they were first embedded in agar. Fixed cercariae were collected by Pasteur pipette and transferred into a 1-ml plastic microcentrifuge tube. The tube was then centrifuged at low speed, causing the cercariae to pellet but not to break apart. The fixative supernatant was removed and fresh 0.1 M NaPO₄ buffer wash was injected into the tube, resuspending the larvae. After a second wash, hot molten agar was quickly squirted into the tube and immediately centrifuged for 30 sec to distribute the larvae through the tube. The agar pellet containing the cercariae was then processed in the same manner as the snail intestines.

Sections for all samples were taken with the Sorvall JB-4 Porter-Blum microtome (Sorvall, Inc., Norwalk, Connecticut) using a glass knife. Sections were dried on glass slides and then stained in aqueous hematoxylin for 24 hr and in anilinetic eosin for 1.5 min. After washing,

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FIGURES 1-4. (1) Closeup video image of a cercaria emerging from the anal region of Cerithidea californica. (A) denotes the anus, (C) denotes cercariae. (2) Identification of microscopic anatomy of C. californica infected intestines viewed at 10× and stained in H&E. The intestinal lumen (L) is surrounded by a layer of columnar epithelium (CE) cells. The base of the columnar epithelium cells is a layer of smooth muscle (SM). The mantle tissue (M) appears to be partly composed of fat cells that stain clear with the nucleus present on 1 side of each cell. Asterisks indicate H. rhigedana cercariae head or tail sections. (3) Longitudinal cross section of H. rhigedana cercariae viewed at 20× and stained in H&E. (4) Section of Euhaplorchis californiensis-infected C. californica intestine viewed at 10× and stained in H&E. (L) Denotes the intestinal lumen and black arrows denote E. californiensis cercariae.

coverslips were mounted onto the slides with Permount Mounting Medium (Fischer Scientific, Pittsburg, Pennsylvania). Pictures of sections were then taken using an Olympus BX51 microscope with an Olympus DP72 camera (Olympus America, Inc., Center Valley, Pennsylvania). Images were captured using Olympus DP2-BSW Software (©1986–2008 Olympus Corporation).

For all snails that were videotaped, cercariae were only observed emerging from a single location, i.e., the area around the anus. Examination of the video images indicated that the cercariae were not exiting the snail via the orifice itself, but rather via the tissue surrounding the anus (Fig. 1). Further, cercariae were commonly observed struggling for a minute or 2 to free themselves from the tissue rather than being forcibly expelled by the host.

Histological examination of intestines from infected snails revealed structures similar in size and shape to those cercariae that were fixed and sectioned after their emergence (Figs. 2–4). These structures were absent from uninfected snails. Further, the cercariae were only observed outside the intestinal lumen. The intestinal lumen was identifiable in all sections by its clearly defined borders of columnar epithelium bound in a layer of smooth muscle. The intestinal walls of both infected and uninfected snails were similar in morphology, indicating that the parasites did not damage these structures. Of those cercariae observed, most were found in a region located beneath the mantle region and above the intestinal lumen. This location is identified as the peri-intestinal sinus, which runs just ventral to the mantle and dorsal to the intestinal lumen (Voltzow, 1994).

The trematode species studied here are known to maintain very specific and predictable temporal patterns of emergence that are believed to facilitate successful transmission of infection between hosts (Fingerut et al., 2003a). Why cercariae follow a pathway via the peri-intestinal sinus may be a matter of finding a balance between their need to control the timing of their emergence, the energy necessary to move through their hosts prior to emergence. Through histological and video analysis, a pathway alongside the intestinal tract of C. californica, primarily in the peri-intestinal sinus, was identified for 2 cercaria species (H. rhigedana and E. californiensis). In the majority of infected snails, cercariae were observed to aggregate dorsal to the intestinal lumen below the mantle, i.e., the location of the peri-intestinal sinus. For those samples in which cercariae were found outside the peri-intestinal sinus (though always still outside the intestinal lumen), the wider distribution may be the result of limited space within the peri-intestinal sinus. Regardless of the cercariae distribution, the intestinal walls of infected snails were always observed to be intact.

The trematode species studied here are known to maintain very specific and predictable temporal patterns of emergence that are believed to facilitate successful transmission of infection between hosts (Fingerut et al., 2003a). Why cercariae follow a pathway via the peri-intestinal sinus may be a matter of finding a balance between their need to control the timing of their emergence, the energy necessary to move through their host, and the damage they do to the host (on which future larvae are dependent) during this process. Riding the peristaltic contractions of the gut would provide cercariae with an energetically efficient pathway through the host. The peristaltic contractions of the gut, however, would either require the cercariae to fight these contractions to maintain control
over when they are expelled from the host or force them to relinquish control over emergence timing. Movement outside the gut lumen through mantle or other tissues would allow cercariae to control their own movement but would require greater expenditure of energy on their part to push through the tissue and could cause damage to the host. The use of the sinus along, but outside, the gut may, therefore, provide a compromise wherein the larvae can control their emergence timing while lowering their energy expenditures and the damage done to the host. The peri-intestinal sinus provides a relatively open area with few impediments to movement, though it does require erupting through the anal tissue, as the sinus ends posteriorly to the anus and does not open directly to the outside environment.

While this study determined that the cercariae pathway was alongside the intestinal tract in the posterior half of the body (anus-intestines), it is still unknown how cercariae exit the gonads and enter this sinus or the time it takes for this journey. Future work, including in vivo staining of the larvae, may provide the answers to both of these questions and lead to a better understanding of how these parasites, and other species that reside in different portions of the host, are able to so successfully and accurately time their emergence.

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LITERATURE CITED


Palaeparasitological Finding of Eggs of Nematodes in Rodent Coprolites Dated at the Early Holocene From the Archaeological Site Cerro Casa de Piedra 7, Santa Cruz, Argentina

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ABSTRACT: The aim of the present study was to examine the parasite remains present in rodent coprolites collected from the archaeological site Cerro Casa de Piedra 7 (CCP7), located in the Perito Moreno National Park (47°57'S, 72°05'W), Santa Cruz Province, Argentina. Eight coprolites obtained from the layer 17, dated at 10,620 ± 40 to 9,390 ± 40 yr B.P., were examined for parasites. Feces were processed whole, rehydrated, homogenized, subjected to spontaneous sedimentation, and examined via light microscopy. Eggs of parasites were measured and photographed. Seven of 8 coprolites possessed 199 eggs of 2, probably new, species of nematodes, including 43 eggs of Heteroxynema sp. Hall, 1916 (Cavioxynura sp. Quentin, 1975) (Oxyurida, Heteroxynematidae), and 156 eggs of Trichuris sp. Roederer, 1761 (Trichinellida, Trichuridae). Heteroxynema sp. is cited for the first time from ancient material worldwide. The finding of Trichuris sp. in both rodents and other host samples from the area under study is indicative of the stability of the biological and environmental conditions for this nematode genus to establish in the Patagonian Early Holocene. The rodent host was assigned to an unknown species of Caviomorpha (Hystricognathi) that lived during the Pleistocene transition in Patagonia.

Rodents act as hosts for numerous parasites, including those of zoonotic relevance (Miyazaki, 1991; Perkins et al., 2005; Morand et al., 2006). Antecedents of paleoparasitological studies carried out in rodent material recovered from archaeological sites of Argentina revealed the presence of eggs of Trichuris sp. in coprolites found in the sediments of the pelvic cavity of 1 human skeleton in the Orejas de Burro 1 archaeological site (Santa Cruz Province), dated at 3,565 ± years B.P. (Fugassa, 2005, 2006a; Fugassa and Barberena, 2006). Eggs from species of Trichuris, Capillaria, and ascaridids (nematodes), and anoplocephalid cestodes have been found in sediments from the pelvic cavity of a human skeleton and in rodent coprolites (Fugassa, 2006b). Eggs of Monococcosus sp. (an anoplocephalid cestode) and species of Trichosomoides and Pterygodermatites (nematodes), dated at 212 ± 35 yr B.P., were reported in rodent coprolites from the archaeological site Alero Mazquiarán (Chubut Province) by Sardella and Fugassa (2009a). A regurgitation pellet of a raptorial bird was found full of rodent bones, along with eggs of Capillaria sp. and Demodex sp. (Acari); it was collected from Cerro Casa de Piedra 5 (CCP5), close to Cerro Casa de Piedra 7 (CCP7), and was dated at 6,540 ± 110 yr B.P. (Fugassa et al., 2007). Eggs from species of Trichuris, capillarids, and Paraspidodera were also recovered from rodent feces from layer 13 of CCP7, dated at 7,920 ± 130 yr B.P. (Sardella and Fugassa, 2009b). Recently, numerous eggs of capillarids, trichurids, and anoplocephalids were found in rodent coprolites from Alero Destacamento Guardaparque, a site close to Cerro Casa de Piedra (CCP) (Sardella et al., 2010).

