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GREGARINES ON A DIET: THE EFFECTS OF HOST STARVATION ON GREGARINA CONFUSA JANOVY ET AL., 2007 (APICOMPLEXA: EUGREGARINID) IN TRIBOLIUM DESTRUCTOR UYTTEBOOGAART, 1933 (COLEOPTERA: TENEBRIONIDAE) LARVAE

Jodi Schreurs and John Janovy, Jr.
School of Life Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska. e-mail: jjanovy1@unl.edu

ABSTRACT: This study was designed to explore the nutritional relationship between Gregarina confusa (Apicomplexa: Eugregarinida) parasites and its coleopteran host, Tribolium destructor, by measuring the cytoplasmic density of gregarines in continuously fed larvae, starved larvae, and larvae refed after starvation. Cultures were maintained in a standard media (whole wheat flour:commercial wheat germ:yeast [30:10:1]). Larvae from control and experimental groups were dissected daily for 3 days then allowed to feed or starve for an additional 3 days. On day 6, the remaining experimental larvae were divided and placed into 2 groups; 1 group remained starved while larvae from the second group were fed a Wheaties® flake. Photographs were taken of the parasites daily and analyzed using ScionImage®. Gregarines from starved larvae were significantly longer and skinnier than those from fed controls, and there was also a significant difference between gregarine deutomerite cytoplasmic densities. Parasites from refed larvae regained cytoplasmic density within 24 hr and showed morphological similarities to those from fed larvae. This study shows that the Tribolium destructor-Gregarina confusa relationship can be manipulated easily through alterations of host diet and thus is an excellent model for use in the study of chemical relationships between parasites and their hosts.

Gregarines are apicomplexans that occur in most, if not all, invertebrate groups, but are especially common in arthropods and annelids. Because their hosts are so numerous, gregarines should probably be considered the most diverse group of parasites (Roberts and Janovy, 2005). A typical gregarine life cycle includes trophont and gamont stages, which participate in the processes of association, syzygy, gametogenesis, gametocyst formation, fertilization, oocyst formation, and dehiscence. Oocyst evisceration, attachment to host intestinal epithelium, penetration of epithelial cells, growth, gamont association, syzygy, and gametocyst formation all occur within the host. Because both hosts and parasites are so diverse, gregarine-invertebrate systems promise to be a rich source of material for exploring the evolution of host-parasite relationships.

There have been numerous cytochemical studies on gregarines (Göhre, 1943; Schrevel, 1970; Amoji, 1975; Ramachandran, 1976; Sathananthan, 1977; Janardanan and Ramachandran, 1987), but none has focused on the effects of host diet on parasite morphology or cytoplasmic contents. Studies by Schawang and Janovy (2001) showed that starvation of adult Tenebrio molitor (Tenebrionidae) results in a halt of gametocyst shedding by Gregarina niphandrides. Thus, by altering diet, the life cycle could be “turned off” at the gametocyst life cycle stage. Once the hosts were refed, gametocyst shedding resumed. These observations suggest that metabolic relationships between host and parasite could be explored with simple techniques. The current study is an attempt to extend the Schawang relationship can be manipulated easily through alterations of host diet and thus is an excellent model for use in the study of chemical relationships between parasites and their hosts.

MATERIALS AND METHODS

Cultures of Tribolium destructor Uyttenboogaart, 1933, were obtained from Andrea Václugurova, Department of Zoology and Ecology, at Masaryk University in the Czech Republic on 8 July 2003, and subsequently maintained in plastic jars 5 cm in diameter and 5 cm tall with perforated plastic lids in an incubator at 27 C with a pan of water provided for moisture. Culture medium consisted of wheat bran, whole wheat flour, commercial wheat germ, and baker’s yeast (40:40:15:5). New cultures were started by transfer of 20 larvae and 20 adults every 2 wk. Under these conditions, offspring larvae became very heavily infected within a month. Host voucher specimens were killed by freezing then glued to pin points (adults) or killed by immersion in boiling water then preserved in 70% ethanol (larvae). Gregarines were identified as Gregarina confusa Janovy et al., 2007, based on size, body proportions, epimerite shape, and oocyst size. For voucher specimens, larval hosts were dissected and stained smears were made from guts that had been teased apart by the methods of Kula and Clotpon (1999). Air-dried smears were fixed in alcohol-formalin-acetic acid, washed in 70% ethyl alcohol, stained in Semichon’s acetocarmine, dehydrated, cleared in xylene, and mounted with Damar balsam (Spectrum, Gardena, California) (Prichard and Kruse, 1982; Kula and Clotpon, 1999). Such smears were also made using guts from larvae dissected in 1:3:5:Mayer’s albumin:water, stained with Lugol’s iodine solution, then dehydrated and mounted as mentioned above. Fresh preparations were made in Tenebrio sp. saline (Belton and Grundfest, 1962). For measurements of living organisms, images were taken with a Nikon Coolpix 995 camera fitted with an Optem 25-70-14 adapter (www.optemintl.com). Digital photograph numbers were recorded, and the actual lengths of specimens were calculated from photographs of a stage micrometer. These photographs were then converted to bitmaps, 15.2 cm x 11.4 cm at 39.4 pixels/cm, and adjusted to auto levels using Adobe Photoshop 7.1® (Adobe Systems Inc., San Jose, California). ScionImage® (Scion Corporation, Frederick, Maryland) image analysis software was then used on these pictures to measure cytoplasmic densities, lengths, and widths of both the proteronite and deutomerite of trophonts and gamonts. Photographs of fixed and stained specimens were taken by the same camera; Adobe Photoshop 7.1® and Adobe Illustrator 10.0.3® were used to prepare the figures.

EXPERIMENTAL DESIGN

Larvae were taken from cultures approximately 1-mo-old and dissected to verify heavy gregarine infections (>500/individual). A single trial involved 3 groups of at least 5, 9–10 mm

Received 14 May 2007; revised 7 October 2007; accepted 10 October 2007.
Figures 1–6. Gregarines from *Tribolium destructor* larvae. (1–3) *Gregarina confusa* of various ages stained with Lugol’s iodine solution, showing increasing accumulations and concentration of polysaccharide with increasing size. (4) Live *G. confusa* from time 0 fed control *Tr. destructor* larva. (5) Live *G. confusa* from *Tr. destructor* larva starved for 7 days. (6) Live *G. confusa* from *Tr. destructor* larva starved for 6 days then refed Wheaties® flake. Bar = 50 μm in all figures.

long, larvae. Included were a time-zero (*T₀*) control group, a starved experimental group, and a continuously fed time-end (*T₇*) control group. The *T₇* group was fed whole wheat flour, commercial wheat germ, and baker’s yeast (30:10:1). All larvae were kept in covered 15 × 60 mm glass Petri dishes on a laboratory shelf for the experiment duration. Each day for 3 consecutive days, 5 larvae were dissected from both the starved and *T₇* groups using the same protocol as for *T₀* larvae. Following the daily dissection, all remaining starved larvae were transferred to clean Petri dishes to minimize the possibility of re-infection. Larvae remained in these dishes for 3 more days; then half the starved group was refed a single Wheaties® flake in a separate Petri dish. On the 7th day, remaining larvae from control fed, experimental starved, and experimental refed groups were dissected using the above protocol. For each group’s dissected larvae, at least 20 pictures were taken of gamonts, and 20 pictures were taken of trophonts, resulting in ~100–200 pictures daily.

These experiments were repeated 3 times. Measurements of lengths, widths, and densities were obtained using image analysis software and copied into Excel® spreadsheets (Microsoft Inc., Redmond, Washington); statistical analysis was performed using the built-in Excel® functions.

Parasite voucher specimens (6 slides stained with acetocarmine, 1 slide stained with Lugol’s iodine) were deposited in the Harold W. Manter Laboratory of Parasitology collection, University of Nebraska–Lincoln, with accession numbers HWML48550 and HWML48550, respectively. Voucher sym-
biotype specimens, 5 adults and 10 larvae, were deposited in the Division of Entomology collection, University of Nebraska State Museum, with accession numbers J. Janovy 55-59 for the adults and J. Janovy 60 for the larvae.

RESULTS

Gregarines stained in Lugol’s iodine exhibited very dark granulation throughout the deutomerite cytoplasm, although protomerite cytoplasm tended to remain relatively clear (Figs. 1–3). Staining was most dense in larger individuals, and the smallest ones, especially single trophozoites, typically had relatively clear cytoplasm. Larger gregarines from continuously fed larvae showed dense cytoplasm, with granules often obscuring the nucleus when observed alive (Fig. 4). In starved larvae, gregarine cytoplasm was generally clearer than in parasites from fed larvae (Fig. 5); refedding of starved larvae produced gregarines with very high cytoplasmic density (Fig. 6). Larvae isolated without food shed many gametocysts the first day, fewer the second day, and by the third day gametocyst shedding stopped. These results were not quantified, but the cessation of gametocyst shedding by starved larvae was obvious.

Table I shows the cytoplasmic densities and P-values from t-tests performed between gregarines from control fed and experimental starved larvae. There was a significant difference between cytoplasmic density for gregarines in starved versus continuously fed larvae. This result was consistent for primitives and satellites of paired gamonts as well as for trophonts. Although gregarines from control fed larvae retained a high cytoplasmic density (between 110 and 165 SCN units) throughout an experiment, gregarines from starved larvae exhibited a drastic decrease in cytoplasmic density (between 87 and 124 SCN units) by day 3 of the experiments. After refeeding, gregarines from previously starved larvae showed a significant increase in cytoplasmic density in less than 24 hr.

Table II shows parasite length/width ratios and P-values from t-tests performed between gregarines from control fed and experimental starved larvae. There was a significant difference between the means of these ratios for gregarines in starved versus continuously fed larvae; the difference was due primarily to the loss of width in parasites from starved hosts. This result was true for primitives and satellites of paired gamonts as well as for trophonts. After refeeding, gregarines from previously starved larvae showed a significant reduction in length/width ratio, or relative increase in width.

Table I: Comparisons of the deutomerites’ mean cytoplasmic density (number of parasites measured, standard deviation) in ScionImage® units between gregarines from control fed versus experimental starved larvae, with day 7 control data taken of gregarines from the refed experimental larvae.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Primite</th>
<th>Satellite</th>
<th>Trophont</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 7 refed</td>
<td>Day 3</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-fed</td>
<td>146.9 (21, 17.7)</td>
<td>143.1 (18, 20.2)</td>
<td>155.0 (21, 17.5)</td>
</tr>
<tr>
<td>Expt-starved</td>
<td>95.1 (22, 22.1)</td>
<td>160.7 (33, 25.0)</td>
<td>122.4 (22, 22.8)</td>
</tr>
<tr>
<td>p/=/*</td>
<td>&lt;0.0001</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control-fed</td>
<td>157.3 (14, 17.1)</td>
<td>151.7 (38, 33.1)</td>
<td>164.7 (14, 18.6)</td>
</tr>
<tr>
<td>Expt-starved</td>
<td>102.4 (26, 21.7)</td>
<td>95.4 (13, 15.9)</td>
<td>124.7 (26, 29.6)</td>
</tr>
<tr>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-fed</td>
<td>137.2 (42, 32.6)</td>
<td>169.8 (20, 23.2)</td>
<td>133.5 (42, 38.0)</td>
</tr>
<tr>
<td>Expt-starved</td>
<td>109.4 (27, 15.9)</td>
<td>102.4 (14, 16.8)</td>
<td>123.9 (27, 21.7)</td>
</tr>
<tr>
<td>p/=/*</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Probability of equality between gregarines from control fed versus starved larvae as determined by t-test; measurements of parasites from all larvae in a group are pooled.

Table II: Comparisons of the deutomerites’ mean length/width ratios (number of parasites measured, standard deviation) between gregarines from control-fed versus experimental-starved larvae, with day 7 control data taken of gregarines from the refed experimental larvae.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Primite</th>
<th>Satellite</th>
<th>Trophont</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 7 refed</td>
<td>Day 3</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-fed</td>
<td>4.9 (21, 0.4)</td>
<td>4.2 (33, 0.7)</td>
<td>4.9 (21, 0.7)</td>
</tr>
<tr>
<td>Expt-starved</td>
<td>6.5 (22, 0.9)</td>
<td>7.3 (8, 0.9)</td>
<td>7.0 (22, 1.0)</td>
</tr>
<tr>
<td>p/=/*</td>
<td>&lt;0.0001</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
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<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-fed</td>
<td>3.6 (14, 0.5)</td>
<td>4.2 (38, 0.7)</td>
<td>3.3 (14, 0.5)</td>
</tr>
<tr>
<td>Expt-starved</td>
<td>6.5 (26, 0.9)</td>
<td>6.1 (13, 1.2)</td>
<td>6.2 (26, 1.1)</td>
</tr>
<tr>
<td>p/=/*</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-fed</td>
<td>5.0 (42, 0.8)</td>
<td>4.2 (20, 0.8)</td>
<td>4.9 (42, 1.2)</td>
</tr>
<tr>
<td>Expt-starved</td>
<td>6.1 (27, 0.9)</td>
<td>6.0 (14, 1.6)</td>
<td>6.9 (27, 1.4)</td>
</tr>
<tr>
<td>p/=/*</td>
<td>&lt;0.0001</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Probability of equality between gregarines from control fed versus starved larvae as determined by t-test; measurements of parasites from all larvae in a group are pooled.
The results from this experiment, as well as those from introducing a Wheaties® flake to the host. This rapid response Length/width ratios are routinely used as taxonomic characters that modify a parasite's development and morphology, or vice versa. It is also important that the long, skinny gregarines from starved larvae become short, robust, and dense within 24 hr of introducing a Wheaties® flake to the host. This rapid response indicates that assimilative metabolism of the parasite can be studied simply by providing the host with certain known dietary components. Although there are still many questions to be answered regarding the observations reported here, the wealth of gregarine species and extreme diversity of their hosts, combined with a novel approach to the study of host-parasite interactions, means that a rich supply of excellent projects awaits future researchers interested in gregarines.

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LITERATURE CITED


DIFFERENTIAL GENE EXPRESSION ANALYSIS IN EUROPEAN EELS (ANGUILLA ANGUILLA, L. 1758) NATURALLY INFECTED BY MACROPARASITES

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ABSTRACT: We analyzed the relationships between the macroparasite community of the European eel and the expression of genes involved in the host physiology during its continental life. The genes studied are implicated in (1) response to environmental stress, i.e., heat shock protein 70 (HSP70) and metallothionein (MT); (2) osmoregulation, i.e., β thyroid hormone receptor (βTHR) and Na+/K+ ATPase; and (3) silvering, i.e., βTHR, freshwater rod opsin (FWO), and deep-sea rod opsin (DSO). All were enumerated by quantitative reverse-transcription polymerase chain reaction. The epizootiological results revealed for 93 yellow eels caught in the Salses-Leucate Lagoon (France) included 11 species: 1 nematode, 2 acanthocephalans, 1 monogenean, and 7 digeneans. The molecular results revealed (1) a significant negative relationship between digenean abundance and the expression level of all the tested genes, except FWO; (2) a significant negative relationship between the abundance of the nematode Anguillicola crassus and the expression level of the Na+/K+ ATPase gene; and (3) a significant positive relationship between the A. crassus abundance and the expression level of the MT gene. Eels infected with digeneans had, on average, a lower level of expressed genes. We hypothesize that the parasites may disturb the eel’s ability to withstand environmental stress and delay their migration to the Sargasso Sea because of degeneration of the gut. We further propose that the effect of the invasive species, A. crassus, on the gene expression was mainly linked to an increased trophic activity of infected eels. Moreover, it is possible that the parasite may have an effect on the fish’s migratory behavior, which is tied to reproductive purposes. Additional work, including an experimental approach, is required to confirm our hypotheses.

Macroparasites are ubiquitous components of natural ecosystems. Their ecological impact on wildlife populations is currently a significant growth area in parasitological research as they may play a role in structuring ecosystems (Thomas et al., 2005). The mechanisms by which macroparasites may regulate natural host populations still require investigation, especially in order to characterize the pathogenic effects at the organismal level.

Proteomics and genomics are fast-moving disciplines that may give a new dimension to host-parasite interaction studies (Biron et al., 2005). Differential gene expression has been recently used in fish-parasite models to compare parasitized and nonparasitized hosts (Collins et al., 2007) and to estimate the immune gene expression related to pathogen and parasite infections (Harms et al., 2003; Lindenstrøm et al., 2004). In both cases gene expression appeared to be a powerful tool to analyze the physiological response of a host infected by a given parasite. However, there is still a lack of knowledge regarding the nature of host functions affected and the consequences for gene expression when hosts are infected with parasites. Although experimental studies with a single parasite species are probably more efficient to detect effects, hosts in the wild are confronted with parasite species that may have synergistic or antagonistic effects on host physiology.

The European eel (Anguilla anguilla) is considered a threatened species (Withr and Bernatchez, 2003). The life cycle of this species is characterized by a growth period in continental waters (yellow stage), followed by a catadromous migration to reach the spawning area in the Sargasso Sea (silver stage) (Schmidt, 1922; Tesch, 1977). The quality of habitat in continental water and the subsequent migration from freshwater to sea water will determine the reproductive success of the species. Among the stresses faced during continental life, the acquisition of a rich parasite fauna (Bruslé, 1994) may have an effect on the survival of the eels. Eels acquire their first macro-parasites after arriving in continental waters, i.e., when they begin to feed. Thereafter, they contend with parasites throughout their growth phase, lasting between 3 and 20 yr (Tesch, 1977). Much attention has been paid on the effects of eel physiology by recently introduced parasite species, namely, the swimbladder nematode Anguillicola crassus and the gill monogeneans Pseudodactylogyrus spp. (Kennedy, 2007). In addition to epizootiological studies and the effects of salinity, nematodes are known to affect the histological structure of the swimbladder (Molnár et al., 1993; Würtz and Taraschewski, 2000; LeFebvre et al., 2002) and blood properties of the eel (Boon et al., 1989; Kelly et al., 2000). Other studies have highlighted a decrease in the host’s swimming performances (Sprengel and Lüchtenberg, 1991) and their ability to withstand stress (Molnár, 1993; Kirk, 2003; Gollock et al., 2004). Previous work on the gill monogeneans Pseudodactylogyrus spp. has mainly focused on their geographical distribution and on the immune response induced by the parasite (Buchmann et al., 1987; Slotved and Buchmann, 1993; Monni and Cognetti-Varriale, 2002).

The present study aims to estimate the macroparasite load and detect if possible a relationship between parasite numbers and the expression of key genes involved in the physiology of the eels during their continental life. This period is characterized by 3 major physiological challenges: (1) the response to environmental stress such as temperature or pollution; (2) effective osmoregulation, which is of some importance in the highly changing ecosystems represented by coastal lagoons; and (3) the metamorphic process (silvering), a fundamental phase in the eel life cycle, which marks the starting point of the migration to the Sargasso Sea for reproduction. The response to environmental stress was tested by estimating the expression of the heat shock protein (HSP) and metallothionein (MT) genes in the liver. The osmoregulation capacity of eels was estimated and gills, respectively. Finally, the success of the silvery process was evaluated by analyzing the expression level of the βTHR and the rhodopsin genes in the liver and eyes, respec-

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vatively. Considered together, we attempted to focus on key genes that, if over- or underexpressed in relation to the presence of the parasite, may alter the host’s homeostatic equilibrium.

**MATERIALS AND METHODS**

**Sample collection**

Ninety-three eels were collected by professional fishermen in Salses-Leucate Lagoon (France, 42°50′N, 03°00′E). Eels were brought to the laboratory alive and maintained in freshwater (for no more than 36 hr after capture) before analysis. It is possible that these holding conditions may influence gene expression. However, since eels were randomly chosen at the time of dissection, we assumed that uninfected and infected eels were both affected by the same stressful conditions.

Each fish was measured (total length) to the nearest millimeter. Age was estimated by counting the winter rings on sagital otoliths (Lecomte-Finger, 1985). The liver, the 4 arches of the same side of the gills, and the digestive tract were examined using a binocular microscope. All the parasites were recovered. For each parasite taxon, some individuals were mounted under a cover slide for further identification. Species identification was done when possible, and classical epizootiological identification was done when possible, and classical epizootiological parameters were enumerated (Bush et al., 1997), i.e., prevalence is the percentage of infected hosts, mean abundance is the total number of parasites were recovered. For each parasite taxon, some individuals were involved in detoxification processes. In teleost fishes, the MT gene expression can be induced by elevated temperatures, heavy metals, pesticides, and hydrocarbons (Smetters, 1993; Iwama et al., 1998). Among the Salmonidae, the HSP70s are involved in the immune response against pathogenic bacteria (Ackerman and Iwama, 2001). MTs are ubiquitous intracellular proteins, which present a strong affinity for metallic ions and thus are involved in detoxification processes. In teleost fishes, the MT gene expression is induced by heavy metals (de Smet and Blust, 2001; Langston et al., 2002), free radicals (Kling and Olsson, 2003), and cold thermal shock (Hermesz et al., 2001).

Target genes

HSPs are ubiquitous molecular chaperones expressed in response to a wide range of stressors (Feder and Hofmann, 1999). In teleost fishes, it has been shown that the HSP70 gene expression can be induced by elevated temperatures, heavy metals, pesticides, and hydrocarbons (Sanders, 1993; Iwama et al., 1998). Among the Salmonidae, the HSP70s are involved in the immune response against pathogenic bacteria (Ackerman and Iwama, 2001). MTs are ubiquitous intracellular proteins, which present a strong affinity for metallic ions and thus are involved in detoxification processes. In teleost fishes, the MT gene expression is induced by heavy metals (de Smet and Blust, 2001; Langston et al., 2002), free radicals (Kling and Olsson, 2003), and cold thermal shock (Hermesz et al., 2001).

In teleost fishes, thyroid hormones (THs) and their receptors (THRhs) are involved in growth control, osmoregulation (Parker and Specker, 1990), and metamorphosis (Inui and Miwa, 1985). βTHR is a protein ligand of T3 and T4 THs that can activate or repress TH gene transcription (Wu and Koenig, 2000). Na+/K+ ATPase is a transmembranous enzyme that uses the energy liberated from adenosine triphosphate (ATP) hydrolysis to exchange extracellular K+ with intracellular Na+ (Møller et al., 1996). In euryhaline fishes such as the European eel, it plays a major role in the osmoregulatory processes (Cutler et al., 2000).

In the European eel, vision adaptation to oceanic water occurs during the silvery. Freshwater rhodopsin (FWO) and the deep-sea rodopsin (DSO) are retinal proteins that permit vision adaptation to the different spectral radiation, respectively, of freshwater and seawater fishes (Pankhurst and Lythgoe, 1983). The differential expression of the FWO and the DSO genes control the progressive switch between these 2 proteins (Wood and Partridge, 1993; Hope et al., 1998; Zhang et al., 2000).

**Molecular analysis**

Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, California) according to the manufacturer’s instructions. RNA concentrations were read spectrophotometrically. Five micrograms of RNA were used to synthesize cDNA in a total volume of 20 μl containing 1× First-Strand Buffer, 0.5 μg oligo dT as primer, 0.5 mM dNTPs, 10 mM DTT, 40 U RNaseOUT (Invitrogen), 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen), and DEPC wa-

**RESULTS**

**Biological, morphological, and epizoological characteristics**

The age and the total length of the 93 European eels sampled ranged from 2 to 8 yr (mean ± standard deviation = 4.5 ± 1.2...
Freshwater opsin

Eyes

Actin

Liver, gills, eyes

AB074846

Actin F

5'-CGGAATTCACGACGACC-3'

205

48

12

Actin R

5'-TCCAGGCTATTGTCGCATT-3'

Heat shock protein 70

Liver

NM_131397

HSP70 F

5'-ACCGAGGCTACCTACCTC-3'

377

67

20

HSP70 R

5'-CTTCAAGGCTAGGCTAGA-3'

Metallothionein

Liver

NM_131075

MT F

5'-CCAAAGCTGAGCTGCAACTG-3'

120

60

10

MT R

5'-CAGCCAGGCGACTGTCG-3'

β thyroid hormone receptor

Liver

AF302241

βTHR F

5'-CAGGAGGCACTGACGACG-3'

229

60

15

βTHR R

5'-GCCGAGTCTAAACTGACG-3'

Na+/K+ATPase

Gills

AJ239317

NaK F

5'-GAATGGAGAGAGAAACGT-3'

157

60

8

NaK R

5'-GGGAAGAGAGAAACGT-3'

Deep-sea opsin

Eyes

AJ249203

DSO F

5'-TTTGTGTCGTCCTGCACTG-3'

446

60

20

DSO R

5'-ACTTCTCTGCACTGACTG-3'

Freshwater opsin

Eyes

AJ249202

FWO F

5'-CATCTCAATCTCTGCTG-3'

319

60

17

FWO R

5'-TGTTGATTATATTGCGG-3'

Expression level of target genes and presence of parasites

Table III shows the ΔCt values of uninfected and infected fish with each of the parasite class for the HSP70, MT, βTHR, Na+/K+ ATPase, FWO, and DSO genes. Expression of the HSP70 gene was lower in the liver of fish when digeneans were present in the stomach as compared to fish without digeneans in their stomach (nuninfected = 40, ninfected = 38, U = 554, P = 0.04) and when at least 1 digenean was present relative to fish without any digenean (nuninfected = 23, ninfected = 55, U = 417, P = 0.02). Fish that were uninfected by digeneans had 1.5 and 1.7 times (reIR) higher levels of liver HSP70 gene expression

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Tissue</th>
<th>Accession no.</th>
<th>Primer*</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
<th>Annealing temp. (C)</th>
<th>Extension time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actine</td>
<td>Liver, gills, eyes</td>
<td>AB074846</td>
<td>Actin F</td>
<td>5'-CGGAATTCACGACGACC-3'</td>
<td>205</td>
<td>48</td>
<td>12</td>
</tr>
<tr>
<td>Heat shock protein 70</td>
<td>Liver</td>
<td>NM_131397†</td>
<td>HSP70 F</td>
<td>5'-ACCGAGGCTACCTACCTC-3'</td>
<td>377</td>
<td>67</td>
<td>20</td>
</tr>
<tr>
<td>Metallothionein</td>
<td>Liver</td>
<td>NM_131075†</td>
<td>MT F</td>
<td>5'-CCAAAGCTGAGCTGCAACTG-3'</td>
<td>120</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>β thyroid hormone receptor</td>
<td>Liver</td>
<td>AF302241</td>
<td>βTHR F</td>
<td>5'-CAGGAGGCACTGACGACG-3'</td>
<td>229</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>Na+/K+ATPase</td>
<td>Gills</td>
<td>AJ239317</td>
<td>NaK F</td>
<td>5'-GAATGGAGAGAGAAACGT-3'</td>
<td>157</td>
<td>60</td>
<td>8</td>
</tr>
<tr>
<td>Deep-sea opsin</td>
<td>Eyes</td>
<td>AJ249203</td>
<td>DSO F</td>
<td>5'-TTTGTGTCGTCCTGCACTG-3'</td>
<td>446</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>Freshwater opsin</td>
<td>Eyes</td>
<td>AJ249202</td>
<td>FWO F</td>
<td>5'-CATCTCAATCTCTGCTG-3'</td>
<td>319</td>
<td>60</td>
<td>17</td>
</tr>
</tbody>
</table>

* F = forward, R = reverse.
† HSP70 and MT primers were designed into conserved regions of at least 4 telost cDNA sequences. HSP70 and MT cDNA sequences of Danio rerio are shown as references.

yr) and from 190 to 480 mm (317 ± 50 mm), respectively. The epizootiological results are presented in Table II. We found 11 species of macroparasites: 1 Nematoda, 2 Acantocephala, 1 Monogenea, and 7 Digenea. The prevalences of the different species of digeneans ranged from 1.1% for species 1 and 2 (for which only 1 fish was found to be infected) to 51.1% for Lecithochirium gravidum. Digeneans were also characterized by having the highest intensities, in particular Prosorchynchus aculeatus (688 parasites in 1 intestine). Of the 7 digenean species, 5 were niche specific (stomach for digenean species 1 and 2, and intestine for Prosorchynchus aculeatus, Bucephalus sp., and Deropristis inflata), and 2 (L. gravidum and Helicometra sp.) were not.

Table II. Epidemiological parameters, calculated according to Bush et al. (1997), of the parasite species found on 93 European eels Anguilla anguilla at Salses-Leucate lagoon (Pyrénées-Orientales, France).

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Class</th>
<th>Species</th>
<th>Niche*</th>
<th>Mean abundance ± SD</th>
<th>Prevalence (%)</th>
<th>Mean intensity ± SD (min-max)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nematoda</td>
<td>Anguillicola crassus</td>
<td>SB</td>
<td>0.32 ± 1.48</td>
<td>10.6</td>
<td>3.00 ± 3.68 (1-13)</td>
<td></td>
</tr>
<tr>
<td>Acantocephala</td>
<td>Acantocephalus anguillae</td>
<td>I</td>
<td>0.10 ± 0.64</td>
<td>4.3</td>
<td>2.25 ± 2.50 (1-6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acantocephalus species 1</td>
<td>I</td>
<td>0.19 ± 0.86</td>
<td>1.1</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Monogenea</td>
<td>Pseudodactylogyrus anguillae</td>
<td>G</td>
<td>1.00 ± 5.97</td>
<td>6.4</td>
<td>15.67 ± 19.67 (1-53)</td>
<td></td>
</tr>
<tr>
<td>Digenea</td>
<td>Lecithochirium gravidum (Hemiuridae)</td>
<td>S</td>
<td>4.19 ± 14.79</td>
<td>44.7</td>
<td>9.38 ± 21.12 (1-134)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>0.09 ± 0.35</td>
<td>6.4</td>
<td>1.33 ± 0.52 (1-2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S + I</td>
<td>4.28 ± 10.63</td>
<td>51.1</td>
<td>8.38 ± 19.91 (1-134)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Helicometra sp. (Opecoelidae)</td>
<td>S</td>
<td>0.18 ± 0.83</td>
<td>6.4</td>
<td>2.83 ± 1.94 (1-6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>0.87 ± 2.67</td>
<td>23.4</td>
<td>3.73 ± 4.53 (1-17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S + I</td>
<td>1.05 ± 2.00</td>
<td>27.7</td>
<td>3.50 ± 4.10 (1-17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prosorchynchus aculeatus (Bucephalidae)</td>
<td>I</td>
<td>20.18 ± 91.45</td>
<td>22.3</td>
<td>90.05 ± 179.54 (1-688)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bucephalus sp. (Bucephalidae)</td>
<td>I</td>
<td>0.65 ± 2.07</td>
<td>13.8</td>
<td>4.69 ± 3.54 (1-13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deropristis inflata (Acanthocephalidae)</td>
<td>I</td>
<td>2.32 ± 12.39</td>
<td>16</td>
<td>14.53 ± 28.81 (1-109)</td>
<td></td>
</tr>
<tr>
<td>Digenea species 1</td>
<td>S</td>
<td>0.05 ± 0.52</td>
<td>1.1</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digenea species 2</td>
<td>S</td>
<td>0.01 ± 0.10</td>
<td>1.1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* G = gills; I = intestine; S = stomach; SB = swimbladder.
† Minimal and maximal values of individual intensity.
than infected fish. Liver MT gene expression was significantly higher when *A. crassus* was present (*n*_uninfected = 69, *n*_infected = 10, *U* = 147, *P* = 0.004). Fish that were infected with *A. crassus* had a MT gene expression level that was 3.8 times higher than that of uninfected fish. Moreover, MT gene expression was significantly lower when digeneans were present in the stomach as compared to fish without digeneans in their stomach (*n*_uninfected = 40, *n*_infected = 39, *U* = 535, *P* = 0.02) and when fish were infected by at least 1 digenean compared to fish without digeneans (*n*_uninfected = 24, *n*_infected = 55, *U* = 433, *P* = 0.02). Fish that were uninfected by digeneans had 2.0 and 2.2 times higher levels of MT gene expression than infected fish.

Liver βTHR gene expression was significantly lower when digeneans were present in the stomach when compared to fish without digeneans in their stomach (*n*_uninfected = 41, *n*_infected = 39, *U* = 455, *P* < 0.001) and when at least 1 digenean was present relative to fish without any digenean (*n*_uninfected = 24, *n*_infected = 56, *U* = 343, *P* < 0.001). Uninfected fish had 2.2 and 2.3 times higher levels of βTHR gene expression than fish infected with digeneans.

The levels of gill Na+/K+ ATPase gene expression were significantly lower when *A. crassus* was present relative to fish without *A. crassus* (*n*_uninfected = 67, *n*_infected = 8, *U* = 93, *P* = 0.003). The uninfected fish had 2.4 times the level of Na+/K+ ATPase gene expression of the infected fish.

The levels of DSO gene expression in the eyes were significantly greater when *A. crassus* was present (*n*_uninfected = 39, *n*_infected = 5, *U* = 42, *P* = 0.04). Fish infected with *A. crassus* had 16.7 times the level of DSO gene expression when compared to uninfected fish. Moreover, DSO gene expression was significantly lower when digeneans were present in the stomach relative to fish without digeneans in their stomach (*n*_uninfected = 20, *n*_infected = 24, *U* = 150, *P* = 0.03) and when at least 1 digenean was present relative to fish without any digenean (*n*_uninfected = 10, *n*_infected = 34, *U* = 99, *P* = 0.04). Fish that were uninfected by digeneans had 5.7 and 13.1 times the levels of DSO gene expression when compared to infected fish. There were no other statistically significant differences in gene expression for the remaining comparisons between infected and uninfected fish.

### Relationships between expression level of target genes and intensity of parasites or biological parameters of the eel

Most of the tested variables were not significantly correlated with levels of target gene expression. When the regression coefficient is positive, the gene expression is negatively correlated with the corresponding variable. It is the opposite when the regression coefficient is negative (Table IV).

We found significant positive correlations between (1) ΔCt_HSP70 and the increasing age of the eels; (2) ΔCt_MT and *Bucephalus* sp. intensity; and (3) ΔCt_βTHR and both the age of the eels and *L. gravidum* intensity.

### DISCUSSION

The parasite richness reported in our study is typical of that found in European eels in Spain (Maillo et al., 2000), Germany (Sures et al., 1999), Italy (Kennedy et al., 1997, 1998), Belgium
Furthermore, there was a significant correlation between the transcriptional level by quantifying the action of the transmembrane protein. Regulation processes during the protein synthesis may have induced the difference between the results.

In the study on silvering, none of the parasites present was found to significantly influence FWO gene expression. However, expression of the DSO gene was significantly lower when digeneans were present. Modification of the host behavior by parasites with complex life cycles has been largely reported when the host is an intermediate host (Moore, 2002; Sasal and Thomas, 2005). Unfortunately, examples when considering fish as the final host are rare. When eels begin their sexual silvering, in addition to the switch between FWO and DSO gene expressions generally observed, there is also a degeneration of the digestive tract (Pankhurst and Sorensen, 1983). We hypothesize that, in terms of number of eggs laid, it should be more advantageous for the digeneans in the digestive tract to delay the more possible the eel migration. Although we did not observe intestine degeneration on the dissected eels, we also believe that the presence of digeneans in eels, coupled with the higher gene expression, could be more a consequence than the cause of this difference. In any case, it is interesting to note that the gene was, on average, 13.1 times more highly expressed in eels uninfected with digeneans; more work is needed to unravel this relationship. Finally, we found that fish infected by A. crassus had higher DSO gene expression than uninfected ones. The high virulence of this invasive nematode (see synthesis in Kirk, 2003) should act as a strong selection pressure on eels. We thus hypothesize that infected eels could anticipate their premigratory metamorphosis while their swimbladder is still functional. An alternative explanation would be that infected eels spend more time in brackish systems or saltwater as the parasite usually prefers freshwater (Kirk et al., 2000). In both cases, there would be an increase of DSO gene expression. An experimental study with changing salinities is required to provide more information on this effect.

ACKNOWLEDGMENTS

This work was financially supported by the French Ministère de l’écologie et du développement durable, the CNRS, and the University of Perpignan. G.F. was financially supported by a grant from the French Ministère de l’enseignement supérieur et de la recherche. Thanks are due to M. Cyprien, professional fisherman in the Salses-Leucate Lagoon, for providing eels.

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| Table IV. Significant parameters of the stepwise multiple regressions. |
|--------------------------|-----------------|-----------------|-----------------|-----------------|
| ΔCt target gene          | Variable†       | N*              | RC              | R²              | P                |
| ΔCt HSP70                | Age             | 74              | 0.56            | 0.22            | <0.001           |
| ΔCt MT                   | Bucephalus sp.  | 76              | 0.25            | 0.07            | <0.05            |
| ΔCt βTHR                 | Age             | 76              | 0.4             | 0.11            | <0.01            |
|                         | L. gravidum (I + S) | 0.08          | 0.2             | <0.001           |
| ΔCt Na⁺/K⁺ ATPase        | None significant| 70              |                 |                 |                  |
| ΔCt FWO                  | None significant| 53              |                 |                 |                  |

* Differences in sample size (N) are due to individuals from which molecular results were not available (high difference between duplicates, nonspecific amplification curves, etc.). † I = intestine; S = stomach; RC = regression coefficient; R² = multiple correlation coefficients.


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ATTACHMENT AND PENETRATION OF CENTROCESTUS ARMATUS (DIGENEA: HETEROPHYIDAE) CERCARIAE TO GILLS OF SECONDARY INTERMEDIATE FISH HOSTS

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ABSTRACT: The attachment and penetration of Centrocestus armatus cercariae into the fish host Zacco temmincki are described in this study. Light and scanning electron microscopy (SEM) were used to examine the topographical features and behavior of cercariae. Histochemistry and transmission electron microscopy (TEM) were employed to trace glandular products and secretions released by cercariae during penetration. Cercariae are first carried into the fish gill chambers via the respiratory currents. The frequency of respiratory-current reversals of fish increased when infected with cercariae. The behavior of cercariae, during breaks in current flow that preceded each current reversal, was observed using a specially devised apparatus. Cercariae produce a mucus-like secretion upon attachment, shed their tail, and employ a brief period of leech-like creeping behavior before penetration. In all cases, the site of penetration was via the surface of the primary gill lamellae. SEM revealed a well-developed anterior penetration apparatus, and a highly contractile body region, that created a driving force for penetration. TEM and histochemistry showed that the mucus observed on the surface tegument of cercariae during attachment were glandular secretions from the parasite. The significance of fish respiratory current reversals to the success of cercariae penetration nicely illustrates the exploitation by the parasite of a host response to a stimulus.

Centrocestus armatus is a heterophyid trematode. The morphological characteristics of the adult worm was described by Woo et al. (1998), and additional detail was provided by Kimura et al. (2007), who also documented the life cycle in the laboratory. The adult worms are usually found in the intestines of fish-eating birds such as Nycticorax nycticorax; sporocysts, rediae, and cercariae develop in freshwater snails, such as Semisulcospira libertina. Metacercariae commonly occur in freshwater cyprinid fishes such as Zacco temmincki and Zacco platypus.

Centrocestus armatus is widely distributed in Japan, Taiwan, the Korean Peninsula, and China, where it has been reported in a wide range of wild and domestic animals (Tanabe, 1922). A number of isolated cases of human infections have also been reported from these same countries (Kurokawa, 1935; Waikagul, 1977; Horii et al., 1988). The epidemiology (Kimura and Uga, 2003, 2005) and the transmission dynamics between the 2 intermediate hosts have recently been described (Paller et al., 2007). In earlier experiments, we also observed that the transmission route of C. armatus cercariae into piscine intermediate hosts was mainly through the gills, in contrast to the previous hypothesis that the larvæ could enter the fish host through penetration of any region of the body (Kimura and Uga, 2005).

Knowledge regarding the sites of entry, and subsequent attachment and penetration of parasites into their hosts, is fundamental to the understanding of the transmission dynamics of any endoparasite. The chemical stimuli for the invasion and attachment response to the teguments of fish-invading cercariae has been studied intensively (Haas and de Nunez, 1988; Haas et al., 1990, 2002; Haas and Roemer, 1998). However, only sparse information is available for cercariae behavior during attachment to, and penetration of, fish hosts via the gills. Furthermore, ultrastructural information addressing the invasive process remains unclear. The purpose of the present study was to fill this gap in information and to provide new insight into the behavior of C. armatus cercariae during attachment to, and penetration of, fish gills, as well as to examine the mechanisms employed during this difficult process in their life cycle.

The interface between C. armatus cercariae and Zacco temmincki gills was investigated at various levels. Initially, observations were made that examined the fish respiratory activity in the presence of C. armatus cercariae released in close proximity to fish hosts. Subsequently, fish gills were excised and inspected for evidence of cercariae attachment and penetration, by using light and electron microscopy. Finally, histology and histochemical tests were performed to trace glandular products and secretions from the cercariae. Together, these procedures have provided a unique perspective and description of the complex attachment and penetration processes.

MATERIALS AND METHODS

Preparation of cercariae

Cercariae were obtained from naturally infected Semisulcospira libertina snails collected from a river that is endemic for C. armatus. The snails were maintained in the laboratory, in an aerated aquarium with dechlorinated tap water, and fed with lettuce ad libitum. When cercariae were needed for an experiment, approximately 30 snails were exposed to bright light at 24 °C; cercariae emerged within 2–3 hr. The harvested cercariae were pooled to make a homogeneous suspension and were used in the experiments within 3 hr of shedding.

To provide a source of uninfected fish for the experiments, juvenile minnows (Zacco temmincki) (mean fork length = 9.83 cm ± 1.28 and mean weight = 5.21 g ± 1.92) were captured from a river known to be free of C. armatus. The fish were maintained in 20-L tanks until needed for experiments.

Observation on the effect of C. armatus cercariae on the fish respiratory activity

In an attempt to determine if the presence of cercariae in the inspired water influenced respiration currents of fish, the following experiment was conducted. Fish were placed individually in 1-L glass beakers containing 400 ml of dechlorinated tap water. The fish were allowed to acclimatize for 15 min in the container before cercariae exposure. Fish were individually infected with a known number of cercariae. Initially, 50 cercariae were introduced into a beaker with a fish. Other fish were exposed to larger numbers of cercariae; the cercariae were increased by increments of 50 until fish were exposed up to a maximum of 300 cercariae. The exposure period was 30 min. Control experiments were...
also carried out using only dechlorinated tap water. All fish were maintained at room temperature (22°C) during the experimental period; at 5-min intervals fish behavior was noted. The fish were subsequently placed in a container devoid of cercariae and further monitored at regular intervals until their behavior returned to normal, i.e., until their behavior was similar to control fish.

Observations on cercariae during penetration of fish gills

In another set of experiments, a specially devised apparatus was used to provide close scrutiny of cercariae behavior on the surface of fish gills. Fish gills were excised and placed in the wide end of a Pasteur pipette (length = 14.5 cm x width = 0.5 cm) and then fixed in place in the constricted waist. The pipette was then firmly fixed to a tube connected to a water faucet. To simulate the respiratory current over the gills of the fish hosts, a suspension of cercariae could then be drawn into, and gently expelled from, the pipettes with the gills fixed in place. Observations of these gills in these artificial gill chambers were made throughout the duration of these experiments using a stereomicroscope. The experiment was repeated, but this time by creating a positive and negative pressure on the gill inside the modified pipette after placing a rubber bulb on one end. Again, the cercariae suspension could be drawn into the pipette and then gently expelled by alternating positive and negative pressure on the bulb. In this case, breaks of 5, 10, and 15 sec were introduced into the middle of intake sequence, leaving the water with cercariae in a stationary condition for the specified time before it was expelled. This process imitated the breaks in current flow just before the onset of each respiratory current reversal observed in fish.

Scanning and transmission electron microscopy

Free-swimming C. armatus cercariae and excised infected gills were fixed in 10% neutral-buffered formalin for at least 24 hr, rinsed in phosphate buffer solution, dehydrated through a graded series of ethanol, and then exchanged with isomyl acetate. The specimens were critical-point dried (Hitachi HCP-2, Hitachi, Tokyo, Japan), coated with gold (Giko IB-3 ion coater, Giko Engineering Co., Tokyo, Japan), and observed using scanning electron microscopy (JSM: T-330A; JEOL, Tokyo, Japan). For transmission electron microscopy, samples were fixed in 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated with an ultramircrotome at 100 nm, and then observed using transmission electron microscopy (Hitachi H-7100; JEOL).

Fixation and preparation of material for light microscopy and histochemistry

Free-swimming C. armatus cercariae and cercariae-infected fish gills were fixed in 10% buffered formalin and washed in distilled water for 1 hr before being dehydrated through an ascending series of ethanol to absolute. Histochemical tests were carried out to trace glandular secretions and were performed according to Mizuguchi (1993). Periodic-Acid-Schiff staining (PAS) was used to localize glycoproteins; the Alcian blue method was used to localize acid mucosubstances; and toluidine blue was used to localize basic proteins.

RESULTS

Centrocestus armatus free-swimming cercariae

The major anatomical features of C. armatus cercariae were observed using light and scanning electron microscopy (Fig. 1). The larvae were pear-shaped, dorso-ventrally flattened, and concave ventrally. Average body length of fixed specimens was 50 μm. The anterior region bore an oral sucker (penetration apparatus) that was occasionally withdrawn inside. This consisted of an oral aperture with 9 large pre-oral hooklets directed towards the posterior and arranged alternatively in 2 rows, 6 sensory papillae (4 located ventrally and 2 anterolaterally), and a number of collared cilia. The first 6 rows of spines at the anterior region were longer and stouter when compared with the body spines. The body was densely covered with small pointed spines (1 μm in length) protruding from the body, but the spines on the posterolateral region of the body were longer and sharper than elsewhere. Long, collared cilia were also found interspersed among these spines. The ventral sucker was located slightly posterior on the median ventral side of the body, but was hardly visible via SEM; however, a suction-like apparatus appeared at the ventral side when folded (Fig. 1C). The average tail length was about 80 μm, with an annulated tegument and a mid-dorsal and mid-ventral crest running longitudinally along its length; it lacked the tail fin folds that are common to some heterophyids.

Effect of C. armatus cercariae on the respiratory activity of fish host

Preliminary experiments of fish exposed to free-swimming C. armatus free-swimming cercariae revealed that the cercariae enter the fish gill chambers via the inhalant respiratory water current. The fish became agitated when placed in the container, but became placid after about 5 min. When cercariae were added in the container, both experimental and control fish again became agitated, but the control fish quickly returned to a placid state. The experimental fish, in contrast, became increasingly agitated over a period of 10 min and their respiratory activity was affected. Close observation revealed that the respiratory rate and current reversal frequency increased compared to control fishes. The respiratory directional change also preceded a period of “still water”. Results showed that the control fishes displayed a respiratory reverse flow of 1 per min, and this pattern was maintained throughout the observation. In experimental fishes, this increased in proportion to the number of cercariae present in the “inhaled” water. The current-reversal frequency increased from 1/min to a maximum of 15/min.

Light and electron microscopic observations of cercarial attachment and the penetration process

The behavior of C. armatus cercariae during attachment and penetration of gills of second intermediate fish hosts was examined via light microscopy. When cercariae were maintained in constant motion over the gill surfaces, no cercariae were found on the gill lamellae. When interruptions in the flow of water over the gills were executed, a number of tailless larvae were observed adhering to the surface of the gill being tested (Fig. 1D). The water current simultaneously induced an increased likelihood of gill attachment of the parasite. However, the cercariae seemed to adhere briefly when the anterior region was in contact, but were quickly swept off by the respiratory current. If the flow of water was interrupted during this contact period, a clear sequence of events followed in rapid succession. Thus, once a cercaria had entered the gill chamber, it collided with the surface of the gill lamellae. The anterior oral sucker most frequently made this contact. The attachment site was most frequently the surface of the primary gill lamellae and occurred in a surface-parallel orientation, with the anterior end directed towards the main gill arch (Fig. 1D). However, cercariae were occasionally observed penetrating the gill inter-lamellar space, which could just accommodate their dimensions. After the onset of attachment processes, the tail was normally shed on the gill lamellae. The tailless larva then exhibited 3 distinct regions: (1) an oral region, which was highly protract-
Figure 1. Attachment and penetration process of Centrocestus armatus cercariae to gills of Zucco tenmineki. (A–C) SEM of free-swimming cercaria showing its anterior penetration apparatus and ventral suction apparatus (Insert photograph in A shows a LM photograph of an unstained, fixed, free-swimming cercaria). Notice the glandular secretions (white arrow in B) and the presence of broad spines at the anterior region. (D) LM observation of cercariae orientation on gill filaments with tails shed off upon attachment. Plates E–L are SEM micrographs of the attachment and penetration process. (E) The now tailless larva has 3 distinct regions: The anterior region bearing the penetration apparatus; the neck, which appeared as a constriction; and the highly contractile hindbody; notice also the mucus strands in arrow. (F) Mucus coat, upon attachment, can also be observed on the surface tegument. The anterior penetrating apparatus of the larva is now engaged with the gill tissue (G), while the contractile movement of the hindbody aided in the penetration process (H, I). It was frequently observed that two or more cercariae penetrated on almost the same site (J). The excretory pore (arrow) could be seen at the posterior opening (K). Notice the minimal tissue damage inflicted on the penetration site (L). CI = cilia; E = eyespot; H = pre-oral hooklets; O = oral aperture; OS = oral sucker; P = oral sensory papillae; S = spines; VS = ventral sucker. Bar = 10 μm.

ible; (2) the neck, which appeared as a circumferential constriction; (3) and a highly contractile hind body (Fig. 1E). The larva entered a short period of leech-like creeping behavior before penetration.

SEM and TEM micrographs (Figs. 1B, E, F, 2B) revealed secretions from the anterior region, and from the surface of the body tegument, that developed as a thin mucus coat upon attachment. The penetration process started with the anchorage of the anterior region to gill tissues, using its penetration apparatus that included the oral sucker, hooklets, and other accessory organs (Fig. 1G). The tailless larva then proceeded to penetrate the gill epithelium (Fig. 1H); muscular contractions of the hind body also facilitated penetration processes. The cercariae had completely entered the gill tissue within a mean of 5 min; the most rapid full penetration occurred within 58 sec (data not shown). After penetration, most cercariae traveled along the central venous sinus and appeared to be migrating towards the main efferent vessel of the gill. TEM micrographs
showed emptied glands upon establishment of the larva in the target organ of the fish (compare Figs. 2D, E).

**Histology and histochemistry**

Observations of histological sections of fish gills infected with cercariae confirmed the observations of SEM examination. Figure 3 shows cercaria entering into the interlamellar space and adhering to the gill tissue via glandular secretions. The gill tissue was digested at the site of penetration and the larva's body constricted in order to force its way through the small opening to the central venous sinus. This activity produced only minimal gill tissue damage by the penetrating parasite. The glands appeared to be darkly stained upon attachment, but became only lightly stained once the parasite was inside the central venous sinus of the gill.

The results of the histochemical tests on sections of the free-swimming cercariae and of the penetrating, tailless larvae are summarized in Table I. The oral and acetabular glands of the free-swimming cercariae produced a strong positive reaction from PAS and toluidine blue staining. The contents of the glands in the penetrating tailless larvae revealed only moderately positive PAS and weak toluidine blue reactions. In contrast, however, the parasite's surface tegument exhibited a strong PAS reaction. No reaction was observed with Alcian blue for either the free-swimming or the tail-less larvae.

**DISCUSSION**

The present work strongly suggests that cercariae of *C. armatus* rely on both physical and chemical means to attach to, and penetrate, a fish's gills. *Centrocestus armatus* cercariae were found to have an apparent preference for entry through the gills. This preference could be explained by the passive sweeping of cercariae into the gill chamber of the fish host via the inhalant respiratory current. However, once cercariae came in contact with a suitable host, they crept in "inch worm style" before penetrating at a suitable site (Haas and Roemer, 1998; Haas et al., 2002).

The behavior of schistosomes (Stirewalt, 1956; Haas and Roemer, 1998) and fish-invading cercariae (Haas and de Nunez, 1988; Haas et al., 1990; Hoglund, 1991) has been studied extensively. However, the studies have only indirect comparative usefulness, with respect to gill tissue invasion, since their larvae penetrate either mammalian or avian skin, or a fish's surface. *Centrocestus armatus* cercariae displayed some similar behavioral patterns, but also exhibited some distinctive characteristics. For example, cercariae of *Schistosoma* and *Diplodostomum* species often retain their tails until they achieve penetration. *Centrocestus armatus* cercariae differ, in that they shed their tails upon attachment and at the onset of penetration. Their tails seem to be of no use for entry; instead, they use their tailless hind bodies to push themselves into the gill tissues. The presence of a suction-like apparatus on the concave ventral side of the body also seems to be a unique characteristic of *C. armatus* cercariae.

The external morphology of *C. armatus* cercariae is first described in the present study; the internal morphology, especially for mucoid and penetration glands, was previously described, in detail, by Ito and Watanabe (1958). In addition to the glan-
dular secretions, *C. armatus* cercariae also demonstrate a highly developed attachment and penetration apparatus at their anterior region, which provides a very efficient means for attachment to, and penetration of, fish gills. The anterior ends of the penetrating, tailless larvae were always seen to be more or less “nestled” into the gill lamellae. The folded, concave position of the ventral region may have facilitated this action. The mechanism of attachment and penetration is different from schistosomes, wherein the larvae first adhere to the host by means of sticky mucus secreted by the glands. However, like the schistosomes, *C. armatus* cercariae appear to penetrate the host gill tissue using both chemical and mechanical means. Entry into the gill tissue was achieved via lysis, induced by the anterior penetration apparatus activity and by the anterior glandular secretions. The constant protractive movements of the oral sucker created an entry site, while the papillae and cilia served as sensory organs. The spines and hooklets provided mechanical support, whereas the anterior glandular secretions were discharged for gill tissue digestion. The broad anterior spines and the hooklets surrounding the oral region also apparently contributed to the invasive process; thrusts of the oral sucker occurred simultaneously with alternating contraction and elongation of the body. The cercaria appears to use muscular contractions to “push” itself into the gill lamella. The presence of bundles of muscles and numerous mitochondria, both in the oral and body regions, indicate high metabolic activity, which corroborate the observed evidence indicating that the organism relies strongly on physical means to penetrate the gill cuticle. Our findings reveal that the time required by *C. armatus* cercariae for full penetration into gill lamellae may be as fast as 58 sec. This indicates a very efficient means of attachment and penetration. If the initial attachment fails, cercariae are lost through the host’s gill opening when water exits.

The results of histology and histochemical tests on sections of free-swimming cercariae and the tailless penetrating larvae reveal an evacuation of glandular secretions during the attachment and penetration process. These results provide a similar pattern of observations to those from SEM and TEM. They also demonstrate that the contents of the glands of the free-swimming cercariae, and the mucus secretions on the surface of the tegument of the tailless penetrating larvae, have very similar histochemical properties, suggesting that the latter have given rise to the former. The presence of glands that secrete mucoid substances onto the outer surface of the cercariae has also been reported for parasites invading crabs such as *Paragonimus miyazakii* (Gyoten, 2000) and *Microphallus primas* (Saville and Irwin, 2005). These 2 studies revealed that some secretions contributed to the formation of a cyst wall. No cyst wall was formed by *C. armatus* cercariae; instead, a mucus coat was

![Figure 3](image-url)

**Figure 3.** Histology sections of Centrocestus armatus cercariae during attachment and penetration to fish gill tissues. (A) Cercaria upon attachment with the glands appeared to be filled with secretions. (B) Cercaria penetrating to gill surface via glandular secretions. Notice the reduction in glandular contents during the penetration process. (C–D) The tailless larva exhibited constriction on its body as it pushed its way into the gill lamellae towards the central venous sinus. AG = acetabular gland; G = primary gill lamellae; V = central venous sinus of the primary gill lamellae. A–B: PAS stained; C–D: Alcian Blue stained. Bar = 50 μm.

<table>
<thead>
<tr>
<th>Table I. Histochemical tests on free-swimming <em>Centrocestus armatus</em> cercariae and gill penetrating tailless larvae.</th>
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<td>Tests*</td>
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<tr>
<td>PAS</td>
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<tr>
<td>Alcian Blue</td>
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<td>Toluidine blue</td>
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* ++++, very strong reaction; ++++, strong reaction; ++, moderate; +, weak; 0, no reaction.
observed upon attachment to fish gill tissues. These secretions have also provided important clues about the ecological requirements of gill-penetrating cercariae.

When cercariae attempt to gain entry into fish host via the gills, they must overcome the double obstacles of the gill cuticle and the effects of water current. The presence of penetrating Centrocestus armatus cercariae that affect the respiratory and current reversal frequency rates in juvenile Z. temmincki is a remarkable observation. This may simply be a stress-related response by the fish hosts to large numbers of invading cercariae. The extent of this reaction suggests a more complex interaction between the invading parasites and the response of the host. Douglas and Lanzing (1981) reported that the presence of suspended particles in the water column increased respiratory rate and current reversal of fish, and further stated that it was a spontaneous, natural rhythm of gill ventilation. The physical impacts of C. armatus cercariae on fish gills, like particulate matter, also increased fish respiratory and current reversal frequency rates. After penetration into the gill vasculature, the parasites may thus reduce hemolymph flow, resulting in progressive hypoxia and leading to further reversal frequency (Saville and Irwin, 2005). These actions may also increase opportunities for C. armatus cercariae to attach and penetrate, resulting in greater infection success.

As can be ascertained, no other investigations, similar to the present study, have been performed. Hoglund (1991) investigated the cercarial transmission route of Diplostomum spathaceum to its secondary intermediate fish host, Onchorynchus mykiss, but only employed ultrastructural observations and radiometric assays in various regions of the fish host. Whyte et al. (1991) also described the infectivity of this parasite on different regions of the fish host, but they did not describe the mechanisms involved in attachment and penetration, in particular for gill tissues. Our observation and description of C. armatus cercariae attachment to, and penetration of, gills of secondary intermediate fish hosts could perhaps be common to other gill-penetrating heterophyids. It could represent an efficient means of overcoming the difficult obstacle in the life cycle of these parasites.

ACKNOWLEDGMENTS

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LITERATURE CITED


ABSTRACT: Parasite life-history characteristics, the environment, and host defenses determine variation in parasite population parameters across space and time. Parasite abundance and distribution have received little attention despite their pervasive effects on host populations and community dynamics. We used analyses of variance to estimate the variability of intensity, prevalence, and abundance of 4 species of lice (Insecta: Phthiraptera) infecting Galapagos doves and Galapagos hawks and 1 haemosporidian parasite (Haemoproteidae: Haemoproteidae) infecting the doves across island populations throughout their entire geographic ranges. Population parameters of parasites with direct life cycles varied less within than among parasite species, and intensity and abundance did not differ significantly across islands. Prevalence explained a proportion of the variance (34%), similar to infection intensity (33%) and parasite abundance (37%). We detected a strong parasite species-by-island interaction, suggesting that parasite population dynamics is independent among islands. Prevalence (up to 100%) and infection intensity (parasitemias up to 12.7%) of Haemoproteus sp. parasites varied little across island populations.

The abundance and distribution of parasites have received little attention despite their pervasiveness, diversity, and potential impacts on host population and community dynamics (Windsor, 1998; Poulin, 1999; Moturisen and Poulin, 2005). Parasite life-history characteristics, together with their biotic and abiotic environments and the antiparasite defenses of their hosts, determine variation in parasite abundance, prevalence in host populations, and intensity of infection in individual hosts across space and time (Poulin, 1998; Krasnov et al., 2006). Unlike prevalence, parasite infection intensity and abundance per host have been found to vary less within, than among, parasite species (Arneberg et al., 1997; Krasnov et al., 2006; Poulin, 2006). Whether these patterns can be generalized to all parasites will depend on additional observations over a range of parasite and host species. Parasites analyzed previously have complex life cycles, wherein some free or vectored life stages might be affected by environmental conditions, which subsequently influence encounter rates between parasites and hosts (Poulin, 2006). In contrast, species with direct life cycles, such as lice (Insecta: Phthiraptera), are less likely to face the challenges imposed by free-living stages and, as such, the variation in population parameters might differ from that presented by parasites with indirect life cycles.

We studied 4 louse species and 1 vector-borne haemosporidian parasite (Haemoproteus sp.). Lice have direct life cycles, i.e., they complete all their developmental stages on the host, but they vary in feeding strategies, host specificity, and mobility. Of the 2 louse species that parasitize endemic doves (Zenaida galapagoensis), Columbicola macrourae (Ischnocera: Philopteridae) is commonly found on wing and tail feathers, and it is more mobile than Physconelloides galapagensis (Ischnocera: Philopteridae), which feeds on body feathers. The better dispersal capacity of C. macrourae lice has been confirmed by straggling events, (movement of parasites between species), population genetics analyses, and coevolutionary studies (Johnson et al., 2002; Clayton and Johnson, 2003; Whiteman et al., 2004). The other 2 louse species parasitize endemic Galapagos hawks (Buteo galapagoensis). Colopcephalum turbinatum (Amblyceran: Menoponidae) feeds on epidermal tissues and blood over most regions of the host’s body, and it has a strong dispersal capacity (Whiteman and Parker, 2004). Degeeriella regalis (Ischnocera: Philopteridae) feeds mainly on keratin of feathers and dead skin, and disperses poorly. Host defenses against these lice are restricted to preening in the case of D. regalis, but they include immune defenses in the blood-feeding C. turbinatum (Marshall, 1981; Whiteman and Parker, 2004; Whiteman et al., 2006). Haemosporidian parasites infect the endemic dove, but they have not been reported from hawks (Padilla et al., 2006; Parker et al., 2006). These parasites have an indirect life cycle, which includes dipteran vectors, in which the macrogametes undergo fertilization to form zygotes, and meiosis takes place with subsequent development of infective sporozoites. Hippoboscid flies are implicated in the transmission of Haemoproteus spp. infecting Columbiformes (Valkiunas, 2005).

The Galapagos dove is the only columbiform species in the archipelago. It occurs on all the major islands, including the 2 northern and the somewhat isolated islands of Darwin and Wolf (Z. g. exsul), and as the main part of the island group (Z. g. galapagoensis) (Santiago-Alarcon and Parker, 2007). Genetic evidence suggests a high degree of historical gene flow among populations of the southern subspecies, which is supported by low and not significant $F_{ST}$ values (Santiago-Alarcon et al., 2006). $F_{ST}$ values also indicate some degree of genetic structure between southern and northern populations (data not shown). The Galapagos hawk is widely distributed among the larger islands, and it is the top predator in the archipelago. Unlike the endemic dove, hawk populations are highly structured, and they have low levels of genetic diversity, as well as behavioral and morphological differences across islands (Bollmer et al., 2003, 2005, 2006; Whiteman et al., 2006).

The Galapagos Islands represent the only Pacific Ocean archipelago that still preserves its entire avifauna (Tye et al., 2002). Some bird populations are declining, however, and we have detected several infectious agents, e.g., Haemoproteus sp. and Chlamydophyila psittaci, with interspecific transmission potential, infecting the endemic Galapagos dove (Z. galapagoensis) (Padilla et al., 2004). In addition, a vector of avian malaria
parasites (Plasmodium sp.), Culex quinquefasciatus, has been reported on the islands (Whiteman et al., 2005), which is worrisome due to the negative impacts that this blood parasite has had on Hawaiian endemic birds (van Riper et al., 1986; Atkinson et al., 2000; Atkinson, Dusek, and Lease, 2001; Atkinson, Lease et al., 2001). Furthermore, we have previously shown that lice can straggle from doves to hawks when the later preys on the former (Whiteman et al., 2004). Thus, the possibility of interspecific transmission of infectious agents among Galápagos endemic birds highlights the importance of analyzing the variability of parasite population parameters across the archipelago.

In the present study, we analyzed the spatial variation of intensity, abundance, and prevalence of 4 species of lice (Insecta: Phthiraptera) infecting Galápagos doves and Galápagos hawks and 1 haemosporidian parasite (Haemosporida: Haemoproteidae) infecting the doves across island populations throughout their entire geographic ranges. "Intensity is defined as the number of conspecific parasites living in (or on) an infected host, and abundance is defined as the number of conspecific parasites living in (or on) any host individual (intensity > 0, abundance ≥ 0)" (Rózs et al., 2000). Prevalence refers to the proportion of the host population that is infected (Bush et al., 1997). These measures are related by abundance = intensity × prevalence. Thus, abundance is the infection intensity averaged over all individuals in the host population, whether infected or not.

Parasite species were selected because of previously reported negative effects of closely related parasites on the condition of individuals of other host species such as rock pigeons (Columba livia; Booth et al., 1993; Brown et al., 1995) and honeycreepers (Drepanididae) from Hawaii (Atkinson et al., 2000, Atkinson, Dusek, and Lease, 2001; Atkinson, Lease et al., 2001). We also collected mites and hippoboscid flies, but these were not included here because of low sample sizes.

**MATERIALS AND METHODS**

We live-captured 199 Galápagos hawks from 8 islands (Fig. 1; May–August 2001: Española, n = 8; Isabela, n = 25; Marchena, n = 26;
Santa Fe, n = 13. May–July 2002: Santiago, n = 58. May–July 2003: Fernandina, n = 28; Pinta, n = 31; Pinzón, n = 10) and 139 Galápagos doves from 6 islands (Fig. 1: May–July 2002: Santiago, n = 27; Santa Cruz, n = 23; Santa Fe, n = 24; Española, n = 24. June–July 2004: Genovesa, n = 21; July 2005: Wolf, n = 20). We sampled endemic doves using hand nets and mist nets following the guidelines in Ralph et al. (1996); hawks were captured using techniques described in Bollmer et al. (2005).

For haemosporidian parasites, we took blood samples (30 μl) by venipuncture from 25 doves each from Santa Cruz, Santa Fe, and Española Islands, 30 each from Santiago and Genovesa Islands, and 29 from Wolf Island (Fig 1). We visited San Cristobal during 2002 and Darwin during 2005, but, due to small sample sizes (n = 2 and 4, respectively), these islands were not included in our analysis. We prepared 2 thin blood smears from each sampled Galápagos dove. Smears were air-dried, fixed in methyl alcohol, and stained with Giemsa. Intensity of infection in blood parasites was quantified from blood smears by counting the number of parasites observed in 10,000 red blood cells for each individual (Varkiññas, Bensch et al., 2006).

Ectoparasites were quantitatively sampled using the dust-ruffling technique (Walther and Clayton, 1997) by applying pyrethroid insecticide (Zema® Flea and Tick Powder for Dogs, St. John Laboratories, Harbor City, California). Ectoparasites were subsequently stored in vials containing 70% ethanol. Dust-ruffling is the method of choice for ectoparasite quantitative sampling when hosts cannot be killed. This method is known to predict parasite abundances well (Clayton and Brown, 2001).

To determine parasite spatial variation in population parameters, we conducted model III analyses of variance (ANOVA) (SPSS 14.0, SPSS Inc., Chicago, Illinois) with parasite species as random factor and island as fixed factor. To determine the variation explained among groups, we followed the procedures described in Underwood (1997). Intensity and abundance values were log$_{10}$ transformed, and prevalence was arcsine$\sqrt{\text{P}}$ transformed to fulfill assumptions of parametric tests. Prevalence after transformation was not normally distributed and, due to the unbalanced sample sizes, we decided to use a categorical model using the general linear model (glm) procedure in R version 2.4.1 with a quasibinomial error structure (Crawley, 2005).

Intensity of blood parasite infection was analyzed by means of a glm with negative binomial errors (Wilson et al., 1996) using the glm.nb procedure of the MASS library of Venables and Ripley (2002) in R version 2.4.1. We report prevalence, mean, and median intensity, and mean abundance (Bush et al., 1997) for the different island populations of the 4 louse species calculated using the program Quantitative Parasitology 3.0 (Röss et al., 2000). We provide 95% confidence intervals estimated by ≥2,000 bootstrap replicates for the different population parameters.

RESULTS

Our results indicate that population parameters of louse parasites are less variable within, than among, parasite species. The amount of variation explained among parasites for intensity was 33.2% ($F_{4,564} = 7.863, P = 0.002$), for abundance was 36.7% ($F_{4,568} = 8.16, P = 0.002$), and for prevalence was 34.4% ($z = 5.24, P < 0.001$). We identified a significant interaction effect between parasite species and island for both intensity and abundance ($F_{4,568} = 6.4, P < 0.001$ and $F_{4,568} = 6.7, P < 0.001$), which suggests independent parasite–host interactions among island populations. Island alone did not have a significant effect on lice intensity and abundance ($P \geq 0.14$). There was a significant effect of island on parasite prevalence, however, but this was driven by low values from Genovesa Island ($z = -4.83, P < 0.001$) represented by the 2 louse species (C. macrorae and P. galapagensis) infecting doves.

Both prevalence and intensity of Haemoproteus sp. infections differed significantly among islands ($z = 2.86, P < 0.01$ and $z = 17.0, P < 0.001$, respectively). This effect was produced by low values of both prevalence (37%) and intensity (0.009–0.84%) on Genovesa Island ($z = -3.2, P < 0.01$ and $z = -6.32, P < 0.001$, respectively), which is the same pattern observed for the 2 louse species infecting doves. Intensities of blood parasites ranged from 0.008 to 12.7% (Table I).

DISCUSSION

In the present study, we have analyzed the variation of population parameters of 5 parasite species (4 lice [Insecta: Phthiraptera] and 1 haemosporidian blood parasite [Sporozoa: Haemosporidial] infecting the endemic Galápagos dove (Z. galapagoensis) and the endemic Galápagos hawk (B. galapagoensis) across their entire geographic range. Our results revealed no significant differences in parasite population parameters among island populations (excluding Genovesa), although a significant island × species interaction indicated independent variation in the populations of both louse species and the haemosporidian parasite among islands. Also, parameter values from different populations of the same parasite species are more similar to each other than to those of other parasite species. Previous studies have shown that infection intensity and abundance of parasites can be considered parasite species-specific traits to the extent that these parameters vary less within than among parasite species, i.e., they are repeatable across parasite populations (Poulin, 2006). Although the variability explained by parasite infection parameters in our system was lower than that reported previously for other parasite systems, i.e., metazoan parasites of fishes and mammals (Arneberg et al., 1997; Krasnov et al., 2006; Poulin, 2006), our analysis corroborates that intensity and abundance are less variable within, than among, parasite species. Previous studies have suggested that prevalence is too variable to be considered a species-specific trait (Arneberg et al., 1997; Poulin, 2006) because it depends on the transmission of parasites among hosts and is sensitive to variable environmental conditions affecting free-living or vector stages (Poulin, 2006). In contrast, when the life cycle of the parasite species occurs entirely on the host, as in louse species (Insecta: Phthiraptera), environmental variability would be more likely have less effect on the transmission dynamics of the parasite. Accordingly, our results showed that 34% of the variation in prevalence was explained by differences among louse species, which is similar to the 36 and 33% explained for abundance and intensity, respectively. This suggests that intensity, prevalence, and abundance in parasites with direct life cycles are equally variable, in contrast to previous studies conducted on parasites with indirect life cycles, where parasite species explained less variation in prevalence than in intensity and abundance. The results of studies by Arneberg et al. (1997), Poulin and Mouritsen (2003), and Poulin (2006) have shown that repeatability of parasite infection parameters among host species is rather weak, or insignificant, meaning that infection population parameters represent traits of parasite species and not of host species. Longitudinal data are necessary, however, to generalize these patterns across parasite species.

Haemosporidian parasites from Caribbean Islands infecting several host species showed the same pattern of variation in prevalence as the blood parasite studied here, where there was no, or little, island effect, and there was a significant island × species interaction (Apanius et al., 2000; Fallon et al., 2003). However, some parasite lineages presented significant differ-
Table I. Estimated population parameters for 5 parasite species ( Columbicola macrourae, Physconelloides galapagensis, Colpocephalum turbinate, Degeeriella regalis, and Haemoproteus sp.) infecting either the endemic Galapagos dove (Zenaida galapagoensis) or the endemic Galapagos hawk (Buteo galapagoensis). Values in parentheses are 95% bootstrap confidence intervals estimated by ≥2,000 bootstrap replicates.

<table>
<thead>
<tr>
<th>Island</th>
<th>Parasite species</th>
<th>Mean intensity*</th>
<th>Median intensity</th>
<th>Mean abundance</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
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<td>Santa Cruz</td>
<td>Haemoproteus sp.</td>
<td>0.008–9.4</td>
<td>11 (7–24)</td>
<td>18.3 (11.74–28.22)</td>
<td>95 (76–99)</td>
</tr>
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<td></td>
<td>C. macrourae</td>
<td>20.05 (13.38–31.76)</td>
<td>7 (4–10)</td>
<td>8.13 (5–14.7)</td>
<td>91 (72–98)</td>
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<td></td>
<td>P. galapagensis</td>
<td>9.35 (6–16.7)</td>
<td>30 (19–42)</td>
<td>30.33 (22.44–39.19)</td>
<td>92 (81–99)</td>
</tr>
<tr>
<td></td>
<td>C. macrourae</td>
<td>31.5 (23.77–40.65)</td>
<td>17 (10–27)</td>
<td>31.6 (22.44–44.41)</td>
<td>92 (76–98)</td>
</tr>
<tr>
<td></td>
<td>D. regalis</td>
<td>6.48 (4.52–10)</td>
<td>3 (1–5)</td>
<td>4.69 (3.17–7.22)</td>
<td>72 (59–83)</td>
</tr>
<tr>
<td>Española</td>
<td>Haemoproteus sp.</td>
<td>0.017–3.8</td>
<td>16 (10–19)</td>
<td>15.8 (11.88–22.46)</td>
<td>95 (79–99)</td>
</tr>
<tr>
<td></td>
<td>P. galapagensis</td>
<td>22 (16.95–33.25)</td>
<td>13.5 (6–20)</td>
<td>13.13 (8.5–20.54)</td>
<td>83 (62–94)</td>
</tr>
<tr>
<td></td>
<td>C. turbinatum</td>
<td>161.63 (75.5–350.5)</td>
<td>128 (57–171)</td>
<td>161.3 (75.5–350.5)</td>
<td>100 (63–100)</td>
</tr>
<tr>
<td></td>
<td>D. regalis</td>
<td>55.63 (31.63–87.83)</td>
<td>56.5 (4–82)</td>
<td>55.63 (30–84)</td>
<td>100 (63–100)</td>
</tr>
<tr>
<td>Santa Fe</td>
<td>Haemoproteus sp.</td>
<td>0.01–1.6</td>
<td>13.9 (9.73–19)</td>
<td>12.75 (8.58–17.46)</td>
<td>91 (73–98)</td>
</tr>
<tr>
<td></td>
<td>C. macrourae</td>
<td>13.9 (9.73–19)</td>
<td>12 (4–19)</td>
<td>12.75 (8.58–17.46)</td>
<td>91 (73–98)</td>
</tr>
<tr>
<td></td>
<td>C. turbinatum</td>
<td>206.46 (120.3–353)</td>
<td>128 (57–171)</td>
<td>206.46 (120.3–353)</td>
<td>100 (77–100)</td>
</tr>
<tr>
<td></td>
<td>D. regalis</td>
<td>21.5 (12.75–32.1)</td>
<td>16 (6–35)</td>
<td>19.85 (11.15–30.54)</td>
<td>92 (65–99)</td>
</tr>
<tr>
<td>Genovesa</td>
<td>Haemoproteus sp.</td>
<td>0.009–0.84</td>
<td>10.83 (6.5–17)</td>
<td>9.5 (2–19)</td>
<td>6.19 (3.24–10.86)</td>
</tr>
<tr>
<td></td>
<td>C. macrourae</td>
<td>6.71 (3.1–10.43)</td>
<td>8 (1–15)</td>
<td>2.24 (0.81–4.62)</td>
<td>33 (15–55)</td>
</tr>
<tr>
<td></td>
<td>P. galapagensis</td>
<td>62.6 (37.5–115.1)</td>
<td>12 (2–19)</td>
<td>18.39 (10.29–32.68)</td>
<td>82 (64–92)</td>
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<tr>
<td></td>
<td>C. turbinatum</td>
<td>48.56 (32.4–76.52)</td>
<td>27 (23–53)</td>
<td>48.56 (32.4–76.52)</td>
<td>100 (86–100)</td>
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<tr>
<td>Marchena</td>
<td>C. turbinatum</td>
<td>8.79 (5.25–12.1)</td>
<td>5 (3–7)</td>
<td>7.48 (5–11.28)</td>
<td>96 (80–99)</td>
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<tr>
<td></td>
<td>D. regalis</td>
<td>114.88 (65.32–195.32)</td>
<td>46 (16–104)</td>
<td>110.46 (63–184.58)</td>
<td>96 (81–99)</td>
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<td>D. regalis</td>
<td>89.9 (56.13–154.77)</td>
<td>36 (13–63)</td>
<td>87 (52.9–143.5)</td>
<td>96 (82–99)</td>
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<tr>
<td></td>
<td>D. regalis</td>
<td>20 (11.96–36.18)</td>
<td>5.5 (2–10)</td>
<td>18 (9.45–36.58)</td>
<td>90 (74–97)</td>
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<td>Pinzón</td>
<td>C. turbinatum</td>
<td>101.8 (69–141.4)</td>
<td>98 (30–166)</td>
<td>101.8 (69–141.4)</td>
<td>100 (70–100)</td>
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<tr>
<td></td>
<td>D. regalis</td>
<td>30.7 (19.2–43)</td>
<td>28 (10–57)</td>
<td>30.7 (19.2–43)</td>
<td>100 (70–100)</td>
</tr>
<tr>
<td>Wolf</td>
<td>Haemoproteus sp.</td>
<td>0.02–12.7</td>
<td>50.5 (38.75–64.75)</td>
<td>39 (28–66)</td>
<td>50.5 (38.75–64.75)</td>
</tr>
<tr>
<td></td>
<td>C. macrourae</td>
<td>60.44 (36.6–92.56)</td>
<td>42 (12–75)</td>
<td>48.35 (28.3–78.65)</td>
<td>80 (57–92)</td>
</tr>
</tbody>
</table>

* Haemoproteus sp. intensity was measured as the number of erythrocytes infected in 10,000 examined red blood cells per dove individual for each island. We report the percentage of infected cells of the 10,000 examined as is conventionally done for haemosporidians. Blood parasites were analyzed only in Galapagos doves because to this date, we have not detected haemosporidian infections in endemic hawks (see Parker et al., 2006).

ences across islands (Fallon et al., 2003). The lack of strong island effects is expected due to the indirect life cycle of haemosporidians. The presence of a vector may increase the susceptibility of these parasites to environmental conditions, thus making infection parameters more variable across populations. In fact, other studies reported a high degree of heterogeneity among populations of blood parasites infecting the same host (e.g., Freeman-Gallant et al., 2001; Valkiūnas and Lezhova, 2001). The significant interaction effect detected between parasite species and island suggests that infection parameters undergo independent dynamics among islands (see Fallon et al., 2003 for an example of the same pattern in the Caribbean Islands). This could be explained by differences in both biotic and abiotic conditions among islands of the Galápagos, where extreme effects on populations can be observed over short distances (e.g., Wikelski and Trillmich, 1997). For example, the degree of inbreeding within island populations of the Galápagos Hawk positively co-varies with louse abundances and intensities and negatively co-varies with variation in host natural antibody responses (Whiteman et al., 2006). However, because these parameters are less variable within, than among, parasite species, biological features of parasites seem to override local environmental conditions to some degree and maintain fluctuations within narrow species-specific limits (Poulin, 2006). Only if long-term studies confirm the specific nature of parasite population parameters, can we hope to use such parameters as predictive tools for population and community analyses. In particular, when different parasite species are infecting the same host, dynamics can be complex depending on the nature of the interaction, and the outcome is not always intuitive (Schjørring and Koella, 2003; Pedersen and Fenton, 2006). Moreover, using parasite species that infect different host species will allow us to determine if these population parameters can represent host–parasite interaction characters (Poulin, 2006).
might explain its extreme deviation from the other samples due to seasonal or interannual variation in environmental conditions influencing lice or hippoboscid vectors. The range of relative humidity on all the islands during the sampling periods (>64%; data obtained from the Charles Darwin Research Station) was substantially above the level considered to impact lice infecting rock pigeons (C. livia), mourning doves (Zenaida macroura), and Incas doves (Columbina inca), i.e., 40% (Moyer et al., 2002). Thus, relative humidity cannot explain the low abundance of lice on Genovesa Island. In the case of haemosporidian parasites, Sol et al. (2000) have demonstrated that vector abundance is the main factor influencing the spatial variation in prevalence, but not intensity, of blood parasites infecting rock pigeons in populations of western Europe. It is interesting to note that Sol et al. (2000) found a parallel geographic pattern to the one reported here. Prevalence at 4 of 5 localities was 100%, and the single sample with low prevalence (14.8%) had few vectors present in that population. Two experiments confirmed that vector abundance limits parasite transmission, ruling out host individual variation, i.e., susceptibility, as an alternative factor (Sol et al., 2000). Hence, lower abundance of vectors for parasite transmission on Genovesa Island might have caused the low parasite prevalence. Unfortunately, we do not have data to support this hypothesis. Alternatively, the low prevalence observed in Genovesa blood parasites might represent sampling during a low point in a seasonal cycle of parasite prevalence, common to many haemosporidans (e.g., Bensch et al., 2007, but see Fallon et al., 2004 for an example of temporal stability of a community of insular blood parasites). This still would not explain the observation that the 3 parasite species infecting endemic doves showed the same pattern of variation across islands, which suggests that these parasites, despite their different life cycles, might be responding in parallel to the same factors. In addition, this temporal variability further supports the need for longitudinal studies that can confirm the specific nature of parasite population parameters.

Finally, we observed high prevalence and parasitemia of Haemoproteus sp. in the dove host (Table I). This suggests that doves are highly susceptible to the local blood parasites. Experimental infections on Hawaiian endemic birds have shown that enemics are highly susceptible and present high parasitemias (>40%) of Plasmodium relictum (mitochondrial cytochrome b gene lineage GRW4), leading in most cases to host death (Atkinson et al., 1995, 2000; Yorinks and Atkinson, 2000; Atkinson, Dusek, and Lease, 2001; Atkinson, Lease et al., 2001). Species of Haemoproteus are often considered to be relatively benign in their avian hosts (Bennett et al., 1993). However, some haemoprocts have been reported to cause diseases in birds (Miltgen et al., 1981; Atkinson et al., 1986, 1988; Carron et al., 2002) and to affect their fitness (Nordling et al., 1998; Merino et al., 2000; Marzal et al., 2005; Valkiūnas, 2005; Valkiūnas, Zickus et al., 2006). High parasitemias as those presented by the endemic Galápagos dove are uncommon in the wild (Valkiūnas, 2005). There are some examples of wild birds with high parasitemias, but these are the exception rather than the rule (Valkiūnas, 2005). Therefore, experimental studies on the fitness effects of these parasites on the endemic dove are desirable.

Summarizing, our data suggest (1) that infection parameters of louse parasites are, for the most part, homogeneous across islands; (2) that prevalence has the same degree of variation as intensity and abundance in louse parasites; (3) that intensity and prevalence of Haemoproteus sp. parasites across island populations are similar, with the exception of Genovesa Island; and (4) that the endemic Galápagos dove is susceptible to the Haemoproteus sp. parasite, with prevalence up to 100% in some island populations and with intensities up to 12% in some individuals.

ACKNOWLEDGMENTS

We thank all the people who provided help during the different stages of the field season, particularly G. Buitron, A. Iglesias, I. Rose, J. Bollmer, J. Merkel, and J. Rabenold. We are grateful to the staff of the Charles Darwin Research Station for invaluable help and logistical support during the course of this study, especially P. Robayo. N. Cuevas, and J. Freire who helped with dove sampling at Tortuga Bay, Santa Cruz. Members of Dr. Parker’s lab group commented on earlier versions of the manuscript. Two anonymous reviewers provided comments that improved the quality of the manuscript. Permits for sample collection were provided by the Galápagos National Park authorities. Financial support was provided by The Whitney R. Harris World Ecology Center, The Saint Louis Zoo FRC 05-2, and E. Des Lee Collaborative Vision in Zoological Research.

LITERATURE CITED


SOIL-TRANSMITTED HELMINTHS IN RELATION TO HEMOGLOBIN STATUS AMONG SCHOOL CHILDREN OF THE KASHMIR VALLEY

Showkat Ahmad Wani, Fayaz Ahmad, Showkat A. Zargar*, Zubair Ahmad Dar, Parvaiz Ahmad Dar, Hidayatullah Tak, and Bashir Ahmad Fomdat†

P. G. Department of Zoology, University of Kashmir, Srinagar, India 190-006. e-mail: showkatajish@yahoo.co.in

ABSTRACT: Soil-transmitted helminths (STHs) remain a major threat to the health of children throughout the world, mostly in developing nations. The aim of the present study was to determine any relationship between STHs and hemoglobin status in school children of Kashmir Valley (India). Stool and blood samples were collected from 382 male and female school children in the age group of 5–15 yr from all 6 school districts of the Kashmir Valley. Finger-prick blood samples were used to collect the hemoglobin, which was then measured on-site by Sahli’s acid hematin method; stool samples were processed using both simple smear and zinc sulphate concentration methods. Of the 382 children surveyed, 299 (78.27%) were infected with Ascaris lumbricoides, Trichuris trichiura, or both. Children infected by STHs were found to have lower mean values of hemoglobin than uninfected children. The present study reveals that STHs are abundant among school children of Kashmir Valley, creating a negative effect on the hemoglobin values and indicating the necessity of implementing control measures.

Soil-transmitted helminths (STHs) continue to be a major public health burden throughout many countries of the world, but especially where both sanitation and hygiene are poor and access to anthelmintics is limited (Savioli et al., 2002). Among the effects associated with these parasites are growth retardation, intestinal obstruction, hepatic and biliary diseases, impaired cognitive development, and nutritional difficulties, such as iron deficiency anaemia (Ramdath et al., 1995; Awasthi et al., 2003). Among the parasitic infections within the Indian subcontinent, helminth problems are the most common. According to WHO (1981), the level of helminth infection can be viewed as an index of a community’s progress toward a desirable level of sanitation. Although several studies have been carried out on the prevalence of STH infections in children of the Kashmir Valley (Khuroo, 1996; Ahmad et al., 2002; Wani, 2007), the impact of these helminths on hemoglobin levels has not been ascertained. Therefore, the present study was undertaken to determine the relationship between STHs and hemoglobin (Hb) status among school children.

MATERIAL AND METHODS

The Kashmir Valley, situated at an altitude of approximately 2,000 m, constitutes the major portion of the Jammu and Kashmir State, India, and includes the 6 districts of Anantnag, Baramulla, Budgam, Kupwara, Pulwama, and Srinagar, with about 26 Tehsils and 33 towns (Gupta, 2005). The study was carried out between May 2006 and November 2006 in all 6 districts. Official meetings with the personnel from health services, city councils, and schools, as well as parents and school children from the study sites, were conducted to explain the protocol of the study. In total, 382 children, including 219 males and 163 females between the ages of 5 and 15 yr ([X ± SD] = 9.2 ± 2.3) participated in the study (these children had no apparent disabilities and were not receiving drug therapy for parasitic infections). Written consents were required from both parents in order for the children to participate. Children requiring medical assistance were properly treated or referred for medical attention. The children’s ages were obtained through school records.

Finger-prick blood was analyzed for Hb on-site using Sahli’s Acid Hematin method (Rai et al., 2004). In addition, 5 g of fresh morning stool samples were collected in nylon containers containing 10 ml of 10% formaldehyde. The containers were labeled and immediately transported to the parasitology laboratory, Department of Zoology, University of Kashmir, for further processing. The stool specimens were examined using direct smear and zinc sulphate concentration techniques. A computer program (SPSS v.10.05 for Windows, Microsoft) was used for data analysis. The descriptive data were given as a mean ± standard deviation (SD). Student’s t-test was used for the analytic assessment. The differences were considered to be significant when the P-value obtained was less than 0.05. Following the WHO ethical guidelines of “no survey without service,” all children enrolled in the survey received 1 tablet of 400 mg albendazole as a treatment protocol for STHs.

RESULTS

Among 382 children subjected to stool examination, data revealed that 299 (78.27%) were infected with Ascaris lumbricoides, Trichuris trichiura, or both. Single and mixed infections were observed in almost equal proportions. Thus, 149 (39.0%) children were infected by a single species of helminth, Ascaris lumbricoides was found in 91 (23.82%) and T. trichiura in 58 (15.18%) children. Mixed infections were observed in 150 (39.26%) children.

As shown in Table I, infected children had significantly lower values of hemoglobin than uninfected children (P < 0.05). It was also observed that children infected with T. trichiura had lower hemoglobin values than children infected by A. lumbricoides (P < 0.05).

DISCUSSION

Results of the present study indicate a prevalence of 78.27% (299 positive cases among the total of 382 children screened). When compared with other parts of the world, the data show that Kashmir Valley is a highly endemic region for intestinal helminthiasis. For example, studies conducted on the frequency distribution of gastrointestinal helminths by Bundy et al. (1988) showed a high overall prevalence of 62% of helminth infections among the urban slum children of Malaysia, while Rodriguez et al. (2000) reported a high prevalence of 72% among the school children studying in a public institution in Maracaibo, Venezuela. Legesse and Erko (2004) noted the high prevalence of 88.2% among the school children in rural Ethiopia, while Kabaterine et al. (2001) reported an overall prevalence of 56% among the school children of southern Uganda. The high prevalence of STH infections is undoubtedly a consequence of a low standard of living, poor sanitation, lack of personal hy-

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for the institution of control measures, including treatment of infected individuals, improvement of sanitation practices, and provision of clean water. The impact of each measure would be maximized through a health education program directed at school children, and their mothers in particular, and to communities in general.

ACKNOWLEDGMENT
We are indebted to all the children, and their parents and teachers for their wholehearted cooperation.

LITERATURE CITED


Table I. Mean hemoglobin values (g dl⁻¹) in infected and uninfected children.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>95% CI</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>Infected</td>
<td>10.54 ± 1.5</td>
<td>5.6–14.5</td>
<td>10.37–10.71</td>
<td>0.02</td>
</tr>
<tr>
<td>Not infected</td>
<td>11.92 ± 1.35</td>
<td>6.8–14.0</td>
<td>11.62–12.21</td>
<td></td>
</tr>
<tr>
<td>Single-species infection</td>
<td>10.81 ± 1.50</td>
<td>5.6–14.5</td>
<td>10.5–11.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Multiple-species infection</td>
<td>10.27 ± 1.30</td>
<td>5.6–13.0</td>
<td>10.04–10.49</td>
<td></td>
</tr>
<tr>
<td>Infection by A. lumbricoides</td>
<td>11.27 ± 1.24</td>
<td>8.5–14.5</td>
<td>11.0–11.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Infection by T. trichiura</td>
<td>10.17 ± 1.7</td>
<td>5.6–13.5</td>
<td>9.7–10.6</td>
<td></td>
</tr>
</tbody>
</table>

giene, traditional methods of agriculture, indiscriminate defec­
tion, use of night soil as fertilizers, and other occupational hazards. Similar factors have also been found responsible for high prevalence of infection by Okay et al. (2004) and Ulukanligil and Seyrek (2003).

In the present study, STH infection and Hb concentration was correlated, with infected children possessing lower mean Hb than uninfected children. It was also observed that children with multiple infections had much lower Hb levels than those with single species infections. The reasons for latter difference are many. For example, because of poverty, children are already at the risk of having low Hb, and, when infected by intestinal helminths, the condition is aggravated. The present results are supported by Persson et al. (2000) and Gabrielli et al. (2005), who showed that cases of iron deficiency anemia were significantly greater among children with helminth infections. Chak­ma et al. (2000) demonstrated that continued presence of worms in marginally nourished children could contribute significantly to blood loss in the intestine, with resultant anemia.

In the present study it was observed that T. trichiura-infected children had comparatively lower levels of hemoglobin than children infected with A. lumbricoides. Adult T. trichiura caus­es the loss of approximately 0.005 ml/day/worm of blood from the colon, leading to anemia in already malnourished children (Annanthakrishnan et al., 1997). Wanachiwanawin et al. (2005) suggested that intensity of T. trichiura infection with worms producing the equivalent of 500 EPG or greater may also be associated with intestinal bleeding, leading to anemia. Baigi (1963) reported a microcyte hypochromic anemia in T. trichiura infection. Layrisse et al. (1967) measured blood loss using 15 Cr-tagged red cells in heavily infected children. They reported a loss ranging from 0.8 to 8.6 ml/day and concluded that infec­tions of more than 800 parasites can induce anemia in chil­dren.

Based on the majority of studies, it is clear that helminth infections associated with low nutritional conditions lead to anemia in children. Light infection by A. lumbricoides and T. trichiura does not produce a harmful effect, while moderate to heavy infections certainly lead to anemia and other nutritional problems (Annanthakrishnan et al., 1997). The range of Hb concentration in uninfected children in the present study was between 6.8 and 14 g/dl, indicating that some children in the uninfected group were also anemic. Endemic malnutrition is the reason for this low Hb, and when STHs are present, the con­dition is exacerbated.

The present study reveals that STHs are abundant among school children of Kashmir Valley and have a negative impact on the hemoglobin concentration. This situation strongly calls for the institution of control measures, including treatment of

TABLE 1. Mean hemoglobin values (g dl⁻¹) in infected and uninfected children.


TWO NEW SPECIES OF AFRICAN *HAEMAPHYSALIS* TICKS (ACARI: IXODIDAE), CARNIVORE PARASITES OF THE *H. (Rhipistoma) leachi* GROUP

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United States National Tick Collection, Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, Georgia 30460-8055. e-mail: dapanaskevich@georgiasouthern.edu

**ABSTRACT:** Two new tick species belonging to the African *Haemaphysalis* (Rhipistoma) *leachi* subgroup, namely *H. (R.) colesbergensis* n. sp. and *H. (R.) oliveri* n. sp., are described. *Haemaphysalis* (R.) *colesbergensis* adults are easily differentiated from the other species of the *H. (R.) leachi* subgroup, including *H. (R.) oliveri*, by the spur on coxa IV, which is considerably longer than that on coxa III. The adults of the 2 new species are equal in size, but the dental formula of the hypostome of *H. (R.) colesbergensis* is 4/4 compared to 5/5 for *H. (R.) oliveri*. The dental formula of *H. (R.) oliveri* also distinguishes it from other ticks in the subgroup, namely *H. (R.) leachi*, *H. (R.) elliptica*, *H. (R.) moreli*, and *H. (R.) punctaleachi* (4/4 in these species), but not from *H. (R.) paraleachi*, which has a 5/5 dental arrangement. However, the average total length and width of *H. (R.) oliveri* males (2.47 X 1.20 mm) are considerably shorter and narrower than those of *H. (R.) paraleachi* males (3.81 X 1.79 mm). Similar differences in size apply to the females. Nymphs and larvae of *H. (R.) colesbergensis* and *H. (R.) oliveri* can be distinguished from those of other members of the *H. (R.) leachi* subgroup, as well as from each other, by a combination of the following characters: size and measurement ratios, length of posterodorsal and posteroventral spurs on palpal segment II, and number of denticles per file on the hypostome. *Haemaphysalis (R.) colesbergensis* is known only from South Africa, where it has been collected from domestic cats and dogs and medium-sized wild felids. *Haemaphysalis (R.) oliveri* is recorded only from Sudan, where it has been collected from small- to medium-sized wild felids and canids and an antelope. The hosts of the immature stages of *H. (R.) colesbergensis* are unknown, while nymphs of *H. (R.) oliveri* have been collected from rodents.

There are currently 5 species in the *H. (R.) leachi* subgroup of ticks (Camicas et al., 1998). These are *H. (R.) leachi* (Audouin, 1826), *H. (R.) elliptica* (Koch, 1844), *H. (R.) moreli* Camicas, Hoogstraal and El Kammah, 1972, *H. (R.) punctaleachi* Camicas, Hoogstraal and El Kammah, 1973, and *H. (R.) paraleachi* Camicas, Hoogstraal and El Kammah, 1983 (Hoogstraal, 1958; Camicas et al., 1972, 1973, 1983; Apanaskevich et al., 2007). Not only are these ticks, in many respects, morphologically similar, but their adults all prefer carnivores as hosts; the immature stages of species for which these are known prefer rodents. At least 1 of the ticks in this subgroup is of economic importance, namely *H. (R.) elliptica*, the vector of the pathogenic *Babesia canis rossi*, which causes babesiosis in domestic dogs in southern Africa (Apanaskevich et al., 2007). The adults of the latter tick [then thought to be identical to *H. (R.) leachi sensu stricto*] frequently attach to humans (Horak et al., 2002), and it, or *H. (R.) leachi sensu stricto*, has been incriminated as a vector of *Rickettsia conorii*, the cause of Mediterranean spotted fever in humans (Kelly, 2001).

Two new species belonging to this subgroup of ticks have recently been discovered, 1 in the Karoo regions of South Africa and the other in southeastern Sudan. All stages of development of the South African species and the adults and larvae of the Sudanese species are described here. The adults of the first species parasitize domestic and wild carnivores, while the hosts of its immature stages are unknown. Adults of the second species parasitize wild carnivores, and there is also a single record from an antelope. Several field-collected nymphs have

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**Figure 1.** *Haemaphysalis (Rhipistoma) colesbergensis* n. sp., male. Conscutum. Bar = 1 mm. All setation is omitted.
TABLE I. *Haemaphysalis* (*Rhipistoma*) *colesbergensis* n. sp., material examined.

<table>
<thead>
<tr>
<th>No. of ticks*</th>
<th>L.</th>
<th>N.</th>
<th>Date</th>
<th>Collector†</th>
<th>USNTC No.</th>
</tr>
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<tr>
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<td>June 2005</td>
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<td>23</td>
<td>5</td>
<td>Domestic dog Arundel, near Colesberg</td>
<td>June 2005</td>
<td>NMW</td>
<td>123891</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td><em>C. caracal</em> Arundel, near Colesberg</td>
<td>1 December 2004</td>
<td>NMW</td>
<td>123898</td>
</tr>
<tr>
<td>23</td>
<td>31</td>
<td><em>F. silvestris</em> Colesberg</td>
<td>July 1989</td>
<td></td>
<td>123903</td>
</tr>
<tr>
<td>South Africa, Eastern Cape Province</td>
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<td></td>
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<tr>
<td>1</td>
<td><em>C. caracal</em> Bloemhof</td>
<td>24 May 1984</td>
<td>IGH</td>
<td>123895</td>
<td></td>
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<td>11</td>
<td>6</td>
<td><em>C. caracal</em> Bloemhof</td>
<td>31 May 1984</td>
<td>IGH</td>
<td>123894</td>
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<tr>
<td>29</td>
<td>13</td>
<td><em>C. caracal</em> Mountain Zebra National Park</td>
<td>5 June 1984</td>
<td>IGH</td>
<td>123896</td>
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<tr>
<td>1</td>
<td><em>C. caracal</em> Mountain Zebra National Park</td>
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<td></td>
<td>LCM</td>
<td>123901</td>
</tr>
<tr>
<td>1</td>
<td><em>C. caracal</em> Speelmanskop, near Cradock</td>
<td>13 August 1984</td>
<td>LCM</td>
<td>123892</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td><em>C. caracal</em> Garslandskloof, near Cradock</td>
<td>16 October 1984</td>
<td>LCM</td>
<td>123893</td>
</tr>
<tr>
<td>1</td>
<td><em>C. caracal</em> Spioenkop, near Cradock</td>
<td>14 May 1984</td>
<td>LCM</td>
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<td>South Africa, Western Cape Province</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>Domestic cat Prince Albert</td>
<td>28 September 2004</td>
<td>HS</td>
<td>123904</td>
</tr>
</tbody>
</table>

* L, larvae; N, nymphs.  † HS, H. Swanepoel; IGH, I. G. Horak; LCM, L. C. Moolman; NMW, N.-M. Wium.  ‡ Reared specimens.

**TABLE II.** *Haemaphysalis* (*Rhipistoma*) *oliveri* n. sp., material examined.

<table>
<thead>
<tr>
<th>No. of ticks*</th>
<th>L.</th>
<th>N.</th>
<th>Date</th>
<th>Collector†</th>
<th>USNTC No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sudan, Upper Nile Province, Paloich District</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>57</td>
<td><em>C. adustus</em> Khor Adar</td>
<td>14 April 1960</td>
<td>HH, DH, SG</td>
<td>092982</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td><em>C. adustus</em> Khor Adar</td>
<td>14 April 1960</td>
<td>HH, DH, SG</td>
<td>092981</td>
</tr>
<tr>
<td>2</td>
<td>&quot;Antelope&quot;</td>
<td>Khor Adar</td>
<td>14 April 1960</td>
<td>HH</td>
<td>092826</td>
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<tr>
<td>59</td>
<td>19</td>
<td><em>C. adustus</em> Gelhak forest</td>
<td>23 May 1962</td>
<td>HH</td>
<td>092881</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td><em>F. silvestris</em> Gelhak forest</td>
<td>23 May 1962</td>
<td>HH</td>
<td>092777</td>
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<tr>
<td>1</td>
<td><em>I. albicauda</em> Gelhak forest</td>
<td>23 May 1962</td>
<td>HH</td>
<td>092883</td>
<td></td>
</tr>
<tr>
<td>14‡</td>
<td>19</td>
<td><em>A. niloticus</em> Tir (2 miles N of)</td>
<td>19 March 1962</td>
<td>HH, SG</td>
<td>092856</td>
</tr>
<tr>
<td>43</td>
<td>35</td>
<td><em>C. adustus</em> Tir (1 mile S of)</td>
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<td>HH, SG</td>
<td>092991</td>
</tr>
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<td>2</td>
<td><em>F. silvestris</em> Tir (near)</td>
<td>23 February 1961</td>
<td>HH, SG</td>
<td>092973</td>
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<td>38</td>
<td>8</td>
<td><em>C. adustus</em> Niayok</td>
<td>23 January 1960</td>
<td>HH, SG</td>
<td>092899</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td><em>G. genetta</em> Niayok</td>
<td>12 January 1961</td>
<td>HH, SG</td>
<td>092887</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td><em>L. serval</em> Paloiok (8 miles S of)</td>
<td>14 December 1960</td>
<td>HH, SG</td>
<td>092979</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td><em>C. adustus</em> Paloiok (5 miles S of)</td>
<td>13 December 1960</td>
<td>HH, SG</td>
<td>092893</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td><em>F. silvestris</em> Paloiok (5 miles W of)</td>
<td>11 December 1960</td>
<td>HH, SG</td>
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<td>10</td>
<td>6</td>
<td><em>F. silvestris</em> Paloiok (2 miles W of)</td>
<td>14 December 1960</td>
<td>HH, SG</td>
<td>092972</td>
</tr>
<tr>
<td>Sudan, Upper Nile Province, Malakal District</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>5</td>
<td><em>C. adustus</em> Malakal, Taufikia</td>
<td>25 February 1961</td>
<td>HH, SG</td>
<td>092990</td>
</tr>
<tr>
<td>9</td>
<td><em>F. silvestris</em> Malakal (6 miles N of)</td>
<td>24 March 1962</td>
<td>HH, SG</td>
<td>094963</td>
<td></td>
</tr>
<tr>
<td>Sudan, Blue Nile Province</td>
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<tr>
<td>354</td>
<td>176</td>
<td><em>A. cahirinus</em> Khor Yabus (vicinity)</td>
<td>22 April 1960</td>
<td>HH</td>
<td>092893</td>
</tr>
</tbody>
</table>

* L, larvae; N, nymphs.  † DH, D. Heynaman; HH, H. Hoogstraal; SG, S. Gaber.  ‡ Reared specimens.
been taken from rodents. These *Haemaphysalis* Koch, 1844, spp. are thus the sixth and seventh taxa to be recognized in the African *H. (R.) leachi* subgroup. Only time will tell whether either of these ticks is a vector of any pathogenic organisms.

**MATERIALS AND METHODS**

The material examined is summarized in Table I and Table II. Both field-collected and laboratory-reared ticks were studied. The specimens of both species that were examined are deposited in the United States National Tick Collection (USNTC) (Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, Georgia). Some of the paratypes of both species will be deposited in the collections of the Zoological Institute, Russian Academy of Sciences (ZIN) (St. Petersburg, Russia), and the Gertrud Theiler Tick Museum of the Onderstepoort Veterinary Institute (OVI) (Onderstepoort, South Africa).

The immature stages and the more delicate structures of the adults were mounted on glass slides and examined using light microscopy, and the macrostructures of males and females were examined under a stereomicroscope. The spiracular plates of the adults and nymphs were studied using scanning electron microscopy (SEM). Measurements for the male and female are given in millimeters (mm), and those for the immature stages are reported in micrometers (µm). The measurements are arranged as follows: minimum–maximum (average ± standard deviation, *n* = number of specimens measured); their schematic layout is shown in Apanaskevich et al. (2007). The measurements and their ratios that are summarized in Table III and Table IV for the immature stages of *H. (R.) elliptica* and *H. (R.) leachi* are also from Apanaskevich et al. (2007). The measurements for the males of *H. (R.) moreli*, *H. (R.)

**TABLE III. Measurements (in µm) and their ratios* for nymphs of the *Haemaphysalis* (*Rhipistoma*) *leachi* subgroup of species.**

<table>
<thead>
<tr>
<th>Characters</th>
<th><em>H. colesbergensis</em></th>
<th><em>H. elliptica</em></th>
<th><em>H. leachi</em></th>
<th><em>H. moreli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length (L)</td>
<td>1,402–1,561 (1,489)</td>
<td>1,366–1,683 (1,543)</td>
<td>1,293–1,427 (1,344)</td>
<td>1,240–1,280 (1,267)</td>
</tr>
<tr>
<td>Total width (W)</td>
<td>817–939 (867)</td>
<td>756–988 (896)</td>
<td>707–817 (755)</td>
<td>600–660 (622)</td>
</tr>
<tr>
<td>Ratio total L:W</td>
<td>1.62–1.81 (1.72)</td>
<td>1.60–1.85 (1.73)</td>
<td>1.66–1.84 (1.78)</td>
<td>1.92–2.10 (2.04)</td>
</tr>
<tr>
<td>Scutum W</td>
<td>461–500 (480)</td>
<td>421–549 (427)</td>
<td>402–446 (416)</td>
<td>350–366 (358)</td>
</tr>
<tr>
<td>Ratio scutum L:W</td>
<td>0.94–1.00 (0.98)</td>
<td>0.92–1.08 (1.00)</td>
<td>0.95–1.02 (0.99)</td>
<td>1.13–1.24 (1.18)</td>
</tr>
<tr>
<td>Ratio gnathosoma L:Combined palpal W</td>
<td>0.67–0.74 (0.71)</td>
<td>0.69–0.79 (0.71)</td>
<td>0.64–0.72 (0.70)</td>
<td>0.75–0.79 (0.76)</td>
</tr>
</tbody>
</table>

* The measurements are arranged as follows: minimum–maximum (average).

**TABLE IV. Measurements (in µm) and their ratios* for larvae of the *Haemaphysalis* (*Rhipistoma*) *leachi* subgroup of species.**

<table>
<thead>
<tr>
<th>Characters</th>
<th><em>H. colesbergensis</em></th>
<th><em>H. oliveri</em></th>
<th><em>H. elliptica</em></th>
<th><em>H. leachi</em></th>
<th><em>H. moreli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length (L)</td>
<td>691–740 (716)</td>
<td>637–696 (674)</td>
<td>657–755 (704)</td>
<td>608–681 (652)</td>
<td>588–672 (634)</td>
</tr>
<tr>
<td>Total width (W)</td>
<td>500–529 (516)</td>
<td>465–500 (481)</td>
<td>461–529 (498)</td>
<td>431–559 (457)</td>
<td>389–417 (405)</td>
</tr>
<tr>
<td>Ratio total L:W</td>
<td>1.35–1.42 (1.39)</td>
<td>1.31–1.43 (1.40)</td>
<td>1.31–1.53 (1.41)</td>
<td>1.14–1.50 (1.43)</td>
<td>1.50–1.66 (1.56)</td>
</tr>
<tr>
<td>Scutum L</td>
<td>230–242 (236)</td>
<td>206–240 (229)</td>
<td>220–269 (237)</td>
<td>196–225 (208)</td>
<td>208–228 (218)</td>
</tr>
<tr>
<td>Ratio scutum L:W</td>
<td>0.67–0.73 (0.69)</td>
<td>0.65–0.75 (0.71)</td>
<td>0.66–0.80 (0.73)</td>
<td>0.62–0.74 (0.69)</td>
<td>0.76–0.81 (0.78)</td>
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<tr>
<td>Gnathosoma L</td>
<td>144–152 (148)</td>
<td>122–152 (144)</td>
<td>126–159 (143)</td>
<td>117–140 (128)</td>
<td>134–150 (142)</td>
</tr>
<tr>
<td>Combined palpal W</td>
<td>202–218 (209)</td>
<td>176–214 (200)</td>
<td>189–221 (206)</td>
<td>177–205 (192)</td>
<td>192–212 (204)</td>
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<tr>
<td>Ratio gnathosoma L: Combined palpal W</td>
<td>0.67–0.74 (0.71)</td>
<td>0.65–0.77 (0.72)</td>
<td>0.65–0.76 (0.70)</td>
<td>0.61–0.73 (0.67)</td>
<td>0.65–0.77 (0.70)</td>
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<tr>
<td>Palpi W</td>
<td>77–87 (81)</td>
<td>69–85 (79)</td>
<td>74–87 (82)</td>
<td>67–80 (73)</td>
<td>76–84 (81)</td>
</tr>
<tr>
<td>Ratio palpi L:W</td>
<td>1.21–1.36 (1.29)</td>
<td>1.13–1.32 (1.24)</td>
<td>1.12–1.37 (1.21)</td>
<td>1.18–1.44 (1.32)</td>
<td>1.27–1.42 (1.33)</td>
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<tr>
<td>Hypostome L</td>
<td>64–73 (68)</td>
<td>54–69 (64)</td>
<td>57–74 (66)</td>
<td>52–62 (57)</td>
<td>58–64 (60)</td>
</tr>
<tr>
<td>Ratio hypostome L:W</td>
<td>2.07–2.40 (2.27)</td>
<td>2.29–2.72 (2.50)</td>
<td>2.08–2.70 (2.38)</td>
<td>1.92–2.74 (2.28)</td>
<td>2.61–2.82 (2.69)</td>
</tr>
<tr>
<td>Genu L</td>
<td>120–129 (124)</td>
<td>103–129 (121)</td>
<td>99–129 (112)</td>
<td>103–130 (114)</td>
<td>102–110 (107)</td>
</tr>
<tr>
<td>Genu W</td>
<td>53–60 (56)</td>
<td>46–62 (56)</td>
<td>48–51 (54)</td>
<td>48–60 (54)</td>
<td>48–52 (50)</td>
</tr>
<tr>
<td>Ratio genu L:W</td>
<td>2.11–2.39 (2.23)</td>
<td>1.92–2.43 (2.17)</td>
<td>1.83–2.22 (2.06)</td>
<td>1.88–2.38 (2.11)</td>
<td>2.11–2.21 (2.16)</td>
</tr>
</tbody>
</table>

* The measurements are arranged as follows: minimum–maximum (average).
Figure 2. *Haemaphysalis (Rhipistoma) colesbergensis* n. sp., male. (A) Genital apron and postgenital sclerite. Bar = 200 μm. (B) Spiracular plate. Bar = 200 μm. Arrows show orientation of spiracular plate (a—anterior; d—dorsal). (C) Gnathosoma dorsally. Bar = 200 μm. (D) Gnathosoma ventrally. Bar = 200 μm. (E) Hypostome. Bar = 100 μm. (F) Coxae. Bar = 500 μm. All setation is omitted.
apex; lateral margin of spur straight; posteroventral spur relatively small, triangular with tapering apex; lateral margin of spur straight. Segment III (or genu) ca. 1.4 times as broad as long; ca. 0.6 times as long as segment II; ventral spur of segment III narrowly elongate, blunt apex at level of anterior one-third of length of segment II. Hypostome (Fig. 2E) slightly shorter than palpi; dental formula 4/4; denticles in files of 6 or 7. Coxae (Fig. 2F) I–III each with short, subtriangular, more or less bluntly pointed spur, extending somewhat beyond coxal margin; spur of coxa IV considerably longer than that of coxa III, triangular, tapering at apex.

Female (Figs. 3, 4): Length from palpal apices to posterior margin of scutum 1.24–1.25 (1.54 ± 0.08, n = 131); breadth (of scutum) 0.70–1.02 (0.91 ± 0.05, n = 140), ratio 1.53–1.86 (1.70 ± 0.07, n = 131). Scutum (Fig. 3) ca. 1.3 times as long as broad; margins diverge for anterior one-fifth of total length, then subparallel one-fifth of the length, thereafter gradually converging, bluntly rounded posteriorly; slight angles on either side of posterior tip. Cervical grooves narrow arcs extending two-thirds of total scutal length. Punctations distinct, large, relatively deep, walls sloping on lateral fields, moderately dense, denser on lateral fields, absent on cervical grooves. Genital operculum (Fig. 4A) broadly U-shaped. Spiracular plates (Fig. 4B) variable in size; irregularly sub-oval or sub-circular; dorsal projection short, broadly triangular. Basis capituli (Figs. 4C, D) dorsally ca. 2.4 times as broad as long; external margins slightly diverging anteriorly; cornua very short, broadly triangular, bluntly pointed, ca. one-tenth as long as base of basis capituli; porose areas elongate–ovate, tilted inward, moderate size, wide­ly spaced. Palpi (Figs. 4C, D) broadly salient (leachi type); combined breadth ca. 1.6 times breadth of basis capituli. Segment II ca. 1.4 times as broad as long; dorsomedian margin of segment II gradually widening anteriorly at level of its mid-length; posterodorsal spur relatively small, triangular, blunt at apex; posterolateral margin slightly or convexly concave; posterovernal spur reduced to a gentle curve. Segment III ca. 1.2 times as broad as long; ca. 0.7 times as long as segment II; ventral spur of segment III narrowly elongate, blunt apex at level of anterior one-third to one-half of length of segment II. Hypostome (Fig. 4E) slightly shorter than palpi; dental formula 4/4; denticles usually in files of 9 or 10. Coxae (Fig. 4F) I–III each with short, subtriangular, more or less bluntly pointed spur, extending somewhat beyond coxal margin; spur on coxa IV longer than that of coxa III, triangular with blunt apex.

Nymph (Fig. 5): Length (unengorged) from palpal apices to posterior body margin 1.402–1.561 (1.489 ± 0.38, n = 16); breadth (at widest point of idiosoma) 817–939 (867 ± 38.17, n = 16); ratio 1.62–1.81 (1.72 ± 0.06, n = 16). Scutum (Fig. 5A) sub-circular, length 451–490 (460 ± 9.47, n = 16), breadth 460–500 (480 ± 13.80, n = 16), ratio 0.94–1.00 (0.98 ± 0.08, n = 16). Spiracular plate (Fig. 5B) sub-oval. Capitulum (Figs. 5C, D) length 250–279 (265 ± 7.25, n = 16), breadth (combined palpal breadth) 350–392 (372 ± 10.71, n = 16), ratio 0.67–0.74 (0.71 ± 0.02, n = 16). Basis capituli, dorsally external margins diverging anteriorly; cornua obsolete; ventrally as illustrated. Palpi (Figs. 5C, D) length 171–191 (186 ± 5.40, n = 16), breadth 140–167 (152 ± 6.40, n = 16), ratio 1.15–1.27 (1.22 ± 0.03, n = 16); broadly salient; dorsally anterolateral margin slightly concave. Dorso­median margin of segment II gradually widening anteriorly at level of its mid­length; posterodorsal spur short and rounded; ventral spur distinct and broad; posterolateral margin slightly concave. Ventral spur of segment III short, broadly triangular. Hypostome (Fig. 5D) length 102–115 (104 ± 4.16, n = 16); breadth 46–53 (49 ± 1.85, n = 16), ratio 2.12–2.35 (2.23 ± 0.07, n = 16); nearly as long as palpi; dental formula 2/2; denticles in files of 7–9 (usually 8). Coxa I spur (Fig. 5E) moderate, triangular; spur of coxae II and III small, triangular; no spur on coxa IV. Genu length 186–200 (192 ± 4.17, n = 16), breadth 76–86 (83 ± 3.05, n = 16), ratio 2.24–2.49 (2.33 ± 0.07, n = 16).

 Larva (Fig. 6): Length (unengorged) from palpal apices to posterior body margin 691–740 (716 ± 12.13, n = 30), breadth (at widest point of idiosoma) 500–529 (516 ± 8.84, n = 30), ratio 1.35–1.42 (1.39 ± 0.07, n = 30). Scutum (Fig. 6A) length 416–436 (426 ± 4.16, n = 30), breadth 323–348 (340 ± 4.77, n = 30), ratio 0.67–0.73 (0.69 ± 0.01, n = 30); margins acutely diverging to level of ca. anterior one-third of scutal length, sub-parallel along mid-third, thence abruptly converging, bluntly rounded posteriorly. Capitulum (Figs. 6B, C) length 144–152 (148 ± 2.03, n = 30), breadth (combined palpal breadth) 202–216 (209 ± 4.03, n = 30), ratio 0.67–0.74 (0.71 ± 0.01, n = 30). Basis capituli dorsally sub-rectangular; cornua as very slight marginal bulges; ven­
Figure 4. *Haemaphysalis (Rhipistoma) colesbergensis* n. sp., female. (A) Genital operculum and vestibular part of vagina. Bar = 200 μm. (B) Spiracular plate. Bar = 200 μm. Arrows show orientation of spiracular plate (a—anterior; d—dorsal) (C) Gnathosoma dorsally. Bar = 200 μm. (D) Gnathosoma ventrally. Bar = 200 μm. (E) Hypostome. Bar = 100 μm. (F) Coxae. Bar = 500 μm. All setation is omitted.
Figure 6. Haemaphysalis (Rhipistoma) colesbergensis n. sp., larva. (A) Scutum. Bar = 200 µm. (B) Gnathosoma dorsally. Bar = 100 µm. (C) Gnathosoma ventrally. Bar = 100 µm. (D) Coxae. Bar = 100 µm. Setation of palpal segment IV is omitted on 6C.
collection data as for holotype except the date of the collection, 9 July 2006. (See also Material examined, Table I.) Some of the paratypes will be deposited in the collections of the ZIN (Russia) and the (OVI) (South Africa).

**Distribution and hosts:** The collection data for *H. (R.) colesbergensis* are listed in Table I. This species is confined to South Africa (Northern Cape, Eastern Cape, and Western Cape provinces). The adults have been collected from the domestic dog, domestic cat, wild cat (*Felis silvestris* Schreber, 1775 [= *F. lybica* Forster, 1780]), and caracal (*Caracal caracal* [Schreber, 1776]). The hosts of the immature stages are unknown but, judging by the hosts of the immature stages of other ticks in this subgroup, are likely to be small mammals such as rodents.

**Etymology:** The species is named after the town of Colesberg (Northern Cape Province, South Africa), the vicinity in which the species was initially identified.

**Remarks**

The collections of *H. (R.) colesbergensis* that were made during 1984 from caracal in the Eastern Cape Province by one of us (I.G.H.) and by L.C. Moolman were at the time identified as *H. leachi* (Horak et al., 1987).

*Haemaphysalis* (*R*) *colesbergensis* adults are easily differentiated from the other species of the *H. (R.) leachi* subgroup by the relatively long spur on coxa IV, which is longer than that on coxa III [shorter or subequal in the other species of the *H. (R.) leachi* subgroup]. One of the other species of the *H. (R.) leachi* subgroup is: male length from palpal apices to posterior margin of conscutum avg. 2.47 mm, breadth of scutum avg. 1.19 mm [3.00 x 1.47 for *H. (R.) elliptica*, 2.45 x 1.06 for *H. (R.) leachi*, 2.00 x 0.90 for *H. (R.) moreli*, 2.65 x 1.29 for *H. (R.) punctaleachi*, 3.81 x 1.79 for *H. (R.) paraleachi*], female length from palpal apices to posterior margin of scutum avg. 1.54 mm, breadth of scutum 0.91 mm [1.73 x 1.02 for *H. (R.) elliptica*, 1.53 x 0.84 for *H. (R.) leachi*, 1.46 x 0.85 for *H. (R.) moreli*, 1.70 x 0.99 for *H. (R.) punctaleachi*], male length from palpal apices to posterior margin of conscutum avg. 2.07 mm, and in the female, the ratio of length from palpal apices to posterior margin of conscutum to width of conscutum is 1.70 [2.30 in male, 1.81 in female for *H. (R.) leachi*; 2.22 in male for *H. (R.) moreli*], punctations of male conscutum and female scutum [smaller in the other species of the *H. (R.) leachi* subgroup], punctations with sloping walls on female scutum [punctations with straight walls in the other species of the *H. (R.) leachi* subgroup], very short dorsal cornua in females, which are one-tenth of the length of basis capituli [one-sixth in *H. (R.) elliptica*, one-fourth in *H. (R.) leachi* and *H. (R.) punctaleachi*, one-fifth in *H. (R.) moreli* and *H. (R.) punctaleachi*], shorter posterodorsal spur of palpal segment II in both sexes, and shorter posteroventral spur of palpal segment II in males [considerably longer in the other species of the *H. (R.) leachi* subgroup]. Additional discriminating characters for *H. (R.) colesbergensis* are width of body: in the male, ratio of length from palpal apices to posterior margin of conscutum to width of conscutum is 2.07, and in the female, the ratio of length from palpal apices to posterior margin of conscutum is 1.70 [2.30 in male, 1.81 in female for *H. (R.) leachi*; 2.22 in male for *H. (R.) moreli*], punctations of male conscutum with straight walls [slope in *H. (R.) moreli*], punctuations of male conscutum and female scutum are moderately dense [very dense in *H. (R.) punctaleachi*], dorsomedian margin of palpal segment II widening gradually anteriorly [abruptly in *H. (R.) leachi* and *H. (R.) moreli*], straight lateral margin of posteroventral spur on palpal segment II in male [concave in *H. (R.) leachi* and *H. (R.) moreli* and *H. (R.) punctaleachi*], 4/4 denticles per file on hypostome [5/5 in *H. (R.) paraleachi*]. nymphs and larvae differ from those of other species of *H. (R.) leachi* subgroup [immature stages of *H. (R.) punctaleachi* and *H. (R.) paraleachi* are unknown] by short posterodorsal and posteroventral spurs of palpal segment II. Additional characters are size and measurement ratios (Table III, Table IV), presence of ventral spur on palpal segment III in nymphs [absent in *H. (R.) moreli*], foldlike ventral spur on palpal segment III in larvae [sharp, triangular in *H. (R.) leachi*, absent in *H. (R.) moreli*], 6 or 7 denticles per file on hypostome [7 or 8 in *H. (R.) elliptica*; 4–6, usually 5 in *H. (R.) leachi*].

**Taxonomic summary**

**Holotype:** Male, from domestic cat, Arundel, near Colesberg, Northern Cape Province, South Africa (30°51'5" S, 23°01' E), June 2005, N.-M. Wiem; deposited in the USNTC (123887).

**Allotype:** Female (USNTC 123886), data and depository as for holotype.

**Paratypes:** Total: 45 ♀, 40 ♂, 30 F 3 nymphs, 70 F, larvae (USNTC 123879, 123882, 123885, 123886, 123887), with the same collection data as for holotype and 34 ♀, 9 ♂ (USNTC 123905) with the same

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**Figure 7.** *Haemaphysalis (Rhipistoma) oliveri* n. sp., male. Conscutum. Bar = 1 mm. All setation is omitted.
teriorly; slight angles on either side of posterior tip. Cervical grooves narrow arcs extending two-thirds of total scutal length. Punctations distinct, moderately dense, denser on lateral fields, absent on cervical grooves, medium size, walls vertical, relatively deep. Genital operculum (Fig. 10A) ovoid, broadly rounded posteriorly. Spiralacular plates (Fig. 10B) variable in size; irregularly sub-oval or sub-circular; dorsal projection short, broadly triangular. Basis capituli (Figs. 10C, D) dorso- ca. 2.2 times as broad as long; external margins diverging ante- roiorly; cornua short, broadly triangular, bluntly pointed, ca. one-fifth as long as base of basis capituli; porose areas elongate-oval, tilted inward, moderately large, widely spaced. Basis capituli ventrally as illustrated. Pal- pi (Figs. 10C, D) broadly salient (leachi type); combined breadth ca. 1.7 times breadth of basis capituli. Segment II ca. 1.4 times as broad as long; dorsomedian margin of segment II abruptly widening anteriorly at level of its mid-length; posterodorsal spur large, triangular; posterolat­eral margin straight; posteroventral spur small, rectangular, rounded at apex. Segment III ca. 1.1 times as broad as long; ca. 0.8 times as long as segment II; ventral spur of segment III narrowly elongate, with bluntly pointed apex at level of anterior one-third of length of segment II. Hypostome (Fig. 10E) nearly as long as palpi; dental formula 5/5; denticles in files usually of 10 or 11. Coxae (Fig. 10F) I-IV each with short, subtriangular, more or less bluntly pointed spur, extending somewhat beyond coxal margin; spur on coxa IV shorter than that on coxa III.

**Holotype:** Male, from side-striped jackal (*Canis adustus* Sundevall), Khor Adar, Palolo District, Upper Nile Province, Sudan (10°22’N, 32°15’E), 14 April 1960, H. Hoogstraal and D. Heynaman, and S. Gürer; deposited in the USNTC (092982).

**Allotype:** Female, data and depository as for holotype.

**Paratypes:** Total: 121♀, 56♂ (USNTC 092982), with the same collection data as for holotype and allotype, and in 1970 200 F, larvae, from wild cat (*F. silvestris* Schreber), Tir, Palolo District, Upper Nile Province, Sudan, 1 February 1961, H. Hoogstraal and S. Gürer (USNTC 092976) (See also Material examined, Table II). Some of the paratypes will be deposited in the collections of the ZIN (Russia) and the OVI (South Africa).

**Distribution and hosts:** The collection data for *H. (R.) oliveri* are listed in Table II. The known distribution of *H. (R.) oliveri* is confined to Sudan (Upper Nile and Blue Nile provinces). The adults of this species have been collected from side-striped jackal (*Canis adustus* Sundevall, 1846), wild cat (*F. silvestris* [= *F. lybica*], serval (*Leptailurus serval* Schreber, 1776), small-spotted genet (*Genetta genetta* Linnaeus, 1758), white-tailed mongoose (*Ichneumia albicauda* [Cuvier, 1829]), and “antelope” (no details as to species). Several adults have been reared from nymphs collected from the African grass rat (*Arvi- canthus niloticus* [Desmarest, 1822]) and Cairo spiny mouse (*Acomys cahirinus* [Desmarest, 1819]). The latter records lead us to assume that small mammals such as rodents are the hosts of the immature stages.
Figure 11. *Haemaphysalis (Rhipistoma) oliveri* n. sp., larva. (A) Scutum. Bar = 200 μm. (B) Gnathosoma dorsally. Bar = 100 μm. (C) Gnathosoma ventrally. Bar = 100 μm. (D) Coxae. Bar = 100 μm. Setae of palpal segment IV is omitted on 11C.
Etymology: The species is named after the eminent ixodologist, Professor Dr. James H. Oliver, Jr.

Remarks
All of the collections of H. (R.) oliveri were made by H. Hoogstraal, D. Heynaman, and S. Gaber during 1960, 1961, and 1962, and the ticks were at the time identified as "Haemaphysalis leachi group."

Haemaphysalis (R.) oliveri adults are easily differentiated from species such as H. (R.) leachi, H. (R.) elliptica, H. (R.) moreli, and H. (R.) punctaleachi that constitute the H. (R.) leachi subgroup by their 5/5 dental formula (4/4 in these species). Adults of H. (R.) oliveri are distinguished from those of H. (R.) paraleachi, which also has a 5/5 dental arrangement, by their being very much smaller. Males: avg. length from palpap apices to posterior margin of scutum 2.47 mm; avg. width of scutum 1.20 mm, compared to 3.81 mm and 1.79 mm for H. (R.) paraleachi [3.00 × 1.47 for H. (R.) elliptica, 2.45 × 1.06 for H. (R.) leachi, 2.00 × 0.90 for H. (R. moreli, and 2.65 × 1.29 for H. (R.) punctaleachi]. Females: length from palpap apices to posterior margin of scutum avg. 1.58 mm, avg. width of scutum 0.91 mm, compared to 2.02 mm and 1.17 mm for H. (R.) paraleachi [1.73 × 1.02 for H. (R.) elliptica, 1.53 × 0.84 for H. (R.) leachi, 1.46 × 0.85 for H. (R.) moreli, and 1.70 × 0.99 for H. (R.) punctaleachi]. Additional discriminating characters for H. (R.) oliveri are width of body: in the male, the ratio of length from palpap apices to posterior margin of scutum to width of scutum is 2.05; in the female, the ratio of length from palpap apices to posterior margin of scutum is 1.74 [2.30 in male and 1.81 in female for H. (R.) leachi; 2.22 in male for H. (R.) moreli]; punctations of male conscutum with straight walls [sloping in H. (R.) moreli]; punctations on male conscutum and female scutum are moderately dense [very dense in H. (R.) punctaleachi]; in both sexes, dorsomedian margin of palpap segment II widening abruptly anteriorly (gradually in H. (R.) elliptica, H. (R.) punctaleachi, and H. (R.) paraleachi); straight or slightly concave lateral margin of posteroventral spur on palpap segment II in male [strongly concave in H. (R.) leachi and H. (R.) punctaleachi]. Larva differs from that of other species of H. (R.) leachi subgroup [immature stages of H. (R.) punctaleachi and H. (R.) paraleachi are unknown] by size and measurement ratios (Table III, Table IV), broad posteroventral spur on palpap segment II [narrow in H. (R.) leachi], presence of ventral spur on palpap segment III in nymphs [absent in H. (R.) moreli], foldlike ventral spur on palpap segment III in larvae [sharp, triangular in H. (R.) leachi, absent in H. (R.) moreli], 6 or 7 denticles per file on hypostome [7 or 8 in H. (R.) elliptica, 4–6, usually 5 in H. (R.) leachi].

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LITERATURE CITED


A NEW GNATHIID (CRUSTACEA: ISOPODA) PARASITIZING TWO SPECIES OF REQUIEM SHARKS FROM LIZARD ISLAND, GREAT BARRIER REEF, AUSTRALIA

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ABSTRACT: Third-stage juveniles (praniza larvae) of Gnathia grandilaris n. sp. were collected from the gill filaments and septa of 5 requiem sharks, including a white tip reef shark, *Triakodon obesus*, and 4 grey reef sharks, *Carcharhinus amblyrhynchos*, at Lizard Island, Great Barrier Reef, Australia, in March 2002. Some juvenile gnathiids were then maintained in fresh sea water until they molted to adults. Adult males appeared 19 days following detachment of juveniles from host fishes, but no juveniles molted successfully into females. The current description is based, therefore, on bright field and scanning electron microscopy observations of adult males and third-stage juveniles. Unique features of the male include the triangular-shaped inferior medio-frontal process, 2 areolae on the dorsal surface of the pylopod, and a slender pleotelson (twice as long as wide) with lateral concavities. The third-stage juvenile has distinctive white pigmentation on the black pereon when alive, while the mandible has 9 triangular backwardly directed teeth. This species has the largest male and third-stage juvenile of any *Gnathia* spp. from Australia and of any gnathiid isopods associated with elasmobranchs.

The Gnatiiidae is a family of isopods characterized by free-living adults and 3 stages of parasitic juvenile stages that feed on fishes. The family is geographically widespread, with more than 180 species known worldwide and more than 50 species in tropical and subtropical waters (Smit and Davies, 2004). Only 12 are known from the Great Barrier Reef, Australia (see Table I in Smit and Davies, 2004). According to Holdich and Harrison (1980), poor sampling effort has contributed to the low number of gnathiid species described from Australia, although their juveniles are among the most common ectoparasites of fishes, especially on coral reefs (Grutter and Poulin, 1998; Smit et al., 2006). Feeding juveniles (praniza larvae) sometimes cause severe tissue damage to infested hosts and may be vectors for fish blood parasites, such as hemogregarines (Smit and Davies, 1999; Davies and Smit, 2001; Davies et al., 2004; Marino et al., 2004; Hayes et al., 2007).

Only third-stage juveniles have been reported from elasmobranchs, the location of the first and second stages being unknown (summarized in Smit and Basson, 2002). However, such third-stage juveniles cannot yet be used directly for species identity, as only free-living male gnathiids are employed for such purposes (see Smit and Davies, 2004). A single species of gnathiid, *Gnathia pantherina* Smit and Basson, 2002, has been described from elasmobranchs, and this species is from South Africa. The current paper reports on the discovery of a second species from 2 species of Australian requiem sharks, the white tip reef shark, *T. obesus* Rüppell, 1837, and the grey reef shark, *C. amblyrhynchos* Bleeker, 1856.

MATERIALS AND METHODS

A single white tip reef shark (1,035 mm) and 4 grey reef sharks, ranging in length from 920 to 1,464 mm (mean ± SE 1,393.5 ± 331.5 mm) were captured by rod and line in March 2002 at Lizard Island, Great Barrier Reef, Australia, as part of an ongoing study on shark age by James Cook University (Great Barrier Reef Marine Park Authority (GBRMPA; permit G02021)). The sharks were killed by a sharp blow to the head and then identified, measured (total length), necropsied, and examined for juvenile gnathiids. The gnathiids were removed with fine forceps, cleaned with a small brush in a few drops of seawater under a dissection microscope, and placed in 50-ml jars with seawater (after Smit and Basson, 2002). One juvenile gnathiid from the white tip reef shark and 20 juvenile gnathiids from the grey reef sharks were allowed to molt into adults at 24–27 °C, while the remainder were fixed in 70% ethanol. Of the 21 juveniles kept alive, 7 died and 14 molted into adult males. For the purpose of this description, specimens were mainly examined by bright field microscopy, using ×10–50 objectives. Specimens were also prepared for scanning electron microscopy following techniques described by Smit and Van As (2000). The anatomical terminologies, as well as the numbering of pereonites and pereopods used by Cohen and Poore (1994), were applied.

RESULTS

All 5 sharks bore only third-stage juvenile gnathiids on their gill filaments and gill septa. The number of gnathiids varied from 2 on the white tip reef shark to 2–41 (14.4 ± 16.7) on the 4 grey reef sharks. A juvenile from the white tip reef shark completed its molt to a male in 19 days, while juveniles collected from the grey reef sharks molted to adult males between 17 and 23 (19 ± 1.8) days postdetachment from their hosts. Molting followed a pattern similar to that described for *G. pantherina* by Smit and Basson (2002) and *Gnathia africana* Barnard, 1914, by Smit et al. (2003), but no juveniles molted to adult females. The current description is based, therefore, on the morphology of free-living males and parasitic third-stage juveniles (praniza larvae).

DESCRIPTION

*Gnathia grandilaris* n. sp.

(Figs. 1–11, 24, 25)

**Diagnosis of adult:** Total length of holotype: 6.8 mm. Total length of other material: 5.7–8.3 mm (7.4 ± 0.8 mm, n = 8). Cephalosome, rectangular, 1.4 times as wide as long, deep dorsal sulcus, narrower than width of median process, extending one-third the length of cephalosome (Figs. 1, 24); lateral margins convex, row of long papoose setae and tubercles extending laterally from under eyes posteriorly-medially reaching

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Gnathia grandilaris n. sp., male.

Frontal border slightly produced, superior fronto-lateral process conical, directed antero-laterally, divided into 2 acute lobes, dorsally 15–18 long pappose setae present on each lobe, not reaching dorsal sulcus (Figs. 2, 24). Medio-frontal process inferior, forming 1 distinctive triangular-shaped lobe, conical and not same length as superior frontolateralis (Fig. 1). Lamina dentata with 8–10 tubercles visible. External scissura shallow. Supraocular lobe pronounced, with 6–8 pappose setae and 4–6 tubercles dorsally.

Antenna 2 longer than antenna 1. Antenna 1 (Fig. 4) with 3 peduncle articles increasing in length distally, with a third article as long as the median tubercle (Figs. 2, 3), posterior margin concave (Fig. 1). Sensory pits, some with a single short pappose seta; pappose setae and tubercles distributed randomly over dorsal and lateral surfaces of cephalosome (Figs. 2, 3, 24); sensory pits not present in dorsal sulcus. Pappose setae, sensory pits, and short simple setae on lateral sides of buccal cavity. Well-developed oval-shaped, bulbous, sessile compound eyes on lateral margin of cephalosome; length of eye slightly less than one-third of cephalosome (Fig. 3). Eight to 10 paraocular tubercles with long pappose setae (Fig. 3). Elliptical posterior median tubercle present (Figs. 1, 24).
first and second articles combined; all 3 peduncle articles covered with short, hairlike simple setae. Flagellum with 5 articles; articles 3 and 4 with 1 aesthetasc seta each. Article 5 terminating in 1 aesthetasc and 3 simple setae. Antenna 2 (Fig. 5) with 5 peduncle articles covered with short, hairlike simple setae. Article 4 with a row of pectinate scales laterally, article 5 largest; flagellum with 7 articles, article 2 largest, article 7 terminating in 4 simple setae (Fig. 5). Articles 2–6 of flagellum with 1 or 2 simple setae on anterior end.

Mandible relatively long, more than half the length of cephalosome; 1.2 times as long as wide, broad basal neck, curved inward with 7–8 processes on dentate blade, tussle of setae between processes (Fig. 2). Apex cylindrical, distally raised in lateral view (Fig. 3). Slight incisor present. Single pappose mandibular seta extending from base of incisor process. Carina not armed, forming ridge on lateral margin extending from basal neck to a third along mandible (Fig. 2). Short, simple hairlike setae distributed randomly on dorsal surface of blade. Internal lobe and pseudoblade absent.

Maxilliped with 5 articles, proximal article largest with short mediiodistal endite not reaching article 3 (Fig. 8). Outer margin of proximal article densely setose. Distal 4 articles bearing plumose setae on lateral margins in order of 6–8–6–9; mesial border, with short simple setae (Fig. 8). Single simple seta on anterior side of articles 2 and 3, as well as a pair of simple setae on article 4. Pulp 1.8 times as long as wide. No coupling hooks.

Pylopod with 3 articles. First article greatly enlarged, convex mesial border fringed with 70 plumose setae; single featherlike seta present distally on first article. A pair of featherlike setae near lateral border and 11 pappose setae distally on ventral surface (Fig. 9). Single proximal, featherlike seta present mesially. Second article oval, 1.2 times as long as wide, margins setose; 10 pappose setae and single simple seta distally on posterior surface (Fig. 9). Two areolae present. Third article minute with fringing setae (Fig. 10).

Pereon 1.4 times as long as wide, wider than cephalosome (Fig. 1), covered with numerous long pappose setae and short, simple hairlike setae. Pereonite 1 fused with cephalosome, dorsally visible, reaching lateral margins, anterior border convex (Fig. 1). Pereonites 2 and 3 of similar size; tubercles present on all pereonites. Tubercles present on dorsal sides of pereonites 2 and 3. Pereonite 4 with prominent anterior constriction separating it from pereonite 3. Long pappose setae on anterior lateral lobe of pereonite 4, median groove present. Pereonite 5 widest part of body, with areae laterales and dorsal sulcus as thin groove. Pereonites 5 and 6 not fused. Pereonite 6 longer than other pereonites, twice as long as wide; posterior margin deeply concave, with lobi laterales, no lobii. Pereonite 7 dorsally visible, small with rounded posterior margin, overlapping first pleonite. Long pappose setae on anterior, lateral, and posterior margins of pereonites.

Pleon and pleotelson less than one-third of total length (Fig. 1). Five subequal pleonites and epimera dorsally visible, long pappose setae and tubercles randomly distributed on pleonites.

Pleotelson triangular, slender, base not wider than length, lateral margins concave; dorsal surface with 6 pairs of long pappose setae (Fig. 6). Distally, 3 pairs of simple setae and single pair of pappose seta.
Many pectinate scales on dorsal surface, distal apex terminating in pair of pappose setae (Fig. 6).

Pereopod 2 basis elongated, oval-shaped, with 2–3 pappose setae, 2 featherlike setae, and 4 simple setae anteriorly, whereas only 3 pappose setae, single featherlike seta, and 3 simple setae posteriorly (Fig. 11). Ischium two-thirds length of basis; single pappose seta, 2 featherlike setae, and 3 simple setae posteriorly. Two pappose setae, 1 featherlike seta, and 3 simple setae present anteriorly. Merus half the length of ischium with anterior bulbous protrusion, pappose and simple setae on bulbous protrusion, posterior margin with 2 pappose and 2 simple setae. Carpus of almost same size and shape as merus, but without anterior bulbous. Anteriorly, 1 denticulated, single simple seta and 2 featherlike setae; posteriorly, pair of pappose and simple setae present on carpus. Propodus about twice the length of carpus, single robust denticulated seta situated in middle; robust seta on posterior side proximal to unguis, few simple setae on dorsal and ventral sides of robust seta. Dactylus half the length of propodus, terminating in sharp, posterior-pointing unguis, prominent protrusion. Pereopods 3–6 similar in basic shape to pereopod 2 (Fig. 11), differ in setation as well as distribution of tubercles. All pereopods with distinct tooth-shaped tubercles on anterior and posterior margin of basis, ischium, and merus. Pereopod 6 with 2 robust, elongated denticulated setae on posterior bulbous protrusion of merus. Dorsal surface of all pereopods covered with pectinate scales.

Exopod of pleopod slightly longer than endopod. Exopod fringed distally with 9 long pappose setae. Sympodite with retinaculae on medial margin. Seven long pappose setae present distally on endopod. All pleopods similar, except pleopod 2 with appendix masculina on endopod; appendix masculina 1.7 times length of rami (Fig. 7).

Uropod rami extending beyond apex of pleotelson, endopod with 7 long pappose setae on distal margin and 2 setae on lateral margin. Exopod with 6 long pappose setae on distal and 11 pappose setae on lateral margin. Endopod contains 2 simple setae and a single featherlike seta distally (Fig. 6). Pectinate scales on lateral areas of uropods. Single featherlike seta present on uropodal basis.