The aim of the present study was to broaden the knowledge of the parasite fauna present in rodent coprolites collected from another CCP layer, older than those previously examined.

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Cerro Casa de Piedra is a hill of volcanic origin in the basin of Río Roble and Lake Burmeister, 900 m above sea level, located in the Perito Moreno National Park (47°57'S, 72°05'W), Santa Cruz Province, Argentina (Fig. 1). It possesses several rock shelters and caves (Aschero, 1982) in an ecotone between a Nothofagus sp. forest and an arbustive steppe (Civalero and Aschero, 2003). The stratigraphic sequence includes 19 levels, with human occupation between ca. 9,700 and ca. 3,400 yr B.P. (Civalero and Franco, 2003).

Eight rodent coprolites obtained from the Area I, sensu Aschero et al. (2007), recovered from layer 17 of CCP7 dated at 9,390 ± 40 (from bones) to 10,620 ± 40 yr B.P. (from dung), were examined for parasites. Average measurements of feces were 10.0 mm in length by 3.93 mm in width, with an average weight of 0.06 g. Coprolites were clear brown in color, with a rough surface and without evidence of plant fibers (Fig. 2). Each coprolite was processed whole, rehydrated with 4 ml of 5% acetic formaldehyde, in a solution of 0.5% trisodium phosphate for 1 wk, followed by homogenization and processing by spontaneous sedimentation (Lutz, 1919). Ten slides were prepared from each coprolite, along with the addition of 1 drop of glycerin to each slide and examined using light microscopy. Parasite eggs were measured and photographed at 40×. Egg measures (range, average, and standard deviation) are given in μm.

Seven of 8 coprolites contained 199 eggs of 2 parasitic nematode species, i.e., *Trichuris* and *Heteroxynema*. There were 156 eggs of what is presumably a new species of *Trichuris Roederer, 1761* (Trichinellida, Trichuridae), found in 7 coprolites; the eggs were lemon shaped, with a smooth surface and polar plugs. The other 43 eggs were assigned to what is also most likely a new species of *Heteroxynema* Hall, 1916, subgenus *Cavioxyura* Quentin 1975 (Oxyurida, Heteroxynematidae). These eggs were thick walled, with an operculum at 1 pole; they were collected from 4 coprolites. They (n = 30) ranged in length (Fig. 3) from 67.5 to 107.5 μm, with a mean of 99.2 ± 4.8, and in width from 45 to 62.5 μm, and a mean of 53.2 ± 4.4. The ranges (mean lengths and widths) of 96 *Trichuris* sp. eggs (Fig. 4) were 67.5 to 77.5 (72.3 ± 2.1) μm, and 40 to 45 (43.1 ± 1.3) μm, respectively.

Species of oxyurid nematodes are monoxenic parasites that live in the posterior third of the digestive tract of various vertebrates and arthropods (Anderson, 2000). Heteroxynematidae includes nematodes that evolved in sciuromorph, caviomorph, and myomorph mammals, with the primitive *Heteroxynema* sp. created by Hall (1916) for *Heteroxynema cucullatum*, a
parasite of the rodent *Eutamias amoenus operarius* from North America. This nematode genus was subsequently divided into 3 subgenera, with *Cavioxyura* spp. as parasites of Neotropical Caviomorpha (Petter and Quentin, 1976). Teixeira de Freitas and Lins de Almeida (1936) reported *Heteroxynema weincki* from the intestine of the cavid *Galea leucoblephara* from the northern area of the Jujuy Province, Argentina, while *Heteroxynema viscaiae* was described by Foster et al. (2002) and by Ferreira et al. (2007) from the wild vizcachas *Lagostomus maximus* from La Pampa and Chaco Provinces, Argentina, respectively. This is the first time that *Heteroxynema* sp. has been found in ancient material.

*Trichuris* spp. includes intestinal parasites of the caecum and colon of mammals, with eggs that mature in soil. Based on the geographic area of the present study, plus the occurrence of rodent feces and appropriate measurements, eggs found here are most similar to those of *Trichuris dolichotis*, a common parasite of *Dolichotis patagonum* Morini, 1955 (Morini, et al., 1955). Although the morphological character and measurements of the coprolites examined in the present study are not compatible with *D. patagonum*, we are still confident of the nematode’s identity.

Based on the macroscopic aspect of the rodent coprolites examined in this study and in the known parasitic specificity of *Heteroxynema* sp. by caviomorphs, the feces were tentatively assigned to a small, or medium-sized, rodent, unidentified at present, but possibly to *Microcavia australis* or *Ctenomys* sp. in samples dated to the Late Holocene. Foster et al. (2002) and by Ferreira et al. (2007) from the wild vizcachas *Lagostomus maximus* from La Pampa and Chaco Provinces, Argentina, respectively. This is the first time that *Heteroxynema* sp. has been found in ancient material.

### LITERATURE CITED


Seroprevalence of Toxoplasma gondii Infection in Tibetan Sheep in Tibet, China

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ABSTRACT: In the present investigation, the seroprevalence of Toxoplasma gondii antibodies in 455 Tibetan sheep in Tibet, China, was examined using an indirect hemagglutination test. Of these, 26 (5.7%) Tibetan sheep were seropositive at the cut-off of 1:64 serum dilution. The seroprevalence ranged from 2.2% to 8.9% among Tibetan sheep of <1-yr-old, 1–3-yr-old, and >3-yr-old, but the differences among the age groups were not significant (P > 0.05). The prevalence in male Tibetan sheep (2.8%) was lower than that in female Tibetan sheep (6.6%), but the difference was not statistically significant (P > 0.05). The results of this survey indicated the presence of T. gondii infection in Tibetan sheep, which may cause economic losses to the local livestock industry and which poses a potential threat to human health in this area.

Toxoplasma gondii is prevalent in a wide range of warm-blooded animals, including humans throughout the world (Dubey, 2010). The main routes of T. gondii transmission include congenital, ingestion of undercooked or raw meat from infected animals, and ingestion of food or water contaminated with oocysts excreted by infected felids (Montoya and Liesenfeld, 2004; Dubey, 2010). Lamb and mutton are consumed widely in many countries, and consumption of uncooked lamb is a risk factor for T. gondii infection in pregnant women (Cook et al., 2000).

Tibetan sheeps are famous for their high quality pelage and their nutritious and delicious meat, and they are recognized as a major source of income for Tibetans. Surveys of T. gondii infection in sheep have been reported worldwide (Dubey, 2009; Chikweto et al., 2011), including mainland China (Wang et al., 2006; Wang et al., 2011). Here, we provide information regarding T. gondii seroprevalence for the first time in sheep from Tibet.

Tibet is located in the southwest part of the Qinghai-Tibet Plateau, with an average altitude of more than 4,000 m. It shares borders with Sichuan and Yunnan Provinces in the east; with Qinghai and Xinjiang in the north; with India, Nepal, Sikkim, and Burma in the south; and Kashmir in the west.

In total 455 blood samples were collected from Tibetan sheep in Nyingchi Prefecture of Tibet, between February and May 2011. Blood samples were transported to the laboratory, left at 37 C for 2 hr, and centrifuged at 3,000 g for 5 min; serum samples were stored at −20 C until assayed. Antibodies to T. gondii were identified in sheep sera by the indirect hemagglutination (IHA) test using a commercially available kit (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province, China), according to the manufacturer’s recommendations. Briefly, sera were added to 96-well V-bottomed polystyrene plates and diluted 2-fold up to 1:2,048, starting at 1:4. IHA titers of 1:64 or higher were considered positive when forming a layer of agglutinated erythrocytes; sera with dubious results were re-tested. Positive and negative controls were included in each test and assayed at the same dilutions of the serum samples.