Penis prominent with 2 contiguous papillae ventrally on pereonite 6. One pair of simple setae present distally to penis (Fig. 25).

Stage 3 juvenile (praniza 3 larva) (Figs. 12–23, 26); Total length of paratypes: 4.3–8.6 mm (6.1 ± 2.3 mm, n = 3). Cephalosome posterior margin straight, slightly wider than anterior margin, almost as wide as long, lateral margins slightly convex (Figs. 12, 13). Many sensory pits. Compound eyes large, well developed, oval shaped, bulbous, on lateral margins of cephalosome; length of eye almost same as length of ce-
phalosome (Fig. 26). No sulcusses or tubercles on dorsal cephalosome. A few simple setae randomly distributed around outer margin of compound eyes. Medio-anterior margin of cephalosome straight, with lateral concave excavations to accommodate first articles of antennae.

Labrum prominent, 2.5 times the length of cephalosome, semicircular with apical process, truncated posterior margin, anterior margin concave (Figs. 13, 26). Ventral part of labrum gutterlike with central groove, covering mandibles dorsally and laterally.

Antenna 1 shorter than antenna 2. Antenna 1 with 3 peduncle articles, 2 pairs of featherlike setae distally on articles 1 and 2; article 3 largest with single featherlike seta and 4 simple setae distally (Fig. 14). Flagellum with 5 articles; first article with single featherlike seta, article 2 largest with 1 simple seta and 1 aesthetasc present distally. Articles 3 and 4 with 1 aesthetasc each, article 5 terminating in 1 aesthetasc and 3 simple setae, few setae on each article (Fig. 14). Antenna 2 with 4 peduncle articles; article 4 largest, setae distributed laterally on article 3 and distally on article 4. Flagellum with 7 articles; article 7 terminating in 3 simple setae, few setae on distal end of each article (Fig. 15). Articles 2–6 of flagellum with 1 or 2 simple setae on anterior end.

Mandible stout, swollen at base, distal margin styliform with 9 teeth on mesial margin, triangular, and backwardly directed, increasing in size from anterior to posterior (Figs. 18).

Paragnaths elongated, gutterlike, terminate in sharp point; no teeth (Fig. 19).

Maxillae not visible. Maxillule long, slender, swollen base, stretching past distal margin of labrum. Seven small teeth on distal inner margin (Fig. 20). Maxilliped large, cylindrical; elongated base with pectinate scales and short, hairlike setae laterally, endite almost reaching palp with single long simple seta and coupling hook. Palp with 3 articles, first article acute with 3–5 small teeth, article 3 with 5 simple setae (Fig. 21).

Gnathopod smaller than pereopods; 7 articles, 2 simple setae on basis, only a few simple setae on other articles (Fig. 22). Dactylus long and strongly hooked.

Pereon almost twice as long as wide, wider than cephalosome (Fig. 12). Pereonite 1 fused with cephalosome, dorsally visible (Fig. 12). Pereonite 2 with anterior constriction separating it medially from pereonite 1. Pereonite 4 twice as wide as long, lateral sides tapering toward rounded posterior margin, posterior margin stretching over pereonite 5; lateral shields at leg attachment. Pereonite 5 consisting of elastic membrane fully expanded in praniza stage with blood meal (Fig. 12), bul-
Pereopod 2 basis elongated, with single featherlike bristle anterior and single simple seta posterior (Fig. 23). Ischium three-quarters length of basis and almost as wide; 2 simple setae present anteriorly and posteriorly. Merus half the length of ischium, with anterior bulbous protrusion. Single elongated denticulated seta and single featherlike seta on bulbous protrusion, posterior margin with pair of simple setae. Carpus of almost same size and shape as merus, but without anterior bulbous; posterior with a simple seta and a featherlike seta. Propodus about twice the length of carpus, single robust denticulated seta situated proximal to posterior side, single featherlike seta present posteriorly, anterior featherlike and simple setae. Dactylus half the length of propodus, terminating in sharp, posterior-pointing unguis, prominent robust seta on posterior side proximal to unguis, 2–3 simple setae on dorsal and ventral sides of robust seta. Pereopods 3–6 (Fig. 23) similar to pereopod 2 in basic form, but differing in setation. Pereopod 3 without elongated denticulated seta; pereopods 4 and 5 with denticulated setae present; pereopod 6 with 2 elongated denticulated setae on bulbous protrusion of merus and a single seta on anterior margin of carpus. All articles of pereopods with pectinate scales and short, hairlike setae.

Pleon and pleotelson slightly half the length of pereon. Five pleonites dorsally visible (Fig. 12). Single simple seta on each posterior lateral pleonite. Pleopod 2 endopod shorter than exopod. Endopod fringed distally with 7 long plumose setae, exopod fringed with 6 plumose setae distally and a single simple seta laterally (Fig. 16). Sympodite with retinaculum, single simple seta on lateral margin, 2 denticulated setae on basis, and short, hairlike setae on dorsal surface and margins. Pleopods 3–5 similar to pleopod 2.

Pleotelson triangular, longer than wide, anterior half of lateral margins concave, posterior half straight, with 6 pairs of simple setae and 1 pair of featherlike setae on dorsal surface; distal apex terminating in a pair of featherlike setae, dorsal surface covered with pectinate scales (Fig. 17).

Uropod rami extending beyond apex of pleotelson. Endopod longer and wider than exopod, both with long, fringing setae; endopod with 5 distal and 8 lateral plumose setae, exopod with 6 distal and 15 lateral plumose setae (Fig. 17). Lateral dorsal areas of both endo- and exopods with pectinate scales; all margins with short, hairlike setae. Distal dorsal area of endopod contains a pair of featherlike setae and 3 simple setae proximally located. Uropodal basis with single featherlike seta.

Externally, live larvae of G. grandilaris with black pereon and distinctive white markings. Dorso-lateral surfaces of pleon with light brown pigmentation patterns, stretching from the posterior rim of pleonite 1 to the anterior margin of pleonite 5. Brownish-yellow pigmentation also present on lateral surfaces of exopod.

**Taxonomic summary**

*Type host:* T. obesus Rüppell, 1837.

*Other host:* C. ambyrhythchos Bleeker, 1856.

*Type locality:* Lizard Island (14°40’S, 145°27’E), Australia.

*Specimens deposited:* Holotype: In the collection of the Queensland Museum, Brisbane, Australia (1 male) (W28356). Paratypes in the collection of the Queensland Museum, Brisbane, Australia (2 males, 3 praniza larvae) (W28357, W28358). Other material in the collection of N.J.S. in the Department of Zoology, University of Johannesburg (5 males).

**Etymology:** The species name is derived from the Latin, *grandilaris*, referring to the large size of both the male and third-stage juvenile.

**Remarks**

The presence of a straight frontal border and frontal processes on the cephalosome of the males of this new species, as well as broad, 3-articled pylopods and denticulated mandibles, support its identity as a species of *Gnathia* (see Cohen and Poore, 1994). Nine of the 12 known gnathiid species from the Great Barrier Reef belong to *Gnathia*, and the remaining 3 to *Elaphognathia*. The *Gnathia* spp. are much smaller than *G. grandilaris*, with adult males all less than 5 mm long. The largest of the known North Australian species, *G. biorbis* Holdich and Harrison, 1980, at 4.8 mm, is still outside the size range of *G. grandilaris* males (5.7–8.3 mm). Not only are *G. grandilaris* males larger, but they also differ in the shape of the frontal border and its associated processes from all known Great Barrier Reef gnathiid species (see Fig. 2 in Cohen and Poore, 1994).

The large size of *G. grandilaris* males is a characteristic shared with the South African species *G. pantherina*, the only other known species associated with elasmobranchs. Both of these species have long pappose setae and tubercles covering their pleon and pereon. However, *G. panth-
*erina* males have a slightly produced frontal border, an inferior frontal process divided into 2 acute lobes, and no fronto-lateral processes (Smit and Basson, 2002), whereas the frontal border of *G. grandilaris* males is produced, with the superior fronto-lateral process forming 2 distinct conical lobes and an inferior medio-frontal process having 1 distinctive triangular-shaped lobe.

Since gnathiid taxonomy is based on male morphology, adequate descriptions and illustrations of juvenile stages are often absent from the literature. The third-stage juvenile of *G. pantherina* (Smit and Basson, 2002) is the only such stage described from elasmobranchs to date, and similarities between this and the juveniles of *G. grandilaris* are in their basic shape and the few setae found on the pereon and pleonites. The most prominent differences are the larger size of the third-stage juvenile of *G. grandilaris* (4.3–8.6 mm), and its pleotelson, with concave lateral margins having 6 pairs of simple setae and 1 pair of featherlike setae on the dorsal surface. In contrast, *G. pantherina* juveniles range from 3.9 to 5.8 mm long; the pleotelson has only slightly concave lateral margins and a pair of simple setae on its dorsal surface (Smit and Basson, 2002). Live *G. pantherina* juveniles also lack the distinct white markings present on the black pereon of *G. grandilaris* juveniles (N. Smit, pers. obs.). Poor illustrations and descriptions of the larval stages make comparisons between *G. grandilaris* juveniles and those of other Australian species impossible.

**ACKNOWLEDGMENTS**

Many thanks to the Lizard Island Research Station Staff and Will Robins (James Cook University) and Tom Lisney (University of Queensland) for collecting the sharks. This study was funded by an Australian Research Council Discovery Grant, and the Sea and Coast II Programme of the National Research Foundation of Southern Africa.

LITERATURE CITED


PREVALENCE AND ABUNDANCE OF FLEAS IN BLACK-TAILED PRAIRIE DOG BURROWS: IMPLICATIONS FOR THE TRANSMISSION OF PLAGUE (YERSINIA PESTIS)

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ABSTRACT: Plague, the disease caused by the bacterium Yersinia pestis, can have devastating impacts on North American wildlife. Epizootics, or die-offs, in prairie dog colonies (Cynomys ludovicianus) occur sporadically and fleas (Siphonaptera) are probably important in the disease’s transmission and possibly as maintenance hosts of Y. pestis between epizootics. We monitored changes in flea abundance in prairie dog burrows in response to precipitation, temperature, and plague activity in shortgrass steppe in northern Colorado. Oropsylla hirsuta was the most commonly found flea, and it increased in abundance with temperature. In contrast, Oropsylla tuberculata cyanomuris declined with rising temperature. During plague epizootics, flea abundance in burrows increased and then subsequently declined after the extirpation of their prairie dog hosts.

Plague, the disease caused by the bacterium Yersinia pestis, was introduced into the United States circa 1900. In the Great Plains, plague can cause local extinctions of black-tailed prairie dog (Cynomys ludovicianus) populations (Cully and Williams, 2001; Pauli et al., 2006). Prairie dogs are important to the ecology of North American grasslands, so the local decimation of their populations has potential ramifications for many other species (Antolin et al., 2002). Plague activity seems sporadic at any particular location; however, the mechanism of persistence of plague between epizootics is not well understood. Although plague can be transmitted between hosts pneumatically in aerial droplets, or septicemically through direct contact with infected tissue, transmission by fleas (Siphonaptera) is considered to be an important mechanism of the disease’s spread (Gage and Kosoy, 2005).

Temporal or geographical variation in flea ecology, possibly as a response to changes in weather, or host abundance, or both, may explain the advent of epizootics because of an increased rate of disease transmission (Stapp et al., 2004; Collinge et al., 2005; Gage and Kosoy, 2005). Because mammalian hosts frequently die of plague once infected, fleas may also act as reservoirs of Y. pestis between epizootics. However, the ecology of fleas in prairie dog colonies has not been well studied. On the Pawnee National Grasslands (PNG) in northern Colorado, plague has been documented in areas of die-offs of prairie dog colonies during the past 5 yr, and it has probably been a cause of colony extirpations since the 1940s, when Y. pestis was first detected in the region (Stapp et al., 2004; Adjemian et al., 2007). Here, we investigate prevalence and abundance patterns of fleas inhabiting prairie dog burrows in shortgrass steppe in the PNG over a 3-yr-period. We also relate patterns in flea density and occurrence to weather conditions, colony characteristics, and plague occurrence.

MATERIALS AND METHODS

We sampled fleas on prairie dog colonies on the PNG, which is located in western Weld County, Colorado (40°49’N, 104°47’W; elevation 1,650 m). The climate of the PNG is semiarid and vegetation is shortgrass steppe, characterized by blue grama (Bouteloua gracilis), buffalo grass (Buchloe dactyloides), and prickly pear cactus (Opuntia polyacantha). Prairie dog colonies occupy only a small fraction of the habitat and total acreage of prairie dog colonies in the area fluctuates yearly (Stapp et al., 2004).

Fleas were sampled, or “swabbed,” using 15- × 15-cm squares of flannel cloth attached to a flexible plumbing snake (2 m cable, 1.3 cm in diameter), which was inserted into a prairie dog burrow to at least 1 m in depth, and then removed (Ubico et al., 1988). Swabs were then placed individually into plastic bags, which were then temporarily stored at −20°C to inactivate the fleas and allow them to be transferred to vials containing 2% NaCl solution and 0.001% Tween 80 (ICN Bio- medicals Inc., Irvine, California), and stored at −80°C until identification.

At each prairie dog colony (30 colonies were sampled in total, although not all were currently inhabited), we sampled 20 burrows that showed evidence of prairie dog activity as determined by presence of fresh droppings, tracks or sightings, or a combination. Sampled burrows were at least 30 m apart. However, on colonies where prairie dogs were extirpated by plague, we simply attempted to find 20 burrows that were not collapsed. Swabbing during different sampling sessions was carried out by different individuals, and burrows were not deliberately reswabbed in subsequent sessions. Fleas were sampled between August 2004 and September 2006, during March and May–October, and at least 5 colonies were swabbed during each month. Fleas were identified by light microscopy to the species level, using Stark (1958) and Hubbard (1968), and identification was confirmed at Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado.

The United States Forest Service has monitored the size of prairie dog colonies on the PNG annually since 1981; since 1995, colonies have been mapped using global positioning satellite technology and incorporated into a geographic information system using Arc/INFO 9.0, ArcView 3.3, ArcMap 8.3 or Arc9 (Environmental Systems Research Institute, Redlands, California).

Between 2004 and 2006, plague epizootics resulted in extinction of numerous prairie dog colonies on the PNG (confirmed by the postmortem examinations of prairie dogs at the CDC or by plague positive fleas). Four prairie dog colonies that we sampled (PNG colonies 3, 41, 62, and 74) experienced die-offs between January and June 2004. In 2005, 5 colonies (colonies 5, 8, 35, 82, and 84) experienced or began prairie dog die-offs between June and November, and 3 others were extirpated between February and August 2006 (colonies 13, 30, and 34).

Rainfall and temperature data were from a long-term weather station associated with the NSF Shortgrass Steppe Long-Term Ecological Research project. Soils data were taken from USDA Soil Conservation Service Soil Survey maps (northern Weld County), upon which approximate colony perimeters in 2004 were superimposed visually. We estimated the dominant and codominant soil types on each colony. Of 80 extinct and active colonies that have been monitored on the PNG since 1981, 86% had sandy-loam (SAL) or fine-sandy loam (FSL) soils, 39% had loam (LOM) soils, 15% had clay sandy loam (CSL) soils, and 15% had soils characterized as complex (CMX; P Stapp, unpubl., obs.). For our purposes, we grouped our study colonies into 4 soil groups: those with SAL or FSL types only; those with LOM or CSL soils; those with CMX soils; and a combination of LOM, CSL, and CMX soil types. We compared flea loads and prevalence of infected burrows among soil groups using data collected the year before, or 1 year after, epizootics, and from colonies that never experienced plague during our study pe-
riod (n = 24 colonies). We expected that burrow flea loads and prevalence of infested burrows might be related to soil texture because colonies with fine-textured, clay-sandy loam soils seem to suffer die-offs more frequently than those on other soil texture types (P. Stapp, unpubl. obs.). We calculated burrow density by counting the number of active burrows in 6, 250-m² transects on each colony.

Data analysis
We used data from the 20 burrow swabs to generate summary statistics for each prairie dog colony. We defined prevalence as the number of flea-infested burrows/number of burrows swabbed, e.g., 11 burrows provided fleas from 20 burrows equals a prevalence of 0.55. Mean flea load is the total number of fleas/number of burrows swabbed, whereas infested burrow mean load is the total number of fleas divided by the number of burrows actually infested. Abundance data were transformed before analysis.

For the analysis on changes in burrow flea load as a result of plague-induced prairie dog die-offs, sampling periods were combined into 4 categories based on plague status: pre, pre-epizootic; epizootic, during the period of high prairie dog mortality; post <1 yr, period following all prairie dog mortality for up to approximately 1 yr later; and post 2 yr, 2 yr after epizootic. Only colonies that experienced plague at some point during the study period were included (n = 12). Data were pooled when colonies were sampled more than once in a given time period, e.g., if colonies were swabbed twice in the month preceding an epizootic, the data were pooled to give a single “pre-epizootic” flea abundance data point. For the analysis on changes in burrow flea load as a result of environment or colony area, we only used data from colonies that were unaffected by plague.

RESULTS
Flea numbers in all burrows were highly aggregated with an aggregation coefficient (k) of 0.19 (values of k ~ 20 indicate a Poisson random distribution and values of k < 1 indicate an aggregated distribution; Wilson et al., 2002).

The most common species was Oropsylla hirsuta, followed by its congener O. tuberculata cynomuris (Table I). Several other species of flea were regularly found, including Thrasis focus and Pulex simulans, which have been previously found in prairie dog burrows (Table II). Pulex simulans is also commonly found on mammalian carrion such as coyotes (Canis latrans) and swift foxes (Vulpes velox; Salkeld et al., 2007), as well as on black-tailed prairie dogs (Brinkerhoff et al., 2006). Other flea species were much rarer than Oropsylla spp., and probably reflects the use of prairie dog burrows by other mammalian hosts:

Euhoplopsyllus glacialis is typically a flea of rabbits; T. focus, of thirteen-lined ground squirrels (Spermophilus tridecemlineatus); Pleocetes exilis, of northern grasshopper mice (Onychomys leucogaster); Aetheca wagneri, of deer mice (Peromyscus maniculatus); and Foxella nigra, of gophers (Thomomys sp.) and grasshopper mice (Hubbard, 1968). We found no O. hirsuta on colonies where prairie dogs had been absent for more than a year, i.e., extinct since 2003 or earlier, and instead found only P. simulans and T. focus.

Seasonal and plague-related patterns
Patterns of abundance of O. tuberculata cynomuris and O. hirsuta differed over the course of the year, with the former found mostly in late winter and early spring, and the latter more common from spring through autumn (Fig. 1). Using data from only active colonies (there were no prairie-dog die-offs or suspected plague activity on the colony during the sampling session), we found a weak positive relationship between the monthly total rainfall and prevalence of O. hirsuta-infested bur-

<table>
<thead>
<tr>
<th>Total no. of fleas identified</th>
<th>Percent of total</th>
<th>Max. no. of fleas per burrow</th>
<th>Mean of burden per burrow</th>
<th>Mean of burden per sampled burrow</th>
<th>Mean (SD) no. fleas in infested burrows</th>
<th>No. of colonies inhabited by fleas</th>
<th>No. of colonies sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Oropsylla hirsuta</td>
<td>4,473</td>
<td>0.06</td>
<td>0.06</td>
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<td>0.06</td>
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</tr>
<tr>
<td></td>
<td>Oropsylla tuberculata cynomuris</td>
<td>3,763 (84.1)</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
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<tr>
<td></td>
<td>Pulex simulans</td>
<td>197</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Thrasis focus</td>
<td>12</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
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</tr>
<tr>
<td></td>
<td>Euhoplopsyllus glacialis</td>
<td>10</td>
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<tr>
<td></td>
<td>Pleocetes exilis</td>
<td>9</td>
<td>0.01</td>
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<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Aetheca wagneri</td>
<td>3</td>
<td>0.01</td>
<td>0.01</td>
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<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Foxella nigra</td>
<td>2</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

† Female fleas were difficult to identify with certainty. However, all examined fleas were O. hirsuta and O. tuberculata cynomuris, as they are very similar in appearance.
‡ Some burrows were occupied by more than one species of flea, most common point occupancy was O. hirsuta and O. tuberculata cynomuris.
§ We examined 30 sites in total, although some were not currently active with prairie dogs.

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Table I. Summary of fleas found in prairie dog (Cynomys ludovicianus) burrows on the Pawnee National Grasslands, Weld Co., Colorado. We sampled 3,013 burrows, though some of these burrows may be duplicates. An additional 300 fleas remain unidentified.
rows (linear regression, $F_{1,71} = 5.19, r^2 = 0.07, P = 0.027$), although rainfall was unrelated to $O. \text{hirsuta}$ load both across all burrows and all infested burrows. The previous month’s rainfall was also related to prevalence of $O. \text{hirsuta}$ in burrows ($F_{1,71} = 5.08, r^2 = 0.07, P = 0.027$).

Mean monthly temperature was a better predictor of $O. \text{hirsuta}$ patterns, although the large variation in flea abundance makes the explanatory power of this variable poor. Nonetheless, higher mean monthly temperatures were associated with an increased prevalence of infested burrows ($F_{1,71} = 4.50, r^2 = 0.06, P = 0.037$), and mean load across all burrows ($F_{1,71} = 5.55, r^2 = 0.07, P = 0.021$), but they were not related to mean flea load in infested burrows ($F_{1,71} = 3.02, r^2 = 0.04, P = 0.088$). Temperature data from the month before sampling had no relationship with any measure of flea abundance.

$Oropsylla \text{tuberculata cyomuris}$ abundance also varied with environmental conditions, although differently than $O. \text{hirsuta}$. Prevalence of infested burrows seemed to decline with increasing temperature during the month of sampling ($F_{1,71} = 16.32, r^2 = 0.19, P = 0.0001$), and it was also negatively related to temperature the month before sampling ($F_{1,71} = 45.88, r^2 = 0.39, P < 0.0001$). Mean load across burrows was related to the monthly temperature ($F_{1,71} = 23.7, r^2 = 0.25, P < 0.0001$) and to the previous month’s temperature ($F_{1,71} = 51.06, r^2 = 0.42, P < 0.0001$). Also, mean load in infested burrows declined with the monthly mean temperature ($F_{1,71} = 10.69, r^2 = 0.13, P = 0.002$) and to the previous month’s temperature ($F_{1,71} = 41.51, r^2 = 0.37, P < 0.0001$). Precipitation did not influence $O. \text{t. cyomuris}$ prevalence, mean load across burrows, or mean load in infested burrows.

Mean burrow flea loads and prevalence of flea-infested burrows was highest during plague epizootics and decreased dramatically during the year afterward (Fig. 2). Mean flea loads and rates of burrow infestation returned to pre- or nonplague levels 2 yr after epizootics, concurrently with the return of small numbers of prairie dogs. The flea abundance patterns of prairie dog colonies that experienced prairie dog die-offs between June and November in 2005 did not differ from other active colonies, when measured in June 2005, or in the preceding August–September.

**Colony size, burrow density, and soil type**

For $O. \text{hirsuta}$, there was no significant relationship between colony active area ($n = 21$) and prevalence of infested burrows,
mean burrow load, or infested burrow means in September 2004. However, in June 2005, just before colony area measurements were taken, there was a negative relationship between prairie dog colony area (n = 15) and prevalence of burrows infested by O. hirsuta ($F_{1,13} = 7.3, r^2 = 0.36, P = 0.018$). Mean burrow O. hirsuta load was also negatively related to colony size (linear regression, $F_{1,13} = 6.48, r^2 = 0.33, P = 0.024$), and O. hirsuta load in infested burrows showed a similar, although nonsignificant, trend ($F_{1,13} = 3.40, r^2 = 0.21, P = 0.087$). We did not analyze the relationship between area and flea abundance in 2006 because few prairie dog colonies were active in 2006.

The number of active prairie dog burrows per 1,500-m² ranged from 2 to 14 (mean = 6.8, SD = 3.9), and it was negatively related to prevalence of infestation with fleas ($F_{1,18} = 5.11, r^2 = 0.22, P = 0.036$), mean burrow flea load ($F_{1,18} = 5.36, r^2 = 0.23, P = 0.033$), and load in infested burrows ($F_{1,18} = 8.61, r^2 = 0.32, P = 0.0089$). Soil type was not significantly related to O. hirsuta abundance (mean load across burrows, mean load of infested burrows, and prevalence of infested burrows; n = 24; ANOVA, $P > 0.577$).

**DISCUSSION**

Our results closely reflect those of other studies (Table II), which found that the flea species found predominantly in prairie dog burrows are *Oropsylla hirsuta* and *O. tuberculata cynomuris*, making up 97% of fleas identified, followed by less common species such as *Pulex* spp. and *Tharsis futs* (<2% each).

The frequency of prairie dog plague epizootics in Colorado and Montana has been associated with weather variables such as rainfall and temperature (Stapp et al., 2004; Collinge et al., 2005). Similarly, there is a relationship between the frequency of human plague cases in the southwestern United States and values for climatic variables such as precipitation and temperature (Enscore et al., 2002). Previous explanations have suggested that increasing rainfall may influence plant productivity, which increases rodent density, which may influence flea density and, therefore, plague transmission (Enscore et al., 2002; Collinge et al., 2005; Gage and Kosoy, 2005), although the interactions of rodent and flea density and plague transmission are poorly known. Here, we show that flea abundance in prairie dog burrows may be influenced, albeit weakly, by weather. *Oropsylla hirsuta* is more abundant in prairie dog burrows in
warmer weather, whereas the converse is true for O. tuberculata cynomuris. Temperature and humidity have been shown previously to affect flea developmental rates in different ways (Krasnov et al., 2001). This leads to 2 hypotheses regarding plague epizootics. The first hypothesis is that perhaps warmer winters allow higher numbers of O. hirsuta to persist throughout the year, amplifying rates of disease spread due to higher vector abundance the following season. The second hypothesis is that epizootics may be fiercer and faster during warmer months simply because more fleas are able to inhabit burrow entrances, and therefore, after plague-induced deaths of prairie dogs in a coterie, prairie dogs from adjacent coteries or other small mammals that visit vacant burrows may be infested by larger numbers of possibly infectious fleas. Interestingly, 5 epizootics in 2005 all seemed to occur in the warmer months, i.e., from June onward. Oropsylla tuberculata cynomuris may also be an able vector of plague, and it may be responsible for epizootical plague transmission in the winter. Nevertheless, the abundance of fleas did not differ between colonies that did or did not experience prairie dog-die-offs. Our fieldwork was carried out predominantly in the warmer months, however, and more needs to be resolved about this flea’s ecology before we draw definite conclusions.

Unfortunately, because of the difficulties of sampling fleas deep in a prairie dog burrow complex, it is not clear that flea abundance measured in the first 2 m of a burrow is indicative of flea populations in the more subterranean prairie dog habitat. Although our results suggest seasonal changes in the abundance of the prairie dogs’ 2 main flea species, these measures may only reflect the flea’s relative tolerance to temperature and humidity regimes in the burrow. More detailed monitoring of weather, and more frequent sampling of fleas, will determine the true nature of the relationship between flea abundance, weather, and transmission of Y. pestis. Ideally, such-monitoring would be preformed concurrently with investigations of flea loads on the prairie dogs themselves.

During June 2005, we observed a relationship between prairie dog colony size and flea abundance. Although convention dictates that increased colony area is indicative of increased prairie dog abundance (Hanson et al., 2007), such a relationship may not be clear-cut. Although large colonies have more prairie dogs inhabiting them than small colonies, changes in area may not reflect a growing population, but a decreasing density of prairie dogs. That is, as competition for resources increases, the colony must spread out to find adequate forage (Crosby and Franklin, 2006). This may explain why colony area increases during periods of drought, but declines in wetter years. Less frequent use of a particular burrow may explain why larger colonies have fewer infested burrows, and lower burrow flea loads. A previous study of fleas combed from prairie dogs found no relationship between prairie dog density and flea load (Brinkerhoff et al., 2006).

The persistence and transmission of plague during, and between, prairie dog plague epizootics is not well understood, but it is possible that fleas act as reservoirs or sources of infection between die-offs if they continue to inhabit burrows in extirpated colonies, until they are able to infect a new host. Previous studies have shown Y. pestis-positive fleas remaining on sites that experienced epizootics in the year previously (Kartman et al., 1962; Lechleitner et al., 1968), although the ability of these fleas to infect natural hosts in the field remains untested. Oropsylla hirsuta is capable of early phase plague transmission in laboratory mice (Wilder et al., 2008), but earlier laboratory studies using guinea pigs as hosts found infrequent transmission over the longer term (3/70 fleas transmitted during 120 days postinfection; Eskey and Haas, 1940).

The abundance of O. hirsuta in prairie dog burrows increased dramatically during epizootics, presumably as fleas abandon dead and dying prairie dogs, and because newly emerging fleas are unable to find hosts. Flea loads then declined markedly after the disappearance of their principal hosts, most likely due to starvation. In the laboratory, O. hirsuta experience high mortality if not provided maintenance blood meals (Wilder et al., 2008). This phenomenon raises doubts as to whether fleas can act as longer term reservoirs of infection between prairie dog die-offs. Previous reports of high numbers of fleas in colonie mo after prairie dog extirpation probably consisted mostly of fleas that had hatched or emerged since the epizootic and which, therefore, may not ever have been exposed to plague-infected hosts (Lechleitner et al., 1968; Stevenson et al., 2003). Other studies have struggled to find plague-positive fleas in year subsequent to prairie dog extirpation (Holmes et al., 2006), although there is some evidence that plague-positive fleas may be distributed throughout prairie dog colonies independently of epizootics (Hanson et al., 2007). An alternative explanation is that Y. pestis-positive fleas found months after epizootics could have been infected by another species of mammal, although this requires further investigation.

If Y. pestis cannot persist in fleas at plague-extirpated colonies, disease transmission at the landscape scale may occur via fleas on mammalian hosts that move between prairie dog colonies. For example, spread of plague may occur in dispersing prairie dogs, scavenging carnivores, or other small rodents (Stapp et al., 2004; Salkeld and Stapp, 2006; Salkeld et al., 2007). Nonetheless, we advocate further study of flea ecology at a finer temporal scale during epizootics, and also the investigation of whether Y. pestis persists in the fleas in the absence of their primary hosts.

ACKNOWLEDGMENTS

We thank J. Montenieri at the CDC, Fort Collins, Colorado, for helping to identify fleas; M. Lindquist, D. Tripp, and M. Antolini for logistical support; C. Cannon, J. Holm, H. Houghton, C. Knox, J. Kraft, H. Franklin, D. Kite, and C. Wermager for field assistance; and A. P. Wilder for comments on earlier manuscripts. This research was supported by grants from the National Science Foundation (EID-0327052) and by the Shortgrass Steppe Long-Term Ecological Research Project (DEB-0217631).

LITERATURE CITED


B. Seery, M. Lindquist, D. Tripp, and M. Antolin for logistical support; C. Cannon, J. Holm, H. Houghton, C. Knox, J. Kraft, H. Franklin, D. Kite, and C. Wermager for field assistance; and A. P. Wilder for comments on earlier manuscripts. This research was supported by grants from the National Science Foundation (EID-0327052) and by the Shortgrass Steppe Long-Term Ecological Research Project (DEB-0217631).

LITERATURE CITED


VARIATION IN ECTOPARASITE LOAD REFLECTS LIFE HISTORY TRAITS IN THE LESSER MOUSE-EARED BAT *MYOTIS BLYTHII* (CHIROPTERA: VESPERTILIONIDAE) IN WESTERN IRAN

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ABSTRACT: We studied seasonal variation of ectoparasite load (number of parasites per individual bat) in free-ranging populations of the lesser mouse-eared bat *Myotis blythii* in western Iran. Data for 1 species each of batfly (*Nycteribidae*), tick (*Ixodidae*), and mite (*Spinturnicidae*) are reported for a 1 yr period. Patterns of parasite load during this time differed considerably among species. However, the parasite load increased markedly in pregnant females in spring and early summer. During the same time frame, parasite load decreased in solitary males when they roosted apart from maternity clusters. However, in late summer, when bats began swarming, males showed a significant (*P* < 0.05) increase in parasite load. Using the ratio of body mass to length of forearm as an index of body condition, no significant correlation was found.

Advances in community ecology research suggest that life history traits of free-living species can be an important determinant of their coexistence with other members of the community (Thomas et al., 2000). Life history traits have a strong influence on the coexistence of species through resource specialization or resource partitioning (Tokeshi, 1999). For example, timing of reproductive events often constitutes the causal basis for the utilization of different types of resources, which in turn facilitates coexistence between species. In this context, parasites have been hypothesized to play an important role in the evolution of host-related life history traits because they often impose important selective pressures. By altering life history traits, parasites can also alter the population dynamics of their hosts and the community structure.

Most biologists assume that ectoparasites on bats can influence the behavior and health of their hosts, and may act as vectors for disease or cause physical damage to their hosts (Marshall, 1982). However, there are not many studies to support the negative relationship between bats and their ectoparasite abundance. Some authors have observed that there is a harmony between host and parasite reproductive events and have concluded that this may be due to an increasing number of hosts suddenly available when birth takes place (e.g., Foster, 1969; Nelson and Demas, 1996). There are also several studies that have documented the relationship between the number of parasites and the condition of bat health (e.g., Glover, 1992; Christe et al., 2000). The latter authors examined the variation in the intensities of a parasitic mite (*Spinturnix myotis*) in relation to the reproductive cycle and immunocompetence of its bat host (*Myotis myotis*). They found that pregnant females were less immunocompetent and harbored more parasites than non-reproductive females. These studies also demonstrated that differences in the social contact between different segments of a species population, as well as differences in roosting habitats among bat species, populations, and sexes, can cause differences in parasite load. In the present paper, we aim to explore the role of life history traits in hosts and the abundance of their parasites.

MATERIALS AND METHODS

The present study was conducted at Mahidasht cave (33°23′N, 47°30′E) in 2005 and 2006, Karafu cave (47°11′N, 36°15′E) in 2006, and Ghaladidar cave (49°32′N, 36°2′E) in western Iran. Mahidasht cave has a nursery colony of approximately 300–350 *Myotis blythii* (Sharifi, 2004). The cave is not used as a hibernaculum, presumably because of its high temperature in winter (Sharifi, 2004). In winter, there are no visible signs of bats, but gradually from winter until mid-March at least 4 species (*M. blythii*, *Rhinolophus mehelyi*, *Myotis capaccinii*, and *Miniopterus schreibersii*) colonize the cave. Preliminary observations made during 1999–2004 indicated that most *M. blythii* in Mahidasht cave gave birth within a period of 2 wk in mid-May, although neonates were frequently found until early June (Sharifi et al., 2004). Thus, the females create mixed nursery colonies with other species in late spring. Males often roost before the mating season in autumn. Karafu is a very large cave which appears to be used as both a hibernaculum and a nursery colony. Observations made in summer indicate that areas of the inner part of the cave are cold and may be used for hibernation. However, the cave is open to the public for visiting its archeological remains and for this reason an insecure situation has been created for hibernating bats. Ghaladidar is a small cave located in Hamadan Province and has a small population of approximately 20–30 *M. blythii*.

On 10 separate occasions, i.e., 15 April, 6 May, 3 June, 1 July, 19 August, and 15 September in 2005 and 21 April, 4 May, and 19–20 September 2006, we captured a total of 209 *M. blythii* using a hand net. Immediately following capture, bats were placed in individual cloth bags (30 × 50 cm) and were suspended from the ceiling near the cave entrances. Bats were identified as to sex, and body mass and length of forearm were measured. Additionally, all bats were assigned to an age class represented by juveniles (individuals with unfused phalangeal epiphyses) and adults (fused epiphyses). The ratio of body mass (g) to length of forearm (mm) was used as an index of body condition (health status). The number of each ectoparasite species was recorded on the wing membranes of each individual bat, as well as the ventral and dorsal parts of the body. We spent approximately 20 sec searching each bat for ectoparasites by blowing on their pelage. Single factor analysis of variance (ANOVA) was used to test for significant differences between numbers of parasites collected from different colonies.

The conservation status of the lesser mouse-eared bat was not included in the assessment conducted by the IUCN (Hutson et al., 2001), and thus this species in its Iranian range is not listed on any threatened or endangered species categories. Other studies conducted by Sharifi et al. (2000) suggests that *M. blythii* is a very common bat species in Iran.

RESULTS

One species of batfly (*Penicillidia* sp., *Nycteribidae*), 1 tick (*Ixodes* sp., *Ixodidae*), and 1 mite (*Spinturnix* sp., *Spinturniciidae*) were present as ectoparasites on the bats present in the caves we searched. *Penicillidia* sp. and *Ixodes* sp. were frequently observed on the pelage, whereas *Spinturnix* sp. was found on the wing and tail membranes. Table 1 shows total numbers of ectoparasites of various species collected and the mean and standard error (±SE) of parasite loads for male and
female *M. blythii*. Results of a statistical comparison between parasite load in males and females also are present in Table I. Figure 1 shows a graphic representation of parasite load in male and female *M. blythii* in spring and summer 2005 and 2006. The relationship between the number of parasites and body condition index (the ratio of mass to forearm length) for all bats is shown in Figure 2.

Between 15 April 2005 and 20 September 2006, more than 3,000 parasites were counted on 209 *M. blythii*. Of these, almost two-thirds (137) were females ($X_1 = 20.07$, $X_2 = 6.8$, $N_1 = 137$, $N_2 = 72$, $F = 3.88$, $P < 0.05$). A positive correlation ($r^2 = 0.87$; $P < 0.001$; $N = 209$) was found between the number of bats captured and the number of parasites observed and counted (Fig. 1). Relatively low parasite loads were evident during the spring and autumn months and during the period of pregnancy. A rapid increase in parasite load was observed during summer months, with a peak occurring during the lactation period in May and June (Fig. 3). *Spinturnix* sp. was the most abundant parasites species (Fig. 4).

Prevalence of ectoparasite infection on *M. blythii* was 86.1% in males, 100% in pregnant females, and 98.24% in nonpregnant females. Mean number and standard error of ectoparasite in male, and pregnant and nonpregnant females are shown in Figure 5. To evaluate the pattern of interdependence of parasites on their host, we derived a survival curve for 31 *Pencilidida* sp. that we removed from their host (Fig. 6).

DISCUSSION

Major life history traits for the lesser mouse-eared bat are characterized by a rapid emergence from winter hibernacula and the formation of maternity colonies in early March. Parturition occurs in mid-May, and young bats are suckled by their mothers for approximately 6 wk (Sharifi, 2004). Observations made at several caves in western Iran indicate that, similar to other temperate regions, a sudden increase in the numbers of cave-dwelling bats, including *M. blythii*, occurs in early autumn. Following this autumn swarming period, bats seek refuge in caves to hibernate. Spermatogenesis is completed in late summer, and spermatozoa are stored in the cauda epididymides in males until copulation. Once mating occurs and females enter hibernation, sperm is stored during the winter months in the reproductive tract of females.

Few studies have been conducted to assess parasite loading in Eurasian bats. Christe et al. (2000) examined variation in the levels of infestation of a parasitic mite (*S. myoti*) in relation to reproductive cycle and immunocompetence in the greater mouse-eared bat (*M. myotis*). They found that reproductive females were less immunocompetent and harbored more parasites than nonreproductive females. However, during lactation, immunocompetence was positively associated with female body.
mass (Christe et al., 2000). Giorgi et al. (2001) investigated, both in the field and in the laboratory, host–parasite relationships of 1 ectoparasitic mite (S. myoti) and its major hosts, 2 sibling species of bats (M. myotis and M. blythii). That study showed that there was no host specificity for this parasite, as the 2 bat species coexisted intimately in maternity roosts. They found that prevalence of S. myoti was extremely high, i.e., 99.5% for juvenile M. myotis and 99.2% for juvenile M. blythii, respectively (Giorgi et al., 2001).

In our study, the most abundant bat parasite was Spintunix sp. and infestation levels in the colony of M. blythii with Spintunix sp. were relatively high. The mean parasite load exceeded 13 individual Eyndhovenia sp. per bat, and on almost half of the sampling occasions it was between 20 and 30. The number of Spintunix sp. increased in June and July and decreased towards August. Wohland (2000) has reported an average of 10–17 S. myoti during summer months on juvenile and adult female M. blythii and a mean infestation of Eyndhovenia euryalis on the greater horseshoe bat Rhinolophus ferrumequinum of 4–8 mites per bat. Of the 3,240 parasites counted in our study, only 83 (2.4%) were found on the tail membrane and 313 (9.6%) were on the abdomen, whereas 391 (42.9%) and 1,459 (45.1%) were present on the right and left wings, respectively. No significant correlation was found ($r^2 = 0.0009$) between total parasite load and the body condition index of individual bat’s health.

Several factors can influence the abundance of parasites car-
ried by an individual. Small parasites may be better colonizers. Considering the small body size of the most abundant ectoparasite (Spinturnix sp.) compared with 2 other ectoparasites (Nyc- teribiidae sp. and Ixodidae sp.), more than 90 percent of parasite load was associated with this species. With a body size of about almost 1-mm diameter, Spinturnix sp. may be considered to be a relatively large mite, but it is still much smaller than 2 other species of parasites found in this study. A similarly high prevalence of smaller size ectoparasites compared to larger ectoparasites was reported by Marshall (1982). Another factor that might contribute to parasite load is reproductive cycle of the parasite in relation to reproductive activities of the host. The numbers of other parasite species could influence the number of parasites on a host’s body, if they compete for the same resources (space and food). Age of the colony, colony size, and climate are also known to have an influence on levels of ectoparasite infestation on bats (Marshall, 1982; Luean, 2006). Besides interactions between species, several of the host’s characteristics such as sex, reproductive stage, and size are assumed to influence the distribution and abundance of ectoparasites (Marshall, 1982; Moura et al., 2003).

In their survey on grooming behavior and parasite load in R. ferrumequinum, Wohland (2000) reported a significant difference between parasite load on males and females. The same study also showed that males had the lowest levels of infestation, at least for the time they were sampled. Our results for M. blythii are consistent with these findings. The number of ectoparasites on solitary males was always lower than the number we observed on females (Fig. 5). However, we found no difference in parasite load between pregnant and nonpregnant females ($X_1 = 16.58, X_2 = 24.96, N_1 = 1,346, N_2 = 1,404, F = 3.91, P = 0.002$). Our findings contrast with those of Christe et al. (2000) who found that S. myoti was more abundant on pregnant females compared to non-breeding females.

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LITERATURE CITED


A NEW SPECIES OF POLYSTOMOIDES WARD, 1917 (MONOGENEA: POLYSTOMATIDAE) FROM FRESHWATER CHELONIANS (TESTUDESINAE: CHERLIDAE) IN BRAZIL

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ABSTRACT: This report describes the first occurrence of Polystomoides brasiliensis n. sp. (Monogenea: Polystomatidae), a new monogenean species in the buccal and pharyngeal cavities of the freshwater turtles in Brazil. Live monogeneans were collected from Hydromedusa maximiliani and Phrynops geoffroanus at the Mariano Procópio Museum’s lake, in Juiz de Fora, Minas Gerais, Brazil. Polystomoides brasiliensis differs from other species of this genus in having 8–9 genital spines, except for Polystomoides uruguayensis, which has 8–10 genital spines. However, the new species differs morphometrically from P. uruguayensis in the greater size of the outer and inner hamuli, as well as having a testis that is proportionally greater than the pharynx and oral sucker. The current study is the first report of monogeneans in chelonians, and the first record of helminths in H. maximiliani.

Polystomatidae (Monogenea) includes 3 parasitic genera exclusive to chelonians, i.e., Polystomoides Ward, 1917, Neopoly­stoma Price, 1939, and Polystomoidella Price, 1939. Species in this genus differ mainly in the number of hamuli they possess. Thus, Polystomoides spp. have 2 pair of hamuli, Polystomoi­della spp. have 1 pair, and Neopoly­stoma spp. have none (Price, 1939; Sproston, 1946; Yamaguti, 1963; Rohde, 1965; Pichelin, 1995; Platt, 2000).

Species of Polystomoides occur in the buccal and pharyngeal cavities, or the urinary and accessory bladder, of terrestrial tortoises, as well as freshwater and sea turtles (Sproston, 1946; Yamaguti, 1963; Knoepffler and Combes, 1977). In South America, species in this genus have been recorded only in the buccal cavity of freshwater chelonians of the Chelidae in Uruguay (Mañé-Garzón, 1958; Mañé-Garzón and Gil, 1961, 1962; Yamaguti, 1963; Mañé-Garzón and Holcman-Spector, 1968; Knoepffler and Combes, 1977; Kohn and Cohen, 1998). In Bra­zil and other South American countries, there are no records of monogeneans in chelonians (Kohn and Cohen, 1998).

The freshwater turtle, H. maximiliani (Mikan, 1820) (Testu­dines, Chelidae), or Maximilian’s snake-necked turtle, has been reported only in Brazil, where it is endemic to the eastern and southeastern regions; in these areas, the distribution is always associated with mountains (Souza, 2004; Novelli and Sousa, 2007). Phrynops geoffroanus (Schweigger, 1812) (Testudines, Chelidae), also known as Geoffroy’s side-necked turtle, occurs in several countries of South America. It can be found from Colombian Amazonia to the Brazilian state of Rio Grande do Sul, and from Uruguay to the northern parts of Argentina (Lema and Ferreira, 1990; McCord et al., 2001). It inhabits the shallow areas of both lakes and rivers, under the trunks of trees and rocks (Medem, 1960; Pritchard and Trebbau, 1984; Ernst and Barbour, 1989). In Brazil, this species is also common in rivers of urban areas (Souza and Abe, 2001a, 2001b; Brites, 2002; Lisboa et al., 2004).

The present report describes a new species of monogenean Polystomoides from both H. maximiliani and P. geoffroanus, and it is the first occurrence of this monogenean genus in Brazil and from this host species.

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MATERIALS AND METHODS

Live monogeneans were collected from the buccal and pharyngeal cavities of a single specimen of H. maximiliani and 4 specimens of P. geoffroanus from the Mariano Procópio Museum’s lake, in Juiz de Fora, Minas Gerais, Brazil, in 2007.

On removal from their hosts, specimens were killed in distilled water kept in a refrigerator (approximately 7 C) for 24 hr and fixed in AFA (70% ethanol, 93 parts; 37% formalin, 5 parts; glacial acetic acid, 2 parts), under slight cover glass pressure for 48 hr, and preserved in 70% ethanol (Amato et al., 1991). Monogeneans were stained in Delafeld’s hematoxylin or Mayer’s carmalum following the procedure of Amato et al. (1991) and mounted in Canada balsam for examination as whole mounts.

Parasites (31 collected) were observed with an Olympus BX 41 light microscope (Olympus America Inc., Melville, New York). Drawings were made with the aid of photomicrographs derived from an Olympus microscopic system; measurements were made using an ocular micro­meter. Identification and classification of the monogeneans to the ge­neric level were in accordance with Price (1939), Sproston (1946), and Yamaguti (1963). Measurements are given as ranges in micrometers (μm), with mean ± standard deviation in parentheses.

DESCRIPTION

Polystomoides brasiliensis n. sp. (Figs. 1–5)

Measurements of 8 adult specimens (1 holotype and 7 para­types):

Body elongate, oval, 2,450–6,062 (3,600 ± 1,479) in length, including opisthaptor; 545–1,875 (1,100 ± 592) in width at level of vaginal apertures. Opisthaptor digitate, 998–2,200 (1,325 ± 503) in length, 1,100–2,325 (1,503 ± 484) in width, bearing 6 haptor suckers of type 2 (Pichi­lein, 1995), 300–813 (455 ± 179) in diameter (n = 46). One marginal hook in interior of each haptor sucker. Hamuli, 2 pairs; inner pair 55.0–71.3 (65.1 ± 4.9) in length (n = 8), outer pair 62.5–80.0 (72.1 ± 5.5) in length (n = 8). Marginal hooks, 16; ventral, of similar shape and size, 25.0–36.3 (31.9 ± 3.4) in length (n = 63). Mouth subterminal, ventral. Oral sucker 245–490 (312 ± 102) in length by 290–520 (364 ± 89) in width. Pharynx oval, 175–415 (249 ± 96) in length by 205–460 (290 ± 99) in width; esophagus short. Intestinal caeca without anterior diverticula, extending to near anterior margin of opisthaptor; ends not contiguous. Common genital aperture median, posterior to in­testinal bifurcation; genital coronet with 8–9 spines with similar sizes and curved, 11.3–17.5 (12.8 ± 1.6) in length. Testis circular or oval, postovarian, median, prequatorial; with regular margin, 370–1,125 (633 ± 308) in length by 330–1,050 (583 ± 298) in width, with lateral regions in contact with intestinal caeca. Ovary pretesticular, submedian, 95–250 (161.5 ± 63) in length, to right of median line. Uterus absent. Ootype short, not opposite ovary, confined between ovary and testis. Two vaginal apertures ventral, near margins of body, extra caeca, at level of anterior margin of testis. Vitellaria follicular, extend from mid­pharyngeal level and anterior margin of opisthaptor, surround intestinal caeca. Egg ovoid, 195–245 (220 ± 35) in length by 175–195 (185 ± 14) in width.
Figures 1–5. *Polystomoides brasiliensis* n. sp. (1) Whole mount, ventral view; (2) genital coronet; (3) egg; (4) marginal hook; (5) inner hamuli (left) and outer hamuli (right).
Table I. Polystomoides spp. described from South America—Number and size of genital spines.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>Size (μm)</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystomoides uruguayensis</td>
<td>8-10</td>
<td>10</td>
<td>Mañé-Garzón and Gil (1961)</td>
</tr>
<tr>
<td>P. fujuesi</td>
<td>2</td>
<td>56</td>
<td>Mañé-Garzón and Gil (1962)</td>
</tr>
<tr>
<td>P. rohdei</td>
<td>29-32</td>
<td>34-52</td>
<td>Mañé-Garzón and Holcman-Spector (1968)</td>
</tr>
<tr>
<td>P. brasiliensis</td>
<td>8-9</td>
<td>11-17</td>
<td>Current study</td>
</tr>
</tbody>
</table>

Testis length/oral sucker length ratio = 2.02:1.0; testis width/oral sucker width ratio = 1.64:1.0; testis length/pharynx length ratio = 2.54:1.0; testis width/pharynx width ratio = 2.01:1.0; outer hamuli length/inner hamuli length ratio = 1.11:1.0; outer hamuli length/marginal hook length ratio = 2.26:1.0; inner hamuli length/marginal hook length ratio = 2.04:1.0; oral sucker length/pharynx length ratio = 1.25:1.0; oral sucker width/pharynx width ratio = 1.25:1.0.

**DISCUSSION**

In the present study, the characters used for differentiation of *P. brasiliensis* sp. included morphometric data for the outer and inner hamuli, testis, pharynx, and oral suckers (Table II), as well as quantitative and morphometric data for the genital spines (Table I). The morphology of the outer and inner hamuli, the relation of sizes of these structures with size of the haptoral sucker, the morphology and morphometric data for marginal hooks (Table II), the testis size/pharynx size and oral sucker size/pharynx size (Table III) ratios are characters used by Rohde (1965) in the distinction of the several species. The number and size of genital spines are the characters more frequently used in the identification of *Polystomoides* spp. (Rohde, 1965). Thus, the validity of these characteristics in the differentiation of the species of this genus is evident.

In the current study, it was verified that absolute sizes of outer and inner hamuli, marginal hooks, and of haptoral suckers of *P. brasiliensis* are greater than those of *P. uruguayensis* (Table II). However, when ratios of the structures of the opisthaptor were compared, it was determined that only the relative size of the inner hamuli of *P. brasiliensis* is significantly different from the inner hamuli of *P. uruguayensis* (Table III). This became evident in the analysis of the ratios of outer hamuli length/inner hamuli length, haptor sucker diameter/inner hamuli length, and the pharynx. Testis size of *P. brasiliensis* is greater than twice that of the pharynx, while in *P. uruguayensis*, the testis size is less than double the size of the pharynx (Table III). We also observed a morphometric difference between the genital spines of the 2 species (Table I).

### TABLE III. Comparative ratios of *Polystomoides uruguayensis* and *Polystomoides brasiliensis*.

<table>
<thead>
<tr>
<th>Species</th>
<th>P. uruguayensis (Mañé-Garzón and Gil, 1961)</th>
<th>P. brasiliensis (Current study)</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis length/oral sucker length</td>
<td>0.98:1</td>
<td>2.02:1</td>
<td></td>
</tr>
<tr>
<td>Testis width/oral sucker width</td>
<td>0.96:1</td>
<td>1.64:1</td>
<td></td>
</tr>
<tr>
<td>Testis length/pharynx length</td>
<td>1.64:1</td>
<td>2.54:1</td>
<td></td>
</tr>
<tr>
<td>Testis width/pharynx width</td>
<td>1.23:1</td>
<td>2.01:1</td>
<td></td>
</tr>
<tr>
<td>Oral sucker length/pharynx length</td>
<td>1.67:1</td>
<td>1.25:1</td>
<td></td>
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<tr>
<td>Oral sucker width/pharynx width</td>
<td>1.28:1</td>
<td>1.25:1</td>
<td></td>
</tr>
<tr>
<td>Outer hamuli length/inner hamuli length</td>
<td>1.36:1</td>
<td>1.10:1</td>
<td></td>
</tr>
<tr>
<td>Haptor sucker diameter/outer hamuli length</td>
<td>6.05:1</td>
<td>6.30:1</td>
<td></td>
</tr>
<tr>
<td>Haptor sucker diameter/inner hamuli length</td>
<td>8.28:1</td>
<td>6.98:1</td>
<td></td>
</tr>
<tr>
<td>Outer hamuli length/marginal hook length</td>
<td>2.47:1</td>
<td>2.26:1</td>
<td></td>
</tr>
<tr>
<td>Inner hamuli length/marginal hook length</td>
<td>1.8:1</td>
<td>2.04:1</td>
<td></td>
</tr>
</tbody>
</table>

* Mean size, shown as μm.
inner hamuli length/marginal hook length. The other ratios between the structures of opisthaptors of both species are similar in their relative sizes (Table III). Finally, the morphology of inner and outer hamuli in P. brasiliensis (Fig. 5) differs from that of the hamuli of P. uruguayensis represented in the study of Mañé-Garzón and Gil (1961).

Prior to the present study, Polystomoides included 30 species. Of these, 15 had been described as parasites collected in the buccal cavity and pharynx of freshwater turtles in Africa, Asia, Europe, South America, and North America (Ozaki, 1935; Paul, 1938; Mañé-Garzón and Gil, 1961, 1962; Rohde, 1965; Mañé-Garzón and Holman-Spector, 1968; Gonzalez and Mishra, 1977; Knoepffler and Combes, 1977; Combes and Rohde, 1978; Timmers and Lewis, Jr., 1979). In South America, P. uruguayensis, P. fuquesi Mañé-Garzón and Gil, 1962, and P. rohdei Mañé-Garzón and Holman-Spector, 1968, had been previously described, all in Uruguay (Knoepffler and Combes, 1977; Kohn and Cohen, 1998) (Table IV). Since the study by Mañé-Garzón and Holman-Spector (1968), there have been no descriptions of new monogeneans from chelonians in South America. Polystomoides coronatus (Leidy, 1888) was also identified in a freshwater chelonian from South America (Table IV) (Mañé-Garzón, 1958), but this species was originally described from North America. The current study is the first to describe a monogenean in chelonians from Brazil, and the first to identify helminths in H. maximiliani.

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TABLE IV. South American chelonian hosts of monogeneans.

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Parasite</th>
<th>Site of infection</th>
<th>Country</th>
<th>Author</th>
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ROHDE, K. 1965. Studies on the genus Polystomoides Ward, 1917 (Monogenea). I. Description of 4 Malayan species with a key to the known species and a comparison of the sub-cuticular layer in Polystomoides and some digenetic trematodes. Zoologische jaar-


ABSTRACT: Trombiculid mites are known to parasitize a variety of amphibian species, yet few comparisons of mite parasitism among amphibian species have been made. In this study, we investigated patterns of trombiculid mite parasitism among 3 plethodontid salamanders (Desmognathus fuscus, Eurycea cirrigera, and Plectodon cylindraceus) in the western Piedmont of North Carolina. All 3 salamander species were parasitized by a single species, Hannemania Dunnii. Desmognathus fuscus harbored mites more frequently (60.4% of individuals) than E. cirrigera (11.1%) or P. cylindraceus (14.6%). Desmognathus fuscus also had higher parasite loads than E. cirrigera or P. cylindraceus (P < 0.001). Mites on D. fuscus were found more frequently on the limbs than other body locations (P < 0.001). We found no correlation between salamander size and mite abundance (P = 0.689), but salamander collection sites influenced the abundance of mites on D. fuscus (P = 0.002). We found no effect of season on mite abundance in D. fuscus (P = 0.952). Salamander habitat preferences and edaphic or climatic differences among study sites may influence patterns of Hannemania sp. parasitism of salamanders.

Materials and Methods

Salamanders were collected from southern Iredell, northern Mecklenburg, and western Cabarrus counties, North Carolina (Fig. 1). We sampled D. fuscus and E. cirrigera at 4 semipermanent, first-order streams that were surrounded by forested (locations d, f, and g), and urbanized (location a) land. We sampled P. cylindraceus in 6 terrestrial habitats, some in and directly adjacent (2 m) to streams. Plectodon cylindraceus, a completely terrestrial species, and E. cirrigera were collected by searching under logs and other cover objects in forested areas. A pair of drift fences (Rice et al., 2001) provided supplemental captures of E. cirrigera and P. cylindraceus during fall 2006.

We used stereomicroscopy to carefully examine each salamander for the presence of larval Hannemania sp. Mites were apparent due to concentrated redness, inflammation, necrosis, and raised abscesses (Sladky et al., 2000). The mites were identified as H. dunnii (Loomis, 1956) based on larval morphology (W. C. Welbourn, pers. comm.). We recorded the location (head, throat, dorsum, venter, cloacal region, tail, or limbs) and abundance of mites on each salamander examined. To permit easier handling, we anesthetized D. fuscus using 1 g of Maximum-Strength Orajel® (Del Pharmaceuticals, Uniondale, New York) per 1 L of dechlorinated water (Cecala et al., 2007). We measured the snout–vent length (SVL) and total length of each salamander to the nearest millimeter, recorded mass to the nearest 0.01 g, and individually marked each salamander using visible implant elastomer (Nauwelaerts et al., 2000; Northwest Marine Technology) to avoid counting individuals on multiple occasions. Because of difficulties in determining the sex of each salamander, the sexes were not recorded. Salamanders were returned to their point of capture within 2 days after capture.

We compared the abundance of larval H. dunnii between D. fuscus and P. cylindraceus by calculating mean mite abundance per salamander species, which included individuals with no H. dunnii infections. We used a General Linear Model (GLM) and a Duncan’s multiple comparison test (General Linear Model, SAS, version 9.1; SAS Institute, Cary, North Carolina) to compare the abundance of mites found on body locations of D. fuscus and P. cylindraceus. To evaluate the relationship between salamander body length and parasite abundance, we used a linear regression with SVL as the independent variable and abundance...
of mites (i.e., total number on each individual) as the dependent variable. We compared differences in mite abundances among different sample locations by using a GLM and a Duncan’s multiple comparison test to compare the number of mites on individual D. fuscus and P. cylindraceus from 4 and 6 localities, respectively (SAS, version 9.1; SAS Institute). Limited samples of E. cirrigera prevented them from being used in any statistical analysis.

We measured seasonal variation of mite abundances in D. fuscus inhabiting one of the study sites (location g) by sampling the population monthly from October 2005 through May 2006. During this sampling, only the number of mites (i.e., abundance) for each infected adult salamander was recorded. Data among months were compared using a GLM and a Duncan’s multiple comparison test to compare the number of mites on individual (linear regression, D. fuscus; $R^2 = 0.055$; $P = 0.688$; P. cylindraceus; $R^2 = 0.005$; $P = 0.428$). However, we did find significant variation in mite abundance on D. fuscus among the sampled localities (GLM; $F = 5.33$; df = 3; $P = 0.002$; Fig. 4A). Desmognathus fuscus captured at location f had a greater mean abundance per individual than the abundances at other sites (Fig. 4A), but we did not find variation in mite abundance among sample localities for P. cylindraceus ($F = 1.89$; df = 1; $P = 0.182$; Fig. 4B). The frequency of mite infection of D. fuscus was parasitized by, on average, 3.5 $H. dunni$ per individual ($n = 158$ individuals), whereas P. cylindraceus ($n = 48$ individuals) and E. cirrigera were parasitized by 0.5 and 0.4 $H. dunni$ per individual, respectively ($n = 11$ individuals; GLM; $F = 3.04$; df = 2; $P < 0.001$; Fig. 2). Most P. cylindraceus and E. cirrigera lacked parasites (85.4 and 88.9%, respectively), whereas 60.4% of all D. fuscus were parasitized. The abundance of mites among salamander body parts also differed among species. Desmognathus fuscus harbored the greatest concentration of mites on the limbs and tail, with all other body parts having lower parasite abundance (GLM; $F = 22.7$; df = 6; $P < 0.001$; $n = 128$; Fig. 3A). Although mites did not preferentially attach to a particular body location on P. cylindraceus (GLM; $F = 1.86$; df = 6; $P = 0.088$; $n = 48$; Fig. 3B), the greatest mean concentration of mites was found on the limbs (Fig. 3B). We did not detect $H. dunni$ parasitizing salamander nasolabial grooves.

We found no significant relationship between salamander SVL and parasite abundance in D. fuscus or P. cylindraceus.
fuscus did not change from October 2005 through May 2006 (GLM; \( F = 0.300; \) df = 7; \( P = 0.952; \) Fig. 5).

**DISCUSSION**

Larval *H. dunni* seem to be a common ectoparasite on plethodontid salamanders of the western Piedmont of North Carolina, but we found significant variation in *H. dunni* abundance among the salamander species we studied. Duncan and Highton (1979) also found that fully terrestrial, plethodontid salamanders (i.e., *P. glutinosus*, *P. fourceni*, and *P. caudonis*) studied in the Ouachita Mountains of Arkansas and Oklahoma had different prevalences of *H. dunni* infection; larval mites were rarely found on *P. glutinosus*, yet heavy infestations occurred on other species. In our investigation, *D. fuscus*, the most aquatic of the three species studied, harbored the greatest concentration of mites. These results differ from previous studies describing higher parasitism in more terrestrial amphibian species (Regester, 2001; Converse and Green, 2005).

We found significant variation in mite attachment locations among salamander species. The high abundance of *H. dunni* on the limbs and tail of *D. fuscus* and *P. cylindraceus* may indicate that mites have a preference for attaching to the extremities, even though there is more surface area on the dorsal and ventral regions of the animal (Anthony et al., 1994; McAllister et al., 1995; Malone and Paredes-Leon, 2005). Green (2001) also noted that embedded mites are most commonly found on the extremities, which may be explained by *Hannemania* sp. selecting their penetration site (Hyland, 1961; Malone and Paredes-Leon, 2005) because the connective tissue of the extremities may allow for a firmer attachment (Malone and Paredes-Leon, 2005).

Brown et al. (2006) suggested that limb loss may result from high mite abundance. We did not document a direct link between limb loss and mite attachment in our study, but we witnessed clusters of mites on the extremities that seemed to impair toe and foot motility. Mite attachment to these areas could impair activities such as foraging and courtship (Anthony et al., 1994; Maksimowich and Mathis, 2000; Regester, 2001; Malone and Paredes-Leon, 2005), which could have detrimental impacts on salamander populations.

We also found differences in salamander parasitism among sampling locations. Duncan and Highton (1979) also noted variation in infestation rates by *H. dunni* among 20 localities in the Ouachita Mountains. Differences in parasitism rates among localities may be the result of different edaphic or climatic microhabitat characteristics, although we noticed no obvious differences among our study sites. Future studies should determine whether microhabitat conditions affect mite distribution or salamander susceptibility to mite infestation.

The present study describes patterns of *H. dunni* abundance on plethodontid salamanders of the western Piedmont of North Carolina, and provides a greater understanding of the natural history of both larval *H. dunni* and plethodontid salamanders. We have also detected larval *Hannemania* sp. on anurans, including *Rana sphenocephala* and *R. palustris*; future studies should investigate patterns of trombiculid mite parasitism on other amphibian species. Furthermore, studies examining the effects of *Hannemania* sp. parasitism on amphibian survivorship or determining climatic and edaphic influences on parasitism rates would be helpful to fully describe the impacts of trombiculid mite parasitism of amphibians.

**ACKNOWLEDGMENTS**

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LITERATURE CITED


Abstract: We examined tegumental development of the diplostomulum of Ornithodiplostomum ptychocheilus, with respect to structural transformations that have functional relevance to the invasion, migration, and site establishment processes in the brain of the fish second-intermediate host, Pimephales promelas. Using a combination of brightfield, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal microscopy (CM), we demonstrated that the diplostomulum becomes established in the outer region of the optic lobes within 24–48 hr of penetration and continue to grow and transform over a period of 4–14 days. During this period, the J-shaped body consists of 2 distinct regions: (1) a highly motile prosoma with distinctive tegumental spines and (2) an opisthosoma, the tegument of which is elaborated into a dense uniform layer of long, thin microvilli. The prosoma is alternately invaginated into and everted from the opisthosoma, thus constituting a protrusible proboscis. By day 14 postinfection (PI), the body has lost its bipartite structure and has taken on the uniformly flattened form characteristic of metacercariae. The transitory complex structure of the diplostomulum appears to be well suited to burrowing through host tissues (primarily by action of the prosoma), followed by rapid dissociation of host tissue and nutrient accumulation (primarily by action of the opisthosoma) in preparation for metacercaria encystment.

Trematodes such as schistosomatids and many strigeoids migrate great distances through the host from the cutaneous point of entry to the final organ-specific site of adult (schistosomatid), or metacercaria and metacercaria (strigeoids) residence. In this way, these families differ markedly from the majority of trematodes, which typically encyst as metacercariae at, or near, the point of cercariae penetration (Fujino et al., 1979; Hong et al., 1991) or, in some cases, may not even penetrate host tissues (Pike and Erasmus, 1967; Martin and Conn, 1990; Conn and Conn, 1995). Because strigeids and schistosomatids typically penetrate and migrate through the host to a distant site without an intervening period, it is best to view the entire concerted process of penetration plus migration as host “invasion” (Hoglund, 1991). Because this 2-step invasion of a host is confined to certain trematode groups, the migrating stage is often assigned a special name, i.e., “schistosomulum” for schistosomatids (McLaren and Hockley, 1976), and “diplostomulum” for strigeids (Eiges, 1961), to differentiate the migrating stage from the cercaria and the metacercaria stages.

A vast amount of literature exists on the tegumental ultrastructure of trematodes at the metacercaria and adult stages (Bibby and Rees, 1971; Smyth and Halton, 1984; Dezfuri et al., 2007). Much less is known about the ultrastructure of miracidium, intramolluscan, and cercaria stages, and few studies have actually examined tegumental transformations during key developmental events (e.g., see Koie et al., 1977; Fujino et al., 1979; Higgins, 1980; Lee et al., 1982; Bogitsh, 1986; Benjamin and James, 1987; Koie, 1987; Hong et al., 1991; Butcher et al., 2002; Sohn et al., 2002). The major exception is with the human-infecting schistosomatids. Thus, several authors have reported on tegumental transformations during the migration of schistosomula of Schistosoma mansoni (see Bruce et al., 1970; Hockley, 1973; Hockley and McLaren, 1973; Stiweralt, 1974; McLaren and Hockley, 1976; Crabtree and Wilson, 1980; Skel-ly and Shoemaker, 2000), and Schistosoma japonicum (see Irie and Yasuraoka, 1981; Sobhon et al., 1988; Zhou et al., 1997). In contrast, few studies have described changes in the tegument of migrating diplostomulum in their intermediate hosts.

The objective of the present study was to examine morphological changes in the tegument of O. ptychocheilus diplostomulum during migration and establishment in its intermediate host, the fathead minnow (P. promelas). In an earlier study, we demonstrated that the tegument of O. ptychocheilus undergoes radical transformation from a smooth, thin, syntacial structure to a complex microvillar system that encircles the entire body (Goater et al., 2005). This transformation represents an obligate feeding phase that precedes the encysted, resting stages of typical metacercariae. In the present paper, our focus is on the description of changes in the tegument of O. ptychocheilus diplostomulum during the migratory phase and with the initial stages of establishment in the optic lobes. Our overall aim in the combined studies is to provide a comprehensive perspective of changes in the developmental and functional ultrastructure of the diplostomulum tegument relative to the invasion and establishment processes.

Materials and Methods

Methods used to establish the life-cycle of O. ptychocheilus in the laboratory have been described by Sandland and Goater (2000) and Goater et al. (2005). In summary, 1-day-old chicks were fed the brains of naturally infected minnows collected from a lake in central Alberta, Canada. All adult fish in this lake are infected with 20–400 O. pty­chocheilus metacercariae (Sandland et al., 2001). Trematode eggs were collected from the feces of exposed chickens and incubated in aerated water at 20°C. Worm eggs hatched 11–13 days later, releasing free-swimming miracidia. Individual F1 Physa gyrina (3–5 mm) originating from wild-caught parents were exposed to 5 miracidia of O. ptychocheilus for 3 hr. Snails were evaluated for cercariae emergence 25–28 days PI. Cercariae counts were performed by dilution following Sandland and Goater (2000). On 18 July 2005, individual minnows were exposed to 50 cercariae for 2 hr within 60-mm Petri dishes (Sandland and Goater, 2000).

To evaluate developmental changes in the structure of the tegument, exposed minnows were killed at various intervals between 2 and 28 days PI. Minnow brains were dissected from the cranium and then stored in modified Karnovsky’s fixative containing 5% glutaraldehyde,
4% paraformaldehyde, and 0.1 M sodium cacodylate buffer, pH 7.3, at 4°C prior to processing for light microscopy (LM) and electron microscopy.

Samples selected for TEM were rinsed overnight in 0.1 M sodium cacodylate buffer, pH 7.3, and then postfixed for 1 hr in 1% osmium tetroxide in the same buffer. Following stepwise dehydration in ethanol, samples were embedded in Spurr’s resin and then sectioned with a diamond knife at 0.1 μm using a Reichert OM-U2 ultramicrotome (Leica-Reichert, Heidelberg, Germany). The sections were mounted on 200-mesh copper grids and stained in saturated aqueous uranyl acetate for 20 min at 40 C and then in Reynolds lead citrate for 5 min at room temperature before examination in a Hitachi H-600 TEM (Hitachi-High Technologies, Redxale, Ontario, Canada) at an accelerating voltage of 75 kV.

For LM analysis, 1-μm sections were cut from the blocks prepared for TEM and mounted on glass slides coated with an adhesive solution containing gelatin (0.5%) and chrome alum (0.05%). The sections were stained with 1% toluidine blue and examined under brightfield illumination with a Zeiss Axioskop (Carl Zeiss Inc., Oberkochen, Germany). Digital images were captured with a QImaging monochrome Retiga Exi camera (QImaging, Surrey, British Columbia, Canada) fitted with a red-green-blue color filter.