Differences in the seroprevalence of male and female T. gondii-infected Tibetan sheep and between different age groups were analyzed using a chi-square test in SPSS for Windows (release 18.0 standard version, SPSS Inc., Chicago, Illinois). The differences were considered statistically significant when P < 0.05.

Antibodies to T. gondii were found in 26 (5.7%) of 455 Tibetan sheep (Table I). The seroprevalence ranged from 2.2% to 8.9% among <1-yr-old, 1–3-yr-old, and >3-yr-old, but the differences among the age groups were not significant (P > 0.05) (Table I). The prevalence in male Tibetan sheep (2.8%) was lower than that in females (6.6%), but the difference was not statistically significant (P > 0.05).

The overall T. gondii seroprevalence (5.7%) in Tibetan sheep is lower than those observed in other countries (Dubey, 2009), probably because of the cold dry climate or the smaller number of felids in the region. Nothing is known of T. gondii prevalence in cats in Tibet. The serological test employed could also be a factor, since IHA is a relatively insensitive test for the detection of T. gondii antibodies in ovine sera (Dubey, 2009).

In conclusion, the present survey revealed the seroprevalence of T. gondii in Tibetan sheep in Tibet, which can cause economic losses to the local ovine industry; ingestion of uncooked mutton poses a potential threat to human health.

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LITERATURE CITED


Table I. Seroprevalence of Toxoplasma gondii infection in Tibetan sheep in Tibet, China by indirect haemagglutination (IHA) test.

<table>
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<th>No. tested</th>
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ABSTRACT: Nystatin is a membrane-active polyene macrolide antibiotic and a channel-forming ionophore. Nystatin exhibits in vitro activity against Babesia gibsoni infecting normal canine erythrocytes containing low potassium (LK) and high sodium concentrations, i.e., LK erythrocytes. The calculated IC₃₀ value of nystatin against B. gibsoni infecting LK erythrocytes was 31.96 µg/ml. The anti-babesial activity of nystatin disappeared when B. gibsoni in LK erythrocytes were incubated in culture media containing high potassium concentrations (HK). Moreover, when the parasites were harvested in canine HK erythrocytes, which contained high potassium and low sodium concentrations as a result of high Na-K-ATPase activity, the in vitro anti-babesial activities of nystatin also disappeared, apparently due to protection by HK erythrocytes. This suggested that nystatin could show its in vitro anti-babesial activity against B. gibsoni by its ionophoric activity, the same as other ionophores such as valinomycin. Consequently, the effects of nystatin on the host cells were observed. Nystatin could not modify the intracellular concentrations of potassium, sodium, adenosine triphosphate, or glucose in either LK or HK erythrocytes, although it caused weak hemolysis in HK erythrocytes. In addition, nystatin did not affect the survival of canine peripheral polymorphonuclear leukocytes. In conclusion, nystatin destroyed B. gibsoni by ionophoric activity but did not affect either canine erythrocytes or leukocytes in vitro.

Babesia gibsoni is a causative pathogen of canine babesiosis, which is generally treated with diminazene acetate; however, the drug is unable to eliminate the parasites from infected dogs. Moreover, in reports regarding other anti-babesial drugs including atovaquone, clindamycin, metronidazole, doxycycline, and pentamidine, almost no single drug treatment or combined treatment could eliminate the parasites from the peripheral blood (Fowler et al., 1972; Farwell et al., 1982; Wulansari et al., 2003; Suzuki et al., 2007; Sakuma et al., 2009); therefore, the development of novel and effective anti-babesial drugs is necessary.

We previously reported that the ionophorotropic antibiotics valinomycin and salinomycin-Na, exhibit a strong in vitro effect against B. gibsoni (Yamasaki et al., 2009). These ionophorotropic antibiotics can modify the intracellular concentrations of monovalent cations. This action may activate transporters of monovalent cations, such as Na-K-ATPase, and increase the consumption of adenosine triphosphate (ATP), resulting in the depletion of intracellular ATP. Because valinomycin and salinomycin-Na have no specificity, they affect both the parasites and the host cells in which intracellular concentrations of monovalent cations are regulated by the function of an active transporter. They are, therefore, ineffective as therapeutics against B. gibsoni infection.

Nystatin is a membrane-active polyene macrolide antibiotic and an antifungal compound. The drug operates as a channel-forming ionophore and is membraneolytic due to its lipid (in particular sterol)-binding activity. It is thought to permeabilize target membranes by binding to beta-ergosterol, the principal fungal sterol (Wiehart et al., 2006). Toxicity problems encountered with nystatin are attributed to the ability of polyene macrolides to also bind cholesterol in mammalian cell membranes, albeit with lower affinity (Wiehart et al., 2006). Meanwhile, it was reported that erythrocytes infected with the trophozoite stage of Plasmodium falciparum were particularly susceptible to lysis by nystatin (Wiehart et al., 2006). In protozoa, Babesia rodhaini and Theileria parva are inhibited by nystatin in vitro (McColm and McHardy, 1984). Based on these previous reports, it is possible that nystatin may be utilized as a therapeutic against Babesia spp. parasites. Accordingly, in the present study, we attempted to define the effects of nystatin on B. gibsoni, canine erythrocytes, and peripheral polymorphonuclear leukocytes.

Normal canine erythrocytes containing a low concentration of potassium (LK) and a high concentration of sodium, i.e., LK erythrocytes, were obtained from normal dogs. The dogs used weighed 8–12 kg. The present study was carried out in accordance with Hokkaido University guidelines for the care and use of animals, which basically conform to the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The present study was approved by the committee for Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University (approval number: 8099). Blood samples were supplemented with ethylenediaminetetraacetic acid disodium salt (EDTA-2Na; Wako Pure Chemical Co., Osaka, Japan) and washed according to the method of Yamasaki et al. (2000).

Culture media I, II, and III (Table I), containing various concentrations of potassium and sodium, were prepared as described previously (Yamasaki et al., 2009). The concentrations of potassium and sodium, and the osmotic pressures in these media, were measured as described by Yamasaki et al. (2009). The osmotic pressures of the media were almost the same as that of the normal culture medium (Table I). The B. gibsoni used in the present study were maintained in culture as previously described (Yamasaki et al., 2003). The parasites were routinely cultured at 38 C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ in culture medium I; the numbers of erythrocytes were sufficient to yield a packed cell volume of 5%. Every 24 hr, 60% of the culture supernatant was removed and replaced with an equal volume of fresh culture medium I.

At first, the anti-babesial activities of nystatin (Wako Pure Chemical Co.) against B. gibsoni were determined. Babesia gibsoni cultured in LK erythrocytes were incubated in culture medium I containing 0–250 µg/ml nystatin for 24 hr. A thin smear sample was made and percent parasitemia was calculated by counting the number of parasitized cells per 2,000 erythrocytes. Data were analyzed using a Student’s t-test. The level of parasitemia was significantly (P < 0.05) decreased at a dose of 25 µg/ml or more (Fig. 1). The level of parasitemia was almost zero at a nystatin dose of 250 µg/ml. Nystatin exhibited in vitro effects against B. gibsoni in LK erythrocytes. The 50% inhibitory concentration (IC₅₀) was calculated using probit analysis. The IC₅₀ of nystatin calculated from this result was 31.96 µg/ml. The IC₃₀ value of nystatin was higher than those of valinomycin (2.32 ng/ml) and salinomycin-Na (63.92 ng/ml), which we reported previously (Yamasaki et al., 2009).