Samples prepared for SEM were rinsed in sodium cacodylate buffer (0.1 M, pH 7.3) and then stained in 1% toluidine blue to color them and reduce loss during subsequent processing of these very small specimens. Dehydration in a graded ethanol series was followed by chemical drying in hexamethyldisilazane (Bray et al., 1993). Once dried, samples were carefully mounted on aluminum stubs, coated with 70 nm of gold in a Polaron triode sputter coater (Quorum Technologies, New Haven, East Sussex, U.K.), and then examined in a Hitachi S-500 SEM (Hitachi-High Technologies) at an accelerating voltage of 10 kV. Images were captured digitally using Quartz PCI imaging software (Nikon Corporation, Tokyo, Japan).

Whole brains that contained diplostomula were fixed in modified Karnovsky’s fixative and coronally sectioned (100 μm) with a vibratome and mounted on gelatin-coated glass slides. Regions containing diplostomula were optically sectioned at 1-μm thickness using differential interference contrast objectives on a Nikon C1+ confocal microscope. Individual images were selected from these optical stacks for illustration here.

RESULTS

LM analysis revealed that the diplostomula reached the brain of the fish host within 24–48 hr of penetration through the skin. Upon initial entry, the diplostomula tended to occur deep within the brain, and moved quickly to the outer edge of the optic lobes by day 2 PI (Fig. 1A). At this stage, the diplostomula had a simple cylindrical vermiform body, and the impact on host tissue was minimal.

By day 4 PI, the diplostomula had increased markedly in size and had developed a characteristic J-shaped body with 2 clearly distinguishable parts, i.e., the large opisthosoma posteriorly, and a much smaller prosoma at the anterior end (Fig. 1B). This somatic transformation was accompanied by marked dissociation of host cells. In cross section, the bipartite body retained its cylindrical form, but moved intermittently to a more flattened form (Fig. 1C), with the overall shape becoming more flattened as development progressed through day 28 PI (Fig. 1D).

SEM revealed a clear pattern of tegumental structure on the 2 body parts. By day 4 PI, the prosoma tegument was distinctly spiny; it retained the distinctive tegumental spines through day 14 PI. Specimens examined on day 5 PI revealed an elongate body form that alternated between flattened body posture, with the prosoma spread broadly and uniformly shaped relative to the opisthosoma (Fig. 2A), and cylindrical body posture, with the prosoma attenuated into a spined proboscis that was of much smaller diameter than the opisthosoma (Fig. 2B). During this 4- to 14-day period, the prosoma was alternately extended (Figs. 2A, B) and inverted into the opisthosoma (Fig. 2C), thus appearing as an eversible proboscis.

Regardless of the changes in body posture associated with movement of the diplostomula, the 2 body regions had distinct tegumental surface features. The opisthosoma surface was elaborated into a dense uniform layer of long, thin microvilli (Figs. 2A–C). In sharp contrast, the prosoma bore prominent, uniformly spaced, posteriorly directed tegumental spines (Figs. 2A–C). Each spine had a flattened triangular shape, with the apical angle curved posteriorly, i.e., dentate form. The spines were interspersed with tegumental folds, and numerous small pores also occurred among the spines (Fig. 3B). At all stages, many specimens, as viewed by SEM, appeared to be covered by an amorphous mat that obscured the opisthosoma microvilli (Figs. 2B–D). At day 10 PI, the bipartite nature of the body was still apparent and the prosoma was still highly subject to invagination and eversion (Fig. 2C). By day 14 PI, the prosoma had been permanently invaginated and was no longer identifiable as a discrete structure, except for the presence of a few remaining spines visible at the point of invagination into the opisthosoma (Fig. 2D). Thus, the body at this stage had lost its bipartite structure and had taken on a uniformly flattened form similar to that of metamericia and adult trematodes.

TEM revealed that each tegumental spine of the prosoma consisted of a triangular cytoplasmic extension from the tegumental distal cytoplasm (Fig. 3C). The outer cortex of each spine was electron opaque, thus obscuring the structure of the apical plasma membrane. The core of each spine was electron lucent; microfilaments were visible in some spines, extending perpendicular to the body surface. The cortical cytoplasm of the tegumental folds between the spines consisted of a thin electron-opaque layer, resulting from the accumulation of small electron-opaque granules. The underlying distal cytoplasm in both spined and folded areas was electron lucent and granular. The distal cytoplasm was underlain by a thin basal matrix to which it was attached by hemidesmosomes to numerous muscle bundles.

CM corroborated the observations made by standard light and electron microscopy. It further confirmed that the body of each diplostomulum generally had the J-shaped appearance (Fig. 4A) and appeared to exhibit both of the 2 body postures shown in Figure 2. Confocal images also confirmed the distinctive surface structures of the 2 body parts, with spination of the prosoma clearly visible (Fig. 4B).

DISCUSSION

This report corroborates and extends the work of Goater et al. (2005) on invasion of the brain of P. promelas by O. pychoeuleus. Based on the detailed TEM observations of Goater et al. (2005) covering a longer period of development, the amorphous covering of the opisthosoma seen by SEM in the present study consisted of the microvilli interspersed with material within the host–parasite interface. The present study extends the previous report by describing a distinctly bipartite body for the diplostomulum, including the highly motile spiny prosoma with its distinctive tegumental spines that are
FIGURE 1. Brightfield light micrographs of toluidine-blue-stained epoxy sections of *O. pygmaeus* diplostomula in the brain of a fathead minnow. (A) Day 2 PI. Transverse section showing the small size and cylindrical shape of the prosoma (P), with close apposition of host tissue. (B) Day 4 PI. Sagittal section showing the cylindrical body and J-shaped configuration of the body, with the curve of the J delineating the anterior prosoma (P) from the posterior opisthosoma (O). The prosoma is partially invaginated in this section. Note the disassociation of host tissue around the opisthosoma. (C) Day 7 PI. Oblique sagittal section showing the flattened shape of the opisthosoma region. Note the dissociation of host tissues and enlargement of the cavity surrounding the parasite. (D) Day 28 PI. Oblique frontal section of the opisthosomal region showing distinct medullary (Md), subegumental (St), and tegumental (T) areas, with greatly enlarged cavity surrounding the parasite.
Figure 2. SEMs showing surface structure of *O. psychrocheilus* diplostomula on Days 5, 10, and 14 of fish host invasion. (A) Day 5 PI. Anterior end of a diplostomulum in the flattened body configuration, showing the marked distinction and abrupt transition between the posterior microvillous opisthosa (Op) and the spined anterior prosoma (Pr). (B) Day 5 PI. Anterior end of a diplostomulum in the cylindrical body configuration, showing the marked distinction and abrupt transition between the posterior microvillous opisthosa (Op) and the spined anterior prosoma (Pr), with a highly protrusible nature. (C) Day 10 PI. Note that the prosoma is mostly invaginated into itself and the opisthosa, leaving an invagination canal (I). (D) Day 14 PI. Anterior end of a diplostomulum, showing the remnants of tegumental spines (S) at the opening of the invagination canal. Note protruding microvilli (V) of the opisthosa (Op).
Figure 3. SEMs and TEMs showing detailed structure of spined protrusible prosoma of *O. psycholellus* diplostomula on Day 5 of fish host invasion. (A) General SEM view showing abrupt boundary between the spined prosoma (Pr), and the microvillous opisthosoma (Op). (B) High magnification SEM of the prosoma showing the triangular shape of the tegumental spines (S), which are interspersed with tegumental folds (F) and small pores (Po). (C) TEM showing the prosoma with an electron-opaque tegumental cortex (Co), dual-density tegumental spines (S), thin distal cytoplasm (D), thin basal matrix (B), and well-developed muscle bundles (M). The spines extend into the host brain tissue.
shaped in such a way that would facilitate burrowing through host tissues. The pores described here are likely to be associated with tegumental sensory-pit receptors, which may assist in site-finding orientation within the host. Sensory-pit receptors were described in detail for the cercariae of a related diplostomatid trematode, Diplostomum pseudospathaceum, by Czubaj and Niewiadomska (1996). It is unlikely that sensory receptors of cercariae and diplostomula would be functionally equivalent, but further study of the prosomal pores of O. ptychocheilus might shed some light on this. The basic configurations of the prosoma tegument and underlying basal matrix and muscle attachments are similar to those of all platyhelminths (Conn, 1993). The highly muscular nature of this region, similar to that of trematode and cestode sucker regions, is well designed for the high motility of the prosoma, which we observed in live specimens.

The present report is the first of a distinct organ with ultrastructural modifications consistent with penetration activity in a migrating post-cercaria stage of a strigeoid trematode. The tegumental spines on the prosoma noted in the present study are unique to this stage of development and this transitory body region. Thus, they have an ultrastructure that is markedly unlike the tegumental spines that develop on the opisthosoma region of 56-day-old metacercariae of this species (Goater et al., 2005). On the basis of LM, other authors have referred to the anterior end of various strigeoid diplostomula as appearing to have unusual structure or motility consistent with penetration activity. Hoffman (1956) reported that the metacercariae of Crassiphila bulboglossa, a strigeid that causes blackspot under the skin of fish intermediate hosts, possesses a "penetration organ" that "extends slightly forward" as the worm moves. This may be similar to the protrusable spiny organ described in the present study, but comparison is not possible since Hoffman's work was illustrated only with hand drawings. Shoop and Corkum (1984) provided detailed information on the tegumental transformations that occur during migration of the strigeid Alaria marcianae in the cat definitive host. In this, as well as in congeneric species, a distinct mesocercaria stage occurs in

Figure 4. Confocal images of O. ptychocheilus diplostomula migrating through the brain of the fish host. (A) Day 5. The diplostomulum body has J-shaped configuration, a distinctly bipartite form with a large posterior opisthosoma (Op), and a highly active eversible anterior prosoma (Pr). Note the close association with host blood vessels (Hv). (B) Day 8 PI. A marked distinction is apparent between the spinous prosoma (Pr), which is angled toward the viewer, with the opisthosoma (Op), the microvilli of which are not visible in this image. The opisthosoma is surrounded by a cavity, whereas the prosoma intrudes directly into contact with the host brain tissue and host blood vessels containing erythrocytes (E).
the second intermediate and paratenic hosts. However, the diplostomulum of *Alaria* spp. is very short-lived and occurs during the brief migration in the lungs of the definitive host, just prior to development of the adult in the small intestine, and may not be comparable to the stage described here.

Our results indicate that the spinose prosoma of *O. pychocheilus* diplostomulum is transitory, occurring only through about day 14 PI. The appearance and activity of the prosoma correspond with the period when the diplostomulum are deep within the brain tissue, sometimes even still in the brainstem and nerve cord. The disappearance of the prosoma corresponds with the final establishment of diplostomulum along the outer edge of the optic lobes, suggesting an important role in migration within the brain to the final site of metacercariae encystment. In a detailed developmental study of changes in tegumental structure of migrating *S. mansoni* schistosomula, Crabtree and Wilson (1980) showed that new tegumental spines were formed when the schistosomulum reached the hepatic portal system. These authors suggested that this transitory loss of midbody spines during migration through the capillaries facilitated movement through these small vessels, while residual anterior and posterior spines served as anchors to support traction. A spineless protrusible area occurred on the schistosomulum during migration, but was integrated into the anterior oral sucker region after the worms reached their final destination in the hepatic portal vessels. Thus, various structures associated with the tegument of several larval trematodes appear to play a role in migration and site selection. Perhaps it is not surprising that these structures regress following location within particular sites.

Trematodes other than the strigeoids and schistosomatids typically do not migrate extensively through host tissues. A small number of these have been studied with respect to the transformations in their tegument during post-cercariae, pre-adult stages (Koie et al., 1977; Fujino et al., 1979; Higgins, 1980; Lee et al., 1982; Hong et al., 1991; El-Darsh and Whitfield, 2000; Butcher et al., 2002). The common feature of all of these reports is that the tegument is not static, but changes considerably between cercaria and adult, with the most obvious changes involving spination and sensory structures. Unfortunately, most studies on such developmental changes in trematodes have not proposed correlations between changes in tegumental structure and stage-specific function. Our data strongly suggest changes in tegumental structure of *O. pychocheilus* involving prosomal spines that assist in migration (present study), and opisthosomal microvilli that assist in nutrient acquisition and host tissue dissociation (Goater et al., 2005).

Earlier studies on migration of diplostomulum in fish hosts have offered some suggestion of the J-shaped bipartite body form that appears comparable to the prosoma and opisthosoma described in the present study. In a study of diplostomulum of *Diplostomum spathaceum*, Ratnerarat-Brockelman (1974) included light micrographs of a J-shaped body and an anterior "penetration organ" that was attached to the host's retina. Hendrickson (1979) also published light micrographs showing a J-shaped bipartite body of *O. pychocheilus* diplostomulum, but did not explicitly address this structural plan. In both cases, the absence of details at the LM level prevented further analysis and comparisons. Höglund (1991) used TEM to describe the body of migrating diplostomulum of *D. spathaceum*, showing tegumental spines of a "spiny anterior end" and noting that "the apical tuft of spines surrounding the mouth region obviously participated in the invasive process." Höglund's micrographs appear similar to those in the present report, but he did not describe a discrete anterior structure or microvillous opisthosoma. Nevertheless, taken together, these studies appear to corroborate the conclusions of the present report and to extend them to other related strigeoids. Future studies should specifically look for analogous structural features in other species.

Distinguishing between the diplostomulum and metacercaria stages is problematic, and the terminology has been applied inconsistently in the literature (see reviews by Dawes, 1946; Olsen, 1974; Smyth and Halton, 1983). This is primarily because of the absence of a clearly demarcated transition point between the 2 stages, a problem that is even further compounded in strigeoids that have intervening mesocercaria stages in intermediate and/or paratenic hosts, and which may never encyst (Shoop and Corkum, 1984). The present study, combined with other published works, identified points of demarcation correlated with a specific basis in functional morphology, which makes it possible to distinguish 4 discrete stages: (1) the penetrating and early migrating diplostomulum described by Hendrickson (1979); (2) the site-establishment stage described in the present study; (3) the primary stage of diplostomulum growth and host tissue alteration in the final site of prolonged infection described by Goater et al. (2005); and (4) the encysted metacercaria described by So and Wittrock (1982). Based upon joint analysis of all of these studies, we propose that the term metacercaria be used only to describe the encysted stages and that all prior stages be referred to as diplostomulum. Furthermore, given the functional and structural uniqueness of each of the 3 diplostomulum stages, we propose the following terms: prodiplostomulum for the early migration stage en route to the brain; mesodiplostomulum for the bipartite site-establishment stage described in the present study, with spiny eversible prosoma and microvillous opisthosoma; and metadiplostomulum for the growth and tissue-alteration stage preceding metacercarial encystment. Such categorization of life-cycle stages is justified in consideration of the great importance of these transitory stages in the life cycle of the parasite and each stage's distinct pathogenicity to the host.

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GENETIC VARIATION OF FISH PARASITE POPULATIONS IN HISTORICALLY CONNECTED HABITATS: UNDETECTED HABITAT FRAGMENTATION EFFECT ON POPULATIONS OF THE NEMATODE *PROCAMALLANUS FULVIDRACONIS* IN THE CATFISH *PELTOBAGRUS FULVIDRACO*

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**ABSTRACT:** Habitat fragmentation may have some significant effects on population genetic structure because geographic distance and physical barriers may impede gene flow between populations. In this study, we investigated whether recent habitat fragmentation affected genetic structure and diversity of populations of the nematode *Procamallanus fulvidraconis* in the yellowhead catfish, *Pelteobagrus fulvidraco*. The nematode was collected from 12 localities in 7 floodplain lakes of the Yangtze River. Using 11 intersimple sequence repeat markers, analysis of molecular variance showed that genetic diversity occurred mainly within populations (70.26%). Expected heterozygosity (He) of *P. fulvidraconis* was barely different between connected (0.2105) and unconnected lakes (0.2083). Population subdivision (Fst) between connected lakes (0.2177) was higher than in unconnected lakes (0.1676). However, the connected and unconnected lakes did not cluster into 2 clades. A Mantel test revealed significant positive correlation between genetic and geographic distances (R = 0.5335, P < 0.01). These results suggest that habitat fragmentation did not cause genetic differentiation among populations or a reduction of diversity in isolated populations of *P. fulvidraconis*. At least 2 factors may increase the dispersal range of the nematode, i.e., flash flooding in summer and other species of fish that may serve as the definitive hosts. Moreover, lake fragmentation is probably a recent process; population size of the nematode in these lakes is large enough to maintain population structure.

Effects of anthropogenic habitat fragmentation and loss of previously continuous habitats on various species have increasingly attracted attention of researchers (e.g., Valtonen et al., 1997; Stow et al., 2001; Schmitt and Seitz, 2002; Williams et al., 2003; Sumner et al., 2004). Several studies have focused on the effects of these factors on the reduction in species richness and abundance (e.g., Gibb and Hochuli, 2002; Morita and Yamamoto, 2002). Habitat fragmentation can also affect the size of local populations and the dispersal pattern of individuals among local populations (Fahrig and Merriam, 1994), which may increase genetic drift and decrease gene flow and genetic population diversity (e.g., Lande, 1988). Furthermore, fitness within isolated populations is expected to decrease because of the accumulation of mildly deleterious alleles. All these factors can lead to an increased risk of local population extinction.

In general, observed differentiation between fragmented and continuous populations is predicted to be the result of long-term isolation (Cunningham and Moritz, 1998). However, recent studies have revealed the effect of recent fragmentation on genetic structure in free-living animals at a local scale (Mossman and Waser, 2001; Stow et al., 2001; Krauss et al., 2004). Apparent differentiation has been detected in populations in cases of recent anthropogenic fragmentation (Williams et al., 2003; Sumner et al., 2004).

At one time, there were many lakes connected in the floodplain of the middle reaches of the Yangtze River. Since the 1950s, however, most of them became isolated from the river by the construction of dams, sluices, or both, and many larger lakes were subdivided into smaller lakes by road construction (Wang and Dou, 1998). The erection of these artificial barriers has inevitably obstructed the migration of fish between the river and lakes, and even among lakes (Chang and Cao, 1999).

The life history of some fish species that migrate between the river and lakes accordingly has been influenced (Chang and Cao, 1999). The yellowhead catfish, *Pelteobagrus fulvidraco*, is widely distributed in lakes and reservoirs in China, and it is very abundant in lakes of the Yangtze Basin (Cheng and Zheng, 1987). The catfish is also a common species found in the Yangtze River, and the exchange of the fish has normally occurred among the river and river-connected lakes (Duan et al., 2002). Despite the isolation, a genetic difference was not detected between 2 geographic populations of the catfish in the middle and lower reaches of the Yangtze River (Song et al., 2001; Wang et al., 2004). This may imply that isolation has not been long enough for genetic change since its life span ranges from just 5 to 8 yr. Moreover, the life span of fish parasites is normally much shorter than that of their hosts. However, genetic effects of recent habitat fragmentation on fish parasite populations should nonetheless be expected.

*Procamallanus fulvidraconis* is a very common parasite in the catfish, i.e., there is generally a high infection prevalence (93.1%) and mean abundance of 18.3 (Li et al., 2006). The parasite's life cycle includes several species of * Cyclops* as intermediate hosts (Li, 1935; Li et al., 2006). With a life span of 1 yr, genetic differentiation may be expected in different populations of the nematode in these newly isolated lakes.

Recently, a polymerase chain reaction (PCR)-based DNA marker, intersimple sequence repeat (ISSR), has been used to investigate genetic diversity and population genetic structure (e.g., Zietkiewicz et al., 1994; Esselman et al., 1999). The technique involves amplification of genomic segments flanked by inversely oriented, and closely spaced, microsatellite sequences by a single primer or a pair of primers based on simple sequence repeats (SSRs) anchored 5’ or 3’ with 1-4 purine or pyrimidine residues. The ISSR marker has some advantages over other markers. For example, ISSR primers anneal directly to simple sequence repeats and, thus, unlike SSR markers, no prior knowledge of target sequences is required for ISSRs. In addition, the target sequences evolve rapidly, and they are abundant throughout the eukaryotic genome; ISSRs may reveal a much higher number of polymorphic bands per primer than...
random amplification of polymorphic DNA (RAPDs) (Fang and Roose, 1997; Esselman et al., 1999). Furthermore, the ISSRs proved to be more reliable and reproducible compared with RAPDs, and they should be a useful technique for the study of intraspecific genetic variability of parasites (Fonseca-Salamanca et al., 2006). The present study thus used this technique to determine whether habitat fragmentation causes genetic differentiation among sampling locations and possibly a reduction of diversity in unconnected sampling locations.

**MATERIALS AND METHODS**

**Sampling**

Poyang and Dongting lakes, the first and second largest freshwater bodies of water, respectively, in China, are the only 2 lakes still connected with the Yangtze River. Honghu and Liangzi lakes became separated from the Yangtze River in the 1950s and 1970s, respectively (Wang and Dou, 1998). Liangzi Lake, however, was divided by road and railway embankments between the 1950s and 1970s into several smaller bodies of water, including Liangzi, Tangxun, Niushan, and Baoan lakes.

Samples were collected from 12 localities in these 7 lakes, with 3 sites in Poyang Lake, 2 each in Dongting, Honghu, and Liangzi lakes, and 1 each in Tangxun, Niushan, and Baoan lakes (Fig. 1). At least 30 yellowhead catfish were obtained from each sample site (Table I). The fork length of each fish was measured; it was then necropsied and specimens of *P. fulvidraconis* removed for genetic analysis (Moravec et al., 2003). Worms were washed in saline and stored in 70% ethanol at −20°C before the DNA extraction. Samples taken from each lake were considered as a population; Poyang and Dongting lakes are defined as connected populations, and the other 5 lakes are defined as unconnected populations.

**Total DNA extraction**

One large female worm was selected from each piscine host; only 11 to 33 females from each lake were ultimately selected for genetic analysis because of the failure in DNA extraction of some female nematodes. Therefore, 129 worms in total were analyzed (Table I). The worms were placed in DNA extraction buffer for about 12 hr, and then they were transferred to 1-ml Eppendorf tube filled with 200 μl of extraction buffer containing 10% sodium dodecyl sulfate and 15 μl of proteinase K (10 mg/ml). The solution was incubated for 5 hr at 57°C, and then it was extracted twice with an equal volume of phenol and once with chloroform. The solution was adjusted to 0.3 M with sodium acetate and precipitated at −20°C overnight with absolute ethanol at the 2× volume. DNA was pelleted by centrifugation (12,000 g, 20 min, 4°C) the following day, and then it was rinsed with 70% ethanol, dried, and finally resuspended in 50 μl of TE (Tris EDTA containing 10 mM Tris and 1 mM EDTA, pH 8.0 (EDTA is ethylenediaminetetraacetic acid)). This extraction protocol yielded sufficient DNA for approximately 50 reactions.

**ISSR PCR amplification**

Thirty-five primers were synthesized according to microsatellite motifs reported previously (Blair et al., 1999; Wang, 2002). DNA amplification was performed in a thermal cycler PTC-100. Reactions were carried out containing 20–35 ng of genomic DNA, 0.2 nM each dNTP, 1 μM primer, 0.8 U of Taq DNA polymerase, 2 mM MgCl₂, and water to a final volume of 25 μl. Each PCR was then subjected to an initial denaturation step at 94°C for 5 min, followed by 35 cycles of amplification for 30 sec at 94°C, 40 sec at 46 to 55°C (according to the annealing temperature of each primer), 90 sec at 72°C, and ended at 72°C for 5 min. PCR products were analyzed by electrophoresis in 2.0% agarose gels, and they were detected by staining with ethidium bromide. For each primer used, controls with host DNA gave a completely different pattern and ensured that bands of individual worms were not correlated to a potential host DNA contamination. Molecular weights were estimated using a 100-bp DNA ladder.

**Table I. Infection of Procamallanus fulvidraconis in Pelteobagrus fulvidraco and genetic diversity in 7 lakes.**

<table>
<thead>
<tr>
<th>Lake</th>
<th>HN</th>
<th>P (%)</th>
<th>M (±SD)</th>
<th>N</th>
<th>PPL (%)</th>
<th>He</th>
<th>Area (km²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poyang</td>
<td>33</td>
<td>51.3</td>
<td>0.2289</td>
<td>90</td>
<td>92.2</td>
<td>10.2 ± 11.1</td>
<td>2,933</td>
</tr>
<tr>
<td>Dongting</td>
<td>18</td>
<td>40.7</td>
<td>0.1921</td>
<td>60</td>
<td>40.0</td>
<td>3.4 ± 8.2</td>
<td>2,432</td>
</tr>
<tr>
<td>Baan</td>
<td>15</td>
<td>89.4</td>
<td>0.2246</td>
<td>30</td>
<td>100</td>
<td>15.8 ± 9.0</td>
<td>48</td>
</tr>
<tr>
<td>Niushan</td>
<td>11</td>
<td>30.1</td>
<td>0.1808</td>
<td>36</td>
<td>100</td>
<td>12.3 ± 9.9</td>
<td>40</td>
</tr>
<tr>
<td>Liangzi</td>
<td>11</td>
<td>93.8</td>
<td>0.2158</td>
<td>60</td>
<td>98.3</td>
<td>12.9 ± 10.0</td>
<td>304</td>
</tr>
<tr>
<td>Tangxun</td>
<td>11</td>
<td>91.2</td>
<td>0.2121</td>
<td>30</td>
<td>93.3</td>
<td>7.6 ± 6.7</td>
<td>37</td>
</tr>
<tr>
<td>Honghu</td>
<td>19</td>
<td>38.9</td>
<td>0.2077</td>
<td>78</td>
<td>74.4</td>
<td>6.1 ± 6.2</td>
<td>344</td>
</tr>
<tr>
<td>Total/Avg</td>
<td>129</td>
<td>72.57</td>
<td>0.2088</td>
<td>384</td>
<td>83.9</td>
<td>9.3 ± 8.1</td>
<td></td>
</tr>
</tbody>
</table>
TABLE II. Oligonucleotides sequences (5' to 3') of the ISSR primers used to reveal polymorphism and the number of polymorphic bands.

<table>
<thead>
<tr>
<th>Primer code</th>
<th>Sequence of primer</th>
<th>No. of bands</th>
<th>No. of polymorphic bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISSR II</td>
<td>(GACA)$_4$</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>ISSR I5</td>
<td>(GA)$_3$ (C,T,A)</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>ISSR II2</td>
<td>(CA)$_2$ GC</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>ISSR II6</td>
<td>(GAG)$_3$</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>ISSR III1</td>
<td>(CAGT)$_4$</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>ISSR III8</td>
<td>(CA)$_2$</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>ISSR III9</td>
<td>(GAA)$_3$</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>ISSR II1</td>
<td>(CA)$_2$ AG</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>ISSR 15</td>
<td>CCA(GTG)$_3$</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>ISSR 17</td>
<td>GGT(GGGGT)$_2$G</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>ISSR 19</td>
<td>(AG)$_2$ T</td>
<td>13</td>
<td>12</td>
</tr>
</tbody>
</table>

Data analysis

Prevalence and mean abundance, as defined by Bush et al. (1997), were calculated for the parasitic infection levels in the fish host. Differences in prevalence and parasite abundance between different lakes were analyzed statistically using a G-test of heterogeneity (Sokal and Rohlf, 1981) and analysis of variance, respectively.

ISSR bands were scored as present (1) or absent (0) for each DNA sample. Expected heterozygosities, i.e., Nei’s gene diversity, and population subdivision (Fst) were calculated by program AFLP-SURV version 1.0 (developed by Veckemans, 2002 [http://www.ualberta.ca/sciences/lagev/alfp-surv.html]), with the assumption of a Hardy–Weinberg equilibrium (HWE). The genetic distance (Nei, 1978) between any 2 populations was calculated using the program POPGENE version 1.32 (developed by Yeh, R.-C. Yang, and T. Boyle [http://www.ualberta.ca/~ryeh/hwe/]), and a dendrogram was constructed by unweighted pair-group method with arithmetic average (UPGMA) method using MEGA version 3.1 (Kumar et al., 2004). A Mantel test was carried out to detect correlation between Nei’s genetic and geographic distances, which were measured between any 2 sampling localities.

The analysis of molecular variance (AMOVA) adapted to RAPD profiles was used to quantify the amount of variation among groups and within populations using program WINAMOVA version 1.55 (Excoffier et al., 1992).

RESULTS

ISSR patterns and infection of P. fulvidraconis

Of 35 oligonucleotides assayed, 11 primers (Table II) revealed an unambiguous genetic polymorphism between individuals; therefore, they were retained for the present study. In total, 113 loci were screened and 82 polymorphic loci were detected.

Among the 384 yellowhead catfish, the overall mean prevalence of P. fulvidraconis was relatively high (83.9% and 9.3 ± 8.1, respectively; Table I). There were significant differences both in mean abundance ($F = 11.26$, $P < 0.001$) and prevalence ($G = 13.04 > X^2_{0.05[6]} = 12.59$) between different populations.

Population genetic diversity of P. fulvidraconis

In total, 129 different haplotypes were recognized. The percentage of polymorphic loci among the populations and expected heterozygosity within populations was 72.57% and 0.2088, respectively (Table I). Expected heterozygosity within connected and unconnected populations was 0.2105 and 0.2082, respectively (Table III). A significant correlation between Nei’s gene diversity and lake area was not found ($R = 0.1512$, $P = 0.746$). AMOVA revealed that most of the genetic variation (70.26%) occurred within populations (Table IV).

Genetic differentiation of P. fulvidraconis

The total gene diversity ($H_o$) and mean gene diversity ($H_e$) within populations was 0.2608 and 0.2088, respectively. The Fst among populations was 0.1996. Fst in connected lakes (0.2177) was higher than in unconnected lakes (0.1676) (Table III).

Mean genetic distance between populations was 0.0758. Based on the genetic distances, a UPGMA tree was constructed (Fig. 2). Exact tests for genetic differentiation were significant for all populations ($P < 0.001$). The connected and unconnected lakes did not cluster in 2 groups, whereas Poyang Lake and Dongting Lake clustered in a different clade. A Mantel test revealed significant positive correlation between genetic and geographic distances ($R = 0.5335$, $P < 0.001$; Fig. 3).

DISCUSSION

The genetic variation of P. fulvidraconis occurred mainly within populations, which implies that the effective population size of P. fulvidraconis was large enough for analysis in the present study. Genetic diversity and its distribution within, and
among, populations was primarily determined by effective population size and gene flow (Blouin et al., 1995). Gene flow is strongly related to the life history and dispersal mechanisms of both hosts and parasites (Criscione et al., 2005). For example, *Ostertagia ostertagi*, a nematode parasite of livestock with large effective population size, had almost all the genetic diversity (98%) distributed within, rather than among, populations, probably because of the high rate at which livestock was transported throughout the United States (Blouin et al., 1992). Species of ascariid nematodes using seal definitive hosts and invertebrate intermediate hosts had low genetic variation across broad geographic ranges (Nascetti et al., 1993). In contrast, *Heterorhadinus marelatus*, an entomopathogenic nematode with a small effective population size and low vagility, showed low genetic diversity within populations and strong differentiation among populations (78% of the total variation) (Blouin et al., 1999).

The yellowhead catfish is abundant in Chinese lakes and rivers (Cheng and Zheng, 1987), and the mean abundance and infection prevalence of the nematode were high in each lake, which indicated that the effective population size of the nematode was large. Furthermore, the mating system and spatial distribution pattern of the parasitic nematode may explain the higher genetic diversity within populations. Mating of *P. fulvidraconis* is usually via allogamy. Cross-fertilization is a form of genetic recombination that may result in high genetic diversity within populations, being different from self-fertilization in certain cestode species and from asexual reproduction by larval trematodes within intermediate hosts (Nadler, 1995). The nematode is usually overdispersed, which can lead to further heterogeneity in spatial distribution and to the increase in diversity of populations.

For *P. fulvidraconis*, genetic diversity was barely different between connected and unconnected lakes. Population subdivision between connected lakes was higher than in unconnected lakes, and the correlation between genetic and geographic distances was significantly positive. Accordingly, the dendrogram cannot be grouped into 2 distinct clades, i.e., connected and unconnected populations. These results suggest that habitat fragmentation did not cause genetic differentiation among populations, or a reduction of diversity in isolated populations of *P. fulvidraconis*. This is primarily because of the relatively high gene flow of the nematodes between lakes. Although the nematode has low vagility because its transmission is dependent upon the ingestion of the intermediate host (Li, 1935), several factors may increase its dispersal. Most importantly, flooding in the region may promote the dispersal of the intermediate and definitive hosts of *P. fulvidraconis* between lakes. Flooding in the Yangtze River is frequent from June to August every year during which an infection peak of *P. fulvidraconis* was observed (Li et al., 2006). The copepod intermediate host and the yellowhead catfish may thus migrate between lakes with water flow. In addition, *P. fulvidraconis* is also found in several other species of fish, i.e., *Parasilurus asotus*, *Mylopharyngodon piceus*, *Channa argus*, *Leiocassis ussuriensis*, *Hemiculter leuciscus*, and *Luciobrama macrocephalus* (Zhang et al., 1999); these fish may increase the dispersal of the nematode between lakes. Finally, artificial aquaculture of *P. fulvidraco* may serve to disperse the nematode throughout China. These factors may have weakened the genetic effect of habitat fragmentation on *P. fulvidraconis*.

In closing, we estimate that the time of lake fragmentation is not more than 60 yr. Therefore, accumulation of genetic differentiation may not be detected, or detectable, within such a short period. Perhaps the use of genetic markers with greater resolving power could detect genetic effects of recent habitat fragmentation. Finally, female worms with fertilized eggs may cause potential genotyping errors (Anderson et al., 2003) and distorted gene frequency estimates of bands.

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SEROEPIDEMIOLOGY OF *TOXOPLASMA GONDII* IN ZOO ANIMALS IN SELECTED ZOOS IN THE MIDWESTERN UNITED STATES

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**ABSTRACT:** *Toxoplasma gondii* infections in zoo animals are of interest because many captive animals die of clinical toxoplasmosis and because of the potential risk of exposure of children to *T. gondii* oocysts excreted by cats in the zoos. Seroprevalence of *T. gondii* antibodies in wild zoo felids, highly susceptible zoo species, and feral cats from 8 zoos of the midwestern United States was determined by using the modified agglutination test (MAT). A titer of 1:25 was considered indicative of *T. gondii* exposure. Among wild felids, antibodies to *T. gondii* were found in 6 (27.3%) of 22 cheetahs (*Acynonix jubatus jubatus*), 2 of 4 African lynx (*Caracal caracal*), 1 of 7 clouded leopards (*Neofelis nebulosa*), 1 of 5 Pallas cats (*Otocolobus manul*), 12 (54.5%) of 22 African lions (*Panthera leo*), 1 of 1 jaguar (*Panthera onca*), 1 of 1 Amur leopard (*Panthera pardus orientalis*), 1 of 1 Persian leopard (*Panthera pardus saxicolor*), 5 (27.8%) of 18 Amur tigers (*Panthera tigris altaica*), 1 of 4 fishing cats (*Prionailurus viverrinus*), 3 of 6 pumas (*Puma concolor*), 2 of 2 Texas pumas (*Puma concolor stanleyana*), and 5 (35.7%) of 14 snow leopards (*Uncia uncia*). Antibodies were found in 10 of 34 feral domestic cats (*Felis domesticus*) trapped in 3 zoos. *Toxoplasma gondii* oocysts were not found in any of the 78 fecal samples from wild and domestic cats. Among the prosimians, antibodies were detected in 1 of 3 Dama wallabies (*Macropus eugenii*), 1 of 1 western grey kangaroo (*Macropus fuliginosus*), 1 of 2 wallaroos (*Macropus robustus*), 6 of 8 Bennett’s wallabies (*Macropus rufogriseus*), 21 (61.8%) of 34 red kangaroos (*Macropus rufus*), and 1 of 1 dusky pademelon (*Thylapale brunii*). Among prosimians, antibodies were detected in 1 of 3 blue-eyed black lemurs (*Eulemur macaco flavifrons*), 1 of 21 ring-tailed lemurs (*Lemur catta*), 2 of 9 red-ruffed lemurs (*Varecia variegata rubra*), and 2 of 4 black- and white-ruffed lemurs (*Varecia variegata variegata*). Among the avian species tested, 2 of 3 bald eagles (*Haliaeetus leucocephalus*) were seropositive. Among 77 possible risk factors, sex, freezing meat temperature (above −13 C vs. below −13 C), washing vegetables thoroughly, frequency of feral cat sightings on zoo grounds (occasionally vs. frequently), frequency of feral cat control programs, capability of feral cats to enter hay/grain barn, and type of animal exhibit, exhibiting animals in open enclosures was the only factor identified as a significant risk (OR 3.22, *P* = 0.00).

** Collection of serum samples from feral cats
Feral cats trapped within the grounds of zoo B, D, and H were captured using humane methods and anesthetized, and blood samples were collected.

** Collection of felines feces
Sixty-six (58 from individual animals and 8 pooled samples from cagemates) were collected from nondomestic felids in all the participating zoos. This was a cross-sectional study, and samples were collected at 1 time point fresh from the floor of the captive cats’ enclosures during routine cleaning practices and from the cages. Twelve fecal samples were collected directly from the rectum of feral cats. Fecal samples were placed in plastic containers, labeled, and stored at 4 C for no more than 7 days before shipment to the APDL, Beltsville, Maryland, for *T. gondii* examination. Feces (5 g) were floated in sugar solution (sp. gr. 1.18), examined microscopically for oocysts, and mixed with 2% sulfuric acid, then aerated on a shaker for 1 wk. After neutralization, the samples were bioassayed in mice as described (Dubey et al., 2005).

** Epidemiological investigation
Seven risk factors—sex, freezing meat temperature (above −13 C vs. below −13 C), washing vegetables thoroughly, frequency of feral cat sightings on zoo grounds (occasionally vs. frequently), frequency of feral cat control programs, capability of feral cats to enter hay/grain barn, and type of animal exhibit (open vs. closed exhibits)—were evaluated using univariate logistic regression analysis.

** Serological examination
Sera of animals were tested for *T. gondii* antibodies with the modified agglutination test (MAT) as described by Dubey and Desmons (1987). Titors of 1:25 or higher were considered indicative of *T. gondii* exposure.

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**RESULTS**

**Serological prevalence in zoo animals**

Antibodies to *T. gondii* were found in felids, macropods, and prosimians, and data from each zoo are given in Table I and Table II. Among the avian species tested, 2 of 3 bald eagles (*Haliaeetus leucocephalus*) were seropositive with MAT titers of 1:25. Antibodies to *T. gondii* were not detected in 6 Rüppell’s vultures (*Gyps rueppellii*), in 2 each of Cinereous vultures (*Aegypius monachus*), red-tailed hawks (*Buteo jamaicensis*), turkey vultures (*Cathartes aura*), barred owls (*Strix varia*), lappet-faced vultures (*Torgos tracheliotus*), Bali mynahs (*Leucopsar rothschildi*), and 1 each of black vulture (*Coragyps atratus*), peregrine falcon (*Falco peregrinus*), Victoria crowned pigeon (*Goura victoria*), spectacular owl (*Pulsatrix perspicillata*), king vulture (*Sarcorhamphus papa*), and barn owl (*Tyto alba*). Most (88%) birds sampled were birds of prey.

Antibodies to *T. gondii* were not detected in any of the 36 New World monkeys, including 1 black howler monkey (*Alouatta caraya*), 4 black-handed spider monkeys (*Ateles geoffroyi*), 5 Reed titis (*Callicebus moloch donacophilus*), 3 Goeldi’s monkeys (*Callimico goeldii*), 1 black-tufted ear marmoset (*Callithrix kuhlii*), 4 woolly monkeys (*Lagothrix lagothricha*), 1 golded-headed lion tamarin (*Leontopithecus rosalia*), 8 pale headed sakis (*Pithecia pitheca*), and 6 Geoffroy’s tamarins (*Saguinus geoffroyi*).

All fecal samples from felids were negative for *T. gondii* oocysts. Feral cats were observed more frequently in zoos A, B, C, E, and H than in zoos F and G. Most (70.6%) of the feral cats trapped were adults, and all seropositive cats were adults. Neither frequency of feral cat control nor having a cat-proof hay barn was identified as a risk factor for *T. gondii* infection in zoo animals. Nevertheless, zoos that control feral cats based on population density are 15% more likely to have seropositive animals than those institutions that have a weekly active feral cat control program (*P = 0.61*). Furthermore, zoos where cats were able to enter the hay barn were 50% more likely than zoos...
TABLE II. Summary of zoo animals seropositive to T. gondii with respective MAT titers.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. positive/no. tested</th>
<th>MAT titers of seropositive animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:25</td>
</tr>
<tr>
<td><strong>Felids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheetah (Acynonyx jubatus)</td>
<td>6/22</td>
<td>0</td>
</tr>
<tr>
<td>Lynx (Lynx caracal)</td>
<td>2/4</td>
<td>0</td>
</tr>
<tr>
<td>Clouded leopard (Neofelis nebulosa)</td>
<td>1/7</td>
<td>0</td>
</tr>
<tr>
<td>Pallas cat (Otocolobus manul)</td>
<td>1/5</td>
<td>0</td>
</tr>
<tr>
<td>African Lion (Panthera leo)</td>
<td>12/22</td>
<td>0</td>
</tr>
<tr>
<td>Jaguar (Panthera onca)</td>
<td>1/1</td>
<td>0</td>
</tr>
<tr>
<td>Amur leopard (Panthera pardus orientalis)</td>
<td>1/1</td>
<td>0</td>
</tr>
<tr>
<td>Persian leopard (Panthera pardus saxicolor)</td>
<td>1/1</td>
<td>0</td>
</tr>
<tr>
<td>Amur tiger (Panthera tigris altaica)</td>
<td>5/18</td>
<td>0</td>
</tr>
<tr>
<td>Fishing cat (Prionailurus viverrinus)</td>
<td>1/4</td>
<td>1</td>
</tr>
<tr>
<td>Puma (Puma concolor)</td>
<td>3/6</td>
<td>0</td>
</tr>
<tr>
<td>Texas Puma (Puma concolor stanleyana)</td>
<td>2/2</td>
<td>0</td>
</tr>
<tr>
<td>Snow leopard (Uncia uncia)</td>
<td>5/14</td>
<td>0</td>
</tr>
<tr>
<td>Domestic cat (Felis domesticus)</td>
<td>10/34</td>
<td>1</td>
</tr>
<tr>
<td><strong>Macropods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dama wallaby (Macropus eugenii)</td>
<td>1/3</td>
<td>0</td>
</tr>
<tr>
<td>Western gray kangaroo (Macropus fuliginosus)</td>
<td>1/1</td>
<td>0</td>
</tr>
<tr>
<td>Wallaroo (Macropus robustus)</td>
<td>1/2</td>
<td>0</td>
</tr>
<tr>
<td>Wallaby (Macropus rufogriseus)</td>
<td>6/8</td>
<td>1</td>
</tr>
<tr>
<td>Red kangaroo (Macropus rufus)</td>
<td>21/34</td>
<td>3</td>
</tr>
<tr>
<td>Dusky pademelon (Thylodactylus)</td>
<td>1/1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Prosimians</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue-eyed black lemur (Eulemur macaco flavifrons)</td>
<td>1/3</td>
<td>0</td>
</tr>
<tr>
<td>Ring-tailed lemur (Lemur catta)</td>
<td>1/21</td>
<td>0</td>
</tr>
<tr>
<td>Red-ruffed lemur (Varecia variegata rubra)</td>
<td>2/9</td>
<td>0</td>
</tr>
<tr>
<td>Black/white-ruffed lemur (Varecia variegata variegata)</td>
<td>2/4</td>
<td>0</td>
</tr>
</tbody>
</table>

with cat-proof hay barns to have T. gondii–positive animals ($P = 0.14$). Univariate regression analysis demonstrated that animals that are kept at open exhibits were 3.22 times more likely to be positive to the parasite than those held in closed exhibits ($P = 0.00$).

In general, all participating zoos had similar food handling practices. Meat was frozen prior to offering it to the animals. Then again, freezing temperatures varied greatly between zoos, ranging from −2 to −20 °C. Surprisingly, zoos that maintained freezing temperatures below −13 °C were 37% more likely to have infected animals than zoos that preserve meat above this temperature; nonetheless this result was not statistically significant ($P = 0.27$). Although also not statistically significant, zoos that washed vegetables thoroughly before offering them to the animals were 90% less likely to have positive T. gondii animals than zoos that do not follow this practice ($P = 0.75$).

**DISCUSSION**

**Serological prevalence in macropods**

Among the Australasian marsupials, clinical toxoplasmosis is more severe in macropods, especially wallabies (Canfield et al., 1990). There are many reports of severe toxoplasmosis in macropods in zoos worldwide, including from the United States (Boorman et al., 1977; Dubey, Ott-Joslin et al., 1988; Miller et al., 1992; Adkesson et al., 2007). In the present study, all of the marsupials tested were macropods, with 49% having titers of $\geq 1:3,200$. From this group, half of the animals were reported clinically unhealthy at the time of the serum collection. It is likely that many of these unhealthy individuals were suffering an active T. gondii infection at the time the sample was collected. In July 2004 there was an epizootic of toxoplasmosis in zoo D, resulting in the clinical death of 3 of 4 red kangaroos. Sera from 2003 and 2004 were submitted for the study. All 4 animals had negative titers ($\leq 1:25$) in their 2003 samples and had seroconverted with titers of $\geq 1:3,200$ in the 2004 samples.

**Serological prevalence in wild felids**

Clinical toxoplasmosis and oocyst shedding has been reported in several of the 13 species of the wild cats found to have T. gondii antibodies in the present study (reviewed in Dubey and Beattie, 1988; Silva et al., 2001). Of the wild felids, toxoplasmosis in the Pallas cat (Otocolobus manul) is of great significance. The natural habitat for Pallas cats is the high mountains in Tibet, western Siberia, Turkestan, and Mongolia. There are only a few Pallas cats in the zoos in the United States because of high mortality in newborns due to toxoplasmosis (Riemann et al., 1974; Dubey, Gendron-Fitzpatrick et al., 1988; Swanson, 1999; Kenny et al., 2002). Additionally, Pallas cats can shed T. gondii oocysts, further contaminating the zoo environment (Dubey, Gendron-Fitzpatrick et al., 1988; Basso et
al., 2005). Unlike the domestic cat, Pallas cats with high titers before pregnancy can give birth to infected kittens (Basso et al., 2005). In the present study the seropositive Pallas cat had a MAT titer of 1:3,200 or higher.

**Serological prevalence in subhuman primates**

New World monkeys and lemurs are highly susceptible to clinical toxoplasmosis (Dubey and Beattie, 1988). Four of the 6 ring-tailed lemurs (Lemur catta) housed together in the Cincinnati Zoo (one of the participant zoos in the present study) died of acute toxoplasmosis (Dubey et al., 1985). Antibodies to T. gondii were not found in the 2 unaffected lemurs 3 wk after the death of the cage-mates, indicating that there were no infected survivors (Dubey et al., 1985). In this respect, it is of interest that T. gondii antibodies were found in 6 of 37 prosimians in the present study (Table I). Two healthy black- and white-ruffed lemurs (Varecia variegata variegata) at zoo A had high MAT titers of \( \geq 1:3,200 \). Four lemurs from zoo D that had tested negative in 2003 seroconverted by November 2004. From this group, 2 red-ruffed lemurs (Varecia variegata rubra) and 1 black lemur (Eulemur macaco flavifrons) had titers of \( \geq 1:3,200 \). The fourth animal was a ring-tailed lemur (Lemur catta) with a titer of 1:100. The red-ruffed lemurs were asymptomatic, and the black lemur was affected with an acute pneumonia. There was no evidence of toxoplasmosis at necropsy, and histopathology and culture from lungs demonstrated Klebsiella pneumoniae. Spencer et al. (2004) reported acute toxoplasmosis in a captive Lemur catta; the diagnosis was verified by isolation of viable T. gondii from the tissues of the dead monkey.

In our study 56.7% of the lemur evaluated were ring-tailed, with only 1 individual having a MAT titer of 1:100; toxoplasmosis was confirmed at necropsy by immunohistochemistry and histopathology in this animal. Recently, Yabsley et al. (2007) reported T. gondii antibodies in 3 of 52 from free-ranging ring-tailed lemurs from St. Catherine’s Island, Georgia; these animals had MAT titers of 1:50.

Antibodies to T. gondii were not found in any of the 36 New World monkeys tested. Most of these monkeys were kept in closed exhibits inside buildings, thus access from feral cats is less likely. Another explanation is that these animals are highly susceptible to toxoplasmosis, and all infected animals could have died of toxoplasmosis.

**Serological survey in avian species**

Initially we wanted to test seroprevalence in carnivorous and noncarnivorous birds. Unfortunately, because of the small size of the majority of birds and difficulty in handling them, we could not obtain a representative number of plasma from all the groups. As a result, close to 90% of the birds tested were birds of prey; probably because of their larger size, extra plasma was collected and stored. In our study 2 captive eagles tested positive to T. gondii antibodies with a low titer of 1:25. These animals are kept in closed exhibits, protected by a wire mesh; however, small mammals are able to enter the exhibit and may be consumed by the predatory birds. Clinical toxoplasmosis in birds of prey is rare (Dubey, 2002).

**Feral cats**

Feral cats have been incriminated as probable sources of T. gondii infections and outbreaks in captive zoo animals in the United States (Riemann et al., 1974; Boorman et al., 1977; Jensen et al., 1985; Gorman et al., 1986; Patton et al., 1986; Dubey, Ott-Joslin et al., 1988; Stover et al., 1990; Junge et al., 1992; Pertz et al., 1997), but to our knowledge seroprevalence in feral cats in the zoos has not been documented (Spencer et al., 2003). The 29.4% seroprevalence found in feral zoo cats is not different from the prevalence of feral cats in the United States (reviewed in Conrad et al., 2005), including feral cats from Ohio, Iowa, and Illinois (Smith et al., 1992; Dubey et al., 1995, 2002).

Ideally, we wanted to test a larger sample of feral cats but could not obtain permission to trap cats in some zoos.

**Detection of T. gondii oocysts**

Toxoplasma gondii oocysts were not found in any of the samples tested from the captive wild felines and feral cats. These results were expected, since epidemiological studies have shown that in the United States less than 1% of cats are shedding T. gondii oocysts at any given moment; consequently it is difficult to detect shedding in any given cat by coprological methods (Dubey, 2004). Also, all of the wild felids that were sampled for T. gondii oocysts were adult animals, and 42% were seropositive for T. gondii antibodies, so one would expect that oocysts shedding had already occurred (Dubey and Frenkel, 1972).

**Epidemiological survey**

Although sources of infection could not be determined, the finding of 63.3% seropositivity in marsupials suggests that captive animals are primarily being exposed to the parasite through ingestion of oocysts, either eliminated by feral cats or by mechanical transportation of oocysts shed by captive wild felids and passed to the susceptible zoo species through the keeper’s clothing, boots, or cleaning equipment, or by birds and insects serving as transport hosts. Feral cats have been incriminated as probable sources of T. gondii infections and outbreaks in captive zoo animals.

Consumption of unwashed raw vegetables and fruits is indicated as a risk factor for acquiring T. gondii in humans. In our study analysis by logistic regression showed that zoos that wash fruits and vegetables had a reduced risk for T. gondii infection, but the result was not statistically significant (OR = 0.9, \( P = 0.75 \)). Only 3 of 8 zoos reported washing vegetables before offering them to the animals; therefore, this could be a source of infection for herbivores. Toxoplasma gondii oocysts are highly resistant, and there is no easy and convenient method to kill oocysts on fruits and vegetables, other than cooking until the temperature reaches 60 C (Dubey, 1998). Although irradiation, ultraviolet, and high-pressure treatments can kill T. gondii oocysts, such methods have not become routine (Dubey et al., 1998; Lindsay et al., 2005; Wainwright et al., 2007).

Toxoplasma gondii epizootics reported worldwide have implicated ingestion of T. gondii tissue cysts as the most probable source of infection in their animal collection. In our study all of the participating zoos fed their carnivores almost exclusively commercial frozen meat products (Nebraska brand frozen meat...
diet, Central Nebraska Packing, Inc., North Plate, Nebraska, and Dallas Crown, Inc., Kaufman, Texas), supplemented 1 or 2 times a week with shank or knuckle bones. Freezing meat overnight in a household freezer is an efficient means of killing most of the T. gondii tissue cysts (Dubey, 1974). However, the temperature in the freezer and the thickness of the meat samples are important. Under controlled conditions, tissue cysts were rendered noninfective when internal temperature of the meat reached −13 C (Kotula et al., 1991). Nevertheless, it must be noted that holding temperatures in freezers vary from institutions, and some of them reported temperatures of −2 C. All of our zoos also reported seeing birds and small mammals inside the carnivorous exhibits, and this could also serve as a source of infection.

The fact that we found exhibiting animals in open enclosures had such a significant risk factor (OR 3.22, P = 0.00) strengthens the assumption that captive animals housed in our participating zoos are predominantly being exposed through ingestion of oocysts. In all of the zoos visited, macropods were held in open exhibits, in contrast to the other groups that were generally housed in closed exhibits. Large ground-dwelling cats are generally in open areas, but medium and small cats that are able to climb trees are housed in closed exhibits.

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LITERATURE CITED


with an emphasis on the roles of free-living mammals. Veterinary Parasitology 42: 199–211.


ANTIGENIC PROPERTIES OF CYSTATIN-BINDING CYSTEINE PROTEINASES FROM NEODIPLOSTOMUM SEOULENSE

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ABSTRACT: The antigenic properties of cysteine proteinases binding to cystatin were analyzed in Neodiplostomum seoulense, an intestinal trematode that infects humans and rodents in the Republic of Korea. Cystatin was found to effectively capture cysteine proteinases present in the crude extract of N. seoulense. The IgG levels against cystatin-binding cysteine proteinases in sera of mice infected with N. seoulense were higher than those in sera of mice immunized with the crude extract of N. seoulense. The production of IgG antibodies against cystatin-binding cysteine proteinases increased according to the length of infection period. In immunoblots of purified cystatin-binding proteinases, 2 molecules, approximately 50 kDa and 60 kDa, reacted with N. seoulense-infected mouse sera. Of the sera from patients infected with various helminths, those of sparganum-infected patients showed the strongest affinities for cystatin-binding cysteine proteinases of N. seoulense. Cystatin-binding cysteine proteinases of N. seoulense are suggested to be putative antigens for serodiagnosis of human N. seoulense infection.

Neodiplostomum seoulense (Neodiplostomatidae) is an intestinal trematode; the natural definitive hosts are rodents, with tadpoles and frogs being the second intermediate hosts (Seo, 1990). The grass snake, Rhabdophis tigrina, plays a crucial role as paratenic host (Seo, 1990). In total, 26 human infections have been reported in capture ELISA for leishmaniasis (Scharfstein et al., 1998), paragonimiasis (Ikeda et al., 1996), and clonorchiasis (Kim et al., 1998). Since snake eaters are not uncommon in rural areas of the Republic of Korea, it is presumed that there may be a considerable number of undetected cases. However, unless a human is heavily infected, diagnosis of human neodiplostomiasis by fecal egg detection is not easy, because the egg-laying capacity of N. seoulense has been found to be low in experimental rodents (Hong et al., 1983). Therefore, the development of other techniques, such as serodiagnosis, is necessary.

Parasite proteinases, cysteine proteinases in particular, are involved in the invasion of parasites into the host and in the uptake of nutrition within the host (McKerrow, 1989). It is also known that cysteine proteinases are involved in immune evasion through the cleavage of host immunoglobulins (Chung et al., 1997). The proteinases have high degrees of antigenic specificities and sensitivities in enzyme-linked immunosorbent assay (ELISA) for fascioliasis (Cordova et al., 1997; O’Neill et al., 1998), paragonimiasis (Ikeda et al., 1996), and clonorchiasis (Na et al., 2002). Cystatin, a proteinase inhibitor, has been known to have reversible- and tight-binding capacities that are specific to cathepsin B, H, and L (Anastasi et al., 1983; Abrahamson et al., 1987), the papain-like cysteine proteinases. The diagnostic potential of cystatin-binding cysteine proteinases has been reported in capture ELISA for leishmaniasis (Scharfstein et al., 1995), fascioliasis (Tantrawatpan et al., 2005), paragonimiasis (Ikeda, 1998), and clonorchiasis (Kim et al., 2001).

With regard to N. seoulense, only 1 cysteine proteinase has been purified and biochemically characterized (Choi et al., 1999). However, antigenic properties of N. seoulense cysteine proteinases have never been reported. The aim of the present study is to analyze the antigenic properties and serodiagnostic potential of N. seoulense cysteine proteinases using immunoblotting, ELISA, and cystatin-capture ELISA.

MATERIALS AND METHODS

Crude extract of N. seoulense

Two- to 4-wk-old BALB/c (n = 30) and C3H mice (n = 20), a highly susceptible and a less susceptible mouse strain, respectively (Chai et al., 1998), were each infected with 200 metacercariae of N. seoulense. The metacercariae were obtained by an artificial digestion method from the mesentery and omentum of the grass snake, R. tigrina, caught in the Republic of Korea. Adult flukes were recovered from the intestines of 10 BALB/c mice at wk 2, 4, 6, and 8 post-infection (PI), according to a previously described procedure (Chai et al., 1998). The worms were homogenized in 10 mM Tris (pH 7.4) buffer and centrifuged at 12,000 g for 30 min at 4 C. The supernatant was used as a crude extract. Animal experiments were carried out in accordance with the guidelines issued by the Institutional Animal Care and User Committee, Seoul National University College of Medicine (Seoul, Republic of Korea).

Mouse sera

Sera of BALB/c (n = 6) and C3H mice (n = 6) infected with N. seoulense were collected at weekly intervals following infection. The sera were diluted to 1:200 and used for conventional ELISA, cystatin-capture ELISA, and immunoblotting. For immune sera, BALB/c mice (n = 6) were inoculated intraperitoneally with 100 µg of N. seoulense crude extract and complete Freund’s adjuvant. Two wk later, the same amount of N. seoulense crude extract was injected into the tail vein for boosting. Immune sera were collected from mice at wk 2 post-boosting. Sera of uninfected BALB/c mice (n = 6) were used as the controls.

ELISA and cystatin-capture ELISA

The wells of a 96-well plate were coated with 10 µg/ml of N. seoulense crude extract in 0.1 M carbonate buffer overnight at 4 C and were then washed with phosphate-buffered saline (PBS)-TWEEN 20 buffer (Sigma, St. Louis, Missouri). The sera from mice or from helminth-infected humans, diagnosed by microscopy or serology (15 clonorchiasis, 6 paragonimiasis, 20 cysticercosis, 17 metagonimiasis, 17 sparganosis, and 10 uninfected sera), were diluted to 1:200 and added to the wells. The 96-well plate was then incubated at 37 C for 4 hr. After the plate had been washed with PBS-TWEEN 20 buffer, peroxidase-conjugated anti-mouse or anti-human IgG (Cappel Co., St. Louis, Missouri) was applied at 1:2,000 or 1:35,000 dilutions for 2 hr, respectively. The color was developed for 30 min using 3,3’, 5,5’-tetramethylbenzidine (Sigma), and the absorbance was measured at a wave length of 490 nm. The statistical cutoff value for a positive reaction was set by the mean plus 10 SD (n = 10) of absorbances obtained from uninfected human sera. Cystatin-capture ELISA was performed, as described in our previous studies.
Cystatin capture extract of coated with extract. Assays were repeated 3 times with similar results, and the values are expressed as the mean ± SD of triplicate wells in a representative assay. Two μg of the crude extract, pre-incubated with various amounts of cystatin overnight, were used as antigens for conventional ELISA and cystatin-capture ELISA. Absorbances were significantly (P < 0.05) higher for ELISA than cystatin-capture ELISA at all of the different concentrations of cystatin used.

However, cystatin-capture ELISA showed dramatically reduced absorbance in the extract pre-incubated with a large amount of cystatin (1 μg), approximately 54% of that shown by the controls. This result indicates that cysteine proteinases of N. seoulense were effectively captured in a cystatin-capture ELISA.

The wells of a 96-well plate were sensitized with 10 μg/ml of cystatin at 4 C overnight. After washing with PBS-Tween 20, the wells were coated with 100 μg/ml of N. seoulense crude extract and incubated at 4 C overnight. The proteins binding to cystatin were collected by boiling the plate at 85 C for 3 min with the addition of SDS-PAGE sample buffer, as described in a previous report (Kim et al., 2001).

Immunoblot

The proteins binding to cystatin were separated by 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond®ECL®, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.). After masking with 2% skim milk (Sigma), the membrane was incubated with the sera of N. seoulense-infected BALB/c mice diluted with PBS-Tween 20 overnight at room temperature. The membrane was then incubated with peroxidase-conjugated anti-mouse IgG (Cappel) at dilutions of 1:2,000 for 2 hr at room temperature. Color development was performed using 4-chloro-1-naphthol (Sigma) as a substrate.

Statistical analysis

Differences in arithmetic means between groups were statistically evaluated using the Student’s t-test. P-values <0.05 were considered to be significant.

RESULTS

Capturing capacity of cystatin

Absorbance values of conventional ELISA, using the crude extract of N. seoulense pre-incubated with cystatin, showed no significant variation in relation to the cystatin concentration (Fig. 1). Their absorbances, in accordance with 10–1,000 ng cystatin, ranged from 80 to 110% of the controls (0 ng cystatin).

Figure 1. Absorbances (Abs) of ELISA and cystatin-capture ELISA of pooled mouse (BALB/c; n = 3) sera against N. seoulense crude extract. Assays were repeated 3 times with similar results, and the values are expressed as the mean ± SD of triplicate wells in a representative assay. Two μg of the crude extract, pre-incubated with various amounts of cystatin overnight, were used as antigens for conventional ELISA and cystatin-capture ELISA. Absorbances were significantly (P < 0.05) higher for ELISA than cystatin-capture ELISA at all of the different concentrations of cystatin used.

Figure 2. Comparison of absorbances (Abs) between sera of mice (BALB/c; n = 6) infected with N. seoulense (wk 8 PI), sera of mice (BALB/c; n = 6) immunized with N. seoulense crude extract (wk 2 post-boosting), and sera of uninfected control mice (BALB/c; n = 6) by ELISA and cystatin-capture ELISA. Assays were repeated 3 times with similar results, and the values are expressed as the mean ± SD from 6 mice for each group in a representative assay. In ELISA, immunized mouse sera revealed significantly higher (P < 0.05) absorbances than N. seoulense-infected mouse sera. However, in cystatin-capture ELISA, infected mouse sera exhibited significantly higher (P < 0.05) absorbances than immunized mouse sera.

Profile of cystatin-binding proteins

Absorbance values of conventional ELISA, using the crude extract of N. seoulense pre-incubated with cystatin, showed no significant variation in relation to the cystatin concentration (Fig. 1). Their absorbances, in accordance with 10–1,000 ng cystatin, ranged from 80 to 110% of the controls (0 ng cystatin).

However, cystatin-capture ELISA showed dramatically reduced absorbance in the extract pre-incubated with a large amount of cystatin (1 μg), approximately 54% of that shown by the controls. This result indicates that cysteine proteinases of N. seoulense were effectively captured in a cystatin-capture ELISA.

ELISA and cystatin-capture ELISA using mouse sera

Sera of BALB/c mice infected with N. seoulense for 8 wk showed similar absorbance values by ELISA and cystatin-capture ELISA (Fig. 2). Absorbances in ELISA using immunized sera were 2 times higher (P < 0.05) than those using N. seoulense-infected sera. However, in cystatin-capture ELISA, immunized mouse sera showed reduced absorbances similar to those of the controls, while infected mouse sera exhibited significantly higher absorbances (P < 0.05).

IgG production in mice infected with N. seoulense

Production of IgG antibodies against cysteine proteinases binding to cystatin was detected by cystatin-capture ELISA at wk 1 PI and increased according to the length of infection in BALB/c mice (Fig. 3). No significant increases of anti-cysteine proteinase IgG were observed in sera of C3H mice.

Profile of cystatin-binding proteins

Immunoblotting of cystatin-binding proteins with mouse sera

The wells of a 24-well plate were sensitized with 10 μg/ml of cystatin (Sigma) in 0.1 M carbonate buffer overnight at 4 C, and the procedure was performed as described above for ELISA.

Cystatin-binding proteins of N. seoulense

The wells of a 24-well plate were sensitized with 10 μg/ml of cystatin at 4 C overnight. After washing with PBS-Tween 20, the wells were coated with 100 μg/ml of N. seoulense crude extract and incubated at 4 C overnight. The proteins binding to cystatin were collected by boiling the plate at 85 C for 3 min with the addition of SDS-PAGE sample buffer, as described in a previous report (Kim et al., 2001).

Immunoblot

The proteins binding to cystatin were separated by 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond®ECL®, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.). After masking with 2% skim milk (Sigma), the membrane was incubated with the sera of N. seoulense-infected BALB/c mice diluted with PBS-Tween 20 overnight at room temperature. The membrane was then incubated with peroxidase-conjugated anti-mouse IgG (Cappel) at dilutions of 1:2,000 for 2 hr at room temperature. Color development was performed using 4-chloro-1-naphthol (Sigma) as a substrate.

Statistical analysis

Differences in arithmetic means between groups were statistically evaluated using the Student’s t-test. P-values <0.05 were considered to be significant.
ELISA and cystatin-capture ELISA using human sera

To evaluate antigenicities of cystatin-binding cysteine proteinases from *N. seoulense* in humans, ELISA and cystatin-capture ELISA were performed using several helminth-infected human serum samples (Fig. 5). The overall absorbance values of human sparganosis sera were higher than those of other helminth-infected sera in both the conventional ELISA and the cystatin-capture ELISA using *N. seoulense* crude extract as the antigen (Fig. 5). According to the statistical cutoff values, ELISA represented positive reactions in 1 of 15 cysticercosis sera, 1 of 6 paragonimiasis sera, 1 of 20 clonorchiasis sera, and 4 of 19 sparganosis sera (Fig. 5). However, cystatin-capture ELISA showed positive reactions in 1 of 20 clonorchiasis and 4 of 19 sparganosis sera.

DISCUSSION

The major habitat of *N. seoulense* in its definitive host is the duodenum; however, worms may extend to the jejunum and ileum in heavy infections (Hong, 1982; Hong et al., 1983). In infections with *N. seoulense*, gastrointestinal symptoms such as diarrhea and epigastric discomfort or pain may occur (Seo et al., 1982). Pathological changes in the intestines, including villous atrophy and crypt hyperplasia, may influence the symptoms. The tribocytic organ of *N. seoulense* has been shown to dissolve host mucosal tissues (Huh et al., 1990; Huh, 1993) and secrete some types of proteinases.

Serodiagnostic potentials of cysteine proteinases have been reported in *Paragonimus westermani* (Ikeda, 1998) and *Clonorchis sinensis* (Kim et al., 2001) by ELISA using cystatin, a 13 kDa protein that specifically binds to papain-like proteinases, as a capturing agent. The present study was performed to identify the cysteine proteinases of *N. seoulense* that bind to cystatin and to characterize their immunological properties for use as a diagnostic antigen.

Cysteine proteinases of *N. seoulense* were effectively captured by cystatin in a manner similar to that shown in other parasite cysteine proteinases (Kim et al., 2001). With this result, the usefulness of cystatin in capture ELISA for the diagnosis of parasite cysteine proteinases has been confirmed.
of *N. seoulense* infection has been verified. Although the binding mechanisms of cystatin to papain-like proteinases are not fully defined, the N-terminal wedge-like structure of cystatin, complementary to the enzymatic site of papain-like cysteine proteinases, is known to contribute to the binding capacity (Bode et al., 1988; Alvarez-Fernandez et al., 1999).

In conventional ELISA, sera from mice immunized with *N. seoulense* crude extract show about 2 times higher absorbances than *N. seoulense*-infected sera. This may indicate that immunization of mice by intraperitoneal injection leads to absorption of 2 times more cysteine proteinases than infection by worms in the intestinal lumen. By comparison, in cystatin-capture ELISA, sera from immunized mice show the lower absorbances similar to those of the uninfected controls. These results suggest that cystatin-binding cysteine proteinases in the crude extract of *N. seoulense* are highly immunogenic and antigenic to mice.

Cysteine proteinases have been shown to exist in large proportions in excretory-secretory products of trematodes (Chappel et al., 1990). Some cysteine proteinases have been found to cleave the host IgG (Chung et al., 1997; Bastida-Corcuera et al., 2000). A recent report showed that *C. sinensis* excretory-secretory antigen is slightly more antigenic and appropriate for use in diagnosis than the crude antigen in ELISA (Choi et al., 2003). However, cystatin-binding cysteine proteinases in the worm crude extract of *C. sinensis* showed enough antigenicity for serodiagnosis (Kim et al., 2001). In the present study, we did not recruit excretory-secretory product of *N. seoulense*, but equally high absorbances were observed in both ELISA and cystatin-capture ELISA using *N. seoulense*-infected sera. This implies that cystatin-binding cysteine proteinases of *N. seoulense* may be secreted without endogenous lysis in their epitopes. When secreted into the host body, these cysteine proteinases may be highly antigenic.

The chronological worm recoveries after *N. seoulense* infection were lower in C3H mice than in BALB/c mice. This finding suggests that C3H mice are less susceptible to *N. seoulense* infection (Chai et al., 1998). Moreover, among the 3 mouse strains examined (BALB/c, C3H/He, and C57BL/6), C3H/He mice exhibit the lowest worm burden, the highest mortality, and the quickest expulsion of *N. seoulense* (Chai et al., 2000). These reports strongly support the present findings that IgG responses against cystatin-binding cysteine proteinases in *N. seoulense*-infected C3H mice represent no significant increases, unlike those seen in BALB/c mice. Due to a rapid worm expulsion, insufficient and inconsistent amounts of cystatin-binding cysteine proteinases may contribute to the low immune reactions in C3H mice.

The 2 protein bands identified by the cystatin-capture immunoblot are presumed to be cysteine proteinases, although direct evidence is lacking. The 50 kDa protein appeared more antigenic than the other protein, which had a molecular mass of 60 kDa. Until the present time, only 1 cysteine proteinase, 54 kDa in size, has been purified from *N. seoulense* crude extract and biochemically characterized (Choi et al., 1999). The 54 kDa protein possessed proteolytic activities typical of cysteine proteinases and seemed to be secreted into the worm’s environment to play a role in immune evasion from the host or other physiological roles (Choi et al., 1999). The molecular masses of the 2 proteins obtained in the present study, presumably cysteine proteinases, were similar in size to the 54 kDa cysteine proteinase (Choi et al., 1999). If either of the 2 proteins were identical with the 54 kDa cysteine proteinase, it would be an important protein for use as a serodiagnostic antigen with well-characterized enzymatic and biochemical activities (Choi et al., 1999). However, further studies are required to confirm the nature of the 2 proteins as cysteine proteinases.

Conventional ELISA and cystatin-capture ELISA, using several helminth-infected human serum samples, provide good evidence for the usefulness of this technique in detecting *N. seoulense* infection cases. However, we regret that *N. seoulense*-infected human sera were unavailable at the time of the present study because no egg positive or “worm-proven” patients were detected by our Department during the past 10 yr. Thus, sera from sparganosis, which is also contracted through the consumption of improperly cooked snakes, were used for evaluation. Both ELISA and cystatin-capture ELISA revealed relatively high reactivity against *N. seoulense* antigens among sparganosis cases. Since no positive control was used to establish cutoff values, statistical cutoff values were created. With these cutoff values, sparganosis sera show 21% cross-reactivity with *N. seoulense* crude extract in both ELISA and cystatin-capture ELISA. Co-infection with both helminths in humans eating improperly cooked snakes seems highly probable, and both kinds of helminthiases may be chronic and last for many yr. With the exception of the first human case, the succeeding 25 cases of *N. seoulense* infection were asymptomatic; it was assumed that they were chronic and suffered from repeated light infections with low worm burdens (Seo, 1990). With regard to *Fibricola cratera*, a species closely related to *N. seoulense*, an experimental human infection was reported in a volunteer in whom the infection lasted at least 40 mo, as revealed by continuous egg production (Shoop, 1989). However, to confirm the reasons for the cross-reactivity, epidemiological investigations on the co-infection of sparganum and *N. seoulense* are necessary.

Cross-reactions with other helminthic infections appeared in a few cases of cysticercosis, paragonimiasis, and clonorchiasis using ELISA, whereas only 1 clonorchiasis serum sample showed cross-reactivity with *N. seoulense* crude extract. Cystatin-capture ELISA may be more useful for differential diagnosis of trematode infections than conventional ELISA.

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**LITERATURE CITED**


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DIFFERENCES IN CYSTEINE PROTEASE ACTIVITY IN SCHISTOSOMA MANSONI-RESISTANT AND -SUSCEPTIBLE BIOMPHALARIA GLABRATA AND CHARACTERIZATION OF THE HEPATOPANCREAS CATHEPSIN B FULL-LENGTH cDNA

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ABSTRACT: Biomphalaria glabrata snails are known to display a wide range of susceptibility phenotypes to Schistosoma mansoni infection depending on the genetics of both the snail and the invading parasite. Evidence exists for a role of hydrolytic enzymes in the defense of molluscs against invading parasites. To elucidate the role of these enzymes in the outcome of infection in the snail, proteolysis was examined in parasite-resistant and -susceptible snails. Zymographs of extracts from the whole snail or hepatopancreas indicated higher proteolytic activity in resistant, compared with susceptible, snails. Lytic activity coincided with a high-molecular-weight smear (220 to 66 kDa) that was abrogated by the cysteine protease inhibitor trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane. Quantitative flourimetric assays showed 3.5-fold higher activity in resistant than in susceptible snails. From a hepatopancreas cDNA library, several cysteine protease encoding expressed sequence tags including the full-length cDNA for cathepsin B were identified. Sequence analysis revealed that this cathepsin B belonged to the C1A family of peptidases characterized by the presence of the catalytic cysteine–histidine dyad, the “occluding loop,” signal sequence, and cleavage sites for the propro and propeptides. Quantitative real-time reverse transcriptase-polymerase chain reaction showed higher up-regulation of cathepsin B transcript in resistant than in the susceptible snail after parasite exposure.

Biomphalaria glabrata, the major intermediate snail host of the parasitic trematode Schistosoma mansoni in the Western Hemisphere, displays variations in susceptibility to parasite infection (Lewis et al., 2001). Using different combinations of snail and parasite strains for infections, these variations were shown to be genetically controlled, with the genetics of both the snail host and parasite affecting the outcome of infection (Newton, 1955; Richards and Shade, 1987; Richards et al., 1992). From the observed complex compatibility between snail and parasite interactions, snails were categorized as either susceptible or nonsusceptible to parasite infection (Richards and Shade, 1987). In cases where an active defense response against the invading parasite was evident (manifested by encapsulation of the miracidia by hemocytes), snails were categorized as being resistant to infection (Paraense and Correa, 1963; Richards, 1970).

Despite the complex nature of this snail–parasite relationship, the existence of pedigree stocks displaying different susceptibility phenotypes has enabled work toward elucidating mechanisms that determine the outcome of trematode infections in the B. glabrata snail host to progress (Lewis et al., 2001). In this context, studies with resistant and susceptible stocks have shown that in adult snails, resistance is controlled by a single gene trait that follows a Mendelian pattern of inheritance, with resistance being dominant (Richards, 1970). Juvenile snail resistance, in contrast, has been shown to be a polygenic trait involving the interaction of at least 4–5 genes (Richards and Merritt, 1972).

From molecular studies undertaken with snails that are either parasite resistant or susceptible, several factors that may affect parasite development have now been described (Adema et al., 1997; Knight et al., 1998; Davids et al., 1999; Miller et al., 2001; Raghavan et al., 2003; Lockyer et al., 2004, 2007; Vergote et al., 2005; Knight and Raghavan, 2006). Recently, using suppressive subtractive hybridization (SSH) strategies, differential regulation of transcripts showing significant hits to proteolytic enzymes (serine protease and cathepsin L) have been described in resistant B. glabrata snail hemocytes after trematode infection (Bouchut et al., 2007; Lockyer et al., 2007). Nonetheless, despite these advances, few studies have focused on how the snail’s internal environment and biochemistry affect parasite development.

Earlier studies showed that hydrolytic lysosomal enzymes may play a role in the phagocytic innate defense response against pathogens (Cheng et al., 1977, 1978; Cheng, 1978; Kasim and Richards, 1978; Cheng and Dougherty, 1989). By analyzing the segregation of susceptibility phenotypes with different enzyme markers (aconitase, acid phosphatase, esterase, leucylglycylglycine peptidase, and 6-phosphogluconate dehydrogenase), Mulvey and Woodruff (1985) were unable to find linkage with these markers and the resistance phenotype. A recent study that showed the presence of serine protease activity in B. glabrata snails (hemocytes) also failed to find an association with this enzyme and variations in the snail’s susceptibility phenotype (Bahgat et al., 2002). In the literature, certain snails have been described as being “unsuitable” for schistosome infection. Because these snails do not exhibit the obvious nonself cell-mediated encapsulation reaction against the parasite typically seen in resistant snails, the “unsuitability” phenotype is thought to reflect an unfavorable biochemical environment that curtails parasite development in these snails (Sullivan and Richards, 1981).

Most biochemical and molecular studies concerning proteolytic enzymes and their involvement in the schistosome life cycle in general, have been investigated within the context of the parasite’s relationship with the vertebrate (mouse and human) rather than the snail host (Sajid and McKerrow, 2002; Stack et al., 2005; Donnelly et al., 2006). In the present study, we rationalized that because proteases have been shown to play several key roles in the biology and pathogenicity of schistosomes, these enzymes may also be relevant in the biology of the snail stage of the parasite’s development. Specifically, be-
cause proteases, in particular cysteine proteases, have been shown to exist in the invading miracidia and primary sporocyst stages of the parasite (Yoshino et al., 1993; Fryer et al., 1996), we thought it plausible that the presence of similar enzymes, or their natural inhibitors (in the snail), or both, could interfere with development of the parasite when encountered by the invading miracidia.

We analyzed by gelatin gel zymographs the basal levels of protease activity in soluble extracts prepared from either the whole snail or various tissues of parasite resistant (BS-90) and susceptible (M-line, NMRI) snails, i.e., the hepatopancreases, ovotestis, albumen gland, and the cell-free plasma (hemolymph). From this analysis, we report here, for the first time, the occurrence of higher levels of enzyme activity corresponding to cysteine proteases in B. glabrata snails that are resistant to S. mansoni compared with those that are susceptible, suggesting that cysteine proteases in the snail host may be a contributing factor in the dynamics of the complex interaction between B. glabrata and S. mansoni. To facilitate the initiation of molecular studies toward an understanding of the possible involvement of these enzymes in the snail–parasite relationship, we constructed a cDNA library from the hepatopancreas, in which the majority of proteolytic activity was found to reside, and we also report from this library the isolation and characterization of a full-length cDNA encoding the snail cysteine protease cathepsin B.

MATERIALS AND METHODS

Snails

Adult snails, 10–12 mm in diameter, that were either resistant (BS-90) or susceptible (M-line, NMRI) to infection by the NMRI strain of S. mansoni, were used in this study. The resistant snail stock used has been shown to be resistant both as adults and juveniles. The BS-90 stock, also known as the Salvador strain, was isolated in Brazil (Paráense and Correa, 1963), and it has been maintained in the laboratory since then. The susceptible M-line and NMRI stocks have been maintained in the laboratory since they were selected for this phenotype (Newton, 1955). Snails were kept overnight in sterile water containing 100 μg/ml ampicillin. Their shells were removed by crushing between 2 microscope slides. Tissues (headfoot, hepatopancreas, albumen gland, and ovotestis) were dissected from individual snails and processed immediately for soluble protein extraction. Individual snails were exposed to the parasite either as juveniles (4 mm) or adults (10 mm, 6 miracidia/juvenile and 25/adult).

Preparation of extracts and analysis

Soluble protein extracts were prepared from either the whole snail (without shell) or freshly dissected tissues after homogenization in phosphate-buffered saline, pH 7.0, containing 0.5% Triton X-100 (v/v) on ice by using a mechanized Kontes pestle (VWR, West Chester, Pennsylvania). Supernatants from the homogenates were recovered after centrifugation at 10,000 g for 15 min at 4 C. Extracts were either analyzed immediately or aliquoted and stored at −80 C until required. Long-term storage (up to 6 mo) at −80 C had no effect on enzyme activity. Protein concentrations of extracts were measured using the Bradford (1976) assay according to manufacturer’s instructions (Bio-Rad, Hercules, California). Similar amounts of soluble protein extract (25 μg) from resistant and susceptible snail stocks (3 snails each) were analyzed under either reducing or nonreducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie Blue, and destained before subjecting the protein extracts to the gelatin zymograph assay described below.

Proteolytic activity in the soluble extracts prepared from resistant and susceptible snails was determined as described previously (Lockwood et al., 1987). Briefly, samples (100 μg/lane) were diluted (1:1) with 2.5× sample buffer (250 mM Tris-Cl, pH 6.8, 5% SDS, 0.25% bromophenol blue, and 25% glycerol) and loaded onto a 10% SDS-containing polyacrylamide gel copolymerized with 1% gelatin (300 Bloom) as a substrate. Polyacrylamide gel electrophoresis was performed overnight at a constant current of 30 mA in electrode buffer (25 mM Tris-Cl, pH 8.8, 192 mM glycine, and 0.1% SDS [v/v]). After electrophoresis, the gel was immersed in wash buffer (2.5% [v/v] Triton X-100), washed twice for 60 min at room temperature, and immersed for 3 hr in preheated (37 C) incubation buffer (0.1 M sodium acetate, pH 5.5, 1 mM dithiothreitol [DTT]). Proteolytic activity was also examined by immersing gels in incubation buffer at pH 7.0. Evidence of proteolytic activity, indicated by a clear region in the gel, was visualized by staining with Coomassie Blue and destaining in fixative containing 10% acetic acid (v/v) and 10% methanol (v/v).

Inhibition of proteolytic activity

Inhibition of proteolytic activity was performed with various inhibitors to block the development of lysis. Briefly, soluble protein extract (2 mg/ml) was subjected to SDS-gelatin impregnated PAGE as described above but using, in this case, a preparative gel. After electrophoresis, gel strips (1 cm in width) were cut, and each strip was washed (3 times in wash buffer as described above) before incubation in the presence of the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM phenanthroline, 1 mM iodoacetamide, 40 μg/ml leupeptin, 40 μg/ml pepstatin, 1 mM N-α-tosyl-1-lysine chloromethyl ketone, 1 mM ethylenediaminetetraacetic acid (EDTA), or 10 μM trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E64). All inhibitors were dissolved in water except for PMSF and pepstatin. PMSF was dissolved in ethanol, and pepstatin was dissolved in 1 mM dimethyl sulfoxide (DMSO). All reagents were purchased from Sigma-Aldrich (St. Louis, Missouri).

Quantitative assessment of proteolytic activity in snail protein extracts

Protease activity was assessed quantitatively on the same soluble extracts analyzed qualitatively by gelatin zymographs. The fluorogenic substrate Z-Phe-Arg-AMC (AMC is 7-amino-4-methyl-coumarin; Axxora, San Diego, California) dissolved in DMSO and stored at −20 C as a 10 mM stock solution was used in an enzymatic assay. Before finalizing the assay conditions, varying concentrations of protein extracts, substrate, and the inhibitor E-64 were used in different assays to determine the optimal concentration of each of these reagents. Briefly, for the fluorometric assay, soluble protein extracts (15 ng each) from either the resistant or susceptible snails were incubated at 37 C for 60 min with the substrate (0.05 mM in 0.1% Brij 35 solution; J. T. Baker, Phillipsburg, New Jersey) in the incubation buffer containing 100 mM Na2HPO4, 1 mM disodium EDTA, and 2 mM DTT at pH 6.0 (Tchoupe et al., 1991). Reactions were performed in 96-well microtiter plates as described previously (Barrett, 1980). Individual wells containing 16 μl of assay stock buffer and 50 μl of extract containing known concentration (diluted with 0.1% Brij 35 solution) were prewarmed at 40 C. The substrate (20 μl from a 50 μM diluted stock solution) was added to each well with mixing. The total assay volume was 200 μl. Reactions were monitored continuously at 1-min time intervals, and the fluorescence intensity of AMC released into the supernatant was measured in real-time in a Biotek FL 600 fluorescent spectrophotometer set at excitation and emission wavelengths of 360 ± 5 and 460 ± 5 nm, respectively. The amount of AMC released was calculated from a standard curve. One unit of enzymatic activity was defined as the amount of enzyme required to release 1 μmol of AMC per min. Activity was measured in duplicate on protein extracts from individual snails for an incubation period of 60 min with a kinetic interval of 1 min. A similar assay was performed simultaneously in the presence of 1 μM of the inhibitor E-64. The background fluorescence was also calculated continuously for each 1-min interval in an assay reaction containing all components except the protein extract. The background from each time point was subtracted from the corresponding test samples before calculating the final fluorescence of each sample. Before deriving the specific activity, the initial velocity (i) of the enzyme reaction (fluorescence units released/minute) was computed using the formula i = ΔF/Δt, where ΔF is the initial velocity and Δt is rate of release of fluorescence units calculated as the difference in release of fluorescence units (y2–y1) using the linear part of the slope at time periods x2 and x1. Δt denotes the...
difference between the time periods ($x_2 - x_1$). Specific activity of the extract for each assay was then calculated as the amount of fluorescence units released per minute per microgram of protein.

**Isolation of nucleic acids and construction of a hepatopancreas cDNA library**

Genomic DNA and RNA from various tissues of either resistant or susceptible snails were prepared as described previously (Knight et al., 1998). The hepatopancreas cDNA library was constructed with poly(A)$^+$ RNA from tissue samples dissected from normal adult BS-90 snails using the lambda ZAP vector (Stratagene, La Jolla, California) as described previously (Raghavan et al., 2003). Mass excision of non-amplified libraries (from 6 separate ligation reactions) with titer ranging between $0.8 \times 10^8$ and $2.7 \times 10^8$ plaque-forming units/ml were performed as described previously (Raghavan et al., 2003) and individual phagemids, plated on X-Gal/IPTG plaques, were picked and stored as glycerol stocks in 96-well format ordered arrays. Recombinant phagemids (insert sizes ranging from 0.4 to 1.5 kb) from 10 microtiter plates were sequenced, and expressed sequence tags (ESTs) were obtained for 861 clones as described previously (Knight et al., 1998). All 861 hepatopancreas EST sequences have been submitted to GenBank with accession numbers ES491296-ES492156. In addition, all EST sequences mentioned in this article are designated with BRI numbers and clone identification numbers.

**Sequence analysis of the hepatopancreas cathepsin B full-length cDNA**

The hepatopancreas EST single pass sequence with the accession ES491472 was sequenced completely from either end using M13 forward and reverse primers. The snail hepatopancreas cathepsin B gene sequence (CTSB) has been submitted to GenBank (EU035711). DNA sequences were analyzed using EMBOSS (Rice et al., 2000), and comparisons were made with sequences in the protein and nucleic acid public databases using the BLAST algorithm (Altschul et al., 1990). The deduced amino acid sequence of the snail cathepsin B protease was further analyzed using the National Center for Biotechnology Information conserved domain database (Marchler-Bauer et al., 2005) to classify and characterize the type of cathepsin and the family and clan to which it belongs. These data were further analyzed against the Interpro EMBL-EBI (Zdobnov and Apweiler, 2001), a database of protein families, domains, and functional sites in which identifiable features found in known proteins can be applied to unknown protein sequences and also against the Sanger MEROPS release 7.80 (Rawlings et al., 2006) databases. Signal sequence prediction was performed using the Signal P program version 3.0 ( Bendtsen et al., 2004).

**Assessment of differential regulation of snail cathepsin B transcript pre- and postparasite exposure by real-time reverse transcription-polymerase chain reaction (RT-PCR)**

Real-time PCR analysis of the snail hepatopancreas cathepsin B gene (GenBank EU035711) was performed with gene-specific primers (forward [F]: 5'-AGCAAACACATCCACATC-3'; reverse [R]: 5'-ATAGCCTCGTGATCC-3'; RT-PCR of the housekeeping gene myoglobin primers F: GATGTTGCGCAAATGTTCC; R: AGCCATCAAGTTTCCCCAG) was used to assess the comparability of template cDNA was used in each amplification reaction. The RT-PCR reactions were performed using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, California). The reaction was performed in a 1-step format with 80 ng of DNase-treated total RNA. DNase treatment was done with RNase-free DNase (RQ1) according to the manufacturer’s suggested protocol (Promega, Madison, Wisconsin). First-stand cDNA reactions and PCR amplifications were performed in triplicate in a single tube with the Full Velocity SYBR Green QRT-PCR Master mix according to the manufacturer’s instructions (Stratagene). Twenty-five microliter of the final reaction volume consisted of 200 nM primers (50 nM myoglobin primers used for adjusting the difference in concentration of the reverse-transcribed RNA starting material), 300 nM reference dye, 1X of Full Velocity SYBR Green QRT-PCR Master mix containing RT-PCR buffer, SYBR Green I dye, MgCl$_2$, and nucleotides (GAUC). The amplification protocol included an initial incubation at 48 C, 45 min for cDNA synthesis and a 95 C initial denaturation for 10 min followed by 40 cycles with 95 C denaturation for 10 sec, and annealing/amplification at 58 C for 1 min. Detection of the fluorescently labeled product was performed at the end of the amplification period. The -fold increase of gene expression was calculated by comparative Ct method with the formula indicated below (Livak and Schmittgen, 2001; standard error and 95% confidence intervals (CI) were calculated for each sample:

$$\text{-fold increase} = 2^{-\Delta \Delta C_t} = 2^{-\left[ \frac{C_t(\text{nonexposed}) - C_t(\text{exposed})}{C_t(\text{unexposed}) - C_t(\text{myoglobin,unexposed})} \right]}$$

To determine significant difference ($P < 0.05$) in gene expression for the different time points after exposure compared with nonexposed snails within each strain, the CI was subtracted from the mean of the -fold increase value. If this value was greater than 1 (1 = value for nonexposed snails), the -fold increase was considered to be significant.

**RESULTS**

Gelatin zymograph reveals higher proteolytic activity in resistant compared with susceptible snails and locates activity to posterior hepatopancreas region of the snail

Figure 1A shows destained gels representing soluble protein from individual snails (whole snail) that are either resistant (BS-90) or susceptible (M-line) to infection after subjecting to gelatin zymograph analysis. Results showed differences in proteolytic activity (clear region resulting from the degradation of impregnated gelatin substrate) between these stocks, with the individual resistant (BS-90) snail extracts showing more activity compared with the same amounts of extracts from individual susceptible (M-line) snails. Lytic activity in all snails corresponded to a high-molecular-weight smear ranging from approximately 66 to 22 kDa or higher. The size range of the smear remained the same regardless of whether the samples were examined under reducing (sample buffer containing ß-mercaptoethanol; data not shown) or nonreducing conditions. To determine the location of the observed protease activity in the snail, extracts prepared from either the anterior (headfoot) or posterior (hepatopancreas and ovotestis) regions of 2 different stocks representing either resistant or susceptible snails were analyzed. Minor activity was detected in the anterior region of both resistant (BS-90) and susceptible (M-line and NMRI) stocks (Fig. 1B). The majority of proteolytic activity, however, was found in the posterior region, with the resistant stock showing higher levels of activity compared with the susceptible snails. Again, the clear region of lytic activity corresponded to a high-molecular-weight smear ranging from approximately 66 to 22 kDa or higher. Analysis of protein extracts from the hepatopancreas of resistant and susceptible snails showed that higher levels of lytic activity were again present in the resistant compared to the susceptible snail, with activity coinciding with the high-molecular-weight heterogeneous smear mentioned above. The clear region indicative of lytic activity of the gelatin substrate remained a complex smear irrespective of using diluted (titrated) extract to determine whether defined bands would then be visible (data not shown). Lytic activity represented by the high-molecular-weight smear only disappeared by incubating gels in buffer at pH 7.0 (data not shown).

Resistant (BS-90) snails possess higher activity levels of cysteine protease activity than susceptible (M-line) snails

Of all the protease inhibitors used to determine the class of proteolytic enzyme in extracts that was responsible for the pro-
ESTs generated from a hepatopancreas cDNA library reveal the presence of several transcripts encoding proteolytic enzymes, including cysteine proteases

From 861 recombinant clones obtained from the mass excision procedure performed using the nonamplified hepatopancreas library, ESTs were generated by single pass sequencing using the M13 universal reverse primer. Sequence identity/similarity (E-value < 10^-4) of the open reading frames of these ESTs to other homologs analyzed by the BLAST algorithm (Altschul et al., 1990) is shown in Table I. Clones showing significant sequence identity to proteolytic enzymes, in particular to cysteine proteases cathepsin B, cathepsin L, and legumain, were identified from the library. In addition, several clones from this library also showed significant matches to other hydrolytic enzymes, including elastase, disintegrin, and metalloprotease, lysozyme, serine protease, hydrolase, cellulase, α-L-fucosidase, and β-1,3-glucanase.

**Sequence analysis of the B. glabrata hepatopancreas cathepsin B sequence**

The hepatopancreas EST single pass sequence (GenBank ES491472) showing similarity to cathepsin B that contained the initiation codon ATG was sequenced completely from either end using M13 forward and reverse primers. Sequence analysis of the DNA indicated that this clone contained the full-length cathepsin B sequence of 1183 nucleotides encoding 333 amino acids (Fig. 3). The sequence has been submitted to GenBank (EU035711) and shows the characteristic hallmark domains of a cysteine protease. Blast analysis of the nucleic acid and deduced amino acid sequence of this clone against the non-redundant databases showed >60% sequence similarity (E-value < 10^-70) to other cathepsin B sequences. Preliminary analysis of the deduced amino acid sequence of the snail ortholog against the conserved domain database of NCBI indicated the sequence to represent a C1A peptidase cathepsin B. This was further confirmed by analyzing the deduced amino acid sequence against the Interpro EMBL-EBI and the Sanger MEROPS da-
tabases. The deduced amino acid sequence encodes the entire preproprotein of cathepsin B that includes the catalytic Cys 115 (C, bold and indicated by a star) and His 281 (H, bold and indicated by a star) that form the catalytic dyad typical of the C1 family of peptidases. The first 19 amino acids encompass the highly hydrophobic signal sequence that is present in secretory proteins. The arrow at amino acid position 19 denotes the putative cleavage site of signal peptide (amino acids 1–19 indicated by bold underline) of the cathepsin B prepropeptide and the arrow between amino acids 86 and 87 indicates the potential cleavage site of the cathepsin B propeptide (amino acids 20–86). Two other residues, although not a part of the catalytic dyad, play an important role in the catalytic mechanism: Gln 109 (Q, in bold and indicated by dark circle) preceding the catalytic Cys 115 forms an oxanion hole, and Asn 301 (N, bold and indicated by dark circle) orients the imidazolium ring of the catalytic His 281. The “occluding loop” (italics and underlined) unique to cathepsins helps it to act also as an exopeptidase in removing C-terminal dipeptides. The polyadenylation signal AATAAAA for the cathepsin B gene is shown in bold, underlined lowercase letters.

**Real-time quantitative RT-PCR reveals the differential regulation of the cathepsin B encoding transcript in *B. glabrata* upon parasite exposure**

To determine the differential regulation of the cathepsin B encoding gene in resistant or susceptible juvenile (4-mm) snails, the snails were exposed (0 to 48 hr) to 5–6 *S. mansoni* miracidia. RNA was prepared from 3 individual size-matched snail for each snail stock. Real-time RT-PCR analysis of the RNA samples showed (Fig. 4) that the early time point (5 hr after exposure) produced a 12.3-fold increase in the cathepsin B transcript in the resistant snail (BS-90), whereas in the susceptible snails (NMRI and M-line) only a 5.2-fold change was observed. Similarly, at the late time period (48 hr) after exposure, a dramatic 24.8-fold increase was observed in the cathepsin B transcript in the resistant snail, but either no increase or a relatively lower level of induction (7.1) was observed in the susceptible snails (NMRI and M-line).

**DISCUSSION**

The involvement of proteases in the intra-molluscan stage of parasite development has been well documented (Yoshino et al., 1993). Although investigations showed that several aspects of parasite development, e.g., snail penetration, nutrient acquisition, and suppression of the snail defense system, rely on the release of proteolytic enzymes (including cysteine proteases) in excretory-secretory products, reciprocal studies to determine whether the presence of similar enzymes in the snail can interfere with the progression of parasite development have not been as intensely investigated. This study shows that higher levels of cysteine protease activity occurs in parasite resistant *B. glabrata* than in susceptible snails. It is possible, therefore, that these enzymes may be important in determining the outcome of the *S. mansoni*- *B. glabrata* interaction. Although previous studies (Bahgat et al., 2002; Mitta et al., 2005) described the presence of several proteolytic enzymes in the snail (amino-peptidase, hydrolase, lysozyme, and genes encoding serine proteases, cathepsin L, and metalloproteases), none described differences (qualitative and quantitative) in activity of these enzymes between parasite resistant and susceptible snails as shown here for cysteine proteinases. Proteolytic enzymes have been detected in both the humoral and cellular components of the snail’s innate defense system, the hemolymph and hemocytes, respectively, with levels changing relative to either bacteria or schistosome infections (Cheng et al., 1977, 1978; Kassim and Richards, 1978). Granulocytes, a type of hemocyte involved in the cellular encapsulation re-
TABLE I. Hydrolytic enzymes identified from the hepatopancreas EST library of the resistant (BS-90) *B. glabrata*. Each of the column headers represent the unique clone identifier, GenBank accession number (sequence similarity to a known gene in GenBank using Blastx analysis and the number of clones represented from each category in the subset of hepatopancreas EST library (861 clones).

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action typically seen in the nonself reaction against incompatible parasites, were shown to express high levels of acid phosphatase activity in a resistant snail upon exposure to *S. mansoni* miracidia, and they were thus hypothesized by Cheng and Garrabrant (1977) to contribute to parasite destruction mediated by these cells. Other snail tissues, including the headfoot, hepatopancreas, and visceral mass (Cheng, 1978; Cheng and Rodrick, 1980), were also shown to express high levels of proteolytic enzymes. In the present study, although cysteine protease activity was detected in the hemolymph and ovotestis (data not shown), most of the enzyme activity was present in the hepatopancreas. Insignificant proteolytic activity was detected in the headfoot, and the activity in hemocytes remains to be tested. Experiments to test for enzyme activity in these cells by methods described here were hampered by the difficulty in isolating large numbers of hemocytes. However, in a recent study using the SSH approach, Bouchut et al. (2007) were able to show the up-regulation of cathepsin L-like transcripts in these cells from an unrelated resistant *B. glabrata* snail after exposure to the trematode *Echinostoma caproni*. Because it is thought that schistosome sporocysts are not easily destroyed by toxic material present in snail plasma (Bayne and Yoshino, 1989), we can only speculate that our results showing higher (qualitatively and quantitatively) activity of cysteine proteases in resistant compared with susceptible snails may indicate that these enzymes could be indirectly rather than directly involved in mechanisms relating to the processing of molecules that are directly toxic for sporocysts. The presence of higher enzyme activity in the hepatopancreas (in both resistant and susceptible snails) relative to other tissues, also suggests that these enzymes may play...
a significant role in the snail’s digestive process. Natural substrates of these cysteine proteases in the snail remain unknown.

Because of results showing elevated activity of these enzymes in the hepatopancreas, a cDNA library was constructed from this tissue and ESTs generated. As expected, several clones corresponding to *B. glabrata* hydrolytic enzymes (cellulase, elastase, disintegrin and metalloprotease, lysozyme, α-L-fucosidase, and serine protease), including cysteine proteases (cathepsin B and L, and legumain), were isolated. One of the ESTs (accession ES491472) encoding the full-length coding sequence of cathepsin B was sequenced in its entirety (accession EU035711). The snail cysteine protease cathepsin B encoding
333 amino acids has all the hallmark domains that are needed for a functional peptidase. Cysteine proteases have characteristic molecular topologies both in their 2- and 3-dimensional structures where the nucleophile is the sulfhydryl group of a cysteine residue. In addition, they are also divided into clans that are evolutionarily related, and further into families on the basis of the architecture of their catalytic dyad or triad (Barrett and Rawlings, 2001). Based on the above-mentioned criteria, the snail cathepsin B belongs to the MEROPS (accession MER00647; Rawlings and Barrett, 1993) cysteine peptidase family C1 and subfamily CIA similar to papain. The catalytic residues of family C1 have been identified as Cys and His, forming the catalytic dyad (Cys 115 and His 281). Two other active site residues are found, a Gln residue preceding the catalytic Cys and an Asn residue following the catalytic His (Gln 115 and Asn 281). The C1A cathepsin B family may contain both endo- and exopeptidase activities, which allows it to make internal cleavages and also remove the C-terminal dipeptide units from the substrate. E-64 is an irreversible inhibitor of peptidases in family C1 (Barrett et al., 1982). In cathepsin B, the presence of an approximately 20-residue “occluding loop” that carries the histidine residues is important for peptidyl-di­peptidase (exopeptidase) activity, and it is inserted between the catalytic Cys and His residues (Illy et al., 1997). Although we do not know the localization of the snail cathepsin B, the presence of the hyrophobic signal peptide at the amino terminus (residues 1–19) of the preprocathepsin B shows it may be a secreted molecule. In addition, results of proteolysis in the gel zymographs coinciding with a complex high-molecular-weight smear (220 to 66 kDa) is considerably higher than the expected size of cysteine proteases (approximately 30–36 kDa). The snail recombinant cathepsin B that has been deduced from the trans­lated sequence has a potential N-glycosylation site (n = 1), protein kinase C (n = 5), casein kinase II (n = 5) phosphorylation sites, and N-myristoylation (n = 13) sites. It is, therefore, possible that posttranslational modifications accounts for the discrepancies in the sizes of the native enzyme and the deduced amino acid sequence.

The biological role of cathepsins in mechanism(s) relating to the antiparasite function of the snail innate defense system, es-

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**FIGURE 4.** Real-time quantitative RT-PCR analysis of the snail hepatopancreas cathepsin B transcript in resistant (BS-90) and susceptible (NMRI and M-line) pre- and postexposure to miracidia. RT-PCR was performed with the cathepsin B gene-specific primers using RNA from individual snails after various times postexposure. Gene-specific primers for the housekeeping gene myoglobin were used to assess sample uniformity and to confirm that the template cDNA was used in equivalent amounts for each amplification reaction. RT-PCR reactions were performed using an Applied Biosystems 7300 Real-Time PCR System, and detection of the fluorescently labeled product was performed at the end of the amplification period. The -fold increase of gene expression was calculated by comparative Ct method as described in Materials and Methods. A value of >1-fold increase was considered to be significant (l = value for nonexposed snails).
pecially encapsulation, remains unknown. With the availability of several cloned transcripts encoding B. glabrata cysteine proteases and at least 1 full-length cathepsin B, characterization of various activities of this enzyme at both biochemical and molecular levels can be achieved with the expression of the recombinant protein. We hope to express the full-length recombinant enzyme in a prokaryotic expression system to raise polyclonal antisera that will be used to purify the native snail cathepsin B by affinity column chromatography. Future physical and biochemical characterization of the purified enzyme should help to resolve the discrepancy between the sizes of the deduced translated sequence and the native enzyme. Antibodies against the recombinant protein will also be useful in the identification of homologs of cathepsin B from hemocytes and other tissues that are not easy to obtain in large quantities, but that are considered important regarding mechanisms involved in snail/parasite interactions, e.g., cerebral ganglia.

Aside from the identification of several ESTs encoding proteolytic enzymes from a hepatopancreas cDNA library (Table 1), transcripts encoding a natural inhibitor (Kazal-like serine protease inhibitor) were also isolated. Previously, we identified the gene encoding cystatin, a known inhibitor of cysteine protease from a resistant (BS-90) snail cDNA library (Knight et al., 1998). Several recent studies have now shown the quantitative increase of the cystatin transcript after trematode infection of B. glabrata snails (Guillou et al., 2007; Lockyer et al., 2007). The occurrence of the proteinase inhibitor α-macroglobulin has also been shown in the snail hemolymph (Bender and Bayne, 1996; Fryer et al., 1996).

In other studies where the effects of parasite infection on hydrolytic enzyme activity have been investigated, levels of glycosidases were shown to correlate with the progress of infection in schistosome infection of B. glabrata snails (Zelck, 1999). Likewise, in the American oyster, Crassostrea virginica, a significant increase in protease activity was observed after infection with the parasite Perkinsus marinus (Munoz et al., 2003). Together, it is clear that future investigations of the possible cytotoxicity of parasites and their natural inhibitors toward warding off trematode infection in the snail host are warranted. Results from our studies using real-time quantitative RT-PCR showing a higher -fold increase of the corresponding cathepsin B transcript in resistant compared with susceptible snails upon parasite exposure is further evidence that proteolytic enzymes play a significant role in the host–parasite relationship.

In summary, qualitative and quantitative differences in the levels of protease activity have been shown to occur between snail stocks that are either resistant or susceptible to S. mansoni infection, with resistant snails consistently expressing higher protease activity than susceptible snails. The majority of enzyme activity detected corresponded to the presence of cysteine proteases in the hepatopancreas. With the availability of cloned cathepsin B and other cysteine proteases from B. glabrata, we anticipate that the molecular and biochemical pathways involving cysteine proteases in killing of schistosomes in the snail host will soon be unravelled.

ACKNOWLEDGMENTS

We thank Dr. Sara Lustigman for inspiration; Dr. Alex Loukas for helpful discussions at the onset of this work; Drs. David FitzGerald and Diana Pastrana for assistance in providing equipment for the enzyme quantitation assay; Dr. Clarence Lee for support; and Dr. Fred Lewis for encouragement, support, and helpful editing of the manuscript. We also acknowledge the Biowulf PC/Linux cluster at the National Institutes of Health, Bethesda, Maryland (http://biowulf.nih.gov), which was used for the sequence analysis. This work was funded by NIH-R01 AI63480-01A1.

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EXCYSTATION OF THE EXCYSTED METACERCARIAE OF ECHINOSTOMA TRIVOLVIS AND ECHINOSTOMA CAPRONI IN A TRYPSIN-BILE SALTS-CYSTEINE MEDIUM AND MORPHOMETRIC ANALYSIS OF THE EXCYSTED LARVAE

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Abstract: A trypsin-bile salts-cysteine (TBC) medium was used to excyst the encysted metacercariae of Echinostoma caproni and E. trivolvis in the alkaline TB medium of Fried and Roth (1974). This medium has been effective in obtaining a relatively high percentage of excysted metacercariae of both species within 2 hr (Smoluk and Fried, 1994; Ursone and Fried, 1995). In May 2007, the original batches of trypsin 4x pancreatin (control no. 5691) and trypsin 1-250 pig pancreas (control no. 14547) from Nutritional Biochemical Corporation (NBC, Cleveland, Ohio), described in Fried and Roth (1974), were depleted. Consultation with a technical representative at NBC indicated that these batches contained significant impurities and were no longer available. Use of trypsin 1-250 porcine pancreas (lot no. R14459) from ICN Biomedicals Inc. (Aurora, Ohio) or a similar porcine trypsin (lot no. 116171) from USB Co. (Cleveland, Ohio) combined with bile salts (see Materials and Methods) gave inconsistent excystation results for both species in June 2007. Apparently, the trypsin in the original TB medium contained unidentified impurities that facilitated the metacercariae excystation of these 2 echinostomes. Because of our need for a consistent supply of excysted metacercariae of both species for teaching and research purposes, we initiated new studies and determined the number and diameter of the excretory concretions found in E. caproni and E. trivolvis. Morphometric analysis can be used to distinguish structural differences in closely related allopatric species of Echinostoma.

Numerous studies (see review in Fried, 1994) have been done on chemical excystation of the metacercariae of E. caproni and E. trivolvis in the alkaline TB medium of Fried and Roth (1974). This medium has been effective in obtaining a relatively high percentage of excysted metacercariae of both species within 2 hr (Smoluk and Fried, 1994; Ursone and Fried, 1995). In May 2007, the original batches of trypsin 4x pancreatin (control no. 5691) and trypsin 1-250 pig pancreas (control no. 14547) from Nutritional Biochemical Corporation (NBC, Cleveland, Ohio), described in Fried and Roth (1974), were depleted. Consultation with a technical representative at NBC indicated that these batches contained significant impurities and were no longer available. Use of trypsin 1-250 porcine pancreas (lot no. R14459) from ICN Biomedicals Inc. (Aurora, Ohio) or a similar porcine trypsin (lot no. 116171) from USB Co. (Cleveland, Ohio) combined with bile salts (see Materials and Methods) gave inconsistent excystation results for both species in June 2007. Apparently, the trypsin in the original TB medium contained unidentified impurities that facilitated the metacercariae excystation of these 2 echinostomes. Because of our need for a consistent supply of excysted metacercariae of both species for teaching and research purposes, we initiated new studies with the so-called "universal medium" first described by Irwin et al. (1984) and redescribed by Irwin (1997). This medium was first used to excyst the metacercariae of Himasthla leptosoma (Irwin et al., 1984), and then to excyst the metacercariae of 8 other non-echinostomatid digenecans (Irwin, 1997).

Because this medium contains trypsin, bile salts, and a cysteine reductant, we refer to it as the TBC medium. One purpose in the present study was to determine the efficacy of this medium for the excystation of the metacercariae of E. caproni and E. trivolvis.

Previous morphological observations on the excysted metacercariae of E. caproni and E. trivolvis noted incidental and qualitative differences in certain structures in these 2 allopatric species. The structures examined were body organs and excretory concretions (calcareous corporules). The second purpose of this study was to provide a quantitative morphometric analysis of the excysted metacercariae of E. caproni and E. trivolvis. For this purpose, we compared body and organ measurements and determined the number and diameter of the excretory concretions in both species.

Materials and Methods

 Obtaining parasite material

Helisoma trivolvis naturally infected with E. trivolvis larvae were used as a source of E. trivolvis metacercariae (Schmidt and Fried, 1997). To obtain E. trivolvis cysts, cercariae released from H. trivolvis were allowed to infect juvenile Biomphalaria glabrata snails. The cysts were removed from B. glabrata 1–21 days postinfection (PI) and used immediately or after storage in Locke’s 1:1 solution for up to 2 wk. Echinostoma caproni cysts were obtained from experimentally infected B. glabrata snails and used within 1–28 days PI or after storage in Locke’s 1:1 solution at 4°C for up to 2 wk (Fried and Huffman, 1996).

Excystation medium

The TBC medium used was based on the A and B solutions of Irwin et al. (1984) and Irwin (1997). Previous studies did not provide information on the suppliers of the components of the medium; we provide such details here. The bile salts (lot no. 0806) and trypsin 1-250 (lot no. R14459) were purchased from ICN Pharmaceuticals (Costa Mesa, California), and L-cysteine (lot no. 1265042) was purchased from Sigma-Aldrich (St. Louis, Missouri). To prepare solution A, 40 mg of bile and 15 mg of trypsin were added to 5 ml of a NaCl/NaHCO3 solution. The NaCl/NaHCO3 solution was prepared using 40 mg of NaCl and 75 mg of NaHCO3 that were added to 5 ml of deionized water. To prepare a batch of solution B, 40 mg of L-cysteine was added to 5 ml of 0.05 M HCl. Solutions A and B were mixed together in a 1:1 ratio just prior to use.

To test the effect of freezing the TBC medium, 1 batch of medium (A and B) was frozen separately at −20°C for 24 hr. The medium was thawed, and A and B were combined and then used for excystation in 3 separate trials, each with 10 cysts of E. trivolvis. Cysts were maintained in this medium for 1 hr at 41°C.

Excystation

Excystation was usually done in a 2.5-cm diameter petri dish with 3 ml of the TBC medium in an incubator at 41 ± 0.5°C. Twenty to 25 cysts were used per trial. Twenty trials were conducted for each species at both 1 and 2 hr. Excystation was scored after 1 and 2 hr based on the number and percentage of fully excysted metacercariae in the medium. Student’s t-test (P < 0.05) was used to determine significant differences in excystation as a function of the species and time in the TBC medium. In some trials, a single major component was omitted from the medium. Trials were done in a water bath at 37.5, 41, 45, or 50°C. For these trials, cysts were placed in a closed 5-ml test tube containing 3 ml of the TBC medium and the tube was held in the water
bath with a clamp. Some excysted metacercariae of both species were transferred to either Locke's solution or Locke's 1:1 solution to determine their survival in such saline solutions at 41°C for up to 12 hr.

**Morphometric analysis**

To obtain the morphometric data, excysted metacercariae of both species were removed from the medium within 1 hr of excystation, rinsed briefly in Locke's solution, and then fixed in hot 5% neutral buffered formalin. The excysted metacercariae were mounted in glycerin jelly and measurements (in μm) were made of the body length, body width, oral sucker diameter, pharynx length and width, acetabulum diameter, and body area (in μm²) on 20 excysted metacercariae of each species. Measurements (in μm) of the diameter of 200 excretory concretions of each species were made. Counts were also made for the number of excretory concretions in 20 excysted metacercariae of each species. Student's t-test (P < 0.05) was used to determine differences in the measurements between species.

**RESULTS**

**Excystation of metacercariae**

During excystation, the larva of each species was activated, rotated rapidly within the cyst, breached an opening in the cyst wall and then excysted, i.e., was free in the medium: The results of the excystation trials are summarized in Table I. There was no significant difference in the percent excystation of *E. caproni* at 1 hr (68.6%) versus that at 2 hr (65.4%). However, the percent excystation of *E. trivolvis* at 2 hr (57.5%) was significantly greater than that at 1 hr (46.5%). The percent excystation of *E. caproni* at both 1 and 2 hr, 68.6% and 65.4%, respectively, was significantly greater than that of *E. trivolvis* at the same times, 46.5% and 57.5%, respectively.

In excystation trials in which a single ingredient was omitted from the medium, i.e., trypsin, bile salts, or cysteine, the percent excystation was minimal or nil. Trials done in the water bath at 41°C gave excystation percentages for both species comparable to those obtained in the incubator at 41°C. Because of the ease of using the incubator for excystation, the water bath studies were discontinued.

The percent excystation for both species at 37.5 and 45°C was markedly reduced in the TBC medium compared to the results obtained at 41°C. At 50°C, the larvae were killed within the cysts, although some larvae of both species breached the cyst walls.

Most larvae of both species that were removed from the TBC medium within 1 hr of excystation and then transferred to either Locke's full strength or Locke's 1:1 solution survived in such saline solutions at 41°C for up to 12 hr. Of the 30 cysts of *E. trivolvis* that were subjected to the frozen and thawed medium, 17 (57%) excysted.

### Table I. Excystation of *E. caproni* and *E. trivolvis* in the TBC medium at 41°C for 1 and 2 hr.

<table>
<thead>
<tr>
<th>Species</th>
<th>Time (hr)</th>
<th>No. of trials*</th>
<th>Total no. of cysts</th>
<th>Total no. of excysted metacercariae</th>
<th>% of excysted metacercariae, mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. caproni</em></td>
<td>1</td>
<td>20</td>
<td>421</td>
<td>289</td>
<td>68.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20</td>
<td>416</td>
<td>272</td>
<td>65.4 ± 1.2</td>
</tr>
<tr>
<td><em>E. trivolvis</em></td>
<td>1</td>
<td>20</td>
<td>404</td>
<td>188</td>
<td>46.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20</td>
<td>412</td>
<td>237</td>
<td>57.5 ± 0.7</td>
</tr>
</tbody>
</table>

* Each trial used 20–25 cysts.

### Table II. Mean ± SE (in μm) of body organ dimensions in the excysted metacercariae of *E. caproni* and *E. trivolvis* (n = 20 for both species).

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>E. caproni</em></th>
<th><em>E. trivolvis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length</td>
<td>266.6 ± 4.8</td>
<td>265.3 ± 5.2</td>
</tr>
<tr>
<td>Body width</td>
<td>77.2 ± 0.8</td>
<td>75.9 ± 2.1</td>
</tr>
<tr>
<td>Body area*</td>
<td>0.021 ± 0.1</td>
<td>0.020 ± 0.1</td>
</tr>
<tr>
<td>Oral sucker diameter</td>
<td>39.7 ± 0.5</td>
<td>40.7 ± 0.5</td>
</tr>
<tr>
<td>Pharynx length</td>
<td>26.4 ± 0.4</td>
<td>24.8 ± 0.9</td>
</tr>
<tr>
<td>Pharynx width</td>
<td>23.2 ± 0.3</td>
<td>23.1 ± 0.7</td>
</tr>
<tr>
<td>Acetabulum diameter†</td>
<td>53.2 ± 0.7</td>
<td>47.3 ± 1.4</td>
</tr>
</tbody>
</table>

* In mm².
† Statistically significant (P < 0.05).

### Morphometric analysis

Results of the morphometric studies are summarized in Table II. There were no significant differences in body length, body width, body area, oral sucker diameter, pharynx length, and pharynx width of the excysted metacercariae of these species. However, the diameter of the acetabulum of *E. caproni* was significantly greater than that of *E. trivolvis*.

Figure 1 shows differences in the number and distribution of excretory concretions in the excysted metacercariae of both species. As shown (Fig. 1), concretions in *E. caproni* were distributed throughout the tubular excretory system, whereas those of *E. trivolvis* were more localized in the posterior branches. Visual observations of the excretory concretions confirmed that the mean ± SE diameter of the concretions of *E. caproni* (10.8 ± 0.2 μm) was greater than that of *E. trivolvis* (7.5 ± 0.1 μm). There was also a greater number of concretions in *E. caproni* compared to *E. trivolvis*. Not obvious in Figure 1 is the fact that the mean ± SE number of the excretory concretions in *E. caproni* was 36.5 ± 1.6, which was significantly greater than that of *E. trivolvis* (31.9 ± 1.7).

### DISCUSSION

The universal medium of Irwin et al. (1984), i.e., the TBC medium, can be used for the successful excystation of *E. caproni* and *E. trivolvis*. Previous studies (Smoluk and Fried, 1994; Ursone and Fried, 1995) with the TB medium of Fried and Roth (1974) noted differences in the percent excystation and the optimal time for maximum excystation between these 2 species. Similar findings were noted in the present study using the Irwin
et al. (1984) medium for both species, thereby reflecting specific differences in the biology of these 2 allopatric species of *Echinostoma*. The TBC medium is easy to prepare and use when solutions A and B are combined just prior to use for excystation. Irwin et al. (1984) used only freshly prepared media in their excystation trials. However, some trials performed by us with the frozen and thawed TBC medium resulted in the successful excystation of *E. trivolvis*. Further studies are needed to determine the efficacy of freezing the TBC medium for excystation studies on echinostomatid and non-echinostomatid digenaea.

Several media prepared for different species of echinostomes need an obligatory reductant treatment (see review in Fried, 1994). However, the exact role of the reductant in the excystation process remains obscure. When the TBC medium is used for the excystation of *E. caproni* and *E. trivolvis*, the reductant is essential, since its omission here resulted in nil excystation. The synergistic effects of trypsin and bile salts in the excystation of echinostomatid and non-echinostomatid digenaea are well documented (see review in Fried, 1994), but the exact role of the intrinsic and extrinsic factors during treatment is not known.

Following excystation of *E. caproni* and *E. trivolvis* in the TBC medium, it is necessary to transfer the newly excysted larvae to Locke’s solution at 41°C where they continue to survive for at least 12 hr. This is important if the larvae are to be used for physiological, biochemical, or immunological studies. Larvae of both species are inactive within 3–4 hr postexcystation in the TBC medium and need to be transferred to a basal salt solution for continued survival.

The advantages of studying structures in excysted metacercariae are obvious to those who have examined critical morphologic features in the cercaria stage where cystogenous and penetration glands often obscure important structures that are being studied. Thus, obtaining excysted metacercariae for morphometric studies is useful. Excysted metacercariae of related species of echinostomatids may show significant differences in structures when examined by light microscopy. Fried and Reddy (1997) showed such differences in certain structures and in the diameter of the excretory concretions of the excysted metacercariae of *E. caproni* versus *E. paraensei*. In that study, live organisms were used. Our current morphometric study was done on uniformly fixed and mounted excysted metacercariae removed from the excystation medium within 1 hr of excystation. Using fixed specimens within about 1 hr postexcystation reduces the possible loss of excretory concretions that may be voided from the excretory pore into the medium. As a function of time, excysted metacercariae tend to release their concretions into the medium. To obtain reliable counts of excretory concretions, we suggest fixing these larvae as soon as possible after excystation so that the excretory concretion count is indicative of the number of concretions present at the time of chemical excystation.

It is apparent that the optimal temperature for excystation of both species is 41°C rather than 37.5 or 45°C. This is probably related to the fact that avian hosts with a body temperature of about 41°C are typically the preferred hosts of many species of *Echinostoma*.

The morphologic differences between the excysted metacercariae of these species relate to the significantly greater diameter of the acetabulum and significantly greater diameter and number of excretory concretions in *E. caproni* versus *E. trivolvis*. Likewise, the excretory concretions of *E. caproni* were dispersed throughout the excretory system, whereas those of *E. trivolvis* were mainly restricted to the posterior aspect of that system.

In conclusion, the TBC medium is effective for the excystation of both *E. caproni* and *E. trivolvis*, and morphometric studies on the fixed metacercariae are useful for distinguishing these 2 allopatric species.

**LITERATURE CITED**

HELMINTH PARASITES IN CHAUNUS MARINUS AND CRANOPSIS VALLICEPS (ANURA: BUFONIDAE) FROM LAGUNAS YALAHAU, YUCATAN, MEXICO

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ABSTRACT: Eight helminth taxa were found parasitizing Chaunus marinus (n = 40) and Cranopus valliceps (n = 40) from the Parque Estatal Lagunas Yalahau, Yucatan, Mexico. Seven taxa (2 digeneans: Langeronia macrocirra, Mesocoelium monas; 1 acanthocephalan: Oncicola sp.; 3 nematodes: Rhadbias fuelleborni, Aplectana itzocanensis, Cruzia morleyi; and a nematode larva) were present in C. valliceps. Nematodes, particularly A. itzocanensis, showed high prevalence, mean abundance, and mean intensity values for both species of amphibians. The occurrence of R. fuelleborni, M. monas, L. macrocirra, and C. morleyi in these amphibians from the Yucatan Peninsula confirms their neotropical distribution, while the presence of A. itzocanensis increases its geographical distribution, suggesting a preference by neotropical, rather than Nearctic areas.

In Mexico, of the 285 amphibian species known to be present (Flores-Villela, 1998), only 14% have been studied in terms of helminth parasites (Pérez-Ponce de León et al., 2000). Thirty-six amphibian species occur in the Yucatan Peninsula, but only 2 species belong to the Bufonidae, i.e., Chaunus marinus (Linnaeus, 1758) and Cranopus valliceps (Wiegmann, 1833). The helminth geographic distribution for these species includes North America (McAllister et al., 1989) and the Caribbean (Goldberg et al., 1995; Linzey et al., 1998; Ragoo and Omah-Maharaj, 2003). In Mexico records exist for Veracruz (Guillén-Hernández, 1992), Jalisco (Galicia-Guerrero et al., 2000), and Nuevo León (León-Reganón et al., 2005). However, until now there was no record for the helminth fauna of these species for the Yucatan Peninsula. The aim of the present work was to elucidate the prevalence, mean intensity, and mean abundance of the helminths parasitizing C. marinus and C. valliceps.

MATERIAL AND METHODS

Field work for sampling amphibians was in the Parque Estatal Lagunas Yalahau region of the Yucatan Peninsula (89°13′41″, and 89°15′00″W, 20°39′16″, and 20°36′5″N) during 2000 (March), 2005 (February, August, September, and October), and 2006 (April). Individuals were collected, either by hand or by seine nets, and kept alive for transport to the laboratory for further examination, which was carried out within 24 hr after capture. Chaunus marinus was collected around a lagoon, and C. valliceps was found in a forest habitat. In the laboratory individuals were killed with an overdose of sodium pentobarbital, and all organs were examined directly using stereomicroscopy. Helminth parasites were removed and counted. To conduct taxonomic identification, parasites were fixed by sudden immersion in hot 70% alcohol and stored in vials. Nematodes were cleared in glycerin, while digeneans and acanthocephalans were stained with Mayer’s paracarmine. Voucher specimens of parasites were deposited in the Colección Nacional de Helminitos (CNHE), Universidad Nacional Autónoma de México (UNAM), Mexico City. Terminology and calculations for prevalence, mean intensity, and mean abundance followed those by Bush et al. (1997).

RESULTS

In total, 40 C. marinus and 40 C. valliceps were examined. Individuals of both host species were collected at the same time, except in April 2006, when C. valliceps was absent. Eight helminth taxa were found parasitizing these amphibians (Table I). At least 5 of these taxa are considered as new records for the Yucatan Peninsula. Seven taxa were found in C. marinus, with only 4 in C. valliceps. Nematodes were the dominant group in terms of abundance. Digeneans (2 species), an acanthocephalan (1 taxon), and nematodes (4 taxa) were found in C. marinus, but only nematodes (4 taxa) were found in C. valliceps. Prevalence, mean intensity, and mean abundance values for each amphibian species are shown in Table II. In general, nematodes (particularly A. itzocanensis) showed high prevalence, mean abundance, and mean intensity values for both species of amphibians.

DISCUSSION

In Mexico 36 helminth species have been previously recorded in C. marinus (Guillén-Hernández, 1992; Lamothe-Argumedo et al., 1997; Galicia-Guerrero et al., 2000; Pérez-Ponce de León et al., 2000; Goldberg and Bursey, 2002) and 6 in C. valliceps (Guillén-Hernández, 1992). In the present study, Oncicola sp. in C. marinus and R. fuelleborni, A. itzocanensis, and Oswaldocruzi sp. in C. valliceps represent new host records. Lagunas Yalahau represents a new locality record for Mesocoelium monas and Cruzia morleyi. Unfortunately we could not identify the nematode larvae because of damaged conditions of the material.

The geographic distributions of C. marinus and C. valliceps are wide, including Nearctic and Neotropical (Lee, 1996; Campbell, 1998); consequently, their parasite fauna also consists of Nearctic and Neotropical species. With respect to the parasite species of nearctic distribution, previous records in C. marinus include Cephalogonimus americanus, Haematoloechus medioplexus (Guillén-Hernández et al., 2000), Langeronia jimenezi (León-Reganón et al., 2005), and Clinostomum attenuatum (Goldberg and Bursey, 2002). In addition, Mesocoelium monas, Langeronia macrocirra, Catadiscus rodriguezi, Gryphelminthins facioi, and G. parva, are considered Neotropical species (Peréz-Ponce de León et al., 2000). Our results confirm the neotropic distribution of M. monas and L. macrocirra. However, we did not find C. rodriguezi, G. facioi, or G. parva in C. marinus, C. valliceps, or Rana brownorum (Yañez-Arenas, 2007), or in any tree frog species in the state of Yucatan (Guillén-Hernández, pers. comm.). Their absence may be related to other factors, such as the absence of an appropriate intermediate host.

Rhadbias fuelleborni has been previously recorded in both Nearctic (Galicia-Guerrero et al., 2000; León-Reganón et al., 2005) and Neotropic (Goldberg et al., 1995; Linzey et al., 1998;
TABLE I. Helminth taxa according to amphibian species infected and location of parasite infection in \textit{C. marinus} and \textit{C. valliceps} from Parque Estatal Lagunas Yalahau, Yucatan, Mexico.

<table>
<thead>
<tr>
<th>Helminth taxa</th>
<th>Parasite location</th>
<th>CNHE accession number*</th>
<th>Host species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Digenean</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Mesocoelium monas}† (Rudolphi, 1819)</td>
<td>Middle intestine</td>
<td>5862</td>
<td>\textit{C. marinus}</td>
</tr>
<tr>
<td>\textit{Langeronia macrocifra} (Caballero and Bravo, 1949)</td>
<td>Anterior and middle intestine</td>
<td>5863</td>
<td>\textit{C. marinus}</td>
</tr>
<tr>
<td><strong>Acanthocephalan</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Oncicola} sp.†</td>
<td>Mesenteries</td>
<td>5864</td>
<td>\textit{C. marinus}‡</td>
</tr>
<tr>
<td><strong>Nematodes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Rhabdias fulleborni}† (Travasos, 1926)</td>
<td>Lungs</td>
<td>5865</td>
<td>\textit{C. marinus}</td>
</tr>
<tr>
<td>\textit{Aplectana itzocanensis}† (Bravo, 1943)</td>
<td>Posterior intestine</td>
<td>5867</td>
<td>\textit{C. marinus}</td>
</tr>
<tr>
<td>\textit{Cruzia morleyi}§ (Pearse, 1932)</td>
<td>Middle intestine</td>
<td>5869</td>
<td>\textit{C. marinus}</td>
</tr>
<tr>
<td>\textit{Ozwaldoeruzia} sp.</td>
<td>Mesenteries</td>
<td>5870</td>
<td>\textit{C. valliceps}‡</td>
</tr>
</tbody>
</table>

* Reference number of the Coleccion Nacional de Helmintos, UNAM.
† New record for Yucatan.
‡ New host record.
§ New locality record.

Ragoo and Omah-Maharaj, 2003) localities; however, \textit{A. itzocanensis} (Goldberg and Bursey, 2002) has been reported only in the nearctic. The presence of \textit{R. fulleborni} in amphibians from the Yucatan Peninsula reflects its wide geographical distribution. Additionally, the occurrence of \textit{A. itzocanensis} increases its geographic distribution and, due to its high prevalence, mean abundance, and mean intensity in local amphibians, suggests a preference for neotropic habitats. \textit{Cruzia morleyi}, previously recorded for Veracruz (Caballero-Deloya, 1974) and Yucatan (Pearse, 1936), can also be considered as neotropic species.

Low values in prevalence, mean abundance, and intensity of digeneans and acanthocephalans in \textit{C. marinus} and \textit{C. valliceps} (McAllister et al., 1989; Goldberg et al., 1995; Galicia-Guerrero et al., 2000; Guillen-Hernández et al., 2000) may be related to the terrestrial preference of these amphibians. The presence of digeneans (\textit{M. monas, L. macrocifra}) and acanthocephalans (\textit{Oncicola} sp.) in \textit{C. marinus} and the absence of these species in \textit{C. valliceps} may be related to the host feeding behavior and the habitat where they spend more time. \textit{Chaunus marinus} preys on aquatic insects (adults and larvae), which are involved in the life cycle of digeneans (in addition to snails) and acanthocephalans, and spend more time close to the aquatic environment than \textit{C. valliceps}, which prefers a terrestrial habitat and feeds mainly on insects (Duellman and Trueb, 1986).

High values of prevalence, mean abundance, and mean intensity of \textit{A. itzocanensis} in both host species may be related to its unique transmission strategy. This parasite can infect its definitive host via skin penetration or via predation. Tadpoles obtain the infection when they feed on the nematode larvae; however, an adult toad can also become infected if it consumes

TABLE II. Infection levels for \textit{C. marinus} and \textit{C. valliceps} from Parque Estatal Lagunas Yalahau, Yucatan, Mexico.*

<table>
<thead>
<tr>
<th>Helminth taxa</th>
<th>\textit{C. marinus}</th>
<th>\textit{C. valliceps}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P (%)</td>
<td>Ma ± SD</td>
</tr>
<tr>
<td><strong>Digenean</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{M. monas}</td>
<td>2.5</td>
<td>0.12 ± 0.79</td>
</tr>
<tr>
<td>\textit{L. macrocifra}</td>
<td>7.5</td>
<td>0.65 ± 2.73</td>
</tr>
<tr>
<td><strong>Acanthocephalan</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Oncicola} sp.</td>
<td>5</td>
<td>0.72 ± 3.85</td>
</tr>
<tr>
<td><strong>Nematode</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{R. fulleborni}</td>
<td>10</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>\textit{C. morleyi}</td>
<td>17</td>
<td>1.12 ± 4.46</td>
</tr>
<tr>
<td>\textit{A. itzocanensis}</td>
<td>50</td>
<td>57.35 ± 90.45</td>
</tr>
<tr>
<td>\textit{Ozwaldoeruzia} sp.</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Larvae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.3 ± 4.2</td>
</tr>
</tbody>
</table>

* Ir = intensity range; Ma = mean abundance; Mi = mean intensity; P = prevalence; SD = standard deviation.
an infected tadpole. Moreover, nematode larvae on the ground can also penetrate an amphibian’s skin (Anderson, 2000).

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LITERATURE CITED


RISK FACTORS FOR TOXOPLASMA GONDII INFECTION IN WILD RODENTS FROM CENTRAL COASTAL CALIFORNIA AND A REVIEW OF T. GONDII PREVALENCE IN RODENTS

Haydee A. Dabritz*, Melissa A. Miller†, Ian A. Gardener‡, Andrea E. Packham, E. Robert Atwill§, and Patricia A. Conrad||

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ABSTRACT: Sera from 523 wild rodents were tested for Toxoplasma gondii antibodies using either an indirect fluorescent antibody test (IFAT) (rats and mice, with titer ≥80 considered positive) or a latex agglutination test (LAT) (Spermophilus beecheyi, were seropositive. Fourteen percent (23/161) of rodents captured in trap sites next to Morro Bay (California) and 15% (16/109) of rodents from sites adjacent to riparian habitats had antibodies to T. gondii, compared to 19% (49/253) of rodents captured in habitats not associated with water; this difference was not statistically significant (P = 0.32). Significantly fewer rodents were captured <200 m from residential housing compared to locations further away (11% vs. 30%, respectively). Factors associated with an increased risk for T. gondii seropositivity in rodents were capture location ±200 m from residential housing and adult age.

The protozoan parasite T. gondii infects most species of warm-blooded vertebrates, including rodents. Like humans, rodents serve as intermediate hosts for T. gondii. Parasites form a latent cyst stage in the tissues of intermediate hosts, notably in the striated muscle and brain. The definitive hosts for T. gondii are domestic and feral cats (Felis catus) and other members of the Felidae (Dubey et al., 1970; Aramini et al., 1999; Kenny et al., 2002). Cats also serve as intermediate hosts. Toxoplasma gondii undergoes sexual reproduction in the feline intestine, resulting in the production of millions of environmentally resistant oocysts that are shed in cat feces (Dubey et al., 1970). Rodents become infected after ingesting soil, vegetation, or water contaminated with T. gondii oocysts. Toxoplasma gondii can be transmitted horizontally between warm-blooded animals, including humans, when they consume the raw or undercooked flesh of intermediate or definitive hosts (Tenter et al., 2000). Vertical transmission also occurs and is of particular concern for humans, because infection in utero can cause abortion or congenital defects in the fetus (Jones et al., 2003). Both modes of transmission appear to be important to ensure the survival of T. gondii in nature.

Small rodents play an important role in the life cycle of T. gondii, because they are believed to represent the main source of infection for domestic and feral cats. Studies of cat predation on wildlife suggest that rodents comprise about two-thirds of the prey consumed, although this may vary according to season, because they are believed to represent the main source of infection for domestic and feral cats. Additionally, infection in utero can cause abortion or congenital defects in the fetus (Jones et al., 2003). Both modes of transmission appear to be important to ensure the survival of T. gondii in nature.

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Significantly fewer rodents were captured <200 m from residential housing compared to locations further away (11% vs. 30%, respectively). Factors associated with an increased risk for T. gondii seropositivity in rodents were capture location ±200 m from residential housing and adult age.

The Morro Bay area of central coastal California was selected for study because of the high proportion of southern sea otters (Enhydra lutris nereis) with evidence of T. gondii infection (Miller et al., 2002; Conrad et al., 2005) off the adjacent coastline. In an updated analysis of the risk for T. gondii exposure in 562 live or dead otters (Conrad et al., 2005), otters sampled between San Simeon and Morro Bay were five times more likely to be infected with T. gondii than were otters living along the more remote and rocky Big Sur coastline. An epidemiologic investigation of T. gondii infection prevalence in owned and feral cats was being concurrently conducted in the Morro Bay area (Dabritz et al., 2007). The objectives of this study were to determine demographic and habitat-related risk factors for T. gondii seropositivity in wild rodents and to obtain tissue samples for future T. gondii genotyping. We hypothesized that there would be a greater risk for T. gondii seropositivity in wild rodents captured near residential housing, because of the association with owned domestic cats. A previous study had shown that T. gondii seropositivity in southern sea otters was associated with high freshwater outflow (Miller et al., 2002), so we also hypothesized that rodents captured near bodies of water (Morro Bay) or water channels would be more likely to be serologically positive.
MATERIALS AND METHODS

Rodent trapping

Trapping was conducted at 18 different locations in the Morro Bay area between July 2004 and February 2006. Study sites were selected to be either <200 m from residential housing or feral cat colonies, where cat densities were expected to be high (n = 13), or situated on public lands ≥200 m from private residences (n = 5). Sites were classified as being adjacent to Morro Bay (n = 3), adjacent to riparian habitats (n = 6), or neither (n = 9). Trapping occurred twice in each wet (December–May) and dry (June–November) season. Each site was trapped for a minimum of 2 nights. In the first trapping session of 2004 (July–August), only mice were sampled; other species were released. Subsequently sampled species were expanded to include rats and voles. Trapping of S. beecheyi (California ground squirrels) began in 2005 and was conducted at 4 sites. At 2 of these sites, ground squirrels were the predominant species trapped. Ten to 60 traps were set per site, depending on the space available. For nocturnal rodents, kangaroo rat-sized folding live traps (HB Sherman, Tallahassee, Florida) were set in a transect layout 5–15 m apart within 2 hr of sunset and baited with hamster/rat feed (Radco, San Jose, California). If temperatures were expected to fall below 12 C, traps were bedded and over-baited. Traps were collected within 3 hr of sunrise. At 4 sites, Tomahawk 48 × 15 × 15 cm live traps (Tomahawk Live Trap, Tomahawk, Wisconsin) were set during daylight hours and monitored for 2–3 hr for the presence of ground squirrels. Rodents were identified to species using a key (Ja­‎mieson and Mears, 1993). The location of each captured animal was recorded on a handheld Garmin GPS locator (Olathe, Kansas). All animals were humanely handled in accordance with an animal use pro­­tocol approved by the Institutional Animal Care and Use Committee at the University of California (UC) Davis, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. In 2004 and 2005, captured rodents were killed using CO2 asphyxiation and immediately bled by intracardiac puncture. In 2006 only, animals trapped on nights 1 and 2 were bled via the retro­­orbital sinus, ear-tagged, and released; rodents captured on the third night were killed and bled via intracardiac puncture. After collection, whole blood was allowed to clot and was centrifuged, and serum was aliquoted into separate vials. Serum was either frozen and transported to UC Davis on dry ice for testing or refrigerated until transport and shipped in coolers at 4 C. Serum was frozen at −20 C until tested.

IFAT for Peromyscus spp., Mus musculus, Reithrodonromys megalotis, Neotoma fuscipes, and Rattus spp.

Sera were diluted in phosphate-buffered saline (PBS), pH 7.4 (Diaz­medix, Miami, Florida) in 2-fold serial dilutions from 1:40 to 1:160. Titer was characterized as the inverse of the dilution of the last well, and was considered as seropositive (Tenter, 1987). Positive controls consisted of sera from experimentally infected T. gondii and were considered infected if they had an IFAT titer ≥80 or LAT titer ≥32, and were otherwise considered uninfected. Fixed effects considered in the model were rodent age class (adult or juvenile), sex, and the site characteristics defined above, and season (wet or dry). Capture location was modeled as a random effect. The variables were first assessed singly, and those significant at P < 0.20 were selected for inclusion in a multivariable model. Different models were compared using the likelihood ratio test. Fit of the selected model was assessed by examining the deviance residuals and Hosmer–Leme­show goodness-of-fit statistic. Statistical tests were considered signifi­cant if the P value was <0.05.

RESULTS

In total, 623 wild rodents were trapped in 3,682 trap-nights (including rodents released without sampling and those captured more than once in 2006), for an overall trap success rate of 16.9%. Of these, 523 individuals were serologically tested for T. gondii infection and 479 were necropsied. Eighty-eight

Table I. Toxoplasma gondii seroprevalence by species for rodents trapped in the Morro Bay area of California.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. tested</th>
<th>No. seropositive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peromyscus maniculatus</td>
<td>74</td>
<td>25 (34)</td>
</tr>
<tr>
<td>Peromyscus boylii</td>
<td>29</td>
<td>9 (31)</td>
</tr>
<tr>
<td>Peromyscus californicus</td>
<td>214</td>
<td>50 (23)</td>
</tr>
<tr>
<td>Peromyscus truei</td>
<td>11</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Spermophilus beecheyi</td>
<td>37</td>
<td>3 (8)</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>15</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Microtus californicus</td>
<td>25</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Neotoma fuscipes</td>
<td>50</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Perognathus californicus</td>
<td>14</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Reithrodonromys megalotis</td>
<td>44</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Rattus rattus</td>
<td>10</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>523</td>
<td>88 (17)</td>
</tr>
</tbody>
</table>

* For Peromyscus spp., Rattus spp., N. fuscipes, R. megalotis, and M. musculus, a positive test was defined as an IFAT titer ≥80; for voles, squirrels, and P. californicus, a positive test was defined as an LAT titer ≥32.
(17%) of the 523 rodents with serum available for testing had antibodies to T. gondii. The proportion of rodents seropositive by species is shown in Table I. Peromyscus californicus comprised the majority (40%) of the species captured. For the 3 habitat classifications, 14% (23/161) of rodents from sites adjacent to Morro Bay and 15% (16/109) of rodents from riparian sites tested seropositive, compared to 19% (49/253) of rodents captured at all other locations. There was no significant difference in the proportion of seropositive rodents amongst habitat types ($P = 0.32$). At 3 sites adjacent to residences, no rodents were captured. Sites located near residential housing had significantly lower trap success (11% vs. 30%) compared with sites remote from residential housing ($P < 0.001$).