To investigate the mechanism of action of nystatin on B. gibsoni, the parasites were incubated in culture media II and III. When the parasites were incubated in these media without nystatin for 4 hr, the level of parasitemia was lower than that in culture medium I (Fig. 2). Interestingly, when the parasites were incubated in either culture medium II and III containing 31.96 µg/ml nystatin, the levels of parasitemia were almost the same as those in the media without nystatin (Fig. 2), indicating that the effect of nystatin on B. gibsoni disappeared, the same as with valinomycin (Yamasaki et al., 2009). Moreover, canine erythrocytes contained high potassium (HK) and low sodium concentrations as a result of high Na-K-ATPase activity, i.e., HK erythrocytes, were used as host cells. Inherited HK erythrocytes were obtained from 3 dogs. These dogs had no clinical signs and lived normal, healthy lives (Maede et al., 1983); the dog strain with HK erythrocytes has been maintained since 1986 in our laboratory. When HK erythrocytes infected with B. gibsoni were incubated in culture medium I containing 31.96 µg/ml nystatin for 24 hr, the level of parasitemia was decreased from 4.47 ± 0.71% to 3.47 ± 0.64% and there was no significant difference. The anti-babesial activity of nystatin seemed...
TABLE I. Ion composition and osmotic pressure of high potassium media.

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<tr>
<th>Measurement items</th>
<th>Medium no.</th>
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<tr>
<td>Sodium ion (mEq/L)</td>
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<tr>
<td>Potassium ion (mEq/L)</td>
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<tr>
<td>Chloride ion (mEq/L)</td>
<td>106</td>
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<tr>
<td>Osmotic pressure (mOsm/kg)</td>
<td>278</td>
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</table>

*Significant difference from the values without nystatin.

to be almost counteracted by Na-K-ATPase activity in HK erythrocytes. These results suggested that nystatin could also show an in vitro effect against *B. gibboni* by its ionophorous activity.

Subsequently, the effects of nystatin on the host cells were observed. HK and LK erythrocytes without the parasites were incubated in culture medium I containing 31.96 μg/ml nystatin for 24 hr to observe the effects of nystatin on canine erythrocytes. After incubation, the intracellular sodium ion, potassium ion, ATP, and glucose concentrations within erythrocytes, and the extracellular hemoglobin concentration were measured as previously described (Yamasaki et al., 2009). When LK erythrocytes were incubated in the medium containing nystatin, intracellular concentrations of potassium, sodium, ATP, and glucose, and the extracellular concentration of hemoglobin with nystatin were not significantly different from those without nystatin (Table II). It has been reported that nystatin could lyse human erythrocytes by forming channels in the erythrocyte membrane (Knopik-Skrocka and Bielawski, 2002); however, nystatin did not lyse canine HK erythrocytes at 31.96 μg/ml in the present study.

When HK erythrocytes were incubated in the medium containing nystatin, intracellular concentrations of potassium, sodium, ATP, and glucose were also not significantly different from those without nystatin (Table III). The extracellular hemoglobin concentration in HK erythrocytes suspension with nystatin (0.12 ± 0.00 g/dl) was significantly higher than without nystatin (0.04 ± 0.00 g/dl) (Table III). In the previous investigation, valinomycin and salinomycin-Na immediately modified their intracellular concentrations, causing hemolysis by their ionophorous activity (Yamasaki et al., 2009). The extracellular hemoglobin concentration in the HK erythrocytes suspension with valinomycin and salinomycin-Na were 0.16 ± 0.07 g/dl and 4.52 ± 1.61 g/dl, respectively. From the present results and the previous report, it is suggested that the activity of monovalent cation exchange of nystatin would be weaker than those of valinomycin and salinomycin-Na and that the activity of hemolysis of nystatin would be weaker than that of salinomycin-Na. To inhibit the activity of Na-K-ATPase in HK erythrocytes, ouabain was added to culture medium I at a final concentration of 0.1 mM. When HK erythrocytes were incubated in medium containing both nystatin and ouabain for 24 hr, the intracellular potassium concentration was significantly decreased and the intracellular sodium concentration was significantly increased; however, the extracellular concentration of ATP and glucose in HK erythrocytes with ouabain was almost the same as without ouabain, despite the presence of nystatin (Table III). Additionally, the hemolysis of HK erythrocytes was stopped when ouabain was added to the culture medium together with nystatin (Table III). Previously, when ouabain was added together with valinomycin, hemolysis of canine HK erythrocytes did not occur, although the intracellular concentrations of potassium and sodium were modified (Yamasaki et al., 2009). It was speculated that the activation of Na-K-ATPase and the consequent ATP consumption was caused by valinomycin and was suppressed by the inhibition of Na-K-ATPase. Similarly, the inhibition of Na-K-ATPase resulted in the cessation of hemolysis of HK erythrocytes by nystatin, indicating that ATP consumption by nystatin was suppressed, although the changes in ATP concentration were not significant.

Peripheral polymorphonuclear leukocytes (PMNs) were prepared according to Yamamori et al. (2000). The isolated PMNs were resuspended in culture medium I to yield a cell count of 1,500 μl and incubated in culture medium I containing 0–100 μg/ml nystatin for 24 hr to observe the effects of nystatin on canine PMNs. After incubation, dead PMNs were stained by 0.3% trypan blue solution (Wako Pure Chemical Industries, Ltd.).

When LK erythrocytes were incubated in the medium containing nystatin, intracellular concentrations of potassium, sodium, ATP, and glucose were measured 24 hr after LK RBCs had been exposed to 31.96 μg/ml nystatin (open columns) and without nystatin (closed columns) for 4 hr. Percentages of parasitized cells were calculated. *Significant (P < 0.05) difference from the values without nystatin.

![FIGURE 1. Babesia gibsoni-infected LK erythrocytes were incubated in normal medium (culture medium I) and media including high potassium concentrations (culture medium II and III) with 31.96 μg/ml nystatin (open columns) and without nystatin (closed columns) for 4 hr. Percentages of parasitized cells were calculated. *Significant (P < 0.05) difference from the values without nystatin.

![FIGURE 2. Babesia gibsoni-infected LK erythrocytes were incubated in normal medium (culture medium I) and media including high potassium concentrations (culture medium II and III) with 31.96 μg/ml nystatin (open columns) and without nystatin (closed columns) for 4 hr. Percentages of parasitized cells were calculated. *Significant (P < 0.05) difference from the values without nystatin.

TABLE II. Influence of nystatin on intracellular sodium ions, potassium ions, ATP and glucose concentrations in LK RBCs. Intracellular sodium ions, potassium ions, ATP, and glucose concentrations were measured 24 hr after LK RBCs had been exposed to 31.96 μg/ml of nystatin. Data are expressed as the mean ± SD (n = 3).

<table>
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<tr>
<th>Measurement items</th>
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<th>Nystatin</th>
</tr>
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<tr>
<td>Sodium ion (mEq/L)</td>
<td>172.4 ± 28.6</td>
<td>154.0 ± 45.7</td>
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<td>Potassium ion (mEq/L)</td>
<td>3.58 ± 1.29</td>
<td>3.29 ± 1.29</td>
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<tr>
<td>ATP (μg/g Hb)</td>
<td>1.08 ± 0.38</td>
<td>1.51 ± 0.30</td>
</tr>
<tr>
<td>Glucose (μmol/g Hb)</td>
<td>115.7 ± 24.9</td>
<td>130.0 ± 27.9</td>
</tr>
<tr>
<td>Extracellular hemoglobin (g/dl)</td>
<td>0.03 ± 0.023</td>
<td>0.03 ± 0.046</td>
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</table>
TABLE III. Influence of nystatin on intracellular sodium ions, potassium ions, ATP, and glucose concentrations in HK RBCs and extracellular hemoglobin concentration as an indication of hemolysis of HK erythrocyte suspension. All of the above were measured 24 hr after HK erythrocytes had been exposed to 31.96 μg/ml nystatin. Data are expressed as the mean ± SD (n = 3).

<table>
<thead>
<tr>
<th>Measurement items</th>
<th>Control</th>
<th>Nystatin</th>
<th>Ouabain*</th>
<th>Nystatin + ouabain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium ion (mEq/L)</td>
<td>83.6 ± 3.8</td>
<td>83.5 ± 2.5</td>
<td>106.9 ± 9.3†</td>
<td>115.2 ± 7.9†</td>
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<tr>
<td>Potassium ion (mEq/L)</td>
<td>83.5 ± 2.2</td>
<td>84.0 ± 6.2</td>
<td>53.9 ± 2.1†</td>
<td>41.0 ± 7.3‡</td>
</tr>
<tr>
<td>ATP concentration (μmol/g Hb)</td>
<td>1.84 ± 0.31</td>
<td>1.64 ± 0.43</td>
<td>1.91 ± 0.65</td>
<td>2.16 ± 0.20</td>
</tr>
<tr>
<td>Glucose concentration (μmol/g Hb)</td>
<td>89.7 ± 38.0</td>
<td>81.2 ± 27.3</td>
<td>112.1 ± 47.0</td>
<td>124.5 ± 52.5</td>
</tr>
<tr>
<td>Extracellular hemoglobin (g/dl)</td>
<td>0.04 ± 0.00</td>
<td>0.12 ± 0.00‡</td>
<td>0.07 ± 0.02</td>
<td>0.07 ± 0.05</td>
</tr>
</tbody>
</table>

* Ouabain (0.1 mM) was also added to HK RBC suspension.
† Values are significantly (P < 0.05) different from each value without ouabain.
‡ Values are significantly (P < 0.05) different from each value without nystatin.

Co.) and the percentage of live PMNs was calculated by counting the unstained cells per 500 PMNs. The percentage of live cells did not decrease, whereas that at 100 μg/ml nystatin was 95.5 ± 2.1%, suggesting that nystatin did not affect the survival of canine PMN; therefore, the present results showed that nystatin did not affect either canine erythrocytes or leukocytes.

In conclusion, it was shown that nystatin could destroy B. gibsoni by its ionophorous activity. Nystatin should not strongly affect either canine erythrocytes or leukocytes, indicating that nystatin may be an antibabesial drug; however, nystatin is not given intravenously to dogs because of its toxicity. It is possible that adequate doses of nystatin to destroy the parasites could not be administered intravenously to dogs. Formulation of nystatin into liposomes by Lopez-Berestein and coworkers in the 1980s established that nystatin could be administered intravenously to mice with good evidence of activity and reduced toxicity (Mehta, Hopfer, Gunner et al., 1987; Mehta, Hopfer, McQueen et al., 1987); however, the effect of liposomal nystatin on B. gibsoni is still not determined. To use nystatin for the treatment of canine babesiosis, further study of the administration of nystatin to dogs in vivo will be necessary. In addition, nystatin could not destroy B. gibsoni infecting HK erythrocytes. Because dogs having HK erythrocytes are living normal healthy lives, they cannot be distinguished from dogs having LK erythrocytes by its clinical feature. Accordingly, intracellular concentrations of potassium and sodium in canine erythrocytes should be determined before the treatment with nystatin, if it could be given intravenously to dogs.

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LITERATURE CITED


Genetic Characterization of *Toxoplasma gondii* Isolates From Pigs in Southwestern China

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**ABSTRACT:** The genetic diversity of *Toxoplasma gondii* varies in different geographical regions. Isolates of *T. gondii* in South America, for example, are genetically and biologically divergent from those in North America and Europe, where the population structure is highly clonal and composed mainly of 3 distinct lineages, i.e., Types I, II, and III. However, little is known of the *T. gondii* genotypes in the People’s Republic of China. *Toxoplasma gondii* infection in pigs causes significant economic loss and presents a risk for human infection. We conducted a survey to determine the genetic diversity of this parasite in slaughtered pigs from Yunnan Province, southwestern China. In total, 412 DNA samples were extracted from hilar lymph nodes and livers of pigs from slaughterhouses in Yunnan Province in southwest China, 56 of which were found to be positive for the *T. gondii* SAG3 gene. These positive DNA samples were typed at 10 genetic markers, including 9 nuclear loci, i.e., SAG1, SAG2, SAG3, BTUB, GRA6, L358, PK1, c22-8, c29-2, and an apicoplast locus Apico. Of these, 5 isolates were genotyped with complete data for all loci. Only 1 genotype (ToxoDB 9) was identified, previously reported as a widespread lineage from pigs, cats, and human patients in China. The results indicate that this genotype may be the major *T. gondii* lineage in China and possibly all of eastern Asia. This is the first report of genetic typing of *T. gondii* isolates from pigs in China’s southwestern Yunnan Province, the results of which have implications for the prevention and control of *T. gondii* infections in humans and other animals.

*Toxoplasma gondii* is an obligate intracellular zoonotic protozoan, with a worldwide distribution, infecting an estimated 30% of the human population (Weiss and Dubey, 2009). While fortunately benign in most people, toxoplasmosis can be life threatening in immunosuppressed patients. Infection in pregnant women may lead to abortion, still birth, or other serious congenital consequences in newborns. Toxoplasmosis is not only of great importance on pig farms, it is also potentially a public health problem owing to its possible transmission to humans via consumption of under-cooked pork (Dubey, 1988; Hill et al., 2006). A number of ethnic minorities living in Yunnan Province in southwestern China have their own unique habits of eating raw meat and half raw meat that increases the risk of *T. gondii* infection by consumption of contaminated pork. It has been reported that seroprevalence of *T. gondii* in blood donors of Dai (28.8%), Bai (22.8%), and Miao (20.3%) minorities are higher than in other nationalities (Zhu et al., 2007). However, there is limited information on *T. gondii* infection and no genetic information from pigs in Yunnan province; it is, therefore, difficult to evaluate the risk of pork consumption in relation to human *T. gondii* infection in this geographic location.

The majority *T. gondii* isolates from humans and animals in North America and Europe have been grouped into 3 clonal lineages, i.e. Types I, II, and III, by multi-locus enzyme electrophoresis, PCR-RFLP, and microsatellite typing (Durdé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002). However, *T. gondii* isolates from South America are biologically and genetically different from those in North America and Europe (Dubey et al., 2002; Lehmann et al., 2006; Dubey et al., 2008), suggesting the overall diversity in *T. gondii* populations is high.

Although we have previously reported limited genetic information for *T. gondii* pig isolates from central and south China and identified 2 genotypes in the process (Zhou et al., 2009, 2010), there are no data on *T. gondii* isolates from pigs in southwestern China, where animal biodiversity is rich and there is a warm and humid climate. In the present paper, we report the genetic characterization of *T. gondii* isolates in pigs from Yunnan Province, southwestern China.

The present study was designed to genetically characterize *T. gondii* isolates from pigs in slaughterhouses from Yunnan province (Fig. 1), with a reported *T. gondii* seroprevalence of 22.3% (Zou et al., 2009). Tissue samples, including liver and hilar lymph nodes, were collected from 412 slaughtered pigs randomly; all pigs were raised in semi-intensive systems and mainly used for sausage preparation in 8 slaughterhouses at 8 locations (Fig. 1), including Baoshan City (BS, 70 liver samples), Nuijiang Lisaw Autonomous Prefecture (NJ, 47 liver samples), Diqing Tibetan Autonomous Prefecture (DQ, 37 hilar lymph node samples), Lijiang City (LJ, 29 hilar lymph node samples), Puer City (PE, 29 hilar lymph node samples), Lincang City (LC, 80 hilar lymph node samples), and Wenshan City (WS, 50 hilar lymph node samples).

Genomic DNA was extracted from 3–5 g tissues of the liver or lymph nodes by sodium dodecyl sulfate/proteinase K treatment, column-purified (Wizard™ DNA Clean-Up, Promega, Beijing, China), and eluted into 40 μl of H2O according to the manufacturer’s recommendations. A nested PCR targeting the *T. gondii* SAG3 gene was performed to detect possible infection with *T. gondii* (Grigg et al., 2001). DNA samples giving positive SAG3 amplification were then used for genetic characterization.

Genetic characterization of *T. gondii* isolates was carried out using the multi-locus PCR-RFLP method according to Su et al. (2010) and Zhou et al. (2010). Multiplex PCR-amplified products were 1:1 diluted in sterile, double-distilled water and then used for nested PCR amplifications with internal primers for each marker, separately. The nested PCR products were digested with restriction enzymes to reveal their genotypes by the method described previously (Velmurugan et al., 2009; Zhou et al., 2009).