The univariable analysis of risk factors for T. gondii seropositivity in wild rodents is shown in Table II. Only a multiple logistic regression model incorporating main effects was employed, because the random effect could not be estimated. Two risk factors were significant in the multiple logistic regression analysis (Table III): adult age and capture in locations ≥200 m from residential housing (sites assumed to have low impact due to domestic cats). In this model, the odds ratio for being seropositive was 3.10 (95% CI = 1.09–8.83, $P = 0.034$) for adults compared to juveniles, and 0.62 (95% CI = 0.39–0.99, $P = 0.049$) for capture in sites <200 m from residential housing, compared to sites ≥200 m away from residences. Check of model fit using the Hosmer–Lemeshow goodness-of-fit test ($P = 0.93$) and deviance residuals indicated that the model fit was adequate.

**DISCUSSION**

Seventeen percent of the wild rodents had antibodies to T. gondii. There was considerable variability in the proportion of rodents infected among different species, as has been reported in other studies worldwide (see summary in Table IV). The prevalence of 26% in Peromyscus spp. was higher than in any previous U.S. reports for Peromyscus spp. since 1985 (range 0–15.5%, typically <7%, under subheading Peromyscus spp., Table IV). Detecting T. gondii antibodies only in Peromyscus spp. and California ground squirrels could be attributable to variable test sensitivity or the duration of immunity to T. gondii. The LAT used to test pocket mouse, ground squirrel, and western harvest mouse sera has not been validated in these species and has been shown to be less sensitive for detecting T. gondii antibodies in pigs and cats (Dubey and Thulliez, 1989; Lappin and Powell, 1991; Dubey, Thulliez et al., 1995). Therefore, seroprevalence in these species could have been underestimated. For practical purposes, the LAT was the only test available, since secondary antibodies for these rodent species were not commercially available. Furthermore, duration of immunity may vary, depending on the species. For example, in congenitally infected rats, antibody titers wane over time and may not be detectable by 87 days postinoculation (Dubey, Shen et al., 1997).

In the present study, the odds of T. gondii seropositivity were about 3-fold greater for adults compared to juveniles, suggesting that the risk for acquiring toxoplasmosis is related to a higher probability for being exposed to oocysts in the environment over the animal’s lifetime. A similar relationship between age and infection has been detected in sea otters (Miller et al., 2002), as well as in humans (Jones et al., 2001). The greater odds for seropositivity in adult mice in the present study may be related to the sensitivity of the different methodologies for detection of T. gondii infection, since congenitally infected mice can test seronegative while harboring parasites in their

### Table II. Univariable logistic regression analysis of risk factors for T. gondii seropositivity in rodents from the Morro Bay area of California.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Description</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximity to residential housing</td>
<td>Far</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Near</td>
<td>0.63</td>
<td>0.39–1.01</td>
<td>0.055</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>1.06</td>
<td>0.67–1.67</td>
<td>0.82</td>
</tr>
<tr>
<td>Age class</td>
<td>Juvenile</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>3.04</td>
<td>1.07–8.61</td>
<td>0.037</td>
</tr>
<tr>
<td>Season</td>
<td>Dry</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wet</td>
<td>0.82</td>
<td>0.52–1.30</td>
<td>0.39</td>
</tr>
<tr>
<td>Site proximity to water</td>
<td>Not</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Next to bay</td>
<td>0.68</td>
<td>0.39–1.16</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Riparian</td>
<td>0.72</td>
<td>0.39–1.33</td>
<td>0.30</td>
</tr>
</tbody>
</table>

### Table III. Multivariable logistic regression model of risk factors associated with T. gondii seropositivity in rodents from the Morro Bay area of California.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Description</th>
<th>Parameter estimate (SE)</th>
<th>Adjusted odds ratio</th>
<th>95% CI</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximity to residential housing</td>
<td>Far</td>
<td>$-0.4749$ ($0.2417$)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Near</td>
<td>$-0.4749$ ($0.2417$)</td>
<td>0.62</td>
<td>0.39–0.99</td>
<td>0.049</td>
</tr>
<tr>
<td>Age class</td>
<td>Juvenile</td>
<td>0.00</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>1.1322 ($0.5335$)</td>
<td>3.10</td>
<td>1.09–8.83</td>
<td>0.034</td>
</tr>
<tr>
<td>Intercept</td>
<td></td>
<td>2.4353 ($0.5260$)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table IV. Prevalence of *T. gondii* in wild-caught rodents worldwide reported from 1985 to 2006.

<table>
<thead>
<tr>
<th>Location</th>
<th>No. positive/no. tested</th>
<th>% Positive</th>
<th>Test (cutoff)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peromyscus spp.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iowa pig farms, U.S.</td>
<td>0/21</td>
<td>0.0</td>
<td>MAT (32)</td>
<td>Smith et al., 1992</td>
</tr>
<tr>
<td>Kansas, U.S.</td>
<td>5/171</td>
<td>2.9</td>
<td>MAT (25)</td>
<td>Brillhart et al., 1994</td>
</tr>
<tr>
<td>Missouri, U.S.</td>
<td>1/15</td>
<td>6.7</td>
<td>DT (8)</td>
<td>Smith and Frenkel, 1995</td>
</tr>
<tr>
<td>Humpback Reservoir, British Columbia, Canada</td>
<td>5/80 (&gt;3 km from residence)</td>
<td>6.3</td>
<td>MAT (25)</td>
<td>Aramini et al., 1999</td>
</tr>
<tr>
<td>Washington Co., Rhode Island, U.S.</td>
<td>4/391</td>
<td>1.0</td>
<td>MAT (25)</td>
<td>DeFeo et al., 2002</td>
</tr>
<tr>
<td>New England, U.S.</td>
<td>2/33</td>
<td>6.1</td>
<td>MAT (10)†</td>
<td>Lehmann et al., 2003</td>
</tr>
<tr>
<td><strong>House mice (M. musculus)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iowa pig farms, U.S.</td>
<td>2/588</td>
<td>0.3</td>
<td>MAT (32)</td>
<td>Smith et al., 1992</td>
</tr>
<tr>
<td>Missouri, U.S.</td>
<td>0/17</td>
<td>0.0</td>
<td>DT (8)</td>
<td>Smith and Frenkel, 1995</td>
</tr>
<tr>
<td>Kansas, U.S.</td>
<td>0/11</td>
<td>0.0</td>
<td>MAT (25)</td>
<td>Brillhart et al., 1994</td>
</tr>
<tr>
<td>Illinois pig farms, U.S.</td>
<td>26/1,243</td>
<td>2.1</td>
<td>MAT (25)</td>
<td>Dubey, Weigel et al., 1995</td>
</tr>
<tr>
<td>Panama City, Costa Rica</td>
<td>2/571</td>
<td>0.04</td>
<td>MAT (NS)‡</td>
<td>Frenkel et al., 1995</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>9/934</td>
<td>1.0</td>
<td>DT (4)</td>
<td>Hejíček et al., 1997</td>
</tr>
<tr>
<td>Illinois pig farms, U.S.</td>
<td>7/465§</td>
<td>1.5</td>
<td>MAT (25)</td>
<td>Mateus-Pinilla et al., 1999</td>
</tr>
<tr>
<td>New England, U.S.</td>
<td>6/390</td>
<td>1.5</td>
<td>Bioassay†</td>
<td></td>
</tr>
<tr>
<td>Manchester, U.K.</td>
<td>118/200</td>
<td>59.0</td>
<td>SAG/ PCR</td>
<td>Marshall et al., 2004</td>
</tr>
<tr>
<td>Manchester, U.K.</td>
<td>53/100</td>
<td>53.0</td>
<td>SAG/ PCR</td>
<td>Hughes et al., 2006</td>
</tr>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India (northern)</td>
<td>Bandicoot rat 18/186</td>
<td>9.7</td>
<td>IHAT (64)</td>
<td>Chhabra et al., 1985</td>
</tr>
<tr>
<td>Baltimore, Maryland, U.S.</td>
<td><em>Rattus norvegicus</em> 54/109</td>
<td>49.5</td>
<td>IFAT (32)</td>
<td>Childs and Seegar, 1986</td>
</tr>
<tr>
<td>Strathblane, Scotland</td>
<td><em>R. norvegicus</em> 5/65</td>
<td>7.7</td>
<td>DT (10)</td>
<td>Jackson et al., 1986</td>
</tr>
<tr>
<td>Kobe Zoo, Japan</td>
<td><em>R. norvegicus</em>, <em>R. rattus</em> 0/55</td>
<td>0.0</td>
<td>LAT (64)</td>
<td>Murata, 1989</td>
</tr>
<tr>
<td>Guangdong, P. Rep. China</td>
<td>Rats unspecified spp. 9/955</td>
<td>1.0</td>
<td>IHAT (64)</td>
<td>Lin et al., 1990</td>
</tr>
<tr>
<td>Mantova, Italy</td>
<td><em>R. norvegicus</em> 14/20</td>
<td>70.0</td>
<td>IFAT (40)</td>
<td>Gench et al., 1991</td>
</tr>
<tr>
<td>Iowa pig farms, U.S.</td>
<td><em>R. norvegicus</em> 0/9</td>
<td>0.0</td>
<td>MAT (32)</td>
<td>Smith et al., 1992</td>
</tr>
<tr>
<td>Kansas, U.S.</td>
<td><em>Neotoma floridana</em> 2/28</td>
<td>7.0</td>
<td>MAT (25)</td>
<td>Brillhart et al., 1994</td>
</tr>
<tr>
<td>U.K. farms</td>
<td><em>R. norvegicus</em> 84/235</td>
<td>35.7</td>
<td>LAT (10)</td>
<td>Webster, 1994</td>
</tr>
<tr>
<td>Missouri, U.S.</td>
<td><em>S. hispidus</em>, <em>R. norvegicus</em> 0/5</td>
<td>0.0</td>
<td>DT (8)</td>
<td>Smith and Frenkel, 1995</td>
</tr>
<tr>
<td>Panama City, Costa Rica</td>
<td><em>R. norvegicus</em> 52/226</td>
<td>23.0</td>
<td>MAT (NS)‡</td>
<td>Frenkel et al., 1995</td>
</tr>
<tr>
<td>Czech Republic</td>
<td><em>R. norvegicus</em> 1/84</td>
<td>1.0</td>
<td>DT (4)</td>
<td>Hejíček et al., 1997</td>
</tr>
<tr>
<td>Taiwan</td>
<td>Rat 1/13</td>
<td>7.7</td>
<td>LAT (32)</td>
<td>Fan et al., 1998</td>
</tr>
<tr>
<td>New England, U.S.</td>
<td><em>R. norvegicus</em> 1/2</td>
<td>50.0</td>
<td>MAT (10)†</td>
<td>Lehmann et al., 2003</td>
</tr>
<tr>
<td>Manchester, U.K.</td>
<td><em>R. norvegicus</em> 19/45</td>
<td>42.2</td>
<td>SAG/ PCR</td>
<td>Hughes et al., 2006</td>
</tr>
<tr>
<td><strong>Voles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strathblane, Scotland</td>
<td>Clethrionomys glareolus* 6/68</td>
<td>8.8</td>
<td>DT (10)</td>
<td>Jackson et al., 1986</td>
</tr>
<tr>
<td>Kansas, U.S.</td>
<td><em>Microtus ochrogaster</em> 1/2</td>
<td>50.0</td>
<td>MAT (25)</td>
<td>Brillhart et al., 1994</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>Clethrionomys glareolus* 3/304</td>
<td>1.0</td>
<td>DT (4)</td>
<td>Hejíček et al., 1997</td>
</tr>
<tr>
<td>Czech Republic</td>
<td><em>Arvicola terrestris</em> 0/10</td>
<td>0.0</td>
<td>DT (4)</td>
<td>Hejíček et al., 1997</td>
</tr>
<tr>
<td>Czech Republic</td>
<td><em>Microtus</em> spp. 15/1,643</td>
<td>0.9</td>
<td>DT (4)</td>
<td>Hejíček et al., 1997</td>
</tr>
<tr>
<td>Hunan Province, P. Rep. China</td>
<td><em>Microtus fortis</em> 36/124</td>
<td>29.0</td>
<td>MAT (20)</td>
<td>Zhang et al., 2004</td>
</tr>
<tr>
<td><strong>Miscellaneous spp.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strathblane, Scotland</td>
<td><em>Apodemus sylvaticus</em> 10/98</td>
<td>10.2</td>
<td>DT (10)</td>
<td>Jackson et al., 1986</td>
</tr>
<tr>
<td>Kansas, U.S.</td>
<td><em>Dipodonomys ordii</em> (kangaroo rat) 0/15</td>
<td>0.0</td>
<td>MAT (25)</td>
<td>Brillhart et al., 1994</td>
</tr>
<tr>
<td>Kansas, U.S.</td>
<td><em>Reithrodontomys megalotis</em> (harvest mouse) 1/2</td>
<td>50.0</td>
<td>MAT (25)</td>
<td>Brillhart et al., 1994</td>
</tr>
<tr>
<td>Kansas, U.S.</td>
<td><em>Ondatra zibethicus</em> (muskrat) 7/42</td>
<td>17.0</td>
<td>DT (8)</td>
<td>Smith and Frenkel, 1995</td>
</tr>
<tr>
<td>Czech Republic</td>
<td><em>Apodemus</em> spp. 24/2,165</td>
<td>1.1</td>
<td>DT (4)</td>
<td>Hejíček et al., 1997</td>
</tr>
<tr>
<td>Czech Republic</td>
<td><em>O. zibethicus</em> 105/437</td>
<td>24.0</td>
<td>DT (4)</td>
<td>Hejíček et al., 1997</td>
</tr>
<tr>
<td>Korea</td>
<td><em>Apodemus agrarius</em> 15/1,008</td>
<td>1.5</td>
<td>ELISA (0.18)</td>
<td>Jeon and Yong, 2000</td>
</tr>
</tbody>
</table>
tissues (Jacobs, 1964; Owen and Trees, 1998). Alternatively, the higher risk for infection in adults compared to juveniles in the present study suggests that congenital transmission is rare or fatal in California mice. Nonetheless, congenital transmission based on the presence of tissue cysts in the brain was documented in 2 of 4 pups born to an experimentally infected *P. maniculatus* (Dubey, 1983). High frequencies of congenital transmission have also been detected by PCR in urban populations of naturally infected mice, but the earlier study did not address pup survival since DNA amplification was performed on fetuses (Marshall et al., 2004). Studies of congenitally infected mice suggest that *T. gondii* infection results in motor and behavioral deficits that would make them more susceptible to predation in the wild (Hay et al., 1983, 1984, 1985; Vyas et al., 2007). To our knowledge, other than the aforementioned experimental infection of *P. maniculatus* (Dubey, 1983), there have been no other published studies in *Peromyscus* spp. of transmission and survival probability for pups born to mice with primary or chronic *T. gondii* infection. Experimental studies in *P. californicus* or other *Peromyscus* spp. would be helpful to assess the impact of congenital transmission on behavior and survival in these species.

Three (13%) of the 23 ground squirrels trapped at Morro Rock were seropositive. A 30- to 40-member feral cat colony had been removed from this site 10 yr earlier. The squirrels at this site live in rocks adjacent to the mouth of Morro Bay in a tidal zone and are fed items such as peanuts and bread by the public, which the squirrels retrieve from the ground. Oocysts transported via water and deposited in the tidal zone or disseminated in the soil by cats defecating in the area could serve as a source of infection for these squirrels. California ground squirrels could also become infected with *T. gondii* if they scavenge dead carrion, as has been reported for this species (Jameson and Peeters, 1988). The *T. gondii* seroprevalence in squirrels detected in the present study is comparable to the 18% seroprevalence in squirrels from Kansas (Smith and Frenkel, 1995). However, only 2 studies of *T. gondii* seroprevalence in squirrels have been conducted since 1985 (Table IV).

In the present study, there was no association of *T. gondii* seropositivity with rodents sampled near riparian habitats or the inlet of Morro Bay, despite the fact that one hypothesized route of transmission of *T. gondii* oocysts to sea otters is via fresh-water outflow (Miller et al., 2002). The results of the present study suggest that transport mechanisms other than, or in addition to, freshwater outflow may be involved. *Toxoplasma gondii* oocysts could reach coastal waters in non-point-source runoff from storm drains or infiltrate aquifers connected with the intertidal zone. The lack of association of *T. gondii*-infected rodents with streams and water bodies could also be related to the fact that rodents consume most of the water they require from their food (Jameson and Peeters, 1988) and that water courses transport oocysts to the near-shore marine environment with little dissemination to the surrounding habitat. When *T. gondii* seroprevalence in *P. maniculatus* around the Humpback Reservoir, British Columbia, Canada, was studied following a human waterborne toxoplasmosis outbreak in 1995 (Aramini et al., 1999), the overall seroprevalence in mice sampled in riparian habitats was 10.6% (subheading *Peromyscus* spp., Table IV). This is comparable to the 15% seroprevalence for rodents sampled near streams in the present study. However, all rodents in the British Columbia investigation were sampled in riparian environments, so no comparison with non-riparian habitats was possible. The association of bodies of water and streams with rodent infection may require a closer association with water than that of the land-dwelling rodents in the present study. Rodents that swim in water (muskrats or pacas) or wade through low-lying semiaquatic environments (capybaras) appear to have higher infection prevalences (ranging from 17 to 60%) than do rodents inhabiting more arid environments (see subheading Miscellaneous spp., Table IV). Oocysts can survive for long

<table>
<thead>
<tr>
<th>Location</th>
<th>No. positive/no. tested</th>
<th>% Positive</th>
<th>Test (cutoff)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washington Co., Rhode Island, U.S.</td>
<td>Wild rodents, miscellaneous spp.</td>
<td>0.8</td>
<td>MAT (25)</td>
<td>DeFeo et al., 2002</td>
</tr>
<tr>
<td>New England, U.S.</td>
<td><em>Blarina brevicauda</em> (shrew) 0/2</td>
<td>0.0</td>
<td>MAT (10)†</td>
<td>Lehmann et al., 2003</td>
</tr>
<tr>
<td>New England, U.S.</td>
<td><em>Zapus hudsonius</em> (jumping mouse) 0/1</td>
<td>0.0</td>
<td>MAT (10)†</td>
<td>Lehmann et al., 2003</td>
</tr>
<tr>
<td>Sao Paulo, Brazil</td>
<td><em>Hidrochoerus hydrochoerus</em> (capybara) 63/149</td>
<td>42.3</td>
<td>MAT (25)†</td>
<td>Canon-Franco et al., 2003</td>
</tr>
<tr>
<td>French Guiana</td>
<td><em>Myoprocta acouchy</em> (acouchy) 1/26</td>
<td>4.0</td>
<td>MAT (40)</td>
<td>De Thoisy et al., 2003</td>
</tr>
<tr>
<td>French Guiana</td>
<td><em>Dasyprocta agouti</em> (agouti) 8/45</td>
<td>18.0</td>
<td>MAT (40)</td>
<td>De Thoisy et al., 2003</td>
</tr>
<tr>
<td>French Guiana</td>
<td><em>Coendou prehensilis</em> (porcupine) 0/19</td>
<td>0.0</td>
<td>MAT (40)</td>
<td>De Thoisy et al., 2003</td>
</tr>
<tr>
<td>French Guiana</td>
<td><em>Agouti paca</em> (paca) 22/37</td>
<td>60.0</td>
<td>MAT (40)</td>
<td>De Thoisy et al., 2003</td>
</tr>
<tr>
<td>Squirrels</td>
<td><em>Sciurus</em> spp.</td>
<td>18.0</td>
<td>DT (8)</td>
<td>Smith and Frenkel, 1995</td>
</tr>
<tr>
<td>Kansas, U.S.</td>
<td><em>Sciurus vulgaris</em> 0/3</td>
<td>0.0</td>
<td>DT (4)</td>
<td>Hejliecek et al., 1997</td>
</tr>
<tr>
<td>Czech Republic</td>
<td><em>Sciurus pygmaeus</em></td>
<td>2/11</td>
<td>DT (8)</td>
<td>Smith and Frenkel, 1995</td>
</tr>
</tbody>
</table>

* DT, dye test; ELISA, enzyme-linked immunosorbent assay; IFAT, indirect fluorescent antibody test; LAT, latex agglutination test; MAT, modified agglutination test; SAG1 PCR, polymerase chain reaction amplification of *T. gondii* surface antigen gene DNA confirmed by sequencing.
† All seropositive rodents had titers ≥40.
‡ Cutoff for a positive MAT was not specified for rodents.
§ Seroprevalence varied by year after a *T. gondii* vaccination program for cats on the farms was initiated.
|| Inoculation of homogenates of heart and brain tissue into mice for bioassay.
# Two of the 6 positive were *Microtus* spp. (voles); 4 were *Peromyscus* spp.
¶ 104/149 (69.8%) seropositive by IFAT using 1:16 as the cutoff for a positive test.
** Five gray squirrels, 5 fox squirrels, and 1 thirteen-lined ground squirrel.

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DABRITZ ET AL.—RISK FACTORS FOR *T. GONDII* INFECTION IN WILD RODENTS 679

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periods in water and moist soil, especially if they are protected from ultraviolet light (Yilmaz and Hopkins, 1972; Dubey, 1998; Lindsay et al., 2003).

Rodents in the present study were about 40% less likely to be seropositive for *T. gondii* (OR 0.62) if sampled near residential housing. This finding was unexpected, because the greater density of cats in peri­domestic habitats is likely to increase the potential for contamination of the soil with *T. gondii* oocysts. The finding of a lower risk for *T. gondii* infection in rodents sampled near residential housing differed from findings of rodent surveys in British Columbia, Canada (Ararimi et al., 1999), and the United Kingdom, where 59% of mice living in urban environments were infected with *T. gondii* compared to 46% of mice captured in rural habitats (D. Thomasson, pers. comm.). In the British Columbia study, deer mice sampled <3 km from residences were more likely to be infected with *T. gondii* than were deer mice sampled elsewhere (subheading *Peromyscus* spp., Table IV). The prevalence of *T. gondii* (tested by PCR) in urban house mice in Manchester, U.K. (59%) was one of the highest prevalences ever reported for mice (subhead­ing house mouse, Table IV). Reasons for the lower sero­ prevalence in mice sampled from peri­domestic environments in the present study may be related to detection bias and the close proximity (<3 km) of all but one site to large areas of residential housing. The only site that was >3 km from the community boundaries (the easternmost site in Fig. 1) was home to an owned domestic cat with a high *T. gondii* titer. There were no sites where the presence of cats or wild felids could be completely ruled out, and all sites were potentially located within the home range of owned and feral domestic cats.

Trap success was significantly lower at sites impacted by human populations or close to feral cat colonies. Low trap success may be a result of homeowner rodent extermination efforts and/or cat predation. Experimental studies have documented behavioral changes in *T. gondii*-infected rodents that may make them more susceptible to predation (Hutchison et al., 1980; Berdoy et al., 1995, 2000; Vyas et al., 2007). Infected mice spend more time moving around in familiar environments, demonstrate impaired motor performance, and are less reactive to new stimuli than are uninfected controls (Hutchison et al., 1980). Compared to their uninfected counterparts, *T. gondii*-infected rats approach novel stimuli more readily, exhibit greater physical activity, and lose their aversion for cat urine (Berdoy et al., 1995, 2000; Vyas et al., 2007). *Toxoplasma gondii*-infected rodents may, therefore, be more easily captured by cats compared with *T. gondii*-infected rodents, and could be less likely to be detected in sites where cats are abundant.

*Toxoplasma gondii* infection in rodents probably involves the interaction of complex ecological elements that include abiotic factors such as climate and oocyst survival, and biotic factors such as differing susceptibility of rodent species to *T. gondii* infection and genotype, different patterns of congenital transmission in mice and rats, and changes in host behavior that make infected hosts more susceptible to predation. It may, therefore, be difficult to elucidate risk factors that are strongly associated with *T. gondii* infection in rodents, even when sentinel species such as sea otters along the adjacent coastline suggest high levels of *T. gondii* transmission in coastal terrestrial fauna.

**ACKNOWLEDGMENTS**

This work was supported by the Morro Bay National Estuary Pro­gram, the Schwall Medical fellowship, and National Science Foundation Grant 0527565. The authors thank Ethan App, Dan Berman, Bruce Buel, Vince Cicero, David Dabritz, Dick Davis, Mike Harris, Dave Jessup, Douglas Kelt, Ray Smith, and Lou Zatt for technical assistance or facilitating access to properties.

**LITERATURE CITED**


**FIGURE 1**. Rodent trapping sites (2004–2006) in the Morro Bay area, California. Communities are designated by shaded areas. Trapping site adjacent to Morro Bay (+), adjacent to riparian habitat (*), or adjacent to neither (\(\Delta\)).


JEON, S. H., and T. S. YONG. 2000. Serologic observation of Toxoplas-


INTESTINAL HELMINTHS OF FRESHWATER STINGRAYS IN SOUTHEASTERN PERU, AND A NEW GENUS AND TWO NEW SPECIES OF CESTODE

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ABSTRACT: Helminths of the spiral intestine of neotropical freshwater stingrays (Potamotrygonidae) were examined in Peru for the first time. The stingrays examined for helminths included Paratrygon arietova, Potamotrygon motoro, and Potamotrygon cf. castexi. Present in P. arietova were the cestodes Nandecostus guarnicutsus (Marques, Brooks, and Lasso, 2001) n. gen. n. comb., Rhinebothrium copianuallum n. sp., Rhinebothrium sp. 1. Rhinebothrioides sp., Potamotrygonococestus cf. fitzgeraldiae, and 1 species each of Cucullanus and Rhadobolona, Nandecostus n. gen. is erected to house N. guarnicutsus, which is formally transferred from Anindobothrium Marques, Brooks, and Lasso, 2001. The new genus is unique among phyllobothridii in its possession of circum- medullary vitelline follicles and a submarginal genital pore, in combination with bothridia with a single apical sucker and marginal bothridial loculi. The helminths of P. motoro included the cestodes Paraconomages arietava, Acanthobothrium peruvianis n. sp., Acanthobothrium cf. ramiroti, Rhinebothrium sp. 1, Rhinebothrioides sp. 1, Potamotrygonococestus sp., the nematode Brevimultiplicimus caecum regoi, a species of Cucullanus, and a species of the digenean superfamily Diplostomoidae. The helminths of P. cf. castexi included the cestodes P. arietava, N. guarnicutsus n. gen. n. comb., Acanthobothrium cf. peruviensi, Potamotrygonococestus sp., Rhinebothrium sp. 1, Rhinebothrioides sp. 2, the nematode species Echinocephalus daileyi and B. regoi, 1 species each of Cucullanus, Rhadobolona, and Procamallanus, and a species of the digenean superfamily Hemirrioidea. All taxa were examined via light microscopy; the cestode taxa were also examined using scanning electron microscopy. Each helminth species recorded in this study is a first report from Peru. The study suggests that the diversity and host specificity of the cestodes in potamotrygonid stingrays may be greater than previously thought. The known numbers of genera and species of tetraphyllidean cestodes parasitizing neotropical freshwater stingrays are now 6 and 22, respectively.

The potamotrygonids are a family of freshwater stingrays endemic to South America (Rosa, 1985). They are unique among elasmobranchs in that they have both invaded and radiated within rivers of South America which drain into the Atlantic and the Caribbean (Thorson et al., 1983; Rosa, 1985; Lovejoy, 1996). Like their marine counterparts, potamotrygonids host a diversity of metazoan parasites. The major metazoan parasite groups that have been reported from South American freshwater stingrays are as follows (excluding incidental infections): 3 species of branchiurans from the skin (Peralta et al., 1998); 1 species each of pentastome (Rego, 1979; Sprent, 2001; Mayes, Brooks, and Thorson, 1981 b; Brooks and Amato, 1998); 1 species each of pentastome (Rego, 1979), 'a nematode (Rahdeg and Henle, 1841), Potamotrygon motoro (Muller and Henle, 1841), and a species of Potamotrygon Garman, 1877 referred to as Potamotrygon castexi Castello and Yagolowski, 1969 by Rosa, 1985, but referred to here as P. cf. castexi (M. de Carvalho, pers. comm.). This study represents the first investigation of the intestinal helminths from potamotrygonids in the Madeira subbasin, and Peru in general.

MATERIALS AND METHODS

Potamotrygonids were collected with hook and line. All collections were made in the Madre de Dios Department of Peru close to Boca Manu, either at or near the point where the Manu River flows into the Alto Madre de Dios River (12°16.350'S, 70°55.789'W), at an elevation of 308 m. Four specimens of P. arietava (2 males, 2 females), 7 specimens of P. motoro (all males), and 14 specimens of P. cf. castexi (11 males, 3 females) were collected. Stingrays were caught in May 2001, January 2002, and May 2003, and examined for intestinal helminths after being killed by cranial concussion under the authorization of University of Connecticut IACUC protocol No. C010 0202. Following examination for parasites, representative stingray specimens were fixed in 10% formalin diluted with 0.6% saline, stored for several days, and subsequently transferred to 70% ethanol. Representative specimens of each of the 3 stingray species have been deposited at the Museu de Zoologia da Universidade de Sao Paulo, Brazil (MZUSP). Stingrays were identified with the aid of taxonomic keys (Rosa, 1985), and identifications were verified by potamotrygonid experts. Images of the specimens of each of the 3 stingray species collected in this study are available online (Marques and Domingues, 2006).

The spiral intestine of each stingray was removed, opened with a midventral incision, and examined for helminths with a dissecting microscope in the field, and again in the laboratory. Each helminth encountered was removed, counted, and either fixed in 10% formalin diluted with 0.6% saline, stored for several days, and subsequently transferred to 70% ethanol, or were fixed in 95% ethanol. Cestode and di-
genean specimens prepared for light microscopy were hydrated in a graded ethanol series, stained in Delafield’s hematoxylin, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted on glass slides in Canada balsam. Nematodes prepared for light microscopy were dehydrated in a graded ethanol series, placed in lactophenol for examination as wet mounts, and subsequently stored in lactophenol. In addition, the stomach of each stingray was removed, opened with a longitudinal incision, and the contents were removed and stored in 95% ethanol.

Specimens for histology were embedded in paraplast and sectioned at 8 μm intervals using a CUT4060 retracting rotary microtome (Olym­pus). Sections were mounted on glass slides flooded with 2.5% sodium silicate and dried on a slide warmer for 4–8 hr. Cross sections of mature proglottids and longitudinal sections of scolecis were prepared separately from specimens for which sufficient material was available. Sections were stained with haematoxylin and eosin (H&E) according to conventional techniques.

Scolecis of 1 or more specimens of each cestode species, and free proglottids of 3 species, were prepared and examined using scanning electron microscopy (SEM). Specimens were hydrated in a graded eth­anol series, transferred to 1.5% osmium tetroxide overnight, dehydrated in a graded ethanol series, and placed in hexamethyldisilazane (HMDS, Ted Pella Inc., Redding, California) for 15 min. They were allowed to air dry and were subsequently mounted on carbon tape and ground with carbon paint on aluminum stubs. For examination of eggs, gravid proglottids were crushed once placed on an aluminum stub. Specimens were sputter-coated with approximately 200–300 Å of gold/palladium and examined with a DSM 982 Gemini Field Emission Scanning Electron Microscope (LEO/Zeiss).

Representative whole mounts of each cestode, nematode, and digenean specimen encountered in each host species were examined with a compound microscope, measured with an ocular micrometer, and identi­fied. Measurements of described cestode species are presented paren­thetically as ranges with the mean, standard deviation, number of spec­imens examined and, if >1 measurement per specimen, number of mea­suresments, respectively. All measurements are in micrometers unless otherwise specified. For specimens of Rhinebothrium Linton, 1890, measurements are for terminal proglottids in which the tests have been dem­onstrated. In such cases, tests data were taken from subterminal proglottids. Hook measurements taken for specimens of Acanthobothrium von Beneden, 1850, follow Ghoshroy and Caira (2001).

To facilitate comparison of the new species of Acanthobothrium with other species in the genus, the categorization system of Ghoshroy and Caira (2001) was used. Categorization of the new species of Acantho­bothrium using this system limited comparisons only to other species receiving the same category designation (see Ghoshroy and Caira 2001). Species in the same category as the new species were determined based on the designations for Acanthobothrium species provided by Ghoshroy and Caira (2001), Ivanov (2005), Fyler and Caira (2006), and Reyda and Caira (2006). The Global Cestode Database (Caira et al., 2006) was queried to compile information on each existing species of Acanthobothrium. Line drawings were made using a camera lucida (model name/number, manufacturer name, city, state/country).

Abbreviations for the museums in which helminth specimens were deposited, or from which specimens were borrowed, are as follows: CHIOC, Colección Helmintológica do Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; LRP, Lawrence R. Penner Parasitology Collection, University of Connecticut, Storrs, Connecticut, United States; MHNLS, Museo de Historia Natural La Salle, Caracas, Venezuela; MHNP, Museo de Historia Natural, Lima, Peru; MZUSP, Museu de Zooloigia da Universidade de São Paulo, Brazil; USNPC, United States National Parasite Collection, Beltsville, Maryland, United States.

RESULTS

Stingrays

Individuals of all 3 stingray species were often found in sympatry, occupying the same habitat at the same location, e.g., near beaches during the dry season or in deep pools sheltered from the main river current during the wet season. The specimens identified here as P. cf. castexi (identified as P. castexi by Reyda and Olson, 2003) may actually represent an undescribed species of Potamotryon (M. de Carvalho, pers. comm.).

Voucher specimens are deposited as: P. aierea, MZUSP No. 95406 (=field code PU-10); P. motoro, MZUSP Nos. 95403, 95411, and 95415 (=field codes PU-3, PU-20, and PU-24); P. cf. castexi, MZUSP Nos. 95404–95405, 95407–95410, and 95412–95414 (=field codes PU-7, PU-8, PU-13, PU-16, PU-17, PU-19, PU-21, PU-22, and PU-23).

Intestinal helminths

Table I summarizes the helminth species encountered in the spiral intestines of the 3 host species examined, along with the number of individuals infected and prevalence and intensity of infection. Cestode species designated with numbers are likely new species, based on preliminary morphological data (see Reyda, 2007), but were not formally described here because of insufficient material. Each of the helminth species listed in Ta­ble I is reported here for the first time for Peru.

In addition to the helminth species listed in Table I, proteo cephalidean larvae were present as hyperparasites in the fol­lowing tetrathyridial cestode species: N. guarticus n. gen. n. comb., Rhinebothrium copeianum n. sp., Rhinebothrium sp. 1, and Potamotrygonocestus cf. fitzgeraldae in P. aierea; Rhine­bothrioides sp. 1 in P. motoro, and Rhinebothrioides sp. 2 in P. cf. castexi (reported in Reyda and Olson, 2003).

DESCRIPTIONS

Nandocestus n. gen.

Diagnosis

Phyllobothriidae. Worms euapolytic. Scolex with 4 sessile bothridia; each bothridium with rim, single apical sucker, and numerous marginal loculi, lacking facial septa. Cephalic peduncle with indistinct posterior boundary; cephalic peduncle and strobila scutellate; scutes irregularly overlapping, consisting of densely packed, long filiriches. Terminal proglottid longer than wide. Tests medullary, anterior to ovary, 1–2 rows deep in cross section. Genital pores submarginal. Cirrus sac elongate oval, containing cirrus with filitriches and spinitriches. Vaginal opening anterior to cirrus sac into genital atrium; seminal receptacle present. Urinary ventral in proglottid, extending from ootype to level of genital atrium. Ovary lobulated, H-shaped in frontal view, tetralobed in cross section. Vitellaria follicular; follicles circummedullary, partially interrupted by uterus, ovary, and cirrus sac. Eggs spherical or semi­spherical, with punctate shell. Parasites of potamotrygonid stingrays. Type and only species: Nandocestus guarticus Marques, Brooks and Lasso, 2001 n. gen. n. comb.

Nandocestus guarticus (Marques, Brooks and Lasso, 2001) n. comb. (Cestoda: Tetrathyrididea) (Figs. 1–17)

Redescription

Redescription based on holotype and 1 paratype of Anindobothrium guarticus and the following newly collected voucher material: whole mounts of 7 worms, 3 free mature and 4 free gravid proglottids, cross sections of 1 scolex and 2 free proglottids, longitudinal sections of 1 scolex, 3 scolecis and 2 proglottids prepared for SEM. Worms (Fig. 3) euapolytic, 13–28 (21 ± 5; 7) mm long, greatest width at level of scolex or near terminal proglottid; 89–132 (105 ± 17; 8) proglottids per worm. Scolex (Figs. 1, 7) 2,300–4,850 (3,218 ± 827; 7) long, consisting of scolex proper and extensive cephalic peduncle with inconspicuous posterior boundary. Scolex proper 580–900 (721 ± 103; 8) wide, maximum width at midlevel, bearing 4 sessile bothridia; each bothridium with rim, 400–600 (470 ± 69; 7) long by 290–420 (363 ± 46; 6) wide, with single apical sucker and 35–43 (38 ± 3; 6; 7) marginal loculi. Apical
### TABLE I. Host-parasite list for helminths found parasitizing the spiral intestine of stingrays in southeastern Peru.

<table>
<thead>
<tr>
<th></th>
<th>No. individual stingrays examined</th>
<th>No. individuals infected (% prevalence)</th>
<th>Minimum-maximum intensity (mean intensity ± SD)*</th>
<th>Museum specimens deposited</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paratrygon aiereba:</strong></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cestoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nandrocestus guarticus</em> n. gen. n. comb.</td>
<td>3 (75%)</td>
<td>5−35 (18.3 ± 15.3)</td>
<td>see text</td>
<td></td>
</tr>
<tr>
<td>Rhinebothrium copianullum n. sp.</td>
<td>3 (75%)</td>
<td>9−12 (10.3 ± 1.5)</td>
<td>see text</td>
<td></td>
</tr>
<tr>
<td>Rhinebothrium sp. 1</td>
<td>4 (100%)</td>
<td>1−36 (19.5 ± 14.5)</td>
<td>LRP Nos. 4092–4099</td>
<td></td>
</tr>
<tr>
<td>Rhinebothriodes sp.</td>
<td>4 (100%)</td>
<td>1−3 (1.5 ± 1.0)*</td>
<td>LRP No. 4100</td>
<td></td>
</tr>
<tr>
<td>Potamotrygonocestus cf fitzgeraldae</td>
<td>4 (100%)</td>
<td>2−5 (2.8 ± 1.5)</td>
<td>LRP Nos. 4101–4105</td>
<td></td>
</tr>
<tr>
<td>Nematoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cucullanus</em> sp. 1†</td>
<td>2 (50%)</td>
<td>1−2 (1.5 ± 0.7)</td>
<td>LRP No. 4106</td>
<td></td>
</tr>
<tr>
<td><em>Rhabdochona</em> sp. 1†</td>
<td>1 (25%)</td>
<td>1</td>
<td>LRP No. 4107</td>
<td></td>
</tr>
<tr>
<td><strong>Potamotrygon motoro:</strong></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cestoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acanthobothrium peruviense n. sp.</td>
<td>3 (43%)</td>
<td>1−25 (9.0 ± 13.9)</td>
<td>see text</td>
<td></td>
</tr>
<tr>
<td>Acanthobothrium cf ramiroi</td>
<td>2 (29%)</td>
<td>2.0 ± 0</td>
<td>LRP No. 4112</td>
<td></td>
</tr>
<tr>
<td>Rhinebothrium sp. 1</td>
<td>1 (14%)</td>
<td>3*</td>
<td>LRP Nos. 4113–4114</td>
<td></td>
</tr>
<tr>
<td>Rhinebothriodes sp. 1</td>
<td>6 (86%)</td>
<td>4−506 (142.0 ± 190.3)</td>
<td>LRP Nos. 4115–4120</td>
<td></td>
</tr>
<tr>
<td>Potamotrygonocestus sp.</td>
<td>1 (14%)</td>
<td>1</td>
<td>LRP No. 4121</td>
<td></td>
</tr>
<tr>
<td>Paraoncomesaraya (Woodland, 1934) Campbell, Marques &amp; Ivanov, 1999</td>
<td>5 (71%)</td>
<td>21−91 (50.2 ± 29.9)</td>
<td>LRP Nos. 4122–4128</td>
<td></td>
</tr>
<tr>
<td>Nematoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cucullanus</em> sp. 1†</td>
<td>2 (29%)</td>
<td>1.0 ± 0</td>
<td>LRP No. 4129</td>
<td></td>
</tr>
<tr>
<td>Brevimulticaecum regoi Sprent, 1990</td>
<td>3 (43%)</td>
<td>1−9 (3.7 ± 4.6)</td>
<td>LRP Nos. 4130–4132</td>
<td></td>
</tr>
<tr>
<td>Digenea</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diplostomoidea sp.†</td>
<td>1 (14%)</td>
<td>1*</td>
<td>LRP No. 4133</td>
<td></td>
</tr>
<tr>
<td><strong>Potamotrygon cf castexi:</strong></td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cestoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nandrocestus guarticus</em> n. g. n. comb.†</td>
<td>1 (7%)</td>
<td>1*</td>
<td>LRP No. 4134</td>
<td></td>
</tr>
<tr>
<td>Acanthobothrium cf peruviense</td>
<td>3 (21%)</td>
<td>1−3 (2.0 ± 1.0)</td>
<td>LRP Nos. 4135–4136</td>
<td></td>
</tr>
<tr>
<td>Potamotrygonocestus sp. 1</td>
<td>4 (29%)</td>
<td>2−7 (3.5 ± 2.4)</td>
<td>LRP Nos. 4137–4144</td>
<td></td>
</tr>
<tr>
<td>Rhinebothrium sp. 1</td>
<td>1 (7%)</td>
<td>8</td>
<td>LRP Nos. 4145–4146</td>
<td></td>
</tr>
<tr>
<td>Rhinebothriodes sp. 2</td>
<td>6 (43%)</td>
<td>1−91 (20.2 ± 35.1)</td>
<td>LRP Nos. 4147–4152</td>
<td></td>
</tr>
<tr>
<td>Paraoncomesaraya (Woodland, 1934) Campbell, Marques &amp; Ivanov, 1999</td>
<td>1 (7%)</td>
<td>97</td>
<td>LRP No. 4153</td>
<td></td>
</tr>
<tr>
<td>Nematoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cucullanus</em> sp. 1†</td>
<td>3 (21%)</td>
<td>1−3 (2.0 ± 1.0)</td>
<td>LRP No. 4154</td>
<td></td>
</tr>
<tr>
<td>Brevimulticaecum regoi†</td>
<td>3 (21%)</td>
<td>1−4 (2.0 ± 1.7)</td>
<td>LRP Nos. 4155–4156</td>
<td></td>
</tr>
<tr>
<td><em>Rhabdochona</em> sp. 1†</td>
<td>6 (43%)</td>
<td>1−7 (3.3 ± 2.9)</td>
<td>LRP Nos. 4157–4158</td>
<td></td>
</tr>
<tr>
<td>Echinoccephalus dalleyi Deardorff, Brooks &amp; Thorson, 1981†</td>
<td>1 (7%)</td>
<td>7</td>
<td>LRP No. 4159</td>
<td></td>
</tr>
<tr>
<td>Procamallanus (Spirocamallanus) sp.†</td>
<td>1 (7%)</td>
<td>1</td>
<td>LRP No. 4160</td>
<td></td>
</tr>
<tr>
<td>Digenea</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemiuroida sp.†</td>
<td>1 (7%)</td>
<td>1*</td>
<td>LRP No. 4161</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates immature specimen(s).
† Indicates a new host record.

sucker 70−90 (78 ± 6; 6; 12) in diameter; marginal loculi 45−80 (53 ± 15; 5) wide. Cephalic peduncle 2,000−4,500 (2,882 ± 817; 7) long, slightly expanded at junction with scolex proper.

Proximal surfaces of bothridia covered with moderately long filitriches. Proximal surfaces of marginal loculi covered with moderately long filitriches and cyrillate (jig-shaped) spinitriches (Fig. 11). Edges of bothridial rims covered with long filitriches (Fig. 10). Distal surfaces of bothridia, apical suckers, and marginal loculi covered with long filitriches and serrated spinitriches (Fig. 8). Cilia distributed throughout bothridial rim and distal bothridial surfaces (Fig. 9). Cephalic peduncle scutellate; scutes irregularly overlapping (Fig. 12), consisting of densely packed long filitriches.

Proglottids craspedote. Terminal mature proglottids 830−1,400 (1,024 ± 177; 7) long by 330−870 (573 ± 223; 7) wide, length-to-width ratio 1.1−2.8 (2 ± 0.6; 7). Genital pores submarginal, irregularly alternating, 60−80% (71 ± 7; 7) of proglottid length from posterior end. Testes irregularly oval, 40−110 (64 ± 15; 11; 26) long by 30−75 (45 ± 12; 11; 26) wide, 1−2 layers deep, 141−190 (169 ± 18; 8) in total number.
Figures 1–4. Line drawings of *Nandocestus guariticus* (Marques, Brooks and Lasso, 2001) n. gen. n. comb. (1) Scolex. (2) Free mature proglottid. Arrows indicate locations of sections shown in Figures 5–6. (3) Anterior (A) and posterior (B) portions of whole worm. Arrow indicates approximate posterior extent of cephalic peduncle. (4) Egg.
Taxonomic summary


Type host: Paratrygon aiereba (Müller and Henle, 1841), Discus ray.

Additional host: Potamotrygon cf. castexi.

Type locality: Caño Guaritico, Hato El Frío, Orinoco Basin, Venezuela (07°52′N, 69°20′W).

Additional locality: Madre de Dios River at Boca Manu, Madre de Dios Department, Peru (12°17′04″S, 70°53′08″W).

Site of infection: Spiral intestine.

Prevalence of infection: Three of 4 host individuals sampled in this study.

Museum specimens examined: Holotype (MNHN No. 6215) and paratype (MNHN No. 6216) of A. guaricus; paratype (HWML No. 20265) of Anindobothrium anaconum (Brooks, 1977) Marques, Brooks, and Lasso, 2001; paratype (HWML No. 16379) of Anindobothrium lisanus Marques, Brooks, and Lasso, 2001.

Remarks: Anindobothrium Marques, Brooks, and Lasso, 2001, was erected by Marques et al. (2001) to house 3 species possessing sessile or pedicellated bothridia that either possess or lack marginal loculi, with poorly differentiated apical suckers. Anindobothrium included the marine phyllobothrid Caulobothrium anaconum Brooks, 1977, which became the type species of the genus, as well as 2 additional species, A. lisanus, from Potamotrygon orbignyi (Castelnau, 1855) from the Rio Negro, and A. guaricus, from P. aiereba from the Orinoco River system. The specimens collected in the current study are morphologically consistent with the holotype and paratype of A. guaricus, and were also collected from the type host P. aiereba, but from the Amazon River Basin.

Examination of the holotype and 1 of the paratypes of the species described as A. guaricus by Marques et al. (2001) suggests that a number of contradictions exist between the morphology of the scolex, proglottids, cirrus sac, and ovary as reported in the original description, and the conditions of these features seen in the type specimens. For example, the scolecids of 2 specimens in the type series had fewer marginal loculi than the approximately 46 reported by Marques et al. (2001); there are 39 marginal loculi in the holotype and 43 in the paratype examined here. The proglottids were craspedote rather than acras-
pedote, as stated by Marques et al. (2001). The anteroventral lobes of the ovary do not converge anteriorly, as stated in the original description but not illustrated in the line drawings, and the ovary is lobulated, rather than follicular.

The type specimens of A. guaricus are morphologically inconsistent with the generic diagnosis for Anindobothrium in 6 characteristics. The bothridia of the type specimens lack transverse loculi, rather, they possess only marginal loculi. The vas deferens in the type specimens are not expanded to form a seminal vesicle; rather they are highly coiled on the median side of the cirrus sac (see Fig. 2). In addition, the vas deferens in the type specimens enter the cirrus sac medially (aporally) (see Fig. 2), not laterally (aporally) as suggested by Marques et al. (2001). Anindobothrium is also diagnosed as possessing lateral vitelline follicles, a vaginal sphincter, and marginal genital pores. However both type specimens examined possess circummedullary vitelline follicles, lack a vaginal sphincter, and exhibit submarginal genital pores. Unlike the type specimens of A. guaricus, the paratypes of A. anacolum and A. lisae that were examined conform to the generic diagnosis of Anindobothrium in possessing transverse loculi and lateral vitelline follicles.

These inconsistencies call into question the generic placement of this species, the morphology of which is also inconsistent with that of all other known phyllobothrid genera. Among phyllobothrid genera, it most closely resembles Cardiobothrium McKenzie and Caira, 1998, Anthocephalum Linton 1890, and Orectolobicestus Ruhnke, Caira, and Carpenter, 2006 in its possession of a single apical sucker and marginal bothridial loculi. However, it differs from all of these taxa in its possession of circummedullary, rather than lateral, vitelline follicles. It further differs from species of Cardiobothrium in lacking facial loculi. Nandocestus further differs from species of Anthocephalum in its possession of a submarginal, rather than lateral, genital pore, and in its possession of a uterus that extends only anteriorly to the level of the genital pore, rather than to the anterior margin of the proglottid. In addition, Nandocestus possesses an extensive cephalic peduncle, where as species of Anthocephalum lack a cephalic peduncle. Its morphology most closely resembles that of species of Orectolobicestus, but further differs from this taxon in having a submarginal rather than lateral genital pore, and in having serrated spinitriches rather than massiform spinitriches on the distal bothridial surfaces. As a consequence, Nandocestus is erected here to house this species. The new genus is monotypic at this time. As a result of this action, Anindobothrium currently consists of 2 species, A. anacolum and A. lisae.

**Rhinebothrium copianulum n. sp. (Cestoda: Tetraphyllidea)**

(Figs. 18–32)

### Diagnosis

Based on whole mounts of 10 worms, 2 free gravid proglottids, 2 egg mounts, cross sections of 3 proglottids, longitudinal sections of 1 scolex, and 2 scolices and 5 proglottids prepared for SEM. Worms (Fig. 21) curopalypotic, crespedate, 30–68 (49 ± 12; 9) mm long, greatest width 830–1,250 (1,089 ± 127; 8) at level of scolex; 456–880 (643 ± 152; 6) long, perigon proglottids per worm. Scolices (Figs. 18, 24) consisting of scolex proper bearing 4 stalked bothridia and inconsiderable cephalic peduncle. Bothridia with muscular rims, constricted at center, 900–1,000 (941 ± 33; 6) long, 410–460 (443 ± 22; 4) wide, divided by 39–43 (41 ± 2; 3) transverse septa and 1 longitudinal septum into 79–87 (83 ± 4; 5) loculi; anterior and posterior halves of each bothridium approximately equal in width. Longitudinal septum extending from posterior margin of anteriormost loculus to posterior margin of bothridium; anteriormost loculus single, 35–45 (42 ± 6; 3) long by 45–57 (52 ± 5; 4) wide; widest loculus 205–234 (227 ± 4; 8) long by 215–250 (234 ± 16; 5) wide; posteriormost loculi double, 40–45 (42 ± 3; 2; 5) long by 30–37 (34 ± 3; 2; 5) wide. No marginal loculi observed. Stalks 81–150 (125 ± 33; 4) long by 200–309 (238 ± 42; 4; 5) wide, attached to bothridia at middle or slightly posterior to middle. Cephalic peduncle 100–250 (169 ± 54; 6) long.

Proximal surfaces of bothridia covered with long filitriches and blade-like spinitriches (Fig. 25), except for edges and except for narrow bands that correspond to position of transverse septa on distal bothridial surfaces (Fig. 26), which bear only long filitriches. Distal surfaces of bothridia with long filitriches and spinitriches throughout, including surfaces of longitudinal and transverse septa (Fig. 27), with edges of loculi bearing only long filitriches. Bothridial rim with long filitriches. Stalks with long filitriches and blade-like spinitriches (Fig. 28) and strobila (Fig. 29) with long filitriches.

Greatest proglottid width 550–1,200 (699 ± 200; 9) in posterior third of strobila. Majority of proglottids wider than long; posteriormost 1–17 (9 ± 7; 8) proglottids longer than wide; mature proglottids 55–122 (85 ± 28; 8) in number, including 0–49 (20 ± 18; 7) proglottids with egg-filled vas deferens.

Terminal proglottid (Fig. 20): 610–1,150 (880 ± 197; 9) long, 360–510 (418 ± 63; 9) wide, length-to-width ratio 1.2–3.0 (2.2 ± 0.7; 9) (Fig. 20). Genital pores marginal, irregularly alternating, 67–79% (72 ± 4; 8) of proglottid length from posterior end. Testes often atrophied in posteriormost proglottids; testes in terminal proglottids irregularly oval, 60–115 (87 ± 15; 8; 27) long by 40–90 (63 ± 16; 8; 27) wide, all in primary field, 6–12 (8 ± 2; 10; 29) in total number, 1–2 layers deep, in as many as 4 irregular columns, extending from near anterior margin of proglottid to anterior margin of ovary. Vas deferens in terminal proglottids conspicuously coiled; coils spanning from anterior margin of proglottid posteriorly to ovarian isthmus. Vas deferens entering cirrus sac at anterior margin. Cirrus sacs elongate oval or triangular, slender in subterminal mature proglottids (Fig. 19), extending medially to, or well past, midline of proglottid, extending posteriorly to anterior ovarian margin or to ovarian isthmus, containing coiled cirrus with filitriches and spinitriches on base. Cirrus sac in terminal proglottids 280–380 (328 ± 37; 9) long by 140–190 (163 ± 15; 9) wide. Everted cirrus (Fig. 30) 260–440 (374 ± 65; 6; 7) long including expanded base; base 90–170 (114 ± 29; 6; 7) wide, with long filitriches and with large scolopate (thornlike) spinitriches; large scolopate spinitriches of cirrus base 8.5–15 (9.0 ± 0.4; 2; 6) in length (Fig. 31), distal portion of cirrus 50–75 (65 ± 11; 5; 6) wide, with long filitriches and short scolopate spinitriches (Fig. 32); small scolopate spinitriches on cirrus distal portion approximately 1.3 long. Vagina thick-walled, sinuous, varying in width, with anterior kink at point where it turns posteriorly, with darkly staining cells in walls, extending laterally from common genital atrium, then posteriorly along medial line of proglottid to ootype; vaginal sphincter observed. Proximal portion of vagina expanded into seminal vesicle. Ovary not illustrated in proglottids, usually the type specimens examined possess circummedullary vitelline follicles, but not illustrated in the line drawings, and the ovary is lobulated, rather than follicular. Vitelline follicles of ovary do not converge anteriorly, as stated in the original description.

Type host: Paratrygon aiereba (CHIOC 31.213a, c-e) and para­type (CHIOC 31.213a, b) of Rhinebothrium paratrygoni Rego and Dias, 1976.

Type locality: Madre de Dios River at Boca Manu, Madre de Dios Department, Peru (12°17.047’S, 70°53.086’W). Additional locality: None.

Site of infection: Spiral intestine.

Prevalence of infection: Three of 4 host individuals sampled in this study.

### Museum specimens examined:

Holotype (CHIOC 31.213a) and para­types (CHIOC 31.213a, c-e) of Rhinebothrium paratrygoni Rego and Dias, 1976. Holotype: USNPC No. 99943. Paratypes: USNPC No. 99944; LRP Nos. 4082–4091 (including whole mounts, SEM specimens, and histology vouchers); MZUSP Nos. 6392a–6392d; MHNPI Nos. 2333–2334.

### Etymology:

This species is named for its copious number of proglottids.
FIGURES 18–23. Line drawings of Rhinebothrium copianulum n. sp. (18) Scolex. (19) Subterminal mature proglottid. (20) Terminal mature proglottid with sperm filled vas deferens. (21) Anterior (A), Middle (B), and Posterior (C) portions of whole worm. Arrows: 21B, first proglottid with visible genitalia; 21C, left arrow, first mature proglottid; right arrow, first mature proglottid with sperm filled vas deferens. (22) Cross section through terminal mature proglottid with sperm filled vas deferens, at anterior portion of ovary. (23) Egg. Cirrus sac (CS); ovary (O); uterus (U); vitellaria (V); vagina (VA); vas deferens (VD).
Remarks

*Rhinobothrium copianullum* n. sp. can be readily distinguished from most species of *Rhinobothrium* considered valid by Healy (2006b) in its possession of a much greater number of proglottids. Whereas *R. copianullum* n. sp. possesses 456–880 proglottids, 30 species of *Rhinobothrium* each possess <120 proglottids.

*Rhinobothrium copianullum* n. sp. can be readily distinguished from the other 9 species of *Rhinobothrium* as follows. It exhibits a greater...
number of bothridial loculi than Rhinebothrium ceylonicum Shipley and Hornell, 1906. Rhinebothrium denticulatum Shipley and Dailey, 1977, Rhinebothrium maccallumii Linton, 1924, Rhinebothrium monodi Euzet, 1954, and Rhinebothrium walga (Shipley and Hornell, 1906) Euzet, 1959 (79–87 vs. 40, 48–54, 31, 17, and 48, respectively). Rhinebothrium copianullum n. sp. is longer (30–68 vs. up to 5 mm) and has a larger cirrus sac (280–380 long and 140–190 wide vs. 160 long and 90 wide) than Rhinebothrium euzeti Williams, 1958. Rhinebothrium copianullum n. sp. has fewer testes than Rhinebothrium chilensis Euzet and Carvajal, 1973 and Rhinebothrium leiblei Euzet and Carvajal, 1973 (6–12 vs. 35–45 and 36–46, respectively).

Rhinebothrium copianullum n. sp. most closely resembles R. parastrygoni, the only species of Rhinebothrium reported from South American freshwater stingrays, in its possession of a large number of proglottids (456–880 vs 682 as measured in current study) and in that most of its mature proglottids are wider than long. The holotype of R. parastrygoni is immature; therefore, comparisons were made based on the paratypes. Rhinebothrium copianullum n. sp. can be distinguished from R. parastrygoni in its possession of larger bothridia (900–1,000 long by 410–460 wide vs. 675 long by 420 wide), a wider strobila (maximum width 550–1,200 vs. up to 300), and much larger blade-like cirrus spin- itriches (8.3–9.5 vs. 2–3 as measured in current study).

Free proglottids of R. copianullum n. sp. were frequently observed in copula in the present study.

**Acanthobothrium peruvianum n. sp. (Cestoda: Tetrahyllideidae)** (Figs. 53–43)

**Diagnosis**

Based on whole mounts of 7 worms and 2 worms prepared for SEM. Worms (Fig. 36) euapolytic, 6–10 (± 1; 5 mm) long, greatest width at level of scolex; 34–57 (43 ± 9; 5) proglottids per worm. Scolex (Figs. 33, 38) consisting of scolex proper and conspicuous cephalic peduncle. Scolex proper with 4 bothridia (15; 3) long by 440–600 (513 ± 85; 4) wide. Bothridia free posteriorly, 190–240 (222 ± 25; 5) wide; each with specialized anterior region in form of muscular pad and 3 loculi; muscular pad with free lateral margins, 115–130 (123 ± 8; 3) long by 125–150 (134 ± 12; 4) wide, weakly triangular in shape (Fig. 39), bearing apical sucker and 1 pair of hooks; apical sucker 50–62 (54 ± 7, 3) long by 50–70 (56 ± 8; 5) wide; anterior loculus 225–250 (241 ± 12; 4; 3) long; middle loculus 65–75 (73 ± 5; 4) long; posterior loculus 70–112 (98 ± 9; 4; 6) long; loculus length ratio 1.0:2.6–3.2 (0.29 ± 0.03; 3) 0.33–0.38 (0.36 ± 0.04; 2); maximum width of scolex at level of posterior portion of anterior loculus. Velum present. Hooks (Figs. 34, 39) delicate, bipronged, hollow, with tubercle on proximal surface of axial prongs; internal channels of axial and abaxial prongs continuous, smooth; axial prongs conspicuously longer than abaxial prongs; lateral and medial hooks approximately equal in size. Hook bases enclosed in a dark granular material. Lateral hook measurements: A = 43–53 (46 ± 4; 5), B = 98–105 (101 ± 3; 3; 4), C = 70–78 (74 ± 3; 4), D = 133–143 (136 ± 5; 3; 4). Medial hook measurements: A′ = 43–48 (45 ± 2; 4; 5), B′ = 100–113 (104 ± 6; 3; 4), C′ = 63–70 (66 ± 4; 3), D′ = 138–150 (142 ± 6; 3; 4). Bases of lateral and medial hooks approximately equal in length, abutting along medial axis of bothridium. Thin layer of tissue covering proximal region of each prong of both hooks. Cephalic peduncle 240–480 (354 ± 92; 5) long.

Apex of scolex and distal bothridial surfaces with short filitriches (Fig. 40). Long filitriches observed along bothridial rims (Fig. 41) at level of anterior and middle loculus. Proximal bothridial loculi, 28–30 (29 ± 1; 2) in diameter. Vitellarian follicular, consisting of 2 lateral fields; each lateral field consisting of 1 dorsal and 1 ventral column; fields extending from level of ovarian isthmus to near anterior margin of testes, interrupted by vagina and cirrus sac. Vitelline follicles 5–15 (11 ± 3; 5; 13) long by 2–10 (6 ± 2; 13) wide. Uterus thick walled, saciform, extending from level of ovarian isthmus anteriorly, stopping short of anterior margin of testes. Excretory ducts lateral. Eggs not observed.

**Taxonomic summary**

Type host: Potamotrygon motoro (Müller and Henle, 1841), Ocellate river stingray.

Additional hosts: None.

Type locality: Madre de Dios River at Boca Manu, Madre de Dios Department, Peru (12°16.350’S, 70°55.789’W).

Additional localities: None.

Site of infection: Spiral intestine.

Prevalence of infection: Three of 7 host individuals sampled in this study.


Holotype: USNPC No. 99945.

Paratypes: USNPC No. 99946; LRP Nos. 4108–4111 (including whole mounts and SEM specimens); MZUSP Nos. 6393a–6393b; MNHP No. 2335.

Etymology: This species is named after Peru, the country of the type locality.

**Remarks**

Because it has 34–57 proglottids, A. peruvianum n. sp. falls on the boundary of 1 of the 4 characters used to categorize species of Acanthobothrium (Ghoshroy and Caira, 2001; Character 2: fewer or greater than 50 proglottids) and thus is either a category 1 or category 8 species. There are 29 Category 1 and 3 Category 8 species of Acanthobothrium recognized (Ghoshroy and Caira, 2001; Fryer and Caira, 2006; Reyda and Caira, 2006). Because of its unusual triangle-shaped cirrus sac in mature proglottids, A. peruvianum n. sp. can be distinguished easily from 28 of the 30 Category 1 species of Acanthobothrium for which cirrus sac information is available; these 28 species possess cirrus sacs that are either pyriform or bent posteriorly, or are globose, spherical, or saccate. Acanthobothrium peruvianum n. sp. differs from the remaining Category 1 species of Acanthobothrium, in which cirrus sac data were not available, by other features. Acanthobothrium peruvianum n. sp. differs from Acanthobothrium guptai Shinde and Bhagwan, 2002 in its possession of narrower proglottids (200–280 vs. 485–495) and a longer scolex (440–470 vs. 168). It especially differs from Acanthobothrium paulum Linton, 1890 by having shorter bothridia (310–400 vs. 500–800).

Acanthobothrium peruvianum n. sp. can be differentiated from all 3 of the Category 8 species of Acanthobothrium recognized by Ghoshroy...
and Caira (2001) and by Fyler and Caira (2006) as follows. It has considerably more testes than Acanthobothrium etini Fyler and Caira, 2006 (43–59 vs. 11–20). It has smaller hooks than Acanthobothrium franus Marques, Centritto, and Stuart, 1997 (133–143 vs. 354–465 total lateral hook length). In addition, A. peruvianum n. sp. has a noticeably greater number of testes than Acanthobothrium parvuncinatum Young, 1954 (43–59 vs. 12–14).

Acanthobothrium peruvianum n. sp. belongs to a different category than the 5 other species of Acanthobothrium which parasitize potamoglypnid stingrays (Ghoshroy and Caira, 2001; Ivanov, 2005). It can be distinguished from Acanthobothrium ramiroi Ivanov, 2005 and A. ter- ezae in that the latter 2 species are much larger worms (6–10 mm vs. 50–84.5 mm and 88–110 mm, respectively), and have stubby medial and lateral hooks that are asymmetrical, rather than hooks that are more elongate and symmetrical. Acanthobothrium peruvianum n. sp. can be distinguished from A. regoi in its possession of fewer proglottids (34–57 vs. 87–120), a shorter overall length (6–10 mm vs. up to 45 mm), and a shorter scolex proper (440–470 vs. 700–900). Acanthobothrium
DISCUSSION

The present study is the first to provide data on intensity of infections of helminths from South American freshwater stingrays (Table I). Despite the relatively small number of individuals of each stingray species examined, the data in Table I suggest the following. Cestode species infections generally occurred at greater intensities than nematodes or digeneans, and differed among congeneric cestodes in different host species. For example, Rhinebothroides sp. in P. aicreba had a mean intensity of 1.5, whereas Rhinebothroides sp. 1 in P. motoro had a mean intensity of 142.0. Moreover mean intensities differed among cestode species within the same host species, i.e., Acanthobothrium cf. peruvianus had a mean intensity of 20.0 in P. cf. castexi, whereas Rhinebothroides sp. 2 in the same host species had a mean intensity of 20.2. In addition, the mean intensities of species of onchobothrid cestodes, i.e., species of Acanthobothrium and Potamotrygonocestus, were generally lower than those of species of phyllobothrid cestodes, i.e., spe-

peruvianus n. sp. can be distinguished from A. amazonensis by its smaller size (6–10 mm vs. up to 35 mm in A. amazonensis), and by having fewer proglottids (34–57 vs. 75–100). Also, the cirrus sac of A. amazonensis is pyriform rather than triangular shaped. Among Acanthobothrium species parasitizing South American freshwater stingrays, A. peruvianus n. sp. most closely resembles A. quinonesi. However, whereas the cirrus sac of A. quinonesi is curved anteriorly, it is triangular shaped in A. peruvianus n. sp. In addition, A. peruvianus n. sp. is a smaller worm (6–10 mm vs. up to 25 mm) than A. quinonesi. The terminal proglottids of A. peruvianus n. sp. are narrower than those of A. quinonesi (200–280 vs. 330–390), the bothridia in A. peruvianus n. sp. are narrower (190–240 vs. 282–310) and the anterior (225–250 vs. 158–226) and posterior (95–120 vs. 65–85) bothridial loculi of A. peruvianus n. sp. are longer than those seen in A. quinonesi.

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cies of Nandocestus, Rhinebothrium, and Rhinebothroides. Finally, species of Rhinebothrium had greater mean intensities in *P. aierba* than in *P. motoro* and *P. cf. castexi*, and species of Rhinebothroides had greater mean intensities in *P. motoro* and *P. cf. castexi* than in *P. aierba*.

Two nematode species, *B. regoi* and *E. daileyi*, have been reported from potamotrygonids elsewhere. *Brevimulticaecum regoi* was originally described as *Brevimulticaecum* sp. by Rego (1979) from the stomach of *P. motoro* in a tributary of the Paraguay River and later described by Spret (1990). The present study expands the records of both the geographic range and host distribution of *B. regoi*. Here, *B. regoi* was found in the spiral intestine of the same host. It is also reported from *P. cf. castexi*. This is the first report of *B. regoi* from the Amazon basin. Little is known about the biology of *B. regoi* or its congeners, which are generally parasites of crocodilians and freshwater fish (Moravec, 1998). *Echinoccephalus daileyi* was originally described from the intestine anterior to the spiral chambers of *Potamotrygon circularis* (= *Potamotrygon constellata* [Vaillant, 1880]) in the Amazon River Basin in Colombia, and from *Potamotrygon histrix* (Müller and Henle, 1834) (see Marques, 2000 for comments on host identification) in the Orinoco River Basin in Venezuela (Deardorff et al., 1981). Here, *E. daileyi* was found in *P. cf. castexi*, a new host record. Species of *Echinoccephalus* Molin, 1858, are parasites of rays and other elasmobranchs (Moravec, 1998).

It is unclear whether the other 3 species of nematodes found in the present study represent accidental infections. Only a single specimen of *Procamaillanus* (Spirocamaillanus) sp. was found in this study in *P. cf. castexi*. The other 2 species of *Procamaillanus* Baylis, 1923 reported from potamotrygonids (ex *P. motoro*) were considered accidental by Moravec (1998). This is the first report of species of *Cucullanus* Müller, 1777 from potamotrygonids. Specimens of *Cucullanus*’ sp. 1 were found in each of the 3 stingray species, although no males were found. Species of *Cucullanus* are generally considered as parasites of bony fishes and chelonians (Moravec, 1998). Species of *Rhabdochona Railliet, 1916* are parasites of freshwater fish (Moravec, 1998); none has previously been reported in potamotrygonids before this study.

Prior to the present study, the only species of digenean known from potamotrygonid stingrays was the hemiuriid, *Thometrema overstreeti* (Brooks, Mayes, and Thorson, 1979) Lunaschi, 1989, which was originally reported by Brooks, Mayes, and Thorson (1979) from the stomach of *Potamotrygon macdalenae* (Valenciennes, 1865). The single hemiuriid species encountered here in *P. cf. castexi* may be accidental. The diplostomid that was encountered in a single *P. motoro* is an unusual finding. Diplostomoids typically occur in the gut of mammals, birds, or reptiles (Niewiadomska, 2002). The individual *P. motoro* that was infected with the diplostomid may have acquired the worm by consuming a teleost intermediate host.

The cestode species described here increases the total number of cestode species and genera represented in potamotrygonids to 24 and 7, respectively. *Nandocestus guariticus* is now known from 2 localities, 1 in the Orinoco River Basin (Marques et al., 2001), and 1 in the Upper Amazon River Basin (present study). *Paratrygon aierba*, the only host species in which *N. guariticus* is known to reach maturity, occupies the Orinoco River Basin as well as the Ucayali, Solimões, Amazon, Negro, Branco, Madeira, and Tocantins rivers of the Amazon basin (de Carvalho et al., 2003). *Nandocestus guariticus* may also occur in these rivers. However, the currently known distribution of *N. guariticus* corresponds to the paleo-Amazonas-Orinoco River Basin that was formed during the early Tertiary period (Lundberg et al., 1998), a region that has been exposed to multiple marine incursions throughout its history (Lundberg et al., 1998). *Paratrygon aierba* is considered to represent the most basal lineage of the Potamotrygonidae Garm, 1877 (see Lovejoy, 1996; Lovejoy et al., 1998). *Nandocestus* is monotypic and, although it has several morphological features in common with other phyllobothriid genera such as *Orectolobiceps* (parasites of sharks, see Ruhnke et al., 2006), its phylogenetic relationships with other phyllobothriids or tetraphyllideans are yet to be investigated. Identification of the sister taxon of *N. guariticus* may shed light upon the mechanism of freshwater colonization of this intriguing cestode species.