![FIGURE 1. Map showing Yunnan Province (YN) in southwestern China where pigs in slaughterhouses were sampled from Diqing Tibetan Autonomous Prefecture (DQ), Zhaotong City (ZT), Lijiang City (LJ), Nuijiang Lisaw Autonomous Prefecture (NJ), Baoshan City (BS), Lincang City (LC), Wenshan City (WS), and Puer City (PE). The shaded Yunnan Province is enlarged for better view.](image-url)
Of the 412 DNA samples examined for the presence of *T. gondii* DNA, 56 were amplification positive for the SAG3 gene, with no SAG3 gene positive samples (0/47) in NJ, 1.4% (1/70) in BS, 4.3% (3/70) in ZT, 16% (8/50) in WS, 17.2% (5/29) in LJ, 21.2% (17/80) in LC, 29.7% (11/37) in DQ, and 37.9% (11/29) in PE. Five DNA samples from Lincang (1), Puer (1), Zhaotong (1), and Wenshan (2) showed complete genotyping results (Table I). Owing to low DNA concentration, most of the samples could not be completely genotyped and were, therefore, not useful.

Only 1 genotype (ToxoDB 9) was identified in the 4 areas (with distances between each other greater than 300 km), suggesting that this genotype is the major lineage in these localities. Furthermore, this genotype was also previously observed in pigs, cats, and humans in south China (Zhou et al., 2009) and in pigs from central China (Zhou et al., 2010). These data suggest that this is a major lineage and is obviously widespread throughout mainland China. The climate varies significantly among the regions from which samples were collected, ranging from temperate to subtropical or tropical. The diversification of climatic conditions has produced a great diversity in animal and plant life in this province.

Genotype ToxoDB 9 has been identified in South and North America (Dubey, Cortes-Vecino et al., 2007), as well as eastern Asian countries, such as Sri Lanka, Vietnam, and China (Dubey, Huong et al., 2007; Dubey, Zhu et al., 2007; Dubey et al., 2008), indicating that it may also be a predominant genotype in other Asian countries and is likely widespread in these regions.

The present work provides new genetic information about *T. gondii* infection in farm-raised pigs in southwestern China. Our next effort will be directed at studying *T. gondii* infection and population genetics in other livestock and poultry in Yunnan Province for a better understanding of animal-derived food safety and public health. Furthermore, it is urgent to improve the life style of local people and implement *T. gondii*-inspection during pig slaughtering and processing (Zhou et al., 2008).

Project support was provided, in part, by grants from the National Basic Research Program (973 program of China (Grant No. 2010CB530001), National Natural Science Foundation of China (Grant Nos. 31172316 and 31101812) and the Yunnan Provincial Program for Introducing High-level Scientists (Grant No. 2009C125). Associate Professor Chunlei Su, Department of Microbiology, the University of Tennessee, Knoxville, Tennessee, is thanked for improving the manuscript.

**LITERATURE CITED**


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Call for Papers

The 87th Annual Meeting of the American Society of Parasitologists

Omni Richmond Hotel
Richmond, Virginia, July 13-16, 2012

Richmond, VA on the James River

Lewis Ginter Botanical Garden
The American Society of Parasitologists (ASP) announces its 87th Annual Meeting to be held at the Omni Richmond Hotel, Richmond, VA, July 13-16, 2012. Members and non-members are invited to submit abstracts for papers they intend to present as an oral presentation, poster, or if you are an invited speaker, as a contribution to one of our symposia or workshops. Individuals organizing symposia and special sessions are reminded that they need to send the names of their speakers to the program officers and that all speakers are required to submit an abstract. For all types of presentations, abstracts must be received on or before the deadline: Friday, February 24, 2012. The scientific Program & Abstracts booklet will be prepared from the abstracts received and will be available both on the ASP web site (http://asp.unl.edu) and the Allen Press web site (TBA) prior to the meeting. Everyone registered for the meeting (either in advance or on site) will receive a copy of the Program & Abstracts booklet in their registration packet. Additional copies of the Program may be purchased ($10.00 U.S. per copy) at the registration desk at the meeting or from Allen Press after the meeting. Please read and follow all instructions carefully when submitting abstracts and when registering on-line for the meeting and the hotel. Thank you!

Electronic Registration for the Meeting
On-line registration for the meeting will be available on a dedicated website for the Annual Meeting (http://events.SignUp4.net/asp2012). You may register online using a credit card at this link or you may mail your registration form with a check or credit card number (with original signature) payable to WVTTC c/o ASP 2012, 6601 Kingston Pike, Knoxville, TN 37919. See the ASP web page (http://asp.unl.edu) or send an e-mail to dennism@purdue.edu with the subject line “ASP” to be added to the listserv for on-line registration information as it becomes available. (For hotel reservation information see page iii.)

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<th>Cost</th>
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<td>ASP Member</td>
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‡ Must be accompanied by a Certification of Student Status Form (http://parasitology.winston.wfu.edu).

† Non-members (both post-graduate and students) may join ASP ($85 and $20–50, respectively); the “Join the ASP” link can be found at http://asp.unl.edu; mail completed application to Dennis Minchella, ASP, Dept. of Biological Sciences, Purdue University, West Lafayette, IN 47907.

Important Dates and Deadlines

Friday, February 24, 2012
• Receipt of all abstracts via the website (http://parasitology.winston.wfu.edu).
• ASP Marc Dresden Student Travel Grant and Student Paper Competition applications are due.

Friday, April 20, 2012
• Early Registration forms for meeting ONLY due.

Thursday, June 21, 2012 by 5 p.m.
• Hotel reservations must be made to secure the ASP conference rate for regular rooms.
Omni Richmond Hotel
100 South 12th Street
Richmond, VA 23219
Ph#: 804-344-7000

Our meeting venue this year is the Omni Richmond Hotel located in the historic Shuckoe district of Richmond. The Omni is a business-friendly hotel situated close to the Old Richmond City Hall, the Capitol Building and the current Richmond City Hall. The Omni has 361 guest rooms that feature shower/tub combinations, bathrobes, complimentary toiletries, hair dryers, wireless internet access in each room for a surcharge of $9.95 (see Wi-Fi section). Three dining facilities are located in the hotel: Trevi’s Restaurant, Trevi’s lounge and Trevi’s Market. The restaurant serves breakfast, lunch and dinner and room service is available 24 hours a day. Amenities include an indoor pool, a workout facility, a 4.0 star business center, AV equipment rental, and wireless internet access in public areas of the hotel. Additional amenities include a coffee shop/café, valet parking and a gift shop/newsstand.

Hotel Reservations—General
Hotel reservation is separate from meeting registration, so each individual guest is responsible for making their own hotel reservations. When reserving your room, make sure that you mention that you are part of the ASP meeting group to receive the ASP Special Group Rate. Everyone should be prepared to guarantee a first night room deposit with a major credit card.

ASP Regular Member Link:

ASP Student Link:
http://omnihotels.com/FindAHotel/Richmond/MeetingFacilities/AmericanSocietyofParasitologistsStudents7

ASP has contracted with the Omni for 450 room nights for the meeting dates. It is important that ASP members attending the meeting make their reservations at the Omni Richmond Hotel to fulfill our room block commitment. All meeting rooms for paper sessions and symposia are provided to ASP at no charge contingent on fulfilling our room block commitment. The daily room rate is $125 for regular ASP member and $99 for student members (quads), plus 13% tax. The special ASP Conference Rate is guaranteed 3 days prior to and 3 days after the meeting for folks wishing to extend their stay to include a short vacation in the Richmond area. The cut-off date for making reservations into the ASP room block is Thursday, June 21, 2012 by 5 p.m. All reservations made after that date will be subject to the hotel’s regular rates and space availability. Convention rates for the ASP meeting are not available by online wholesalers (e.g., Priceline.com) and you jeopardize fulfilling our guaranteed room block by attempting to use these sources.

Wireless Internet is free in the hotel’s common areas and is also available in guest rooms at a cost of $9.95 per day (rates may vary). However, ASP members can become a member of the Omni Hotels and Resorts Select Guest Program and get free Wi-Fi in your room. We encourage you to enroll at http://omniselectguest.com and take advantage of this FREE service. A host of other benefits are included when you enroll in this membership program.