*Rhinebothrium copianullum* is the second species of the genus reported from potamotrygonids. The only other species, *R. paratrygoni*, was described by Rego and Dias (1976), based on specimens deposited at the Coleção Helminológica do Instituto Oswaldo Cruz (Rio de Janeiro, Brazil) between 1938 and 1940. The type specimens were reportedly collected from the Salobra River (a tributary of the Paraguay River) from a potamotrygonid species referred to as *Elipesurus* sp. (Rego and Dias, 1976). However, *Elipesurus* Jardine, 1843, is now considered to be a genus inquirendum (see de Carvalho et al., 2003). Thus, the type host of *R. paratrygoni* is unknown. Based on their occurrence in the Paraná River Basin (de Carvalho et al., 2003), any of 6 stingray species are potential candidates, i.e., *Potamotrygon brachyura* ( Günther, 1880), *P. cf. castexi*, *Potamotrygon falkneri* Castex and Maciel, 1963, *P. histrix*, *P. motoro*, and *Potamotrygon schuhmacheri* Castex, 1964. Since its original description (Rego and Dias, 1976), *R. paratrygoni* has been reported from 8 additional species of potamotrygonids (Brooks, Mayes, and Thorson, 1981; Brooks and Amato, 1992; Marques, 2000) and from several additional localities in the Paraná River Basin (Brooks, Mayes, and Thorson, 1981; Brooks and Amato, 1992) as well as several other rivers, including the Orinoco (Brooks, Mayes, and Thorson, 1981) and the Tocantins, Xingú, Negro, and Lower Amazon of the Amazon River Basin (Marques, 2000). The discovery of the new species *R. copianullum* raises the possibility that the specimens reported as *R. paratrygoni* in several previous studies are new.

The reportedly low level of host specificity of *R. paratrygoni* strongly contradicts the patterns of host specificity documented for numerous species of tetraphyllideans from marine elasmobranchs (Williams, 1969; Caira, 1990; Ruhnke, 1994; Caira and Jensen, 2001; Healy, 2006a). In fact, data for the tetraphyllidean family Onchobothriidae indicate that the vast majority of the 201 species known in 2001 are oioxenous, i.e., restricted to a single host species (Caira and Jensen, 2001). A similar pattern has been documented for species of *Rhinebothrium* outside of South America. Healy (2006b) compiled a list of the valid species in the genus, which totaled 39. Healy (2006a) noted that, with the exception of *R. paratrygoni*, all species of *Rhinebothrium* parasite 1, or occasionally 2, host species. Data in the current study suggest that *R. copianullum* may be oioxenous, parasitizing only 1 of the 3 potamotrygonid species. However,
a full understanding of the host specificity of *Rhinebothrium* in potamotrygonids requires further taxonomic study of specimens in multiple host species and localities, including specimens of the potentially new species, *Rhinebothrium* sp. 1. Considering the pattern of high host specificity seen in many marine tetraphyllideans, and the observed oioxenous host specificity of *R. copianullum*, it seems possible that some of the specimens that were reported as *R. parastrrygoni* from different host species are not all conspecific. The discovery of *R. copianullum* and the potentially new species, *Rhinebothrium* sp. 1 (Table I) in *P. ariereba* in southeastern Peru, suggests that additional species of *Rhinebothrium* may await discovery in potamotrygonid host species and localities not yet explored.

The 2 species of *Rhinebothrium* parasitizing potamotrygonids, *R. copianullum* and *R. parastrrygoni*, are morphologically distinct from other species of *Rhinebothrium* in numerous features, including their possession of a large number of proglottids (>450), and in their retention of many mature proglottids on the strobila (55–122 in *R. copianullum*), most of which are wider than long. Despite the similarities of *R. copianullum* and *R. parastrrygoni*, the relationships between these 2 freshwater species and their marine congener remain unclear. Brooks, Mayes, and Thorson (1981) and Brooks and Deardorff (1988) provided the first phylogenetic hypotheses of species of *Rhinebothrium* that included *R. parastrrygoni*, the only species of *Rhinebothrium* known from potamotrygonids at that time. More recently, Healy (2006a) conducted morphological and molecular phylogenetic analyses of rhabdocoitine taxa, which, in addition to numerous marine species, included specimens of *Rhinebothrium* sp. 1 and *Rhinebothriodes* sp. 2 (called *Rhineboth­rium* sp. 8 and *Rhinebothriodes* cf. *freitasi*, respectively, in Healy, 2006a), as well as *Rhinebothrium megacanthophallus* Healy, 2006, from Himantarula chaophayra Monkolprasit and Roberts, 1990, a freshwater stingray in Borneo. In the trées resulting from her analyses, Healy (2006a) found that *Rhinebothrium* sp. 1 and *Rhinebothriodes* sp. 2 were consistently supported as sister taxa, whereas *R. megacanthophallus* was more closely related to marine species of *Rhinebothrium* (Healy, 2006a). It is puzzling that *Rhinebothrium* sp. 1 was more closely related to a species of *Rhinebothriodes* than to its congeners, because the proglottid morphology of *Rhinebothriodes* is distinct from the proglottid morphology of species of *Rhinebothrium*, e.g., overall shape, genital pore position, ovarian asymmetry, testes number. Clearly, more extensive analyses, including multiple species of *Rhinebothrium* and *Rhinebothriodes* from potamotrygonids, are needed to elucidate the relationships among freshwater and marine rhabdocoitine species.

*Acanthobothrium peruvianum* is the sixth species of the genus to be described from potamotrygonids in South America. Fyler and Caira (2006) observed that 3 of the *Acanthobothrium* species described from South American freshwater stingrays could be grouped based on several morphological features. Based on its morphology, *A. peruvianum* also belongs within this phonetic grouping, which consists of *A. amazonesis*, *A. quinonesi*, and *A. regoi* (Fyler and Caira, 2006). Each of these species possesses: (1) small vitelline follicles that extend posteriorly only to the level of the ovarian isthmus; (2) an ovary restricted to the posterior portion of the proglottid that does not reach the cirrus sac anteriorly; (3) hook bases encased in a dark granular material; and (4) a triangular muscular pad at the apex of the scolex. The other 2 species of *Acanthobothrium* in potamotrygonids, *A. terezae* and *A. ramiroi*, also possess features (2) and (4), but lack features (1) and (3), and are both large robust worms (50–84.5 mm and 88–110 mm, respectively) with stubby asymmetrical medial and lateral hooks. The morphological differences between *A. terezae* and *A. ramiroi* and the other 4 species of *Acanthobothrium* in potamotrygonids raise the issue of the monophyly of species of *Acanthobothrium* within potamotrygonids. However, the interrelationships among these species and their marine congener remain in question until a comprehensive phylogenetic hypothesis is available for the genus.

The *Acanthobothrium* spp. in those marine elasmobranch species that have been examined for cestodes are remarkably diverse (Euzet, 1959; Brooks, 1977; Marques et al., 1995; Ghoshroy and Caira, 2001; Campbell and Beveridge, 2002; Fyler and Caira, 2006; Reyda and Caira, 2006). As many as 7 species of *Acanthobothrium* have been reported from a single elasmobranch species (see Ghoshroy and Caira, 2001). The data compiled by Ghoshroy and Caira (2001) indicate that, on average, each marine elasmobranch species from the eastern Pacific and western Atlantic oceans that is host to *Acanthobothrium* possesses at least 2 species (Ghoshroy and Caira, 2001). However, this level of diversity has not been observed in the freshwater stingrays in South America. Only 6 species of *Acanthobothrium* have been described from the 14 species of potamotrygonids examined for cestodes to date (Brooks and Amato, 1992; Marques, 2000; Ivanov, 2005; this study). Furthermore, several of the potamotrygonid species known to host *Acantho­bothrium* species possess only a single species, e.g., *P. cf. castexi* hosts *Acanthobothrium cf. terezae* only. Although new species of *Acanthobothrium* will likely be described from potamotrygonids, it seems unlikely that the diversity will be found to parallel that seen in the marine elasmobranchs examined to date.

The total number of species of cestodes parasitizing South American freshwater stingrays likely exceeds the current count of 24. In addition to the taxa described above, several potentially new species of tetraphyllidean cestodes were encountered in this study (*Rhinebothrium* sp. 1; *Potamotrygonocestus* sp. 1; and *Rhinebothriodes* sp. 1 and 2). These taxa are yet to be formally treated. Additional species are needed to more precisely resolve the identities of those taxa that could not be positively identified to species in this study. A greater understanding of the diversity of cestodes in potamotrygonids awaits taxonomic treatment of these potentially new taxa, as well as other taxa in host species and localities yet to be explored.

Likewise, an understanding of the host specificity of cestodes in potamotrygonids is dependent on additional taxonomic progress. In addition to *R. parastrrygoni*, as noted above, many of the tetraphyllidean cestode species parasitizing potamotrygonids appear to be less host-specific than their marine counterparts in that they have been reported from multiple host species (Brooks, Mayes, and Thorson, 1981; Brooks and Amato, 1992; Marques, 2000; Marques and Brooks, 2003; Marques et al., 2003). Although a high level of host specificity (oioxenous) was observed for *R. copianullum*, the host specificity of many of the other cestode species cannot be assessed until the presence or absence data are available for a significantly greater number of potential host species. This approach could also verify patterns of host associations at higher taxonomic levels, e.g.,
the observed affinities of *Rhinebothrium* species to *P. aierba* and *Rhinebothroides* species to *P. motoro* and *P. cf. castexi*.

Although extant potamotrygonids and their cestodes are generally believed to be the descendants of marine ancestors, the history of colonization remains widely contested. This issue has been one of the primary foci of investigations in this host–parasite system for the past 27 yr. In fact, Brooks, Mayes, and Thorson (1981) and Brooks, Thorson, and Mayes (1981) used parasite phylogenies to infer host phylogenies to uncover patterns of colonization. This novel approach sparked much discussion, and resulted in the development of an intriguing set of hypotheses. They proposed that ancestral stingrays and their cestodes colonized a Pacific-draining Proto-Amazon between the early Cretaceous and Mid-Miocene (Brooks, Mayes, and Thorson, 1981; Brooks, Thorson, and Mayes, 1981; Brooks, 1992). Conversely, Lovejoy et al. (1998) and Marques (2000) proposed that ancestral stingrays and their cestodes moved from the Caribbean to the upper Amazon via marine incursions during the Miocene. This issue, at least from the parasitological standpoint, remains largely unresolved because of the preliminary nature of the cestode taxonomy. Some progress has been made towards a more resolved cestode taxonomy by several recent studies (Caira and Orringer, 1995; Marques et al., 2001; Marques and Brooks, 2003; Marques et al., 2003; Ivanov, 2004, 2005), as well as the present study in which new species of tetrathyridids and tetraphyllids from potamotrygonids have been described and previously described species have been redescribed adding clarification to the taxonomy. This progress on the taxonomy of the cestodes of potamotrygonids opens the door for the construction of comprehensive phylogenetic hypotheses to address several biogeographic and evolutionary questions.

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VARIABILITY IN TRIACTINOMYXON PRODUCTION FROM TUBIFEX TUBIFEX POPULATIONS FROM THE SAME MITOCHONDRIAL DNA LINEAGE INFECTED WITH MYXOBOLUS CEREBRALIS, THE CAUSATIVE AGENT OF WHIRLING DISEASE IN SALMONIDS

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ABSTRACT: Myxobolus cerebralis, the causative agent of whirling disease, infects both salmonid fish and an aquatic oligochaete, Tubifex tubifex. Although M. cerebralis has been detected in river drainages throughout the United States, disease severity among wild fish populations has been highly variable. Tubifex tubifex populations have been genetically characterized using sequences from the 16S mitochondrial DNA (mtDNA) gene, the 18S ribosomal RNA gene, the internal transcribed spacer region 1 (ITS1), and randomly amplified polymorphic DNA (RAPD). Our earlier work indicated that large differences in compatibility between the parasite and populations of T. tubifex may play a substantial role in the distribution of whirling disease and resulting mortality in different watersheds. In the present study, we examined 4 laboratory populations of T. tubifex belonging to 16S mtDNA lineage III and 1 population belonging to 16S mtDNA lineage I for triactinomyxon (TAM) production after infection with M. cerebralis myxospores. All 4 16S mtDNA lineage III populations produced TAMs, but statistically significant differences in TAM production were observed. Most individuals in the 16S mtDNA lineage III-infected populations produced TAMs. The 16S mtDNA lineage I population produced few TAMs. Further genetic characterization of the 16S mtDNA lineage III populations with RAPD markers indicated that populations producing similar levels of TAMs had more genetic similarity.

Myxobolus cerebralis, the myxozoan parasite that causes whirling disease in salmonids, has been detected in most regions of the United States where salmonids are present. In the Intermountain West, whirling disease has had devastating effects on a number of wild fish populations (Nehring and Walker, 1996; Vincent, 1996), whereas in other regions the effects of the disease seem to be relatively benign (Modin, 1998; Kaeser and Sharpe, 2006; Kaeser et al., 2006). The life cycle of M. cerebralis involves 2 hosts, a salmonid fish and an ubiquitous aquatic oligochaete, Tubifex tubifex (Wolf et al., 1986), and 2 spore stages, the actinospore and the myxospore. Myxospores of M. cerebralis develop in the cartilage of the infected fish (El-Matbouli et al., 1995), and they are released into the stream environment upon death of the fish. Ingestion of M. cerebralis myxospores by T. tubifex results in the production of the actinospore or the triactinomyxon (TAM) form of the parasite that is infectious for salmonids (Kerans and Zale, 2002; Blazer et al., 2003). Contact between the waterborne TAMs and the salmonid epithelium results in completion of the M. cerebralis infection cycle (El-Matbouli et al., 1995; Kerans and Zale, 2002; Gilbert and Granath, 2003).

Research and monitoring have shown that the distribution of M. cerebralis and severity of whirling disease among wild fish populations in the United States is locally and regionally variable (Kaeser and Sharpe, 2006; Kaeser et al., 2006; Krueger et al., 2006). Differences in disease severity may be attributed to variation in the parasite, the salmonid, or oligochaete hosts, the environment, or to their interactions (Kerans and Zale, 2002). Comparisons of the 18S ribosomal DNA and internal transcribed spacer 1 (ITS1) region of M. cerebralis isolates from North America and Europe showed little or no sequence variation, suggesting that the current distribution of M. cerebralis in the United States is the result of a radiation from a recent common ancestor (Andree et al., 1999; Whipps et al., 2004) and that the parasite likely contributes little to disease variability. However, different life history strategies within wild rainbow trout populations (Downing et al., 2002) and the fact that a number of different salmonid species with varying degrees of susceptibility to whirling disease coexist in many river systems (Hedrick, McDowell, Gay et al., 1999; Hedrick, McDowell, Mukkatira et al., 1999; Hedrick et al., 2001; Solidi et al., 2002, 2003; Vincent, 2002; Wagner et al., 2002; Bartholomew et al., 2003; Blazer et al., 2004) may contribute to some of the local and regional disparities seen in disease severity. Nonetheless, increasing biological (Holmquist, 1983; Anlauf, 1994; Reynolds et al., 1996; Timm, 1996; Anlauf and Neumann, 1997) and genetic evidence (Anlauf and Neumann, 1997; Sturmbaur et al., 1999; Beauchamp et al., 2001; Stevens et al., 2001; Kerans et al., 2004) has suggested that differences in T. tubifex, a cosmopolitan freshwater species (Brinkhurst and Jamieson, 1971) found in a range of habitats from pristine mountain streams to very polluted waters (Milbrink, 1983; Lauritsen et al., 1985), may be responsible for much of the regional and local variability seen in whirling disease severity.

Using mitochondrial 16S DNA sequences, researchers identified 6 major lineages, separated by genetic distances ranging from 5.8 to 13.0%, suggesting the existence of cryptic Tubifex species (Sturmbauer et al., 1999; Beauchamp et al., 2001). These lineages exhibited substantial differences in tolerance to environmental variables such as cadmium concentration (Sturmbauer et al., 1999), and they varied in their susceptibility to experimental infection with M. cerebralis (Beauchamp et al., 2002). Prevalence of M. cerebralis infection among individual oligochaetes also differed between 16S mitochondrial DNA (mtDNA) lineages collected from whirling disease endemic areas of the Colorado River, Colorado (Beauchamp et al., 2002, 2005), and in the tailwater of the San Juan River, New Mexico (Dubey and Caldwell, 2004).

Our earlier work showed that genetically distinct T. tubifex laboratory cultures obtained from regions with different whirling disease histories had significant differences in TAM production after exposure to similar numbers of M. cerebralis myxospores (Kerans et al., 2004). These studies suggested that
**T. tubifex** populations belonging to 16S mtDNA lineage III and having a specific ITS1 sequence type (Kerans et al., 2004) were significantly more compatible with *M. cerebralis* than other 16S mtDNA lineages (compatibility is defined as the extent to which the parasite is able to develop and propagate within its host, and it was determined by enumerating TAM production during the observation period). These cultures also exhibited differences in population growth and biomass accumulation when subjected to different water temperature regimes (Kerans et al., 2005). DuBey et al. (2005) have also shown that 2 laboratory cultures originating from 16S mtDNA lineage III and VI worms responded differently to exposure to *M. cerebralis* myxosporous and that they exhibited growth differences in response to different temperature regimes. Another genetically distinct population of *T. tubifex* isolated from the Great Lakes region is resistant to *M. cerebralis* infection (Beachamp et al., 2002; Steinbach Elwell et al., 2006). Together, these results demonstrate that differences in compatibilities to *M. cerebralis* infection between different lineages, strains, or populations of *T. tubifex* exist that could play a substantial role in the variability we see in the distribution, dissemination, and severity of whirling disease among wild salmonid populations.

In the present study, we sought to extend our understanding of the effects of *M. cerebralis* on populations of the oligochaete host by examining the compatibilities to *M. cerebralis* infection (total number of infectious spores produced and percentage of *T. tubifex* infected) of multiple laboratory cultures representing 16S mtDNA lineage III (hereafter referred to as lineage III) and 1 laboratory culture from 16S mtDNA lineage I (lineage I) after exposure to equivalent numbers of myxosporous. In addition to examining the reproducibility of *M. cerebralis* laboratory infections, we wanted to determine whether the success of worm populations (as measured by the biomass produced by an individual oligochaete worm over the entire experiment) differed among the laboratory cultures and between worms exposed and not exposed to infectious myxosporous.

**MATERIAL AND METHODS**

**Experimental *T. tubifex* populations**

To examine host–parasite compatibility, cultures containing the same number of *T. tubifex* were challenged with *M. cerebralis* myxosporous, and the TAMs released by the cultures were enumerated. All laboratory cultures were established from offspring of uninfected worms that were sexually mature and positively identified as *T. tubifex* using morphological characteristics as described by Kathman and Brinkhurst (1998). The parental or founder *T. tubifex* individuals of these different cultures were collected from the Madison and Gallatin Rivers in Montana and from Mt. Whitney, California as described previously (Kerans et al., 2004). In addition, cultures were established from *T. tubifex* collected in fall 1997 just below Logan Dam on the Logan River in northeastern Utah (UT) and from *T. tubifex* collected in March 1998 at 2 locations, Arroyo Medio and Pisciulharo Colonia (APMC), in Argentina. The Logan River was negative for whirling disease until August 1999 (E. Wagener, pers. comm.), and whirling disease had not been reported in Argentina at the time of collection (K. Johnson, pers. comm.).

**Genetic analysis**

Genomic DNA was isolated from individual oligochaetes using Nucleospin kits purchased from Clontech Laboratories, Inc. (Palo Alto, California). The 16S mtDNA lineage of *T. tubifex* individuals from each culture was determined using the mitochondrial 16S gene primers (Beachamp et al., 2002). Briefly, polymerase chain reactions (PCR) contained 25 pmol of primer I or III, and tub16SR, 250 μM dNTPs, 4.0 mM MgCl₂, 1 unit of Taq polymerase (Promega, Madison, Wisconsin), 1X Redload loading dye (Research Genetics, Huntsville, Alabama), 10 mM Tris-HCl (pH 9.0 at 25 °C), 50 mM KCl, and 0.1% Triton X-100. PCR amplification was performed as previously described (Kaeser et al., 2006) except that primer annealing was at 60 °C for 1 min. PCR products were visualized by gel electrophoresis on 2.25% agarose, 0.02 M Tris-acetate, 0.5 mM EDTA (pH 8.0) gels.

The genetic heterogeneity of each *T. tubifex* culture was also assayed using randomly amplified polymorphic DNA (RAPDs) (Welsh and McClelland, 1990). Between 10 and 20 *T. tubifex* individuals from each culture were analyzed. Five different RAPD primers (5'-CTCGGCCCT-TG-3', 5'-GGGGCCGCT-GA-3', 5'-GGGGCCTTTA-3', 5'-CCGGCCCTT-AC-3', and 5'-CCGGGCCTTAG-3') purchased from the University of British Columbia Nucleic Acid-Protein Service Unit (Vancouver, British Columbia, Canada) were used to assess genetic differences in the cultures established from Logan River, Utah, and Argentina. An additional 2 primers (5'-CTCTGGGGCCT-3' and 5'-CTCTGGGTGGA-3') were used to examine the similarity between the Madison River, Montana, and Logan River, Utah, cultures. The RAPD assay was performed as described previously (Kerans et al., 2004). Banding patterns of 10 individuals from each population using 4 different RAPD primers (5'-CTCTGGGGCTT-3', 5'-CTCTGGGTCCT-3', 5'-CCGGCCATCC-3', and 5'-CCGGGCCTTAG-3') were scored to estimate nucleotide divergence using the RAPDIP program (Clark, 1997).

The ITS1 region of *T. tubifex* individuals from the Logan River, Utah, and AMPC, Argentina, cultures was amplified and sequenced as described previously (Kerans et al., 2004). Two clones from 3 different individuals from the Argentina culture were sequenced. Both clones sequenced from 1 individual were identical (GenBank DQ296051), whereas the clones from the other 2 individuals differed from each other at 8 (GenBank DQ296050 and DQ296052) and 5 nucleotide positions (GenBank EU154064-65). A single ITS1 clone from 1 individual (GenBank DQ296049) and 6 clones from another Logan River individual (GenBank EU154058-63) were sequenced. With the exception of the ITS1 sequences from the Gallatin River, each laboratory population used in this study had only 1 ITS1 haplotype. The ITS1 haplotypes from each laboratory population only differed from each other at single nucleotide positions; therefore, they were aligned using ClustalW in MacVector version 6.5.3 (Accelrys, San Diego, California) to create a consensus sequence that was used in the phylogenetic analysis. The consensus ITS1 sequences for *L. hoffmeisteri* (GenBank AF361102-03), *I. templetioni* (GenBank AF362435), and 3 cultures of *T. tubifex* from Mt. Whitney, California (GenBank AF362436 and AY333365-74), the Gallatin River, Montana (GenBank AF362437-39 and AY334646-72), and the Madison River, Montana (GenBank AF362440-41 and AY334473-91), that had been sequenced previously (Kerans et al., 2004) were also used in this analysis. Three closely related ITS1 haplotypes were found when individuals from the Gallatin River culture were sequenced. Small insertion/deletion events largely accounted for the differences in the 3 types of Gallatin River sequences (Kerans et al., 2004).

Consensus sequences were aligned using ClustalX (Thompson et al., 1997), with default settings including the pairwise and multiple alignment gap-opening penalties of 10/0.10 and 10/0.20, respectively. The alignment was then examined by eye and no adjustments were deemed necessary. The alignment was analyzed by maximum parsimony using PAUP version 4.0 (Sinauer Associates, Inc., Sunderland, Massachusetts) and the following settings: heuristic search, random addition sequence with 100 replicates, tree bissection-reconnection branch swapping, and Multrees on (CI = 0.917 and RI = 0.838).

**Experimental design and statistical analysis**

The experimental design was similar to that described previously (Kerans et al., 2004). Briefly, 700 worms from each laboratory culture were divided into groups of 100, then they were cleaned, weighed, and held in dechlorinated tap water at 15 °C for 24 hr without food. Oligochaetes were then placed in separate 266-ml food containers with 40 ml of sand and 150 ml of water. Myxosporous were extracted from rainbow trout as described previously (Kerans et al., 2005), and 4 replicates were inoculated with the myxospore emulsion at a concentration of 1,000 myxosporous per worm, whereas 3 replicates were inoculated with an equivalent amount of myxospore-free emulsion obtained by processing *M. cerebralis*-free rainbow trout. Worm weights varied among
the different laboratory cultures at the beginning of the experiment. The mean weights of individual worms (mg ± 1 SE) for 3 nonmyxospore- and 4 myxospore-containing replicates were, respectively, as follows: Madison River, Montana (4.68 ± 0.02; 4.76 ± 0.27); AMPC, Argentina (3.7 ± 0.10; 3.73 ± 0.06); Mt. Whitney, California (3.20 ± 0.07; 2.75 ± 0.05); Gallatin River, Montana (2.75 ± 0.07; 2.82 ± 0.07); and the Logan River, UT (1.51 ± 0.06; 1.58 ± 0.08).

All treatments were aerated and held at 15 C. The water was changed, and 0.10–0.12 g of dried *Spirulina* sp. was added weekly. Beginning 70 days postexposure (PE), all water drawn off the samples was filtered through a 20-μm mesh to retain any TAMs. The filter was backflushed with dechlorinated tap water and the resulting solution was examined for the presence of TAMs using a hemacytometer (×100 magnification, phase contrast or dark-field). After TAMs were detected on day 88 PE in the Mt. Whitney cultures, TAMs in 3 of the myxospore-containing replicates for each oligochaete culture were enumerated weekly for 8 wk. The total number of TAMs produced by each replicate during that week was calculated by determining the mean in two 55-μl counts and extrapolating. The total number of TAMs produced by each replicate during the entire experiment was calculated by summing these weekly counts. The water from the myxospore-free replicates from each culture was combined and examined for TAMs as described above.

One replicate from each myxospore-containing treatment group was randomly selected to determine percent infection. At 100 days PE, 60 worms were randomly selected and placed individually in 12-well plates with dechlorinated tap water. Oligochaetes were held at 15 C for 3 days, and then each well was scanned to determine whether that individual was producing TAMs. These oligochaetes were maintained in the incubator at 15 C and scanned again after 7 and 14 days. Only worms surviving the incubation period were scored regardless of whether TAMs were present in the well. We do not believe that worm mortality in the 12-well plates was related to *M. cerebralis* infection because we recovered similar numbers of adults from exposed and unexposed cultures at the end of the experiment. Greater numbers of the worms from the Logan River culture perished during this protocol than worms from the other cultures, possibly due to bleaching contamination in the 12-well plates.

At the end of the experiment (day 150 and 151 PE) the adult oligochaetes in the remaining replicates were removed, cleaned, and weighed. The sediment containing the progeny and cocoons was preserved in Kahle’s solution (Pennak, 1978) and later transferred to 70% ethanol until they were removed from the sediment and weighed. We found few, if any, cocoons or progeny in the lineage III cultures infected with *M. cerebralis*, whereas the unexposed cultures had many progeny. Reproduction occurred in both the exposed and unexposed lineage I replicates. The weight of adults and progeny were combined to determine total biomass in each container. Initial worm biomass was subtracted from the final worm biomass and divided by 100 to calculate the biomass produced by an individual worm.

One-way analysis of variance (ANOVA), followed by Tukey’s honestly significant difference (HSD) test, was used to compare total TAM production among the *T. tubifex* cultures. Residuals from ANOVA using untransformed data were heterogeneous and non-normal; therefore, a natural log transformation was used to homogenize variances and to improve normality. Two-way ANOVA followed by Tukey’s HSD (where appropriate) were used to compare the biomass produced by an individual worm among *T. tubifex* cultures in treatments exposed and not exposed to myxospores. Residuals from ANOVA were normal and variances homogeneous; therefore, no transformation was used.

RESULTS

**Genetic characterization of laboratory populations**

Previously, a number of individuals from cultures established from the Gallatin River, Montana; the Madison River, Montana; and Mt. Whitney, California, were genetically characterized using RAPDs and ITS1 sequences (Kerans et al., 2004). We extended this characterization by determining the 16S mtDNA lineage (Sturmbar, 1999; Beauchamp et al., 2002) for approximately 50 individuals from each of these cultures and 2 additional cultures from the Logan River, Utah, and AMPC, Argentina. Between 18 and 22 individuals from each culture were assayed both before and after the completion of the experiment. Within each culture, 100% of the individuals tested had identical 16S mtDNA lineage types. The Madison River, Mt. Whitney, Argentina, and Logan River cultures were determined to belong to lineage III, whereas the Gallatin River culture belonged to lineage I. The stability of these cultures was also examined by randomly choosing 10 individuals from each culture 3 yr after the experiment was performed and determining that the cultures had maintained their 16S mtDNA lineage.

Characteristics of the ITS1 sequences obtained from individuals from the Madison River (794 base pairs [bp]), Mt. Whitney (848 bp), and Gallatin River (821, 802, and 775 bp) have been described previously (Kerans et al., 2004). To further examine the phylogenetic relationships between these cultures of *T. tubifex*, we compared previously obtained ITS1 sequences to those obtained from 2 individuals from the Logan River culture (785–790 bp) and 3 individuals from the Argentina culture (847–848 bp). Figure 1 shows the most parsimonious tree resulting from cladistic analysis of the ITS1 region. Significant bootstrap and jackknife values indicate that the Gallatin River *T. tubifex* were distinct from the Madison River, Argentina, Logan River, and Mt. Whitney *T. tubifex*. Moreover, the ITS1 sequences indicate that the Mt. Whitney and the Argentina *T. tubifex* were more closely related to each other than to the individuals from the Madison River and Logan River cultures. The average pairwise sequence divergence between the 2 distinct *T. tubifex* ITS1 clades ranged between 9.1 to 14.4%, whereas the average pairwise divergence within each clade ranged from 0 to 6.3%.

Our previous work using RAPD banding patterns indicated that individuals from within each of the Mt. Whitney and Gallatin River cultures were genetically identical. RAPD banding patterns obtained from individuals of the Madison River culture

![Figure 1. Most parsimonious tree resulting from cladistic analysis of the ITS1 locus from 3 oligochaete taxa and 5 populations of Tubifex tubifex (Gallatin River, Montana; Madison River, Montana; Mt. Whitney, California, Logan River, Utah; and AMPC, Argentina). The results of 1,000 bootstrap (above the branch) and jackknife (below the branch) replicates are shown. Trees generated using other alignments (gaps included or excluded) as well as other phylogenetic methods (maximum likelihood and neighbor joining) had the same topology.](image-url)
indicated that a low level of genetic variation existed within this laboratory population. However, comparisons of RAPD banding patterns between the 3 cultures showed that only a few interspecific markers were shared. Therefore, although individuals from within a given laboratory culture seemed to be genetically similar, the RAPD results suggested that substantial genetic differences existed among these 3 laboratory cultures established from *T. tubifex* collected from different geographical areas (Kerans et al., 2004). Using RAPDs, the laboratory culture established from the Logan River appeared to be genetically homogeneous (Fig. 2A). RAPD analysis showed that the individuals from the Argentina culture had a low level of a genetic variation (Fig. 2B). We estimated nucleotide divergence within the Argentina culture to be 0.0022 + 0.0008 (Clark and Lanigan, 1993), which is similar to that previously found for the Madison River culture (Kerans et al., 2004). We then simultaneously compared RAPD banding patterns between all the different cultures used in this experiment. As illustrated in Figure 3, individuals from lineage III shared a number of bands compared with the lineage I culture from the Gallatin River. Strikingly, individuals from the Madison River and the Logan River cultures appeared genetically identical. Intraspecific variation within Madison River population was greater than the interspecific variation between the 2 cultures. Estimates of nucleotide divergence also indicated that the Argentina and Mt. Whitney cultures were more similar to each other than to the Madison River and Logan River cultures, whereas all lineage III populations were more similar to each other than to the lineage I population from the Gallatin River. Estimates of nucleotide divergence (Clark and Lanigan, 1993) confirmed these observations (Table I).

**M. cerebralis** infection of laboratory populations

Tryactinomyxons of *M. cerebralis* were first observed 88 days PE in all the *T. tubifex* cultures except the Gallatin River culture (Fig. 4). The number of TAMs released differed among the laboratory populations of *T. tubifex* by several orders of magnitude (Figs. 4, 5; ANOVA $F_{5,10} = 88.93$, *P* < 0.0001) during the 55-day period of enumeration. During the course of the experiment, the Argentina culture produced approximately twice as many TAMs as the Mt. Whitney culture, which produced slightly more TAMs than the Logan River culture. Total TAM production in the Logan River culture was approximately twice that of the Madison River culture. Statistically significant differences were detected between the number of TAMs released by the *T. tubifex* from Argentina and those released by the *T. tubifex* from the Madison and Gallatin rivers (Tukey’s HSD, *P* < 0.05). The *T. tubifex* from the Gallatin River released fewer TAMs than all of the other cultures (Tukey’s HSD, *P* < 0.05) during the course of the experiment. Triactinomyxons were not found in water drawn from the negative controls.

All of the cultures in the exposed treatment groups contained individuals releasing TAMs except those from the Gallatin River. No TAMs were ever released from an individual Gallatin River *T. tubifex*. Water filtered from only 1 of the mass culture replicates at 1 time point during the course of the experiment contained a small number of TAMs, suggesting very few individuals in the Gallatin River cultures produced *M. cerebralis* actinospores. In contrast, we found nearly all the individuals in the lineage III-infected cultures were producing TAMs (Table II). At the concentration of 1,000 myxospores per worm, between 91 and 98% of the individuals in the lineage III cultures were producing TAMs. Although we did not enumerate the actual production of TAMs per worm, we observed that some wells contained high concentrations of TAMs, whereas other wells contained worms that released few TAMs.

Worm cultures were more successful when they were not exposed to myxospores than when they were exposed to myxospores because the biomass produced by an initial individual *T. tubifex* ([final worm biomass – initial worm biomass]/100) was
lower in exposed treatments than in unexposed treatments (Fig. 6; ANOVA $F_{1,20} = 16.75$, $P = 0.0006$). The biomass produced by an initial individual *T. tubifex* differed among the experimental groups (Fig. 6; ANOVA $F_{4,20} = 15.16$, $P < 0.0001$). The biomass produced by an individual *T. tubifex* was lower in the oligochaetes from Argentina than in all the other cultures and higher in oligochaetes from Utah than all other cultures but the Madison River (Tukey's HSD, $P < 0.05$). In addition, the almost significant interaction effect (ANOVA $F_{4,20} = 2.41$, $P = 0.0831$) probably occurred because biomass produced by individual *T. tubifex* from the Gallatin River were similar between exposed and unexposed treatments, whereas it was lower in exposed than unexposed treatments for all other laboratory populations (Fig. 6).

**DISCUSSION**

Exposing 4 different *T. tubifex* laboratory populations, all belonging to 16S mtDNA lineage III, to similar numbers of *M. cerebralis* myxospores in identical environmental conditions, resulted in substantially different compatibilities. The total number of TAMs produced by the Argentina culture was ap-

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**Table I.** Nucleotide divergence estimates between the *T. tubifex* populations generated using the RAPD-DIP program (Clark, 1997) from banding patterns scored from 4 different RAPD primers (5'-CCTGGGCGTTG-3', 5'-CCTGGGTCCA-3', 5'-CCGGCCCTTAC-3', and 5'-CCGGCCTTATG-3') using 10 individuals from each population.

<table>
<thead>
<tr>
<th>Laboratory culture</th>
<th>Laboratory culture</th>
<th>Divergence</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPC, Argentina</td>
<td>Mt. Whitney, California</td>
<td>0.007</td>
<td>0.002</td>
</tr>
<tr>
<td>AMPC, Argentina</td>
<td>Madison River, Montana</td>
<td>0.026</td>
<td>0.007</td>
</tr>
<tr>
<td>AMPC, Argentina</td>
<td>Logan River, Utah</td>
<td>0.026</td>
<td>0.007</td>
</tr>
<tr>
<td>AMPC, Argentina</td>
<td>Gallatin River, Montana</td>
<td>0.051</td>
<td>0.009</td>
</tr>
<tr>
<td>Mt. Whitney, California</td>
<td>Madison River, Montana</td>
<td>0.036</td>
<td>0.009</td>
</tr>
<tr>
<td>Mt. Whitney, California</td>
<td>Logan River, Utah</td>
<td>0.036</td>
<td>0.009</td>
</tr>
<tr>
<td>Mt. Whitney, California</td>
<td>Gallatin River, Montana</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>Madison River, Montana</td>
<td>Logan River, Utah</td>
<td>0.0008</td>
<td>0.0004</td>
</tr>
<tr>
<td>Madison River, Montana</td>
<td>Gallatin River, Montana</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>Logan River, Utah</td>
<td>Gallatin River, Montana</td>
<td>0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>
proximately 2-fold greater than the Mt. Whitney culture, 2.5-fold greater than the Logan River culture, and 7.5-fold greater than the Madison River culture. The variation in TAM production in these laboratory cultures of lineage III T. tubifex was substantial. These data suggest that there is a wide range of M. cerebralis compatibilities among different laboratory populations of T. tubifex belonging to the 16S mtDNA lineage III. The Gallatin River culture, which belongs to lineage I, produced few TAMs. Previous attempts at infecting the Gallatin River culture with M. cerebralis have shown similar results (Kerans et al., 2004, 2005).

Genetic analysis using the 16S mtDNA lineage primers and RAPD markers indicated that our laboratory populations established from T. tubifex founders collected from different geographic locations with different whirling disease histories were genetically homogeneous and stable through time. The 16S mtDNA lineage remained constant over a period of 6 yr, whereas RAPD banding patterns indicated that there was very little, if any, intraspecific variation within each laboratory culture. Genetic data collected from individuals from the Gallatin River culture, including ITS1 sequences, RAPD banding patterns, and the 16S mtDNA lineage, all indicate that this population of T. tubifex is substantially different from the other T. tubifex cultures used in this experiment.

Phylogenetic analysis of the ITS1 sequences obtained from individuals within these laboratory cultures showed that the Gallatin River population formed a clade that was distinct from that of the ITS1 sequences from the lineage III cultures. Bootstrap and jackknife sampling techniques supported the separate branching of the Mt. Whitney and Argentina cultures from the Madison River and Logan River cultures. Although statistical tests could only detect significant differences in TAM production between the Argentina and Madison River cultures, the Argentina and Mt. Whitney cultures produced the greatest number of TAMs, whereas the Madison River and Logan River cultures produced lower numbers of TAMs. Although RAPD analysis showed that the lineage III laboratory cultures shared a large number of bands, indicating a high degree of relatedness, the Argentina and Mt. Whitney cultures were more similar to each other than to the Madison River and Logan River cultures. Interestingly, RAPD analysis could not distinguish the Logan River culture from the Madison River culture. In addition, analysis of the ITS1 sequences did not show any nucleotide differences between consensus sequences of the Madison River and the Logan River isolates. Although not statistically significant, the Logan River culture produced almost 3 times as many TAMs as the Madison River worms. Increasing the number of replicates in future experiments may increase statistical resolution and aid in determining whether TAM production by these 2 genetically similar cultures is statistically different.

The variability seen in TAM production between replicates suggests that biological processes also affect TAM production. Biological differences between the oligochaetes at the initiation of our experiment could have been responsible for some of the variation seen in TAM production. For example, the Madison

<table>
<thead>
<tr>
<th>Laboratory culture</th>
<th>mtDNA lineage</th>
<th>No. of individuals producing triactinomyxon</th>
<th>Infection %</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPC, Argentina</td>
<td>III (48)</td>
<td>54/57</td>
<td>95%</td>
</tr>
<tr>
<td>Logan River, Utah</td>
<td>III (49)</td>
<td>42/46</td>
<td>91%</td>
</tr>
<tr>
<td>Madison River, Montana</td>
<td>III (51)</td>
<td>58/60</td>
<td>97%</td>
</tr>
<tr>
<td>Mt. Whitney, California</td>
<td>III (53)</td>
<td>59/60</td>
<td>98%</td>
</tr>
<tr>
<td>Gallatin River, Montana</td>
<td>I (51)</td>
<td>0/60</td>
<td>0%</td>
</tr>
</tbody>
</table>
River worms weighed approximately 3 times more on average than the Logan River worms at the initiation of the experiment. If size or life stage is a factor in the initiation or progress of infection, this difference could have affected TAM production. However, one might expect the larger oligochaetes to produce more TAMs, but this was not observed, i.e., the smaller Logan River worms produced greater numbers of TAMs than the Madison River worms. In addition, at the beginning of the experiment, the Madison River worms, which produced the smallest number of TAMs, were similar in size to the Argentina worms, which produced the largest number of TAMs. These observations suggest that the size of the tubificid upon infection has little effect on subsequent infection productivity.

Clearly, the lineage I T. tubifex culture established from Gallatin River responded differently to the M. cerebralis myxospore challenge than the lineage III cultures. It has been suggested that resistant strains of T. tubifex, like those from the Gallatin River, may act as biological filters for M. cerebralis, thereby inactivating the parasite and reducing infection of susceptible strains (Beauchamp et al., 2006; Baxa et al., 2008). However, using a response-surface competition design (Goldberg and Scheiner, 2001, Steinbach Elwell et al. 2006) showed that the presence of resistant oligochaetes did not affect infection prevalence or parasite proliferation in the susceptible strain. More experiments examining the effects that cohabitation of resistant and susceptible strains have on M. cerebralis infection prevalence and TAM production are needed. In addition, other researchers have shown that changes in the substratum types and incubation temperatures also influences TAM production in T. tubifex cultures in a laboratory setting (Arndt et al., 2002; Blazer et al., 2003; Kerans et al., 2005), indicating that numerous biotic and abiotic factors influence TAM production by T. tubifex. Our laboratory studies showed that even under identical environmental conditions, genetically similar T. tubifex populations had significant differences in TAM production. Therefore, in natural river systems, where large differences in environmental conditions exist, one would expect greater variability in TAM production even if T. tubifex populations where genetically homogeneous. Furthermore, studies examining the genetic diversity of T. tubifex populations in river systems indicate that many systems contain multiple 16S mtDNA lineages (Beauchamp et al., 2002; DuBey and Caldwell, 2004; Beauchamp et al., 2005; Kaeser et al., 2006) whose dispersal and relative abundance could also influence levels of TAM production.

Finally, our data show that the biomass produced by an initial individual worm was lower in the lineage III T. tubifex exposed to myxospores than those not exposed to myxospores, indicating that a productive infection by M. cerebralis has a negative effect on the fitness of T. tubifex. A decrease in fecundity seemed to be the major cause of the fitness reduction. This is in agreement with our previous laboratory studies conducted using similar experimental procedures (Stevens et al., 2001; Kerans et al., 2004). However, Steinbach Elwell et al. (2006) showed that adult growth was the only biomass variable that differed between exposed and unexposed cultures of lineage III worms from Mt. Whitney. Differences in experimental procedures may have contributed to the differences in results. Where lower biomass accumulation seemed to be due to a decrease in fecundity, T. tubifex cultures remained in sediment containing myxospores for the duration of the experiment (Stevens et al., 2001; Kerans et al., 2004), whereas when only adult growth was affected, T. tubifex cultures had only been briefly exposed to myxospores (Steinbach Elwell et al., 2006). Reduced fecundity as a result of M. cerebralis infection suggests that some T. tubifex lineages could be under strong selection pressure to develop resistance or life history strategies to avoid the parasite (Minchella, 1985; Ebert and Herre, 1996). However, the reported prevalence of infection in T. tubifex populations in nature is typically less than 10% (Rognlie and Knapp, 1998; Zendt and Bergersen, 2000; Beauchamp et al., 2002; DuBey and Caldwell, 2004), indicating that the probability that a worm will encounter the parasite is low. In addition, T. tubifex is host to a number of other parasites (Kent et al., 2001), suggesting that selection pressure to evolve resistance may be limited by other environmental and biological factors.

In conclusion, this study has shown that although T. tubifex populations belonging to lineage III are susceptible to parasite infection, they have a wide range of compatibilities to M. cerebralis infection as determined by examining parasite proliferation after exposure to equivalent numbers of M. cerebralis myxospores. In our experiments, we were able to detect statistically significant differences in TAM production between the high and low producers belonging to lineage III. Using ITS1 sequences and RAPD banding patterns, we further characterized individuals that belonged to the lineage III laboratory cultures used in this study. Our data suggest that differential propagation of M. cerebralis in T. tubifex populations may, in part, contribute to the differences we see in disease severity among salmon populations. However, although the molecular markers used to study T. tubifex seem to differentiate between lineages and some populations of T. tubifex, none of the loci examined in this or other studies have been shown to be linked to the genes responsible for disease resistance. Therefore, it cannot be assumed with confidence that certain lineages are susceptible and other lineages are resistant to M. cerebralis infection. In fact, a recent study by Baxa et al. (2008) has shown that some laboratory populations established from progeny derived from T. tubifex 16S mtDNA lineage III TAM-producing parents are resistant to M. cerebralis infection. Together, these results indicate that more data are needed to determine how genetically defined T. tubifex populations interact with M. cerebralis myxospores and their environment before genetic-typing of T. tubifex can be used to predict and manage whirling disease within a watershed.

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LITERATURE CITED


HAEMOSPORIDIAN BLOOD PARASITES IN EUROPEAN BIRDS OF PREY AND OWLS

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Leibniz-Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, D-10315 Berlin, Germany. e-mail: Krone@IZW-Berlin.de

ABSTRACT: Avian blood parasites have been intensively studied using morphological methods with limited information on their host specificity and species taxonomic status. Now the analysis of gene sequences, especially the mitochondrial cytochrome b gene of the avian haemosporidian species of Haemoproteus, Plasmodium, and Leucocytozoon, offers a new tool to review the parasite specificity and status. By comparing morphological and genetic techniques, we observed nearly the same overall prevalence of haemosporidian parasites by microscopy (19.8%) and polymerase chain reaction (PCR) (21.8%) analyses. However, in contrast to the single valid Leucocytozoon species (L. toddi) in the Falconiformes we detected 4 clearly distinctive strains by PCR screening. In the Strigiformes, where the only valid Leucocytozoon species is L. danilewskyi, we detected 3 genetically different strains of Leucocytozoon spp. Two strains of Haemoproteus spp. were detected in the birds of prey and owls examined, whereas the strain found in the tawny owl belonged to the morphospecies Haemoproteus noctuae. Three Plasmodium spp. strains that had already been found in Passeriformes were also detected in the birds of prey and owls examined here, supporting previous findings indicating a broad and nonspecific host spectrum bridging different bird orders.

Avian haemosporidian parasites (species of Haemoproteus, Leucocytozoon, and Plasmodium) have been frequently used to investigate host-parasite interactions in birds, partly because of the relative ease of obtaining reliable samples and partly because blood smears allow both qualitative and quantitative assessments. Morphological blood smear analyses have been applied to investigate parasite prevalence among different bird species (Cheke et al., 1976; Earle et al., 1991; Leppert et al., 2004), pathology and morphology (Bennett et al., 1993; Hunter et al., 1997; Markus and Oosthuizen, 1972), and examining hypotheses of sexual selection, immunestatus, and their influence on reproduction (Korpimäki et al., 1995; Dufa, 1996).

Although most studies were conducted on passerine birds, much less is known about birds of prey and owls, which, because of their position on top of the food chain, play a very important role in balance maintenance of the ecosystems in which they occur. In the present study we have investigated a dataset of 14 species of European raptors to add-information on the presence and the genetic variability of haemosporidian parasites in this avian group. Blood parasites have been described as causing disease and even mortality in birds of prey (Remple, 1981; Valentin et al., 1994; Hunter et al., 1997). Pathogenicity is usually considered to be lower in species of Haemoproteus than in those of Leucocytozoon, but may be severe in Plasmodium spp. Some hemoproteids, however, have been reported to cause diseases in birds (Milgen et al., 1981; Atkinson, Greiner et al., 1986; Atkinson, Forrester et al., 1988; Cardona et al., 2002) and thus to affect their fitness (Nordling et al., 1998; Marzl et al., 2005; Valkiūnas, 2005; Valkiūnas, Zickus et al., 2006). The genus Haemoproteus is the largest among avian blood parasites; 6 of the 135 described Haemoproteus species in birds have been morphologically described in members of the Falconiformes (Peirce and Marquiss, 1983; Peirce et al., 1990; Bennett et al., 1994; Valkiūnas, 2005), while 4 Haemoproteus species have been recognized in the Strigiformes (Bishop and Bennett, 1989). Of 36 avian Leucocytozoon species, only 1 species each is accepted in Falconiformes (L. toddi) and in Strigiformes (L. danilewskyi, synonym to L. ziemannii) (Greiner and Kocan, 1977; Valkiūnas, 2005), while 5 of the avian Plasmodium species listed by Valkiūnas (2005) were found in Falconiformes, plus 5 more in Strigiformes. Species identification of blood parasites is based mainly on morphological features, as well as limited experimental data on their vertebrate host specificity. In contrast to Plasmodium spp., blood stages of Haemoproteus spp. and Leucocytozoon spp. are not directly transmissible to other vertebrate hosts, and host specificity is therefore difficult to investigate. Taxonomy of avian blood parasites at the species level is still developing, and the discussion on the number of valid morphospecies is in process (Pierce et al., 1990; Bennett et al., 1994; Valkiūnas, 2005; Kriaunauskiene et al., 2006).

In the last decade, molecular biological tools have been developed to study haemosporidian parasites of birds (Bensch et al., 2000; Ricklefs and Fallon, 2002; Beadell et al., 2004). DNA-based techniques render detection of haemosporidians easier, especially at early stages of infections and during chronic infections when parasitaemia is low and parasites can be overlooked in blood smears (Jarvi et al., 2003). Phylogenetic analyses based on parasite genomes have shown that the current morphologically based taxonomy may not always reflect genetic relationships. Considering genetic variation among individuals within populations and species, molecular diversity is expected to be greater than morphological diversity (Sehgal et al., 2006). However, Martinsen et al. (2006) concluded from their study that morphological identification of haemosporidians was fully supported by genetic data, with the exception of a single species. On the other hand, Valkiūnas, Bensch et al. (2006) showed that polymerase chain reaction (PCR)-based techniques alone may even underestimate simultaneous infections by haemosporidian parasites, so microscopy remains an important tool for these kinds of investigation.

Based on these prior efforts, the aims of our study were (1) to extend the knowledge of haemosporidian infections beyond the Passeriformes to birds of prey and owls, (2) to examine the number of morphologically described species compared to the diversity detected by PCR screening, and (3) to investigate the
phylogenetic relationships of haematozoans from birds of prey and owls and their relationships with already described parasite species parasitizing passeriform birds.

**MATERIAL AND METHODS**

**Sampling**

Sixty-eight birds of prey and owls admitted to rehabilitation centers and bird clinics, as well as 33 nestlings of white-tailed sea eagles (*Haliaeetus albicilla*), were sampled for blood parasites between December 2002 and December 2003. Most birds were sampled from September to February (n = 46) and from March to August (n = 22). The blood samples of the nestlings of white-tailed sea eagles were collected in June and July 2003. All birds originated from 2 different regions in Germany, i.e., Lower Saxony (52°00’ to 53°00’N, 9°50’ to 11°00’E) and Berlin-Brandenburg (52°00’ to 53°25’N, 11°50’ to 14°50’E). Altogether, blood samples from 11 species of birds of prey and 3 species of owls were collected. Blood was taken from the *Vena cutanea ulnaris*, and a sample volume of approximately 20 µl was stored in SET-buffer (Hellgren et al., 2004) and kept frozen at −20°C until further analysis. In addition to the blood sample, 2 thin blood smears were prepared from each bird and air dried. Blood smears were fixed with absolute methanol and stained with Giemsa.

**Morphological analysis**

Each blood smear was scanned for at least 10 min using a Zeiss Axiophot microscope with 25X, 100X, 200X, 400X, and 1,000X magnification for parasite detection and characterization (Krone et al., 2001). Parasites were identified according to Valkiūnas (2005).

**PCR screening**

Genomic DNA from the blood sample was extracted by standard methods, including phenol-chloroform extraction followed by ethanol precipitation (Sambrook et al., 1989). To detect haemosporidian parasites, we used a set of complementary primers from Bensch et al. (2000) and Hellgren et al. (2004). We used the nested PCR approach of Hellgren et al. (2004), with an initial amplification of a 617-bp-long fragment common to all 3 genera of haematozoa (primers HaemF and HaemR3N), with a subsequent nested PCR using combinations of primers from Bensch et al. (2000) and Hellgren et al. (2004) for genus-specific amplification of *Leucocytozoon* (primers HaemFL and HaemR2L) and *Haemoproteus/Plasmodium* (primers HaemF and HaemR2) species.

The initial PCR was performed in 25 µl volumes, which contained 1.5 mM MgCl₂, 2.5 µl 10× buffer, 0.125 mM of each nucleotide, 0.6 µM of each primer, 0.5 units AmpliTag DNA polymerase (all reagents from Applied Biosciences, Darmstadt, Germany), and approximately 25 ng of genomic DNA. The amplification protocol started with 3 min at 94°C, followed by 20 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec, and ended at 72°C for 10 min as final elongation step. For the nested PCRs, we used 1 µl of the PCR products from the initial PCR as template in each of the 2 PCRs. These nested PCRs were performed using the same reagents (except primers, see above) as described for the initial PCR, but ran for 35 cycles. Negative (no DNA) and positive controls (samples from birds with proven haematozoan infections) were used. Amplicons were sequenced bidirectionally with primers HaemF and HaemR2 (*Plasmodium* and *Haemoproteus* spp.) or HaemFL and HaemR2L (*Leucocytozoon* spp.) using the BigDye® cycling sequencing kit (v. 3.1) and an A310 Automated Sequence Analyzer (both Applied Biosciences).

**Test for differences in parasite prevalences**

Fishers exact test was applied to test for differences in the parasite prevalences measured by the 2 screening methods applied here (microscopic analysis vs. PCR screening).

**Phylogenetic analysis**

**Phylogenetic tree constructions:** Obtained sequences were edited and aligned using the software program BioEdit (Hall, 1999). Sequences were compared to a large dataset of mostly haemosporidians of passeriform birds. Maximum parsimony (MP) analysis was performed using the branch-swapping heuristic close-neighbor interchange (CNI) search algorithm as described by Strimmer and von Haeseler (1996) implemented in Puzzle (Schmidt and von Haeseler, 2003). Statistical support for nodes was obtained by 1,000 bootstrap iterations. Maximum-likelihood (ML) analysis was performed using the quartet puzzling algorithm as described by Strimmer and von Haeseler (1996) implemented in MrBayes (v. 3.1.2; Ronquist and Huelsenbeck, 2003). Statistical support was obtained by 25,000 quartet puzzling steps on each topology. The ML analysis was performed specifying the optimal model as reflected by the hierarchical likelihood ratio test (hLRT) as well as by the AIC criteria (Akaike, 1974) implemented in Modeltest (v. 3.6; Posada and Crandall, 1998). Both methods selected the GTR model (Rodríguez et al., 1990), with a gamma distribution shape parameter of α = 0.6518 and a proportion of invariable sites of I = 0.323. The number of gamma rate categories was 8, and the rate matrix parameters were A/C = 14.4014, A/G = 15.9806, A/T = 15.2595, C/G = 13.2010, C/T = 109.9848, and G/T = 1. Sequences were also analyzed using Bayesian inference (BI) as implemented in MrBayes (v. 3.1.2; Huelsenbeck and Ronquist, 2001). Posterior probabilities for the BI were determined by running 3 heated chains (default temperature setting: 0.2) and 1 cold chain for 1,000,000 generations (Ronquist and Huelsenbeck, 2003). The optimal model selected under both hLRT and AIC criteria (see ML analysis above) and its parameters were specified as priors. Each analysis was run twice, and trees were sampled every 100 generations. Stability of likelihood convergence was determined using the “sump” command in MrBayes leading to the exclusion of the first 30,000 samples as burn-in when convergence diagnostics were calculated. Posterior probabilities for nodes were based on the remaining 19,400 topologies. The sequence dataset also included several already published sequences of passerine haemosporidians as references in the construction of the phylogenetic trees.
Number of pairwise nucleotide differences: They were calculated among the sequences of *Leucocytozoon* species within the same bird order using Mega 3 (Kumar et al., 2004). *Haemoproteus* and *Plasmodium* species were not included in the calculations, because only 2 new strains were detected in these parasites.

**RESULTS**

**Morphological analysis**

In the microscopic blood examinations stages of haemosporidian parasites were detected in 20 of 101 individuals (19.8%) (Table I). *Leucocytozoon* spp. infections were most common (10.9%), followed by *Plasmodium* spp. (5.9%) and *Haemoproteus* spp. (4.9%). The only 3 haematozoan parasites that could be morphologically identified to species level were *Haemoproteus noctuae* from a tawny owl (*Strix aluco*), *H. nisi* from a goshawk (*Accipiter gentilis*), and *L. danilewskyi* from a long-eared owl (*Asio otus*).

**PCR screening**

An overall prevalence of 21.8% for haemosporidian parasites was found using the PCR method. The highest prevalence was detected for *Plasmodium* spp. (11.9%), while both *Leucocytozoon* spp. (6.9%) and *Haemoproteus* spp. (2.9%) gave fewer detected infections (Table I). Fourteen of the PCR-positive samples were sequenced, while the remaining 8 samples yielded either only partial or no sequences despite repeated trials. Four strains of *Leucocytozoon* species were found in birds of prey, i.e., *L*-CIAE2 in the European marsh harrier (*Circus aeruginosus*), *L*-ACN1 in the Eurasian sparrow-hawk (*Accipiter nisus*), and *L*-BUBT2 and *L*-BUBT3 in the common buzzard (*Buteo buteo*). In owls we identified 3 *Leucocytozoon* strains (*L*-STAL1 in the tawny owl and *L*-ASOT1 and *L*-ASOT2 in the long-eared owl). As for *Haemoproteus* species, a single strain (*H*-STAL2) was detected in the tawny owl and 1 (*H*-FAT1) in the Eurasian kestrel (*Falco tinnunculus*), whereas the 2 new *Plasmodium* strains were detected in the European marsh harrier (*P*-CIAE1) and in the common buzzard (*P*-BUBT1).

In addition to these 2, 3 more *Plasmodium* strains (previously described in passerines by Waldenström et al., 2002; TURDUSI [AF495576] in the song thrush (*Turdus philomelos*) and SW2 [AF495572] in the sedge warbler (*Acrocephalus schoenobaenus*) and by Hellgren et al., 2004; BT7 [AY393793] in the blue throat (*Luscinia svecica*) were also detected in the rough-legged buzzard (*Buteo lagopus*), common buzzard, Eurasian sparrow-hawk, goshawk (*Accipiter gentilis*), and tawny owl (Table I, last column). The new sequences were submitted to GenBank and assigned accession numbers EF607284–94.

No significant differences were detected between the outcome of the microscopic examination and the PCR screening for species of Haemosporida (Fishers exact test, $P = 0.8625$).

**Phylogenetic analysis**

**Phylogenetic tree:** All but the P-BUBT1 *Plasmodium* spp. sequences clustered together in the phylogenetic tree (Fig. 1) and were grouped with species of the Haemosporida characterized earlier from passerines. The sequences of species of *Leucocytozoon* formed a separate cluster as did sequences from...
0.1 substitutions per site
species of *Haemoproteus* and *Plasmodium*. In the phylogenetic tree, the *Leucocytozoon* sp. sequences from owls clustered together with strains from passerines, while 3 of the *Leucocytozoon* sp. sequences from birds of prey (exception: L-CIAE2) formed a sister cluster to the sequences of *Leucocytozoon* sp. from owls and passerines. Although both *Haemoproteus* sp. sequences, 1 from the tawny owl (H-STAL2) and the other from the Eurasian kestrel (H-FAT1), clustered with the sequences from *Haemoproteus* spp., they appeared separated from the parasite sequences found in Passeriformes (Fig. 1). Of the 2 *Plasmodium* sp. sequences found in birds of prey, the one from the marsh harrier (P-CIAE1) clustered within the *Plasmodium* spp. strains found in passerine birds. The placement of the *Plasmodium* sp. sequence from the common buzzard (P-BUBT1) was unexpected, being positioned outside of the cluster of the *Plasmodium* spp. sequences; support for this placement, however, was very low (data not shown).

**Pairwise nucleotide differences:** The pairwise sequence differences within the *Leucocytozoon* sp. strains of the Falconiformes ranged from 9 to 26%, with 5 of 6 comparisons displaying ≥20% difference. Among *Leucocytozoon* strains of the Strigiformes, the pairwise sequence differences were only 2–6%.

**DISCUSSION**

We applied 2 methods for the detection of blood parasites, 1 based on traditional examination of blood smears (Valkiūnas, 2005) and the other based on amplification of parasite mtDNA (Bensch et al., 2000; Hellgren et al., 2004; Waldenström et al., 2004). Both methods detected haemosporidian parasites at similar levels (Table I). Several earlier studies had stated that molecular-based methods were superior to microscopic examination for detecting haemosporidian infections in birds (Richard et al., 2002; Waldenström et al., 2004; Durrant et al., 2006). In the present study, however, the molecular methods failed to detect some infections, although parasites were observed in the smears and vice versa (Table I). Valkiūnas, Bensch et al. (2006) and Kržanauskienė et al. (2006) reported similar PCR screening problems when trying to detect haemosporidian parasites that had been previously diagnosed morphologically. It should be noted, however, that the primers used here were originally designed to detect haemosporidian parasites of passerine birds (Bensch et al., 2000; Hellgren et al., 2004; Waldenström et al., 2004) and thus may not be equally efficient in detecting infections in more distantly related avian orders. Best congruence was observed for the *Plasmodium* spp., not surprisingly perhaps, because species of this genus are known to be the least host-specific of the investigated parasites (Waldenström et al., 2002). However, recent findings suggest that some species of *Haemoproteus* are less host specific than previously thought (Kržanauskienė et al., 2006). Further development of suitable primers is necessary to determine the real composition (regarding different strains and/or species) of the haemosporidian parasite fauna in each individual host. Because both PCR screening and microscopy have shortcomings in their capability to detect parasites (Valkiūnas, Bensch et al., 2006), we recommend a combination of both methods. It is worth noting that Durrant et al. (2006) reported an approximately 10-fold reduction in sensitivity via microscopy compared to the PCR screening in the diagnosis of haemosporidian infections in birds. Our study and those of others (Kržanauskienė et al., 2006; Valkiūnas, Bensch et al., 2006) do not support these findings. According to our data, both methods detected similar parasite prevalences.

Given the genetic variability within populations and species, the number of strains detected by PCR exceeded the number of *Leucocytozoon* species described morphologically. After the taxonomic revision of the *Leucocytozoon* species described morphologically from Strigiformes and Falconiformes by Greiner and Kocan (1977) and Valkiūnas (2005), numerous species described previously collapsed and were declared to be synonyms of *L. ziemanni* (synonym of *L. danilewskyi*) and *L. toddi*. However, although the sequence diversity of *Leucocytozoon* strains detected by PCR (3 strains in Strigiformes and 4 in Falconiformes) in our dataset was expected as a reflection of the genetic variability within a single species, it may also reflect the existence of an as yet unidentified *Leucocytozoon* subspecies or species in birds of prey. This hypothesis is based on the pairwise comparisons of *Leucocytozoon* sp. sequences among birds of prey, which ranged from 9 to 26%, but only 2 to 4% among owls. Thus, it is feasible that the low nucleotide differences in pairwise comparisons stem from intra-parasite-species variability (such as calculated for all pairwise parasite strain comparisons from owls and in 1 from birds of prey), whereas the high values (≥20%) seen in 5 pairwise parasite sequence comparisons from birds of prey may well represent parasite species differentiation. However, we are currently unable to distinguish between these 2 possibilities. Because of the current lack of reliable morphological characters for separating *Leucocytozoon* spp. from hosts of the Falconiformes and Strigiformes (Greiner and Kocan, 1977; Valkiūnas, 2005), differences in gene sequences may gain more importance as characteristics for taxonomic classification in the future.

One goal of the present study was to correlate the sequences with parasite species that had previously been identified morphologically. Because of the low intensity of haemosporidians and the presence of mixed infections with haemosporidians, we were able to assign the obtained sequence to a parasite in only 1 case. Morphological identification in this instance was pos-

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**Figure 1.** Bayesian inference-based phylogenetic tree of haemosporidian parasites examined in this study and of related species. Numbers at nodes indicate statistical support for the three approaches applied: maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI). BI support values were multiplied by 100 for facility of inspection. The MP tree was obtained by constructing the consensus tree of 31 equally parsimonious trees. Sequences newly obtained in this study are underlined, with sequences of birds of prey additionally marked in bold font. Dotted brackets indicate passeriform hosts. All nonfalconiform and nonstrigiform blood parasite sequences were obtained from the following publications: Bensch et al. (2000), Escalante et al. (1998), Hellgren et al. (2004), Perkins and Schall (2002), and Waldenström et al. (2002). Black triangles and dots mark sequences of *Plasmodium* spp. strains that had previously been described in Passeriformes but were found in this study in birds of prey (triangles) and owls (dot). * = MP support < 50%; — = not resolved in analysis; † = position was basal to P-Parus2 in the ML analysis.
sible because this parasite was visible in the blood smear in sufficient numbers and stages. This was the strain from the tawny owl (H-STAL2), which was identified as originating from Haemoproteus noctuae. Other haemosporidian species were diagnosed in the blood smears, but a correlating sequence could not be amplified (Table 1). The low intensities found in the present study may be caused by the large number of winter birds sampled and by the large number of nestlings examined. In these birds transmission rates are low (Valkiūnas, 2005), despite the prevalences being similar to the ones found in a former study from the same area (Krone et al., 2001). Our results corroborate data from Sehgal et al. (2006) who also detected several different strains of Leucocytozoon sp. in Californian raptors, but were not able to assign these strains to known species. The authors, therefore, suggested a reassessment of the nomenclature. In contrast to Leucocytozoon sp., the number of detected Haemoproteus and Plasmodium strains was much smaller than the number of accepted species. This may be explained by the small sample size of birds examined here, i.e., only 1 strain of Haemoproteus was found in the tawny owl and another in the Eurasian kestrel. Both were clearly assigned to the Haemoproteus spp. cluster, together with sequences from Passeriformes and a dove (H. columbae) (Fig. 1). The only 2 new strains of Plasmodium were found in the European marsh harrier and in the common buzzard. In addition, the 3 Plasmodium strains described earlier from Passeriformes were also found in the birds examined here (Fig. 1), corroborating previous findings (Waldenström et al., 2002) that indicated a broad and unspecific host spectrum bridging different host orders in Plasmodium.

ACKNOWLEDGMENTS

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LITERATURE CITED


**moproteus columbae.** Transactions of the Royal Society of Tropical Medicine and Hygiene 186–187.


A REVIEW OF SPECIES IN THE GENUS RHOPALIAS (RUDOLPHI, 1819)

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ABSTRACT: Currently, there are 6 recognized species in the genus Rhopalias. These parasites are found in the small intestines of marsupials throughout North and South America. Small mistakes in various classical taxonomic works have given rise to recent and numerous misidentifications of these species. In this work, we examine a total of 99 specimens across all species from museum collections in an attempt to determine informative taxonomic characters to distinguish these species. Despite confusion in the literature, accurate identification of these species can be achieved by observing the presence or absence of oral and flanking spines anterior to the oral sucker.

Species of Rhopalias (Trematoda: Rhopalidae) occur in the small intestines of marsupials throughout the Nearctic and Neotropical regions. Six species are currently recognized as valid. Rudolphi (1819) described Distoma coronatum from Didelphis marsupialis Linnaeus, 1758 collected by Johann Natterer in Brazil. Diesing (1850) described Rhopalophorus horridus from Chironectes minimus (Zimmermann, 1780) also collected by Natterer. Stiles and Hassall (1898) recognized that Rhopalophorus was an occupied name, and renamed the genus Rhopalias. Braun (1901) provided formal descriptions of both previously described species, and recognized and described another species, Rhopalias baculifer, from the same material examined by Diesing (1850). Chandler (1932) described Rhopalias macracanthus from North America in Didelphis virginiana Kerr, 1792. Kifune and Uyema (1982) described Rhopalias caballeroi from D. marsupialis and Philander opossum (Linnaeus, 1758) in Brazil. Finally, Rivallis et al. (2004) described the most recent species in this genus, Rhopalias caucensis from P. opossum collected in Colombia.

Skrjabin (1948) reproduced the descriptions of the species in the genus and provided an identification key to the known species of Rhopalias in that work, it is apparent that the labels for the figures referring to R. baculifer and R. horridus were reversed. Travassos et al. (1969) reproduced the work of Skrjabin (1948), along with the mislabeled figures. In the summary work of Yamaguti (1971), Figure 1651 of Rhopalias coronatus is a reproduction of R. horridus after Caballero (1946); this figure is in fact a representation of the currently recognized species R. caballeroi. In the same work (Yamaguti, 1971), Figure 1650B of R. coronatus, after Caballero (1946), but attributed to Braun (1901), also appears to be a drawing of R. caballeroi, but because the tentacles are covering the area where flanking spines would be found, identification to species is not possible from the Figure (although it is likely R. caballeroi). Finally, Radev et al. (2005) offer original figures of R. macracanthus drawn from specimens stored in the Manter Laboratory of Parasitology (HWML 0844, 22422, 22423). These specimens have been verified by 1 of us (T.R.H.) as R. macracanthus, but the figures of these specimens in Radev et al. (2005) lack the defining flanking spines, making them easily mistaken for R. caballeroi.

The species of Rhopalias are some of the most distinctive and easily recognized trematodes of mammals. However, the correct identification of the species in this genus has remained a difficult task, given the flaws in the above references and the lack of a thorough investigation of important taxonomic characters. Here, we intend to provide the most thorough investigation, to our knowledge, of the 6 currently recognized species in this genus.

MATERIALS AND METHODS
Specimens prepared and studied by us were stained in acetic Semichon’s carmine, dehydrated in an ethanol series, cleared in terpineol and xylene, and mounted in Canada balsam or gum Damar (Prichard and Kruse, 1982). All other specimens studied were obtained from museum collections including: the United States National Parasite Collection (USNPC), Beltsville, Maryland; the Harold W. Manter Laboratory of Parasitology (HWML), Lincoln, Nebraska; the Naturhistorische Museum Wien (NMW), Vienna, Austria; the Universidad Nacional Autónoma de México (UNAM), Mexico City, Mexico; the Museo de Historia Natural at the Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Peru; and the Kyushu University Museum (FUK), Fukuoka, Japan. Due to the commonality of misidentified and mislabeled specimens throughout the previous literature and in museum holdings, this paper deals only with specimens that have been studied by the authors. We do not include a comparative table of measurements from previous studies for this same reason. All measurements were taken with a Zeiss Ultraphot microscope equipped with an ocular micrometer and are presented to the nearest micrometer unless otherwise noted; ranges are followed by the mean and the number of characters studied (n) is given if different from the number of specimens studied (N). Canonical discriminant analysis (CDA) was performed on the natural log transformations of the 23 measurements summarized in Tables I and II excluding egg length and egg width using PROC CANDISC in SAS (version 6.12, SAS Institute, Cary, North Carolina).