Hotel Parking is $20.00/day for valet parking. This is the best deal in town since every private parking garage charges a lower daily rate, but you pay each time you enter the lot after using your vehicle. The valet rate is paid only once regardless of how many times you come and go.

Hotel Reservations—Student Quads
Students wishing to make arrangements for a Quad room must contact the ASP Student Representative for assistance with scheduling roommates for quads. He will help you get organized and communicate directly with the local arrangements committee chair and/or our representative at the Omni Richmond Hotel. The individual assigned as the contact person for each quad will be responsible for providing the names of the quad members, for securing the room with a credit card, and for assuming complete responsibility for paying the bill at checkout. Students sharing quads will need to work out their portions of the room costs with each other. The ASP Student Representative is Mr. Kyle Luth, Wake Forest University, Dept. of Biology, 1834 Wake Forest Road, Winston-Salem, NC 27106, Phone: 336-758-5577, email: luthke8@wfu.edu

Transportation Information

Air Transport
The Greater Richmond area is served by the Richmond International Airport located in nearby Sandston, VA, 7 miles (11 km) southeast of Richmond and within an hour’s drive from historic Williamsburg, VA. Nine airlines, providing up to 200 flights daily, including many nonstop flights from major destination markets, are located in the airport.

Ground Transportation
The Omni Hotel does not have its own airport shuttle service, but they do contract with Groome Transportation, a local service, to get people to and from the airport. You may call Groome directly (800-552-7911) to set up your airport transport or alternatively you may set up your transport with Groome via the WVTT registration site when you login to register for the meeting. WVTT will handle contacting Groome and scheduling your airport transport. YOU MUST INDICATE WHEN YOU REGISTER THAT YOU WANT WVTT TO ARRANGE YOUR AIRPORT TRANSPORTATION.

RATES: One person fare is $24 one-way (a round-trip in advance for $43); 2 persons one-way is $26.50 or $48.50 round-trip; 3 person fare is $30.75 one way or $56.75 round-trip; 4 or more the fare is $12 each, each way. All of the ONE WAY fares can be charged to the passenger’s room at the Omni Richmond. Round-trip fares must be purchased in advance. Regardless of whether you use Groome directly or you book transport through WVTT, Groome will need flight times & flight numbers so they can arrange pickup at baggage claim. (All fares are subject to change since these fares are for 2011.)

Richmond also has intercity bus service, 2 Amtrak stations, as well as local and paratransit bus service in Richmond, Henrico and Chesterfield counties, as well as taxi service in the city and at the airport. Richmond is also benefits from an excellent position with reference to car travel, being located at the junction of Interstate-64 east/west and Interstate 95 north/south.

Rental Vehicles
Cars can be rented at the Richmond International Airport. All major car rental services are located at the airport. We do not have, at this time, any discount offers from any rental agencies. If we get any discount offers we will post them on the ASP Website.
About Your Abstract(s)

Abstracts must be submitted online (http://parasitology.winston.wfu.edu) where you will find all the instructions for your submission(s). The deadline for receipt of abstracts is Friday, February 24, 2012. Everything in the meeting Program & Abstracts booklet is entered, formatted, edited, and produced electronically at Allen Press. Please carefully read the directions below and follow them.

Acceptance/Scheduling of Abstracts
Most abstracts, except those designating new taxa, will be accepted. Multiple papers being presented by one author may be restricted to two, with the remainder designated as posters at the discretion of the Scientific Program Officers. Notification of acceptance of and/or scheduling of your paper is not automatic. If you need a letter to present to your administration stating that your abstract has been accepted, please be sure to check that box on the Web submission page. If you need to know the time and date that your paper is scheduled, please e-mail the ASP Scientific Program Officers for this specific information, but not before May 25, 2012. Alternatively, you can check at http://parasitology.winston.wfu.edu or http://asp.unl.edu, where an electronic copy of the final Program & Abstracts booklet will be posted after May 25, 2012.

Preparing Your Abstract(s)
All abstracts MUST be submitted in English. When writing your abstract, both the title and the content are important components. The TITLE should be short, concise, and indicative of the abstract; omit “the” wherever possible. Avoid general words like “Studies on” or “Investigations of.” Use words with index retrieval value. On the Web abstract-submission page, you will be asked to indicate the presenting author of the paper or poster, which will be indicated by an asterisk (*) in the Program & Abstracts. The CONTENT should be a single paragraph of no more than 2,100 characters (including spaces, punctuation and HTML codes introduced by the abstract submission program) containing a concise statement of: (1) the problem under investigation (2) the experimental methods used and (3) the essential results obtained. The text should cite quantitative data from representative experiments or summary data. Scientific names should be written in full when first used, but on repetition abbreviate the genus name with the first letter of the genus only. Italicize all scientific names in abstract (title is handled by editor) as instructed on the Web abstract/submission page. We do not desire to prevent discussion of new taxa, but abstracts and/or titles which constitute the first designation of new taxa will not be accepted. Thus, do not use genus and species names of new taxa anywhere in your abstract.

Submitting Your Abstract(s)
Submit your abstract(s) via our Web page (http://parasitology.winston.wfu.edu): Follow all directions, fill in all the boxes, and press “Submit.” You will receive confirmation and a copy of your submitted abstract via a confirmation Web page; please print this page so as to have your confirmation number. Note: The function to write/correct your abstract(s) and related information will be disabled at midnight on Friday, February 24, 2012.

Scientific Attractions

Symposia and Workshops
The 44th (!) Coccidiosis Conference is being organized by Dr. Anthony Sinai (Univ. of Kentucky College of Medicine) and Dr. Xun Suo, (College of Veterinary Medicine, Beijing, China); Drs. Armand Kuris (UCSB) and John J. Janovy (UNL), our President and Vice President, respectively, are planning the annual President’s Symposium; Drs. Matt Bolek (Oklahoma State University) and Ben Hanelt (University of New Mexico) will host a Nematomorph Workshop; Mr. Kyle Luth
(WFU), our student representative to council, is organizing the annual Student Symposium. This year's student symposium will feature younger ASP members who have recently completed their degrees. They will be discussing the processes, obstacles and challenges they encountered in their search for a permanent position (speakers include Drs. Valerie McKenzie, Matt Bolek, Nick Negovetich, Janet Koprivnikar and Charles Criscione). The 5th Annual Associate Editors Symposium will feature the research of associate editors Ash Bullard (Auburn), Ramon Carreno (Ohio Wesleyan) and Mark Siddall (AMNH).

Awards and Lectures
We anticipate the selection of recipients of the H.B. Ward Medal, the Clark. P. Read Mentor Award, the Ashton Cuckler Young Investigator Award, the Stoll-Stunkard Memorial and the Bueding von Brand Lecture Awards, as well as our Marc Dresden Student Travel Grant and Best Student Presentation Awards. Additional awards will be announced in the program after the relevant committees have met and made recommendations.

Student Travel Grants and Paper Competition

Marc Dresden Student Travel (MDST) Grants/Student Paper Competition
Applications and directions for MDST Grants are on the 2012 ASP Meeting web page (http://parasitology.winston.wfu.edu). The deadline for receipt of MDST Grant applications and the ORAL Student Presentation Award Competition is the same as the deadline for receipt of abstracts, **Friday, February 24, 2012**. Students, you must complete and submit the MDST Grant application form which also certifies your student status and indicates whether or not you wish to have your paper entered into the **oral student presentation award competition**. Once the MDST form has been completed and submitted an email will be sent automatically to your advisor requesting his/her letter of support. For students competing in the oral paper competition, you must paste a copy of your abstract into the MDST Grant application form before it is submitted to the Chair of the MDST Grant Committee (may be a pdf or Word document). Questions about the competition process may be directed to the committee chair, Dr. Kym C. Jacobson, NMFS/NWFSC, Hatfield Marine Center, 2030 South Marine Science Drive, Newport, OR 97365, Ph: 541-867-0375, fax: 541-867-0389, email (kym.jacobson@noaa.gov).

Social Activities

The ASP Welcoming Reception will be held in the Omni Hotel's James River Salon C & D on Friday evening, July 13, 2012 from 7-10PM. Our traditional third day offsite venue this year will be an evening at the **Lewis Ginter Botanical Garden**, a 30 acre botanical garden, located just north of Richmond. Originally the hunting grounds of the Powhatan Indians called "Oughnum", the garden encompasses a beautifully sculptured collection of gardens that feature native plants of the southeast. The Garden is one of the signature attractions for visitors to the Richmond area. The Garden includes the restored home of the Garden's donor, Ms. Grace Arents, called Bloemendaal House, an English Garden, a Children's Garden, a Healing Garden, and a Conservatory that houses a variety of orchid species. **ASP members will have access to the Central Garden area adjacent to the auditorium where the meal is to be served and the Conservatory, but not to the other garden areas.** Our evening at The Lewis Ginter Botanical Garden is destined to be another in a long line of exceptional offsite venues that ASP members have enjoyed in the recent past. **A short program recognizing the contributions of the outgoing Journal Editor, Dr. Gerald (Jerry) W. Esch, will follow the meal.**
Oral and Poster Presentations

Electronic Presentations—MS PowerPoint®

MS PowerPoint® is the presentation standard for the meeting. Speakers should bring their presentation stored on CD-R, USB Flash Drive, or USB Pen Drive only; other storage formats may not be supported. Presenters must download their files to the PC of their session at least 30 minutes before the start of their session. Presenters are encouraged to save their presentation using the “Pack and Go” feature of PowerPoint®, especially if digital MPEGs (Moving Pictures Experts Group) or special fonts are used (detailed instructions for this procedure are in PowerPoint® “Help”). To ensure that each packed presentation is functional before you leave home, you are encouraged to unpack and run the presentation on a different computer than was used to create the presentation. Mac users can use the in-house systems if their files are saved with the “Pack and Go” feature. The onus will be on presenters to verify the compatibility of their files with the computers in use at the Omni Richmond Hotel.

The standard oral presentation is 12 minutes plus 3 minutes reserved for questions and answers, unless otherwise indicated by the moderator. A laser pointer will be provided by the session moderator. Use contrast to your advantage, especially in labels. Blue or red letters in black boxes cannot be seen! White backgrounds are considered by some professionals to be the best. Keep the number of text lines to 9 to 10, or less, per slide. If you have to say, “I know you can’t see this, but …,” then omit it!! Use fonts, colors and features both consistently and judiciously. Your audience will thank you and, if you are giving a student presentation, it will improve your presentation. Remember, your science is on display, not your computer skills.

Poster Presentations

The poster session provides an additional means of substantive discussion of papers for members attending the Meeting. No competing activities will be scheduled during the poster session (unless absolutely unavoidable). Papers scheduled for presentation in the poster session are grouped by category, numbered and listed with their abstract in the Program & Abstracts booklet. Each numbered poster will be allotted a correspondingly numbered 4' x 4' space (122 x 122 cm). Indicate the abstract number, title, and name(s) of the author(s) at the top of the poster so that it may be identified easily; the lettering for this heading should be at least 1 inch (72 points) high. A copy of your abstract should be posted in the upper left-hand corner of your poster. Please Note: Posters can be in English or Spanish, but the abstract posted in the left-hand corner of your poster must be in English.

22nd Annual ASP Student Auction

Yet again, we are asking you to search your attics, basements, out-buildings, coffers or safety deposit boxes for donations to our Annual Auction to raise funds for student travel to ASP meetings. Serious, humorous... whatever you come up with... it’s all grist for the auction mill and will be much appreciated. You may bring your donation(s) with you to the meeting and turn it/them in for documentation when you register. If you would like to mail your auction items ahead (mail to arrive no earlier than 3 days prior to the meeting), please mail them to the following address.

ASP, July 13-16, 2012
ATTN: Ms. Lisa Jeffers
Omni Richmond Hotels & Resorts
100 South 12th Street
Richmond, VA 23219
If you have questions about the auction or about items you wish to donate, please contact: Dr. Kelli Sapp, High Point University, Dept. of Biology (D-10), 833 Montlieu Avenue, High Point, NC 27262; Phone: 336/841-4534, ksapp@highpoint.edu or Ms. Lee Couch, Department of Biology, MSC 03 2020, 1 University of New Mexico, Albuquerque, NM 87131-0001; Phone: 505/277-2400, leouch@unm.edu.

**Local Attractions**

Situated on the James River, Richmond served as a port for many of the agricultural products that were produced in the US during the early years of the country and it is still today a booming port for many of the goods coming from the Tidewater areas of eastern Virginia. And as we all know, Richmond was the seat of the confederacy during the civil war. However, Richmond was also the site of much of the fervor for independence during the American Revolution. The St. John’s Church where Patrick Henry made his famous speech that included the phrase, “Give me liberty or give me death” is also located in Richmond. There are several other historical sites worth seeing in the city as well, including Belle Isle and Brown’s Island, two islands located in the James River, the Edgar Allan Poe Museum, the Hollywood Cemetery where 2 U.S. presidents are buried, the Virginia Museum of Fine Arts where you can see the largest collection of Faberge Eggs outside of Russia and the Reconciliation Statue, built in 2007, which symbolizes the city’s period of slavery. You can stroll through downtown Richmond to many of the shops, museums and eateries, visit Jackson Ward, which was called the “Wall Street of Black America” because of the financial acumen of people such as Maggie L. Walker, who started the St. Luke Penny Savings Bank for African Americans, tour the Arthur Ashe Athletic Center, shop in Carytown or stroll through Shockoe Bottom, formerly a Powhatan Native American fishing village.

**Things to do/places to visit in Richmond and surrounding area**

- Virginia Museum of Fine Arts
- Maymont Park
- Lewis Ginter Botanical Garden
- Virginia Capitol Building
- Virginia Historical Society
- Hollywood Cemetery
- Three Lakes Nature Center and Aquarium
- The Museum of the Confederacy
- Byrd Theater
- Agecroft Hall
- Science Museum of Virginia
- Virginia Holocaust Museum
- St. John’s Episcopal Church
- Edgar Allan Poe Museum
- St. Paul’s Episcopal Church
- Monument Avenue
- The James River
- Black History Museum and Cultural Center of Virginia
- Beth Ahabah Museum and Archive
- Williamsburg
- King’s Dominion
- Charlottesville Wine area
Schedule of Events

Friday, July 13th
ASP Council Meeting ................................................................. 8:00 a.m.-Noon
2–3 oral paper sessions or symposia ........................................... 1:00–5:00 p.m.
Welcoming Reception, The James River Salon C & D ............... 7:00–10:00 p.m.

Saturday, July 14th
ASP President’s Symposium ...................................................... 9:00–11:00 a.m.
Stoll-Stunkard Memorial ............................................................. 11:00 a.m.-Noon
4–5 oral paper sessions/symposia .............................................. 1:00–5:00 p.m.
ASP Student Social ................................................................. 5:00–6:30 p.m.
22nd Annual ASP Auction Preview & Auction ....................... 6:00–9:00 p.m.

Sunday, July 15th
1–4 oral paper sessions/symposia .............................................. 8:30 a.m.–Noon
1–4 oral paper sessions/symposia .............................................. 1:00–5:00 p.m.
Evening at the Lewis Ginter Botanical Garden ....................... 7:00–10:00 p.m.

Monday, July 16th
1–4 oral paper sessions/symposia (as needed) ....................... 8:30–10:00 a.m.
Poster Session ................................................................... 10:30 a.m.–Noon
H.B. Ward Medal Lecture ......................................................... 1:00–1:50 p.m.
Bueding-von Brand Lecture ...................................................... 1:50–2:40 p.m.
ASP Awards & Business Meeting ........................................... 3:00–4:30 p.m.

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MAILING DATES FOR VOLUME 97, 2011

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No. 3, June . . . . . . . mailed 2 June 2011
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COVER CAPTION: Scanning electron micrographs of Haplosporidium tuxtlensis n. sp. Basal view of a spore showing emerging filaments (f) from the wall. Figure 2B, From Veá and Siddall 97: 1062–1066.