RESULTS
Specimens examined
Rhopalias coronatus (22 specimens total): HWML34950 (1 specimen) from Didelphis albiventris, Paraguay; HWML70000 (12 vouchers) from P. opossum, Santa Cruz Department, Bolivia; HWML70002 (1 specimen) from P. opossum, Santa Cruz, Department, Bolivia; HWML70009 (3 vouchers) from P. opossum, Santa Cruz, Bolivia; HWML70013 (1 specimen) from Lutreolina crassicaudata Berisso, Argentina; UNAM4081 (1 specimen) from Didelphis sp., Veracruz, Mexico; USNPC14998 (1 specimen) from unknown host, Panama; USNPC72792 (2 vouchers) from D. marsupialis, El Tlacal, Venezuela.

Rhopalias horridus: V4677 (1 neotype, 1 voucher) from C. minimus, Brazil.

Rhopalias caucensis (4 specimens total): UNAM1225 (2 specimens) from Didelphis mesamericana, Guazacapa, Guate-
Table I. Measurements of *Rhopalias* species including *R. coronatus*, *R. baculifer*, and *R. horridus* by coefficient of variation (CV), distance from the anterior extreme to the anterior margin of the vitellaria (ANTVIT), and distance from the anterior margin of the vitellaria to the posterior margin of the acetabulum (ACEVIT).*

<table>
<thead>
<tr>
<th>Character</th>
<th><em>R. coronatus</em> (N = 22)</th>
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<th><em>R. baculifer</em> (N = 8)</th>
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<th><em>R. horridus</em> (N = 2)</th>
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<td>n</td>
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<td>Range</td>
<td>CV</td>
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<td>Mean</td>
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<tr>
<td>Total length</td>
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<td>47  8</td>
<td>9,369</td>
<td>7,128–12,600</td>
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<td>2,463</td>
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<tr>
<td>Total width</td>
<td>22</td>
<td>735</td>
<td>219–1,584</td>
<td>51  8</td>
<td>998</td>
<td>840–1,162</td>
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<td>489</td>
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<td>Length</td>
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<td>Length</td>
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<td>Length</td>
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<td>Width</td>
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<td>Length</td>
<td>21  169</td>
<td>75–344</td>
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<td>375–1,188</td>
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<td>269</td>
<td>210–319</td>
<td>18  2</td>
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<td>35  8</td>
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<td></td>
<td>Length</td>
<td>22  39</td>
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<td>231 , 8</td>
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<td>0–70</td>
<td>153  2</td>
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<td></td>
<td>Width</td>
<td>21  104</td>
<td>30–244</td>
<td>61  8</td>
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<td>104–200</td>
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<td>Length</td>
<td>20  186</td>
<td>0–606</td>
<td>101  8</td>
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<td>0–0</td>
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<td>22  1,160</td>
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<td>72  8</td>
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<td>&gt;200</td>
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<td></td>
<td>Width</td>
<td>94  90</td>
<td>70–108</td>
<td>9  36</td>
<td>84</td>
<td>68–98</td>
<td>8  10</td>
<td>68</td>
<td>65–72</td>
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</tbody>
</table>

* N; total number of specimens; n; number of measurements. All data is rounded to the nearest mm.

mala; UNAM966 (1 specimen) from *D. marsupialis*, San Jose, Costa Rica; USNPC92122 (1 specimen) from *P. opossum*, Colombia.

*Rhopalias baculifer* (8 specimens total): HWML35933 (2 specimens) from *C. minimus*, Panama; HWML70012 (4 specimens) from *P. opossum*, Catemaco, Mexico; UNAM1137 (2 specimens) from *P. opossum* Alajuela, Costa Rica.

*Rhopalias macracanthus* (25 specimens total): HWML22664 (1 specimen) from *D. virginiana*, Tallahasee, Florida; HWML70001 (2 specimens) from *P. opossum*, Santa Cruz Department, Bolivia; HWML70028 (1 specimen) from *P. opossum*, Santa Cruz, Bolivia; HWML70003 (1 specimen) from *P. opossum*, Santa Cruz, Department, Bolivia; HWML70010 (1 specimen) from *P. opossum*, Santa Cruz Department, Bolivia; UNAM1226 (6 specimens) from *D. mesamericana*, Chiapas, Mexico; UNAM2956 (1 specimen) from *D. virginiana*, Veracruz, Mexico; UNAM2957 (1 specimen) from *D. virginiana*, Veracruz, Mexico; UNAM4622 (1 specimen) from *D. virginiana*, Veracruz, Mexico; UNAM968 (4 specimens) from *D. marsupialis*, Colima, Mexico; UNAM4081 (1 specimen) from *Didelphis* sp.; USNPC5745 (1 specimen) from *D. virginiana*, Washington,
TABLE II. Measurements of *Rhopalias* species including *R. caballeroi*, *R. caucensis*, and *R. macracanthus* by coefficient of variation (CV), distance from the anterior extreme to the anterior margin of the vitellaria (ANTVIT), and distance from the anterior margin of the vitellaria to the posterior margin of the acetabulum (ACEVIT).*

<table>
<thead>
<tr>
<th>Character</th>
<th><em>R. caballeroi (N = 37)</em></th>
<th><em>R. caucensis (N = 4)</em></th>
<th><em>R. macracanthus (N = 25)</em></th>
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<td></td>
<td><em>n</em></td>
<td>Mean</td>
<td>Range</td>
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<td>813–3,489</td>
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<td>Total width</td>
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<td>691</td>
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<td>Acetabulum</td>
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<tr>
<td>Length</td>
<td>37</td>
<td>228</td>
<td>24–319</td>
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<tr>
<td>Oral sucker</td>
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<td>Length</td>
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<td>132</td>
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<td>Width</td>
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<td>135</td>
<td>75–288</td>
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<td>Cirrus sac</td>
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<tr>
<td>Length</td>
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<td>Posterior testis</td>
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<tr>
<td>Length</td>
<td>68</td>
<td>82</td>
<td>53–105</td>
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<tr>
<td>Width</td>
<td>68</td>
<td>45</td>
<td>32–60</td>
</tr>
</tbody>
</table>

*N*; total number of specimens; *n*; number of measurements. All data is rounded to the nearest µm.

DC; USNPC69744 (2 specimens) from *D. virginiiana*, Florida; USNPC75092 (1 specimen) from *D. virginiiana*, Leon County, Florida; USNPC8548 (1 specimen) (paratype) from *D. virginiiana*, Houston, Texas.

*Rhopalias caballeroi* (37 specimens total): FUK264-1 (1 specimen), FUK311 (9 specimens), FUK584 (2 specimens) (paratypes) from *D. marsupialis*, Huanuco, Peru; HWML70021 (8 specimens) from *P. opossum*, Santa Cruz Department, Bolivia; HWML35933 (3 specimens) from *C. minimus*, Panama; UNM531076 (5 specimens) (paratopotypes) from *P. opossum*, Huanuco, Peru; UNM531077 (1 specimen) (paratopotype) from *D. marsupialis*, Huanuco, Peru; HWML70014 (1 specimen) from *L. crassicaudata*, Berisso, Argentina; UNAM4081 (1 specimen) from *Didelphis* sp., Veracruz, Mexico; UNAM965 (1 specimen) from *D. marsupialis*, Venezuela; USNPC92124 (5 specimens) from *P. opossum*, Colombia.

**Description with translation—original from Lamothe-Argumedo (1979)**

*Original description: Rhopalias (Diesing, 1850) Stiles and Hassall, 1898.*
Trematoda con cuerpo alargado, espinoso; la porción anterior excavada ventralmente más amplia que la posterior y con un par de trompas retráctiles armadas con ganchos. Ventosa oral subterminal, con prefaringe, faringe musculosa y esófago; ciegos largos llegando al extremo posterior del cuerpo. Acetábulo mayor que la ventosa oral, cerca del extremo anterior. Testículos de forma variable situados en el tercio medio del cuerpo o más posteriormente, uno atrás del otro; bolsa del cirro larga, claviforme, extendiéndose posteriormente al acetábulo y conteniendo un complejo prostático bien desarrollado. Poro genital medio preacetabular. Con un agujero glandular preacetabular en forma de copa que se abre inmediatamente abajo del poro genital. Ovario esférico, medio, postacetabular y pretesticular; no existe un receptáculo seminal, útero largo o corto, en el campo intercecal, entre el ovario y el poro genital. Vitelógenas foliculares, dispuestas en los campos laterales que pueden o no confluir en el espacio pretesticular, desde el borde posterior del acetábulo hasta el extremo posterior del cuerpo. Parásitos intestinales de marsupiales, ocasionalmente de otros animales. Diagnosis general, con los caracteres de la familia. Especie tipo: *Rhopalias coronatus* (Rudolphi, 1819) Stiles y Hassall, 1898.

**Translation:** Trematodes with a long, thorny body; forebody concave, wider than posterior part and with a pair of armed retractile tentacles with hooks. Subterminal oral sucker, with prepharynx, muscular pharynx, and esophagus; long ceca extending to the posterior end of body. Acetabulum larger than oral sucker, near anterior end. Testicles of variable shape located at midbody, in tandem; cirrus pouch long, claviform, extending beyond the acetabulum and containing a well-developed prostate complex. Genital pore preacetabular at midbody or midline with a glandular preacetabular hole that opens immediately behind the genital pore. Ovary spherical, located at midbody, postacetabular and pretesticular; without seminal receptacle, uterus in intercecal field between ovary and genital pore. Vitelín folicules, arranged in lateral fields may or may not come close together in pretesticular space, from posterior edge of the acetabulum to posterior end of body. Intestinal parasites of marsupials, occasionally of other animals. Generic diagnosis, with the characters of family. Type species: *Rhopalias coronatus* (Rudolphi, 1819) Stiles and Hassall, 1898.

**Remarks**

Although most accounts of species of *Rhopalias* mention the “oral spines,” it is evident that most workers did not always distinguish between the 2 sets of spines present on some of the specimens. We recognize 2 separate sets of spines on the body. One set, herein called the “oral spines,” lies immediately anterior to the oral sucker. These spines are often arranged in 2 rows and 8–16 spines may be visible at any one time. The second set of spines, herein called the “flanking spines,” are a paired set arranged laterally to the oral spines. The number of visible spines from each flanking set may number from 2 to 6. Often, the flanking spines are contrasted from the oral spines by a small physical separation or by being in a different orientation after permanent mounting on a slide.

The presence of the ventral hood in these species can cause problems with various measurements by distorting the width of the specimen and the distance between the anterior extreme of the worm and the acetabulum. The ventral hood is usually more pronounced in larger specimens, and more likely to be prominent in *R. coronatus* than any of the other species.

**Figure 1.** *Rhopalias coronatus*. (A) Entire specimen. (B) Anterior end. Both specimens are HWML70000. Both scale bars = 0.1 mm.

*Rhopalias coronatus* (Rudolphi, 1819)
Stiles and Hassall, 1898
(Fig. 1)

**Synonyms:** Rhopalias dobbini Prod’Hon 1968

**Diagnosis:** Flanking and oral spines present. Between 3 and 11 spines visible within tentacle sacs; spines measuring between 32 and 67 long (56). Size of spines on tentacles varies according to position of that spine on tentacle. Seen clearly on fully everted tentacle: proximal spines shorter than distal spines. Spines concentrated in proximal
half of tentacle. Tentacle sacs very long, reaching far beyond posterior margin of pharynx. Tentacles may or may not reach acetabulum. Specimens 2,160–9,360 (4,440) long by 219–1,584 (735) wide. Acetabulum 150–840 (376) long by 150–816 (350) wide. Oral sucker 93–344 (183) long by 88–325 (180) wide. Cirrus sac 563–2,219 (970) long, terminating near ovary. Testes in tandem, usually overlap, anterior testis 156–625 (333; n = 21) long by 100–281 (167; n = 21) wide and posterior testis 256–919 (499; n = 21) long by 75–281 (149; n = 21) wide. Ovary 75–344 (169; n = 21) long by 88–350 (178; n = 20) wide. Prepharynx absent or present and up to 313 (39) long. Pharynx 115–425 (202) long by 30–244 (104; n = 21) wide. Esophagus absent or present and up to 606 (186; n = 20) long. Specimens with average of 24 eggs in uterus, eggs measure 70–108 (90; n = 84) long by 38–70 (51; n = 95) wide.

Figure 2. Rhopalias horridus. (A) Entire specimen. (B) Anterior end. Both specimens are NMW4677. Both scale bars = 0.1 mm.
**Remarks**

*Rhopalias coronatus* is easily distinguished from almost all other species mainly by the extent of the tentacle sacs. These tentacle sacs in *R. coronatus* extend far beyond the posterior margin of the pharynx, often, but not necessarily, to the acetabulum. Specimens of *R. horridus* share this feature, but these 2 species can be distinguished as the tentacular spines of *R. horridus* are clearly visible, small, and numerous, whereas those of *R. coronatus* are often hard to see, large, and number less than 10 spines per tentacle. Also, *R. coronatus* has both oral spines and flanking spines, whereas *R. horridus* has neither.

*Rhopalias horridus* (Diesing, 1850)  
Stiles and Hassall, 1898  
(Fig. 2)

**Synonyms:** Rhopalias goyanna Komma and Alves, 1974


**Remarks**

Although this species has been reported numerous times from throughout South America, all specimens of *R. horridus* examined by us during this study were misidentifications. The only material available for study for this species comes from the specimens described by Braun (1901). Three vials of specimens were provided to us by the NMW in Vienna, Austria. Only 2 specimens prepared from these vials were identified as *R. horridus*. The rest of the specimens, as noted by Braun (1901), are *R. coronatus*.

There is no mention by Braun (1901) or by Gomes and Vicente (1972), of spines flanking the oral sucker in this species. However, the figures provided by Gomes and Vicente (1972) depicting this species clearly indicate the presence of flanking spines. This observation is repeated in their Table 1. Inspection by 1 of us (S.L-G.) of the specimens listed in the study of Gomes and Vicente (1972) revealed that these specimens were *R. horridus* and that no flanking spines were present.

Komma and Alves (1974) described *R. goyanna* from Didelphis azarae in Brazil. The specimens they describe are attenuated just posterior to the testes. These specimens are actually body spines. The authors refer to in their description are not the oral spines as recognized by possessing short tentacle sacs, i.e., not extending beyond the posterior margin of the pharynx, and the presence of both oral spines and flanking spines.

*Rhopalias baculifer* Braun, 1901

(Fig. 4)

**Diagnosis:** Flanking and oral spines absent. Between 8 and 10 spines visible within tentacle sacs, these spines large and few; average length from 85–138 (113; n = 10). Between 8 and 10 spines observed from each tentacle. Tentacle sacs never reach beyond posterior margin of pharynx. Tentacle sacs 275–320 (293; n = 3) long by 113–140 (123; n = 3) wide. Specimens 1,999–2,471 (2,211) in total length and 576–799 (725) in total width. Acetabulum 210–335 (264) long and 194–290 (242) wide. Oral sucker 125–185 (154) long by 118–175 (147) wide. Cirrus sac 575–775 (685; n = 3) long. Testes in tandem, do not necessarily overlap. Anterior testis 150–225 (196) long and 213–360 (293) wide. Posterior testis 220–388 (308) long by 94–275 (222) wide. Ovary 100–160 (140) long by 150–240 (199) wide. Prepharynx absent or up to 125 (46). Pharynx 120–175 (149) long by 85–100 (92) wide. Esophagus never observed. Specimens average 7 eggs in uterus; eggs 92–98 (95) long by 44–52 (49) wide.

**Remarks**

*Rhopalias caucensis* can be distinguished from other species of *Rhopalias* by possessing short tentacle sacs, i.e., not extending beyond the posterior margin of the pharynx, and the presence of both oral spines and flanking spines.

*Rhopalias baculifer* Braun, 1901

(Fig. 4)

**Diagnosis:** Flanking and oral spines absent. Between 8 and 10 spines visible within tentacle sacs, these spines large and few; average length from 50–120 (86; n = 26). Tentacle sacs 210–319 (269) long by 63–150 (104) wide and never reach posterior margin of pharynx. Specimens 7,128–12,600 (9,369) long and 840–1,162 (998) wide. Acetabulum 406–575 (478) long by 435–575 (508) wide. Oral sucker 190–356 (275) long, 230–331 (249) wide. Cirrus 813–1,290 (981) long and never reaches ovary. Testes lie in tandem, slightly overlap: anterior testis 720–1,488 (1,188) long by 127–336 (257) wide; posterior testis 888–1,848 (1,416) long by 164–312 (243) wide. Ovary 181–394 (302) long and 206–350 (285; n = 7) wide. Prepharynx absent or up to 70 (20). Pharynx 150–281 (215) in length by 104–200 (158) in width. Esophagus never observed in specimens studied. Always more than 200 eggs in uterus, eggs measuring 68–98 (84; n = 36) long by 38–63 (51; n = 36) wide.

**Remarks**

Rivallis et al. (2004) revise the species by describing a “short form” of *R. baculifer*. We believe these specimens represent *R. caballeroi*. The authors used Travassos et al. (1969) in their identification, and likely viewed only the figures. *Rhopalias baculifer* and *R. caballeroi* have no oral nor flanking spines, but these 2 species are easily distinguished by total length, as, from our measurements, the shortest specimen of *R. baculifer* (7,126) is more than twice as long as the longest specimen of *R. caballeroi* (3,489).

*Rhopalias baculifer* is by far the longest species in this group. It is easily distinguished from *R. coronatus* by the tentacle sacs, with these sacs in *R. baculifer* never reaching beyond the posterior margin of the pharynx. Also, the cirrus sac in *R. baculifer* is very short and does not reach the ovary, as in all other species.

*Rhopalias macracanthus* Chandler, 1933

(Fig. 5)

**Synonyms:** Rhopalias louisiana Hearin, 1937

**Diagnosis:** Oral spines absent. Flanking spines present. Between 6 and 10 spines visible within tentacle sacs, spines 83–138 (108; n = 52) in length. Tentacle sacs 150–415 (308) long by 45–190 (139) wide, never reach beyond posterior margin of pharynx. Specimens 969–7,093 (3,683) long and 360–1,508 (966) wide. Acetabulum 155–538 (359) long by 125–475 (341) wide. Oral sucker 88–242 (168) long by 70–264 (185) wide. Cirrus sac 375–2,300 (1,232; n = 24) long, terminates close to ovary. Testes lie in tandem, may or may not overlap. Anterior testis 63–675 (346) long and 58–475 (286) wide; posterior testis 123–806 (523; n = 23) long and 70–430 (275; n = 23) wide. Ovary 88–282 (188) long by 85–300 (213) wide. Prepharynx absent or up to 314 (92). Pharynx 70–230 (170) long by 50–205 (139) wide. Esophagus absent or up to 113 (43). Specimens averaged 22 eggs in uterus; eggs 75–113 (98; n = 67) long by 36–74 (55; n = 67) wide.

**Remarks**

*Rhopalias caucensis* can be distinguished from other species of *Rhopalias* by possessing short tentacle sacs, i.e., not extending beyond the posterior margin of the pharynx, and the presence of both oral spines and flanking spines.
Remarks

_Rhopalias macracanthus_ can be distinguished from other species of _Rhopalias_ by having tentacle sacs that do not extend beyond the posterior margin of the pharynx and by having only flanking spines.

_Diagnosis_: Oral and flanking spines absent. Between 4 and 11 spines visible within tentacle sacs, spines 48–131 (93; n = 76) in length. Tentacle sacs 138–358 (251; n = 36) long by 38–184 (94; n = 36) wide,
wide. Esophagus absent or up to 114 (16; n = 36). Specimens averaged 8 eggs in uterus, eggs 53–105 (82; n = 68) long by 32–60 (45; n = 68) wide.

Remarks

We were able to obtain a majority of the type series for examination and found that the specimens had neither flanking nor oral spines. Kifune and Uyema (1982) remark that these spines are "usually" absent. Rivals et al. (2004) recently collected this species from Colombia, but misidentified it as *R. baculifer*.

Because there has been much confusion in the literature in identification of the species of *Rhopalias*, we provide a key to the species.

**Key to the species of *Rhopalias***

1a. Tentacle spines small, numerous (>30); oral and flanking spines absent                  ................................................. *R. horridus*

1b. Tentacles spines few (<15); large; oral and flanking spines present or absent  ................................................................. 2

2a. Tentacle sacs extending far beyond posterior margin of pharynx  ................................................................. *R. coronatus*

2b. Tentacles not extending beyond posterior margin of pharynx                                                                                                                  3

3a. Oral and flanking spines both absent                                                                 .......................... 4

3b. Some combination of oral and/or flanking spines present ................................................................. 5

4a. Specimen exceeds 6 mm total length ................................................................................................. *R. baculifer*

4b. Specimen less than 6 mm total length ................................................................................................. *R. caballeroi*

5a. Both oral and flanking spines present ................................................................................................. *R. caucensis*

5b. Oral spines absent, flanking spines present ................................................................................................. *R. macracanthus*

**DISCUSSION**

In their description of *R. goyanna*, Komma and Alves (1974) describe the body of the specimen as "pinched," with the specimen missing its body posterior to the testes. This phenomenon was seen by us in a random assortment of *R. coronatus* specimens in the HWML collection. Specimens were seen in various stages of this pinching, which seemed to leave the worm intact, because the specimens did not seem to be leaking fluids. In some specimens, the posterior ends of the body appear shriveled and not pinched, but we believe that both scenarios likely lead to the same conclusion of a truncated body. No single factor, e.g., intra- or interspecific cooccurrence or crowding in the intestines, seemed a commonality with the occurrence of the pinching; a potential source of a specimen's pinching was a trichostrogyloid nematode seen wrapped around the constricted area.

In the past, researchers studying *Rhopalias* species have used various (and often untested) characters to make taxonomic decisions. These characters include, but are likely not limited to, distribution of the body spines (Hearin, 1937), size of the tentacle spines (Prod’Hon, 1968), and relative position of the vitellaria (Miyazaki et al., 1978). We agree with Braun (1901) that it would be unwise to use body spines as a character in species discrimination, as the spines are fragile and are known to fall off during the processes of collecting, fixing, staining, or mounting the specimen (Braun, 1901). Length of spines on the tentacles is also not a good character for species discrimination, especially in *R. coronatus*, as the tentacle spine length in this species can vary widely in a single individual. Our analysis included 2 measurements where we recorded the distribution and anterior and posterior extents of the vitellaria, the distance from the anterior extremity of the body to the vitellaria, and the distance from the posterior margin of the acetabulum to the vitellaria. We include this measurement in Tables I and
II and in the discriminant analysis to determine its potential in species separation.

In our analysis, proper discrimination of species was not achieved using any single quantitative character, but the results of the CDA (Fig. 7) showed that, using a linear combination of all characters, discrimination of species was possible. The first CDA performed included all 6 species. The analysis provided good separation of *R. coronatus* and *R. baculifer* against an indiscriminant cluster of the other 4 species. While the analysis is interesting, little information is gained, as *R. coronatus* and *R. baculifer* are relatively easy to distinguish anyway and most of the confusion from past studies has involved misidentifica-
tions of the other 4 species. Thus, a second CDA was performed on only these 4 species. Based on the results of this analysis, the first 2 canonical variates were significant ($P < 0.001$) and accounted for 89% of the variation in the analysis (see Table III). Each canonical variate is a linear combination of the independent variables (measurements), and each variate is independent of the other. Since the variate is a linear combination of each variable, the canonical loading associated with each variable can be interpreted as the relative contribution that variable has on each variate. Thus, it follows that cirrus length (CIRL), cirrus width (CIRW), pharynx width (PHW), and acetabulum length (ACEL) have the largest relative contribution
to the first canonical variate, and thus species discrimination. The centroid values for the first 2 variates for each species were plotted (Fig. 7), along with a circle representing 1 standard deviation around each centroid. The graph clearly shows that the CDA supports the taxonomic separation of all species in this genus.

The observations and analyses herein show that the confusion with the identification of these species can be eliminated when using taxonomically informative characters. However, with the wide variation in size exhibited by most of the species in this analysis, it would be beneficial to collect more specimens throughout the Neotropics and Nearctic to conduct an analysis on more temporally consistent specimens and determine if this variability can be attributed to geographic distance among populations.

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**TABLE III.** The canonical discriminant loadings of the 23 independent variables for 2 canonical discriminant variates that distinguish 4 species of Rhopalias (R. caucensis, R. horridus, R. caballeroi, R. macracnthus).

<table>
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<th>Variable</th>
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A NEW SPECIES OF EIMERIA (APICOMPLEXA: EIMERIIDAE) FROM THE MARBLED SALAMANDER, AMBYSTOMA OPCUM (CAUDATA: AMBYSTOMATIDAE), FROM NORTHERN LOUISIANA

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ABSTRACT: Between December 2002 and June 2004, 10 marbled salamanders, Ambystoma opacum, were examined for coccidian parasites. Salamanders were collected in Bradley (n = 2), Little River (n = 1), Miller (n = 1), and Sevier (n = 1) Counties, Arkansas; Webster Parish, Louisiana (n = 2); and Bowie (n = 1) and Nacogdoches (n = 2) Counties, Texas. Two of 10 (20%) A. opacum from Louisiana harbored an undescribed species of Eimeria. Oocysts of Eimeria trauthi n. sp. were ellipsoidal, 36.6 × 33.1 (33–40 × 29–37) μm, with a thin, single-layered wall; shape index 1.1. Polar granule(s) and micropyle were absent. Oocyst residuum was composed of hundreds of loosely packed homogenous granules of various sizes enclosing a vacuole. Sporocysts were elongate-ellipsoidal, 20.8 × 8.1 (19–22 × 7–9) μm; shape index 2.6. Sporocyst residuum was spherical and composed of a cluster of granules often membrane-bound. This is the first time a coccidium has been reported from an amphibian species in Louisiana and the second time a coccidium has been described from this salamander host. In addition, the following 26 salamanders from various counties in Arkansas, Oklahoma, and Texas were surveyed during the study period and were negative for coccidia: Ambystomatidae, 4 spotted salamanders (Ambystoma maculatum) and 7 mole salamanders (Ambystoma talpoideum); Cryptobranchidae, 4 Ozark hellbenders (Cryptobranchus alleganiensis bishopi); Plethodontidae, 6 spotted dusky salamanders (Desmognathus conanti) and 3 many-rubbed salamanders (Eurycea multiplicata multiplicata); and Salamandridae, 2 central newts (Notophthalmus viridescens louisianensis).

Upton et al. (1993) provided a taxonomic summary of the coccidian parasites of salamanders (order Caudata) of the world. However, over the last decade or more, little has been documented on additional coccidia infecting salamander hosts. McAllister et al. (2002) reported an Eimeria sp. from 1 of 16 (6.3%) Kiamichi slimy salamanders, Plethodon kiamichi, from western Arkansas; however, few oocysts completed sporulation. More recently, Duszynski et al. (2003) summarized the coccidia of Caudata. Since then, to our knowledge, there have been no new coccidians described from North American salamanders. Herein, we provide a description of a new species of Eimeria from marbled salamanders, Ambystoma opacum, from northern Louisiana.

METHODS AND MATERIALS

Between December 2002 and June 2004, 10 juvenile and adult A. opacum (mean ± 1SE snout-vent length [SVL] = 48.7 ± 6.2, range 36–61 mm) were collected by hand and examined for coccidia. Salamanders were collected in Bradley (n = 2), Little River (n = 1), Miller (n = 1), and Sevier (n = 1) Counties, Arkansas; Webster Parish, Louisiana (n = 2); and Bowie (n = 1) and Nacogdoches (n = 2) Counties, Texas. For comparative purposes, 26 additional salamanders of various taxa were collected and examined for coccidia as follows: 4 spotted salamanders, Ambystoma maculatum, from Bowie (n = 1) and Cass (n = 1) Counties, Texas, and Little River (n = 1) and Miller (n = 1) Counties, Arkansas; 7 mole salamanders, Ambystoma talpoideum, from Greene County, Arkansas; 4 Ozark hellbenders, Cryptobranchus alleganiensis bishopi, from Randolph County, Arkansas; 6 spotted dusky salamanders, Desmognathus conanti, from Ouachita County, Arkansas; 3 many-rubbed salamanders, Eurycea multiplicata multiplicata, from Polk (n = 1) County, Arkansas and McCurtain (n = 2) County, Oklahoma; and 2 central newts, Notophthalmus viridescens louisianensis, from McCurtain County, Oklahoma. Specimens were placed in individual plastic bags on ice and returned to the laboratory within 24 hr for processing. Salamanders were killed by immersion in a concentrated chlorobutanol (Chloretone [Carolina Biological Supply, Burlington, North Carolina]) solution, and a mid-ventral incision was made to expose rectal contents. Feces was collected and placed in individual vials containing tap water supplemented with antibiotic (100 IU/ml penicillin-G, 100 μg/ml streptomycin) and examined directly without sucrose flotation by light microscopy. Following a sporulation period of 5 days at room temperature (ca. 23 C), oocysts were sent to S.J.U. for further study. Oocysts were examined and measured using a calibrated ocular micrometer and photographed using Nomarski interference-contrast optics. Measurements are reported in micrometers (μm), with means followed by the ranges in parentheses. Oocysts were 4 wk old when measured and photographed. Standardized abbreviations for characteristics of oocysts and sporocysts follow Wilber et al. (1998) as follows: oocyst length (L) and width (W), their ranges and ratios (L/W), micropyle (M), oocyst residuum (OR), polar granules (PG), sporocyst length (L) and width (W), their ranges and ratio (L/W), Stieda body (SB), substieda body (SSB), parastieda body (PSB), sporocyst residuum (SR), refractile bodies (RB), and nucleus (N). Photovouchers of sporulated oocysts were accessioned into the Harold W. Manter Laboratory of Parasitology (HWML), Lincoln, Nebraska. Voucher specimens of salamanders are deposited in the Arkansas State University Museum of Zoology (ASUMZ), State University, Arkansas.

RESULTS

Two of 10 (20%) A. opacum were passing coccidian oocysts of an undescribed species of Eimeria, described herein as new. None of the other 26 salamanders was found to be passing oocysts.

DESCRIPTION

Eimeria trauthi n. sp.

(Figs. 1–4)

Diagnosis: Oocyst shape subspherical to ellipsoidal; wall thin, single-layered ~0.8–1.0 thick; L × W (n = 25) 36.6 × 33.1 (33–40 × 29–37); L/W 1.1 (1.1–1.2); M and PG absent; large, spherical, membrane-bound OR present; OR characteristics composed of hundreds of loosely packed homogeneous
Taxonomic summary

**Type host:** Ambystoma opacum (Gravenhorst, 1807), marbled salamander (Caudata: Ambystomatidae), adult male, 49 mm SVL, collected 1 January 2004 by C. T. McAllister. Symbiotype deposited as ASUMZ 28167.

**Other host:** A. opacum, juvenile male, 36 mm SVL, collected 1 June 2004 by C. T. McAllister at the type locality (uncataloged).

**Type specimens:** Photosyntypes deposited as HWML 45798.

**Type locality:** USA: Louisiana, Webster Parish, Lake Bistineau State Park off State Highway 163 south of Doylinel (32°26.3’N, 93°23.1’W).

**Prevalence:** Found in 2 of 10 (20%) of the salamanders examined; 2/2 (100%) Webster Parish, Louisiana; 0/5 Arkansas; 0/3 Texas.

**Sporulation:** Exogenous. All oocysts were passed unsporulated or partially sporulated and became fully sporulated within 5 days at ca. 23 C.

**Prepatent and patent periods:** Unknown.

**Site of infection:** Unknown. Oocysts recovered from rectal contents and feces.

**Etymology:** The specific epithet is given in honor of Stanley Elwood Trauth, Professor, Arkansas State University, in recognition of his numerous contributions to our understanding of the natural history and ecology of Arkansas amphibians and reptiles.

**Remarks**

Upton et al. (1993) described *Eimeria opacum* from *A. opacum* from Grant County, Arkansas. Oocysts of *E. traathi* are much larger than those of *E. opacum* (36.6 × 33.1 vs. 29.4 × 28.0), have a single oocyst wall (bilayered wall in *E. opacum*), and possess an oocyst residuum and Stieda body, which are absent in *E. opacum*. No other salamander eimerian, including *Eimeria ambystomae* Saxe, 1955, originally reported by Saxe (1955) from tiger salamanders (*Ambystoma tigrinum*) from Iowa and by Duszynski et al. (1972) from the same host species from Colorado and New Mexico, and by McAllister and Upton (1987) from smallmouth salamanders (*Ambystoma texanum*) from Texas, has been described with a combination of characters descriptive of the new species. In addition, this is the first time, to our knowledge, that a coccidian has been reported from any amphibian host in Louisiana.

**Discussion**

The coccidians thus far reported from salamanders appear to be somewhat host specific, and the majority of salamanders harboring coccidia are from the Ambystomatidae and Salamandridae (Duszynski et al., 2003). Indeed, very few plethodontids have been reported as hosts, and those that have were passing oocysts of inadequately described coccidians. Furthermore, only a single isosporan has been described from western slimy salamanders, *Plethodon albagula* (Plethodontidae) from Arkansas (Upton et al., 1993). Species of *Ambystoma* appear to be common hosts (Duszynski et al., 2003), and the present study represents the second time a coccidian has been described from *A. opacum*. Interestingly, during the study period herein, 26 additional salamanders were examined for coccidian, and none was found to be passing oocysts, for an overall prevalence of 2 of 36 (5.6%) infected. A similar trend was reported previously by Upton et al. (1993), who surveyed 312 salamanders for coccidia from Arkansas, Missouri, New Mexico, and Texas and reported only 21 (6.7%) to be passing oocysts. As many of the
415 species of salamanders (Pough et al., 1998) are yet to be surveyed for coccidia, further study is certainly warranted, and additional coccidians will undoubtedly be described.

ACKNOWLEDGMENTS

We acknowledge the Arkansas Game and Fish Commission, Oklahoma Department of Wildlife Conservation, and Texas Parks and Wildlife Department for Scientific Collecting Permits issued to C.T.M. We especially want to thank Ben Wheeler, University of Arkansas Community College-Batesville, for providing fecal samples from C. a. bishopti.

LITERATURE CITED


MOLECULAR CHARACTERISTICS OF CAMALLANUS SPP. (SPIRURIDA: CAMALLANIDAE) IN FISHES FROM CHINA BASED ON ITS rDNA SEQUENCES

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ABSTRACT: In the paper, we explored the intra- and interspecific evolutionary variation among species of Camallanus collected from different fish species in various regions of China. We determined the internal transcribed spacers of ribosomal DNA (ITS rDNA) sequences of these nematodes. The divergence (uncorrected p-distance) of ITS1, ITS2, and ITS rDNA data sets confirmed 2 valid species of Camallanus in China, i.e., C. cotti and C. hypophthalmichthys. The 2 species were distinguished not only by their different morphologies and host ranges but also by a tetranucleotide microsatellite (TTGC)n present in the ITS1 region of C. cotti. Phylogenetic analyses of the nematodes disclosed 2 main clades, corresponding to different individuals of C. cotti and C. hypophthalmichthys from different fish species in various geographical locations, although the interior nodes of each clade received poor support.

Species of Camallanus (Spirurida: Camallanidae) occur almost globally in freshwater and marine fishes and even in amphibians (Chabaud and Bain, 1994; Levesen and Berland, 2002; Moravec et al., 2003). In China, 3 species, i.e., C. cotti, C. hypophthalmichthys, and C. zacconis, have been recorded from various freshwater fish. Among the 3 species, C. cotti is a potential pathogen that can affect host behavior and even cause death (McMinn, 1990; Kim et al., 2002). As a generalist parasite, it is frequently found in many species of the Cypriniformes, Siluriformes, and Perciformes. Recent surveys of helminths reveal that the host range and geographic distribution of this species is increasing and that the worm is a potential danger to endemic fishes (Font and Tate, 1994; Wu et al., 2004).

The 3 species have been traditionally distinguished by their morphological characters. However, there are only a few differences among them. Camallanus hypophthalmichthys is distinguished from the other 2 by the presence of 3 small, but prominent, caudal processes (Moravec et al., 2004). There is almost no conspicuous morphological distinction between C. cotti and C. zacconis, and they share many common host species and similar geographic distributions. Therefore, it was proposed that C. zacconis is a junior synonym of C. cotti (Moravec, 1973; Moravec et al., 2004), leaving just 2 Camallanus species, i.e., C. cotti and C. hypophthalmichthys, in China.

In recent decades, several genetic markers, such as internal transcribed spacer of ribosomal DNA (ITS rDNA), have proved to be valuable for determining the phylogenetic relationships of closely related species of nematodes (Hoste et al., 1998; Perlman et al., 2003; Otranto and Traversa, 2004). In the present study, we determined the ITS rDNA sequences of the Camallanus spp. These sequence data were used to explore the intra- and interspecific evolutionary variation among species of Camallanus collected from different fish species in various regions of China.

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MATERIALS AND METHODS

Sample collection, identification, and DNA extraction

The camallanids were sampled from fishes in the Yangtze River from 2002 to 2005. They were removed from the fish intestine, and then they were identified using their respective hosts, as well as morphological characters via dissecting microscopy. Preliminary examination showed that the camallanid from fishes of the Hypophthalmichthyinae was easily identified (as C. hypophthalmichthys) and compared favorably with those found in other fishes. The other camallanids were difficult to distinguish morphologically. Therefore, they were tentatively identified as Camallanus cotti. Information on the respective hosts, geographic localities, and sample codes are listed in Table 1 and Figure 1. Procamallanus fulvidraconis (accession number DQ076698) was used as the outgroup for the phylogenetic analyses.

All nematodes were washed in 0.6% saline before being stored in 85% alcohol. Worms were then soaked in TE buffer (pH 8.0) for 2 days to remove ethanol before DNA was released. Total nematode genomic DNA was extracted from 1 specimen in the respective fish host species using standard proteinase K, phenol/chloroform extraction (Sambrook, 1989). The extraction was then eluted into 25 μl of TE, pH 8.0, and stored at −20°C until use.

Polymerase chain reaction (PCR) amplification and DNA sequencing

The forward primer TW81 (5’-GTTTCGGTAGTTGAACCTGC-3’) and the reverse primer AB28 (5’-ATATGCTGAAGTCAGGGGT-3’), as used by Subbotin et al. (2001), were used to amplify the fragment corresponding to the ITS1 gene in part, ITS1 rDNA, 5.8S gene, ITS2 rDNA, and 28S gene in part. PCR mixtures consisted of 20 ng of worm genomic DNA, 2 μl of each of the 2 primers at 20 mM, 2.4 U of Takara Ex Taq DNA polymerase (TaKaRa Biotechnology Co. Ltd., Dalian, China), 8.0 μl of 2.5 mM dNTPs solution, 10 μl of 10X PCR reaction buffer with 20 mM MgCl2, and double-distilled water to a final volume of 100 μl. The PCR profile consisted of an initial denaturation step of 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 sec, elongation at 72°C for 1 min 10 sec, and a final extension at 72°C for 10 min in a PTC-100TM programmable thermal controller (MJ Research, Watertown, Massachusetts). A negative control was included in each PCR reaction. PCR amplification products were detected on ethidium bromide-stained 1.0% agarose-Tris-acetate-EDTA gels under UV light, and then purified over spin columns (Wizard PCR Prep. Promega, Madison, Wisconsin).

The purified products were cloned into pMD18-T vector following the manufacturer’s protocol. Flanking sequence primers M13+ and M13− were used to determine the plasmid DNA on an automatic DNA sequencer (model 3730, ABI Applied Biosystems, Foster City, California) in both directions. The obtained sequences have been deposited in GenBank database under accession numbers DQ403203–DQ403233.

731
Sequence alignment and analyses

The sequences were aligned initially using Clustal X (Thompson et al., 1997), with the following parameters: gap opening penalty = 10.0 and gap extension penalty = 5.0. Upon completion, the alignments were visually inspected in Seaview (Galtier et al., 1996), and slight modifications by eye were made to improve their accuracy. Alignment files for ITS1 rDNAs, ITS2 rDNAs and the combined ITS (ITS1 and ITS2) rDNAs are available by anonymous FTP from ftp.ebi.ac.uk in directory/pub/databases/emb/align or via the EMBLALIGN database via SRS at http://www3.ebi.ac.uk/Services/webin/help/webin-align/align_SRSHelp.html; under accession numbers ALIGN_001095, ALIGN_001096, and ALIGN_001097. Sequence divergence of the ITS1 rDNAs and ITS2 rDNAs, and the combined ITS rDNAs were implemented by Mega 2.1 (Kumar et al., 2001) after alignments, and the frequency of nucleotide bases of the ITS rDNA was also performed in the program.

Transitions and transversions were plotted against sequence divergence in DAMEBIE 4.0.59 (Xia and Xie, 2001) to evaluate the possibility of sequence saturation. Sequence saturation was inferred from the shape of the trend line, with a linear relationship indicating the sequences were unsaturated and an asymptotic relationship showing the presence of saturation. Different nucleotide sequences have variant DNA evolution substitution patterns. The best-fit model helps to resolve phylogenetic relationships accurately. Modeltest 3.6 (Posada and Crandal, 1998) was used to find the best-fit nucleotide evolutionary model. According to the hierarchical likelihood ratio tests (hLRT), the HKY+G model was selected based on the ITS rDNA sequences, which was then used in the model-based phylogenetic methods (NJ and Bayesian analyses).

Three methods, i.e., maximum parsimony (MP), neighbor-joining (NJ), and the Bayesian approach, were used for the phylogenetic analyses to gauge the robustness of our resulting hypotheses. MP and NJ analyses were implemented in PAUP*4.0b (Swofford, 2002), and the Bayesian approach was performed in MrBayes 3.6 (Huelsenbeck and Ronquist, 2001). Equally weighted MP analyses were computed. The heuristic search setting was 100 replicates of random taxon addition tree bisection-reconnection branch swapping, multiple trees retained, no steepest descent, and accelerated transformation. Gaps were treated as missing data. Bootstrap analysis with 1,000 replicates was performed to assess the support for each branch on the corresponding tree. The NJ algorithm was performed by application of the DNA substitution model generated from Modeltest 3.6, and 1,000 replicates were also used for the bootstrap analysis. The Bayesian approach was used to construct a maximum likelihood tree. Four independent Markov chains were simultaneously run for 1,000,000 replicates by sampling 1 tree per 100
replicates with the Bayesian procedure. The first 1,000 trees were discarded as part of a burn-in procedure, and the remaining 9,000 sampling trees were used to construct a 50% majority rule consensus tree. The combined ITS rDNA sequences were used in the phylogenetic analyses.

RESULTS

Characteristics of ITS1 and ITS2 rDNA sequences

In total, 31 sequences of the *Camallanus* nematodes, *Camallanus cotti*, and *C. hypophthalmichthys*, were obtained from different fish host species collected from different localities in China. The sequences were compared with the rDNA sequence of *Onchocerca volvulus* in GenBank (Morales-Hojas et al., 2001) to determine the boundaries of code and spacer regions. The 33-base pair (bp) 18S rDNA 3' end, 156-bp 5.8S rDNA, and 43-bp 28S rDNA 5' end were determined, in addition to the ITS1 and ITS2 rDNA.

Among the different individuals, of *C. cotti* from 23 fish species and individuals of *C. hypophthalmichthys* from 2 fish species collected at different localities, the length of ITS1 rDNA sequences ranged from 667 to 690 bp and from 637 to 645 bp, respectively. The ITS2 rDNA sequences were consistently 501 bp in the *C. cotti*, but varied from 462 to 469 bp in *C. hypophthalmichthys*. The variations resulted mainly from deletion/insertion nucleotides. In ITS rDNA, the G+C contents varied from 33.2 to 34.4% in the ingroup. In total, 1,305 characters were analyzed, of which 543 were variable and 164 were phylogenetically informative. The divergence (uncorrected p-distance) of various data sets is shown in Table II. When the outgroup taxon was excluded, the saturation plots of uncorrected sequence divergence (K80) against transitions and transversions revealed unsaturated relationships among the sequences (plots not shown). Furthermore, in the ITS1 rDNA region of *C. cotti*, a simple sequence repeated (SSR) polymorphism, a tetranucleotide (TTGC)n was detected with n ranging from 4 (Danjiangkou Reservoir, Hubei; host *Opsarichthys bidens*) to 10 (Jialingjiang River, Chongqing; host *Mystus macropterus*), but commonly 6 or 7 were present. However, this SSR did not occur in *C. hypophthalmichthys*.

Phylogenetic analyses

Two major clades, A and B, within *Camallanus* were identified distinctively by all 3 methods of analysis (Fig. 2). Clade A contained individuals of *C. cotti* from different fish species from 3 localities, whereas clade B only included strains of *C. hypophthalmichthys* in *Hypophthalmichthys molitrix* and *Aristichthys nobilis* from 4 localities, both with high bootstrap values or posterior probabilities. Although major clades were well resolved, the interior nodes of each clade received only poor support by the 3 methods. In the Bayesian tree, however, 4 significant support sub-clades in interior nodes of clade A were determined. The first sub-clade included the parasites from ZP (=Zacco platypus) and CIL (=Culter ilishaeformis) (100%); the second from PF (=Peleobagrus fulvidraco) and HS (=Hypseleotris swinhonis) (100%); the third from GI (=Gnathopogon imberbis), SD (=Saurogobio dabryi), and AS (=Acanthobrama simoni) (95%); and the fourth from CI (=Ctenopharyngodon idella) and MP (=Mylopharyngodon piceus) (100%).

DISCUSSION

Analysis of ITS rDNA sequence of *Camallanus* spp.

Limited sequence data have been available for the ITS rDNA of parasitic nematodes, especially in fish hosts. The data presented herein represent the first report of the ITS rDNA sequences from *Camallanus* spp. The ITS1 region is longer than the ITS2 region, in agreement with similar findings for *Trichostrongylus* spp. (Hoste et al., 1998) and the cyst-forming nematodes of the Heteroderidae (Subbotin et al., 2001). Analogous to the ITS region in many other parasitic nematodes, the G+C content of *Camallanus* spp. is smaller than the A+T content (33.2 to 34.4% vs. 65.6 to 66.8%) (Hoste et al., 1998; Subbotin et al., 2001; Otranto and Traversa, 2004).

Despite the great divergence seen in the ITS region of the genus, there were still several conserved domains (data not shown). Similarly conserved regions are also detected in the ITS1 region of the *Thelazia* species (Hoste et al., 1998) and the ITS region of *Trichostrongylus* species (Otranto and Traversa, 2004). It is generally believed that conserved regions are important to maintain the secondary structure of the pre-rRNA of the spacer(s) and may help to mediate cleavages in the ITS region that occur during rRNA transcription process (Mai and Coleman, 1997; Hoste et al., 1998).

Species validity within *Camallanus* spp.

The ITS rDNA region has been successfully used for phylogenetic study and identification of closely related species of nematodes. Generally, species are regarded as valid if all of the mean variation values of the interspecific ITS sequences are much higher than those of the intraspecies. However, the literature does not suggest how much higher the values need to be to validate species differences. For example, Otranto and Traversa (2004) stated that for species of the spirurid *Thelazia*, the intraspecific variation of the ITS1 region varied from 0.3 to 2.5% and interspecific ranged from 35 to 77%. In contrast, Hoste et al. (1998) found that divergences of the ITS1 region among 3 closely related species of *Trichostrongylus* ranged from 1.3 to 5.7%. Newton regarded *Cooperia oncophora* and *C. surnabada* as synonyms, because the difference between the
ITS2 fragments was no greater than 1.7%. The threshold of 1.7% was established using as standard the difference between valid species in the genus. Recently, research on 9 species of philometrids collected in China revealed that the interspecific divergence was more than 7.32% in the ITS region (Wu et al., 2005). All these studies, and many others, indicated that for parasitic nematodes, there was a significant difference between the variations of intra- and interspecies whether the ITS1, ITS2,
or combined rDNA sequences were used. In the present study, the sequence variations for C. cotti from different localities and hosts are 2.5% (ITS1), 0.6% (ITS2), and 1.5% (ITS), whereas within C. hypophthalmitidis, the differences are only 0.3% (ITS1), 0.4% (ITS2), and 0.4% (ITS). However, the divergences between the two groups are as high as 14.6% (in ITS1), 22.1% (in ITS2), and 20.3% (in ITS). This suggests that C. cotti and C. hypophthalmitidis are most likely different species.

Three Camallanus species have been reported in China from different fish species. Among them, C. zacconis was first recorded in Zacco temmincki from the Jialingjiang River, a branch of the upper reaches of the Yangtze River (Li, 1941). Subsequently, C. zacconis was reported by Wang and Ling (1975) and Wang et al. (1979) from Hemiculter leuciscus, Elpicthtys bambusa, Siniperca chuatsi, Megalobrama terminalis, and Erythroculter ilishaiformis in Fujian Province and Silurus asotus in Poyang Lake, Jiangxi Province. Sun (1988) also recovered C. zacconis from H. leuciscus, Culter erythropterus, Mastacembelus mastacembelus, S. chuatsi, S. asotus, and Pelteobagrus fulvidraco in Wuhan City and Hongzhou Lake. In the present study, although its typical host Z. temmincki was not examined, C. cotti from the congeneric host Z. platypus was found and included. Furthermore, C. cotti was also found in another fish, Mystus macroperus, from the Jialingjiang River, where C. zacconis is typically found. Additionally, many C. cotti individuals were collected from several species of fishes that have been reported as hosts of C. zacconis. In the present molecular phylogenetic tree, C. cotti from different fish hosts from different localities form a single clade with a high bootstrap value, and molecular divergences remained at an intraspecific level, thus supporting the hypothesis, based on morphological characteristics, that C. zacconis is a junior synonym of C. cotti (Morsevic, 1973; Morsevic et al., 2004). We agree that, so far, only 2 Camallanus species (C. cotti and C. hypophthalmitidis) have been validated as parasites of various freshwater fishes in China.

Similar to the finding by Otranto and Traversa (2004) with respect to Thelazia spp. in the ITS1 region, there was also a microsatellite found in C. cotti. The microsatellite makes it easy to distinguish C. cotti from C. hypophthalmitidis. The minimum repeated number of the microsatellite comes from the strain found in O. bidens in Danjiangkou Reservoir, and the maximum repeated number comes from an individual in M. macroperus in Jialingjiang River. This suggests genetic differentiation between the populations. Microsatellites have been widely used as important genetic markers for epidemiology and population structure study on isolates from different geographic areas (McCoy et al., 2001; Otranto and Traversa, 2004). Thus, it is possible to use the microsatellite to examine the population genetics and epidemiology of C. cotti.

In summary, 2 Camallanus nematodes, C. cotti and C. hypophthalmitidis, from different fish host species collected from different locations in China are recognized in this study. However, further study should include more species of Camallanus, as well as other genes, to clarify the evolutionary relationships in this genus. Population genetics and phylogeography of C. cotti should also be examined.

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LITERATURE CITED


DESCRIPTION OF A NEW SPECIES OF *MYXOBOLUS* (MYXOZOA: MYXOBOLIDAE)
BASED ON MORPHOLOGICAL AND MOLECULAR DATA

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ABSTRACT: *Myxobolus ampullicapsulatus* n. sp. was isolated from the gills of *Carassius auratus auratus* (L., 1758) in Chongqing, China. Myxospores were pyriform, measuring 16.5–19.5 μm long × 8.5–10.0 μm wide × 7.0 μm thick. Two equal polar capsules were ampullaceous, measuring 7.0–10.0 μm long × 2.5–4.0 μm wide, containing polar filaments coiled 9–10 turns. Spore length of this species exceeds that of the majority of other *Myxobolus* spp., and those overlapping in this dimension can be differentially diagnosed by other characters. Furthermore, the small subunit ribosomal DNA (SSU rDNA) of *M. ampullicapsulatus* n. sp. is unique among myxozoans sequenced to date. Phylogenetic analyses of the SSU rDNA gene sequence placed this species in a clade composed exclusively of Gill parasites, most closely related to *Myxobolus longisporus*, which also infects the gills of cyprinid fishes in China.

Myxozoans are a group of microscopic metazoan parasites (Siddall et al., 1995; Schlegel et al., 1996; Pote et al., 2000), best known for the infections they cause in freshwater and marine fishes. *Myxobolus* Bürschli, 1882, is the most speciose genus within the Myxozoa, composed of some 792 species (Lom and Dyková, 2006). Spores of *Myxobolus* spp. are diminutive and possess many morphological similarities to one another, making identifications difficult based on morphology alone. To aid with these identifications, molecular systematics has become a mainstream approach in taxonomic and phylogenetic studies on myxozoans. This work has revealed that within some genera, classification schemes based on myxospore morphology are inconsistent with estimates of phylogeny based on molecular sequence data (Smothers et al., 1994; Kent et al., 2001; Fiala, 2006). *Myxobolus* for example, is a paraphyletic taxon. Nonetheless, some studies have shown evolutionary patterns within certain lineages of *Myxobolus* spp., based on either spore morphology (Salim and Desser, 2000) or tissue specificity (Andree et al., 1999; Eszterbauer, 2004; Molnár et al., 2006). Thus, although no one factor can explain the evolution of *Myxobolus* spp. as a whole, patterns within clades may emerge as additional DNA sequence data become available for the numerous *Myxobolus* spp. known.

In the present work, a new *Myxobolus* sp. found infecting the gills of goldfish (*C. auratus auratus*) in China is described with morphological data and SSU ribosomal RNA (rRNA) gene sequence.

MATERIALS AND METHODS

Sample collection and species identification

Fifty specimens of *C. auratus auratus* (Linnaeus, 1758) from Longshui Lake in Chongqing, China, were captured on 21 October 2004. Plasmodia were excised from gills and transferred to microscope slides, where they were ruptured to release spores. Fresh spores were rinsed 3 times with sterile distilled water and pelleted by centrifugation (2,000 g). Identification of species and treatment of specimens were done following the method of Zhao et al. (2001). All specimens were observed and measured at ×1,000 magnification using a NIKON E–600 microscope (Nikon Instrument Inc., Shanghai, China). The illustrations, based on fresh materials, were drawn with the aid of a camera lucida and computer software. Photomicrographs were made using a NIKON–DXM–1200 at ×1,000 magnification. Measurements, based on 25 spores, are given in microns (μm) as the arithmetic mean and standard deviation, followed by the range in parentheses.

DNA isolation, cloning, and sequencing

DNA was extracted as follows: fresh spores were suspended in 500 μl lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM ethylene diamine tetraacetic acid, 0.2% sodium dodecyl sulfate, and 0.2 mg/ml Proteinase K); the mixture was then incubated at 55 C. Following phenol/chloroform extraction, DNA was precipitated and resuspended in 50 μl H2O (Sambrook et al., 1989). A portion of the SSU rRNA gene was amplified with the primer pair MX5-MX3 (5'-CTG CGC AGG GCT CAG TAA ATC AGT-3' and 5'-CCA GGA CAT CTT AGG GCA TCA CAG A-3') (Andree et al., 1999). Polymerase chain reaction (PCR) was performed in 25-μl volumes containing 3 mM MgCl₂, 0.2 mM of dNTP, 0.5 μM of each primer, 15 ng of genomic DNA, and 1.5 U of Taq Ex DNA polymerase (TaKaRa, Otsu, Japan). Cycling parameters were as follows: 5 min initial denaturation at 94 C; then 35 cycles of 1 min at 94 C, 1 min at 56 C, and 2 min at 72 C; followed by a final cycle of an extended elongation step at 72 C for 10 min. Purified PCR products were inserted into a PMD18-T vector (TaKaRa) and 2 clones were sequenced in an ABI Prism 377 DNA Sequencer (Applied Biosystems Inc., Foster City, California).

Phylogenetic analyses

Sequences were selected to include all members of a well-supported lineage of freshwater myxozoan parasites that primarily infect cyprinid form fishes (Fiala, 2006). Additional sequences were included based on Basic Local Alignment Search Tool (BLAST) searches. *Henneguya ic-tulari* and *Henneguya exilis* were used to root the tree. DNA sequence alignments were created with Clustal X (Thompson et al., 1997) and manually edited. Maximum parsimony analyses (MP) were conducted in PAUP*4.0b10 (Swofford, 2003). MP employed a heuristic search with 10 repetitions of random sequence addition, and tree bisection and reconnection branch swapping. Bootstrap confidence values were calculated with a heuristic search using simple sequence addition and 100 replicates. Bayesian analyses were conducted in MrBayes (Ronquist and Huelsenbeck, 2003) under a GTR model, with 100 generations, tree sampling every 100 generations, with a burn-in of 100 trees.

RESULTS

DESCRIPTION

*Myxobolus ampullicapsulatus* n. sp.

(Figs. 1, 2)

Vegetative stage: Small, polysporous cysts, spherical or ellipsoid (1.0–2.0 mm in diameter), found predominantly in second gill arch, located toward base of gill filaments between secondary lamellae (interlamellar-epithelial type LE1 of Molnár, 2002). Replete with spores at various stages of development and deformed spores (Figs. 1E, F).

* Mature spores: Pyriform, with bluntly pointed apex and rounded pos-
Figure 1. Photomicrographs of *M. ampullicapsulatus* n. sp. (A) Spore in frontal view with an intercapsular appendix (arrow). (B) Spore with mucous envelope in sutural view. (C) Spore stained with Lugol's iodine. (D) Sporoplasm after lysis (arrow). (E, F) Spores deformed. Bars = 10 μm.

terior in valvular view. Sutural folds absent. Spore length (LS) = 18.0 ± 0.9 (16.5–19.5), width (WS) = 9.3 ± 0.4 (8.5–10.0) and thickness (TS) = 7.0. Length-to-width ratio 1.92 (± 0.12). Two polar capsules equal, ampullaceous with distinct neck region, anteriorly converged, terminating below spore apex, occupying two-thirds of spore cavity length, measuring 8.5 ± 0.7 (7.0–10.0) long (LP), by 3.0 ± 0.2 (2.5–4.0) wide (WP). Polar filaments coiled with 9–10 turns, perpendicular to capsule length. Intercapsular appendix distinct, large. Transparent mucous envelope on posterior end of spore.


**Taxonomic summary**

*Type host:* Carassius auratus auratus (L., 1758).

*Site of infection:* Plasmodia found in epithelia of the gill filaments, between the secondary lamellae, and toward the gill arch (site LE1 as defined by Molnár, 2002). Cysts most commonly observed in filaments of the second gill arch.

*Prevalence:* Eight of 50 fishes (16%).

*Type locality:* Lake Longshui, Dazu County, Chongqing (29°5′N, 106°5′E), China.

*Other localities:* Natural freshwater bodies, Beibei County and Shapingba District, China.

*Deposition of types:* Syntype (mounted in glycerin-alcohol-formalin) has been deposited in the collection center of the Key Laboratory of Animal Biology of Chongqing, Chongqing Normal University, Chongqing, China (Coll. No. CQ-20041021).

*Etymology:* The name *ampullicapsulatus* refers to the conspicuous

Figure 2. Schematic illustrations. (A) *M. ampullicapsulatus* n. sp. with mucous envelope in frontal view. (B) *M. ampullicapsulatus* n. sp. in sutural view. Bar = 10 μm.

Figure 3. A comparison of spores of 5 species morphologically similar to *M. ampullicapsulatus* n. sp. (A) *M. pseudokoi* from Cone et al. (2005). (B) *M. longisporus* from Dyková et al. (2003). (C) *M. aureatus* from Lom et al. (1992). (D) *M. ampullicapsulatus* n. sp. collected in the present study. (E) *M. bilobus* from Cone et al. (2005). (F) *M. wuili* after Wu and Li (1986). Bars = 10 μm.
morphological feature of the myxospore and is a composite of the Latin *ampulli-* (= ampoulelike, of bottle) and *capsula* (= capsule).

**Remarks**

Of the approximately 800 species of *Myxobolus* described (Eiras et al., 2005; Lom and Dykavá, 2006), *M. ampullicapsulatus* n. sp. displays a superficial similarity to *M. pseudokoi* Li et Desser, 1985; *M. longisporus* Nie et Li, 1992; *M. aureatus* Ward, 1919; *M. bilobus* Cone Yang, Sun et Easy, 2005; and *M. wuli* Landsberg and Hu, 1991, by bearing pyriform spores (Table I; Fig. 3). However, spores of *M. pseudokoi* are smaller (length 11.5–14.0 × width 6.0–7.0 μm) when compared to *M. ampullicapsulatus* n. sp. (16.5–19.5 × 8.5–10.0). *Myxobolus bilobus* has many rounded folds and falls within a well-supported clade of *M. bilobus* spp. that infect the gills of cyprinid fishes (Fig. 4). With the exception of *M. pendula*, which is a parasite of the gill arch, *M. ampullicapsulatus* n. sp. and the remaining species in this clade are found within the gill filaments. There is also some clustering based on geographic location of these intralamellar *Myxobolus* spp., i.e., those from Hungary, Canada, and China form distinct clades (Fig. 4). This is not a consistent pattern outside of this clade, as several intralamellar *Myxobolus* spp. are found throughout the tree, i.e., *M. dispar*, *M. muelleri*, *M. bramae*, and *M. macroupolaris*. We attempted to resolve whether there was any clustering by cyprinid host subfamily, but this was also inconsistent (data not shown). Bayesian analyses strongly supported a clade where the cluster containing *M. ampullicapsulatus* n. sp. and *M. longisporus* formed a sister clade to *M. pendula*, but MP provided weak support for this clade, as indicated by the low bootstrap value of 55% (Fig. 4).

**Molecular characterization**

The partial SSU rRNA sequence generated for *M. ampullicapsulatus* n. sp. was 1,576 bp long (GenBank DQ339482) and unique compared to all *Myxobolus* spp. sequenced to date. Based on GenBank BLAST searches (Altschul et al., 1990), the new species was most similar to *M. longisporus* (AY364637) at 91%, and 89% similar to *M. intimus* (AY325285), *M. hungaricus* (AF448444), and *M. obsesus* (AY325286). Phylogenetically, *M. ampullicapsulatus* n. sp. is a sister species to *M. longisporus* and falls within a well-supported clade of *Myxobolus* spp. that infect the gills of cyprinid fishes (Fig. 4). With the exception of *M. pendula*, which is a parasite of the gill arch, *M. ampullicapsulatus* n. sp. and the remaining species in this clade are found within the gill filaments. There is also some clustering based on geographic location of these intralamellar *Myxobolus* spp. The synopsis of *Myxobolus* spp. by Eiras et al. (2005) lists 15 species found in the gills of goldfish in China alone. The present species, *M. ampullicapsulatus* n. sp., can be distinguished from the others by having distinctly long pyriform spores with a mucous envelope and large intercapsular process. The species we considered morphologically most similar, *M. wuli*, does not possess a mucous envelope and appears to be

**DISCUSSION**

The synopsis of *Myxobolus* spp. by Eiras et al. (2005) lists 15 species found in the gills of goldfish in China alone. The present species, *M. ampullicapsulatus* n. sp., can be distinguished from the others by having distinctly long pyriform spores with a mucous envelope and large intercapsular process. The species we considered morphologically most similar, *M. wuli*, does not possess a mucous envelope and appears to be...
less tissue specific, infecting gills, spleen, and stomach (Table I). Although Salim and Desser (2000) questioned the taxonomic reliability of the mucous envelope, we depend on this feature, together with the difference in tissue specificity, until *M. ampullicapsulatus* n. sp. and *M. wulii* can be compared by other means, i.e., with DNA sequence data. We suspect that there may be other existing *Myxobolus* spp. from goldfish that will prove to be synonymous once additional data can be collected and analyzed. For *Myxobolus* spp. with DNA sequences available, *M. ampullicapsulatus* n. sp. clustered mainly with species that have pyriform spores and infect the gills of cyprinid fishes, i.e., *M. longisporus*, *M. pseudokoi*, *M. bilobus*, *M. dajardini*, *M. intimus*, *M. obesus*, a *Myxobolus* sp., and *M. hungaricus* (Fig. 4). The same observation was made by Eszterbauer (2004), Salim and Desser (2000) suggested that there may be some congruency between morphological and phylogenetic classification within particular clades of *Myxobolus* spp. However, the monophyly of this clade was disrupted by *M. pendula*, which possesses more ovoid spores. Conversely, *M. elegans*, which bears pyriform spores and infects the gills of cyprinids, was not a member of this clade. *Myxobolus* spp. from other families of fishes that bear pyriform spores are even more distantly related, i.e., *M. arcticus* and *M. insidiosus* (Kent et al., 2001). Thus, when taken together, gill-infecting *Myxobolus* spp. of cyprinid fishes with pyriform spores tend to group together, but this is not without exception.

Tissue specificity is often discussed as an important factor in the evolution of myxozoans (Kent et al., 2001; Blaylock et al., 2004; Eszterbauer, 2004; Whipp et al., 2004; Cone et al., 2005; Easy et al., 2005; Fiala, 2006; Molnár et al., 2006; Burger et al., 2007). Here, *M. ampullicapsulatus* n. sp. fell within a well-supported clade of *Myxobolus* spp. that supported the need for special staining techniques and histological analysis. Even with the most conservative subfamily assignments, using only the Cyprinidae and Leuciscinae of Cavender and Coburn (1992), no consistent patterns emerged from our phylogeny (data not shown). Likewise, although there was some pairing of sympatric species through the tree, there were no geographically distinct clades in our analyses (Fig. 4).

These inconsistencies do not discredit the usefulness of morphology and other nonmolecular attributes in the study of myxozoan species. Similarly, DNA sequence data must be used in conjunction with other characteristics to determine boundaries between species. There are no universal criteria that equate differences in SSU rDNA sequences to species boundaries, but intraspecific variation typically ranges from <1% (Easy et al., 2005; Whipp and Kent, 2006) to as much as 2.6% (Schlegel et al., 1996). Here, the SSU of *M. ampullicapsulatus* n. sp. was ≥91% similar in identity to that of *M. longisporus* (AY364637).

### Table I. Comparisons between *Myxobolus ampullicapsulatus* n. sp. and other *Myxobolus* spp. with pyriform spores or overlapping dimensions from cyprinid fishes. Preoccupied names are indicated in parentheses for replacement names erected for homonyms generated as a result of the synonymization of *Myxobolus* and *Myxosoma.*

<table>
<thead>
<tr>
<th>Species</th>
<th>Hosts</th>
<th>Location</th>
<th>Site of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. ampullicapsulatus</em> n. sp.</td>
<td></td>
<td>China</td>
<td>gills</td>
</tr>
<tr>
<td><em>M. auratus</em> Ward, 1919</td>
<td><em>C. auratus auratus</em></td>
<td>USA</td>
<td>fins</td>
</tr>
<tr>
<td><em>M. bilobus</em> Cone et al., 2005</td>
<td><em>Pimephales promelas</em></td>
<td>Canada</td>
<td>gill filament</td>
</tr>
<tr>
<td><em>Notemigenous crysoleucas</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. buchowensis</em> Chen, 1998</td>
<td><em>C. auratus auratus</em></td>
<td>China</td>
<td>gills</td>
</tr>
<tr>
<td><em>M. longisporus</em> Nie et Li, 1992</td>
<td><em>Cyprinus carpio haematopterus</em></td>
<td>China</td>
<td>gills</td>
</tr>
<tr>
<td><em>M. nanyangensis</em> Eiras, Molnár, and Lu, 2005</td>
<td><em>C. auratus auratus</em></td>
<td>China</td>
<td>gills</td>
</tr>
<tr>
<td>(replaced <em>Myxosoma carassii</em> Hu, 1965)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. pseudokoi</em> Li and Desser, 1985</td>
<td><em>Luxilus cornutus</em></td>
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<td><em>M. shulensis</em> Eiras, Molnár, and Lu, 2005</td>
<td><em>Pseudorasbora parva</em></td>
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<tr>
<td>(replaced <em>Myxosoma sinkiangensis</em> Chen and Ma, 1998)</td>
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<td><em>M. wellerae</em> Li and Desser, 1985</td>
<td><em>L. cornutus</em></td>
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<td><em>M. wulii</em> Landsberg et Lom, 1991</td>
<td><em>C. auratus auratus, Hypophthalmichthys molitrix, Pelleobagrus braschnikowi, Opsarichthys bidens</em></td>
<td>China</td>
<td>gills, spleen, stomach</td>
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<tr>
<td>(replaced <em>Myxosoma magna</em> Wu and Li, 1986)</td>
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and 89% similar to M. intimus (AY325285), M. hungaricus (AF448444), and M. obesus (AY325286). These sequence identities are well below what would be considered intraspecific. Ultimately, the decision to define a new species must incorporate multiple lines of evidence, not just morphological or molecular data alone.

In estimating myxozoan phylogeny, it is unclear which morphological characters are most useful for classification of these parasites in a taxonomic scheme that reflects their evolutionary history and is supported by molecular data. As taxon sampling increases, perhaps patterns will emerge. Here, M. amplifilamentos (Myxosporea) was phylogenetically closely related to other Myxobolus spp. with pyriform spores, from the gills of cyprinid fishes. Furthermore, it was recognized as a distinct species both by morphology and DNA sequence data; thus, we describe it as a new species.

ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of China (Project No. 30570221), the Natural Science Foundation of Chongqing (No. 8618), and the Science Research Foundation of the Science Press, Beijing, China, 993 p.

LITERATURE CITED

As a new species. Ocean molecular data alone. increases, perhaps patterns will emerge. Here, TABLE I. Extended.

<table>
<thead>
<tr>
<th>Spore length × width</th>
<th>Polar capsule length × width</th>
<th>Polar filament coils</th>
<th>Intercapsular appendix</th>
<th>Macous envelope</th>
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<td>present</td>
<td>Present study</td>
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<td>absent</td>
<td>Wu and Li (1986)</td>
</tr>
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</table>


INHIBITION BY DICATIONS OF IN VITRO GROWTH OF Leishmania major AND Leishmania Tropica: CAUSATIVE AGENTS OF OLD WORLD CUTANEOUS LEISHMANIASIS

Alexa C. Rosypal, Karl A. Werbovetz*, Manar Salem†, Chad E. Stephens‡, Arvind Kumar§, David W. Boykin†, James E. Hall, and Richard R. Tidwell||

School of Medicine, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599. e-mail: tidwell@med.unc.edu

ABSTRACT: Old World cutaneous leishmaniasis is caused by infection with Leishmania major and Leishmania tropica. Pentamidine and related dications exhibit broad spectrum antiprotozoal activity. Based on the previously reported efficacy of these compounds against related organisms, 18 structural analogs of pentamidine were evaluated for in vitro antileishmanial activity, using pentamidine as the standard reference drug for comparison. Furans and reversed amine compounds were examined for activity against L. major and L. tropica promastigotes. The most active compounds against both Leishmania species were in the reversed amine series. DB745 and DB746 exhibited the highest activity against L. major and DB745 was the most active compound against L. tropica. Both of these compounds exhibited 50% inhibitory concentrations (IC50) below 1 nM for L. major. Ten reversed amidines were also tested for their ability to inhibit growth in an axenic amastigote model. Nine of 10 reversed amine analogs were active at concentrations below 1 nM. These results justify further study of dicationic compounds as potential new agents for treating cutaneous leishmaniasis.

Old World cutaneous leishmaniasis (OWCL) is commonly caused by infection with Leishmania major and Leishmania tropica. Cutaneous leishmaniasis is primarily treated with antimonials and pentamidine is used in parts of South America (Blum et al., 2004; Roussel et al., 2006). OWCL sometimes self-resolves with scarring after several months. Lesions and scars, however, can be disfiguring or disabling depending on their location (Desjeux, 2004). Over 90% of CL cases occur in 8 countries, including Iraq and Afghanistan (Mishra et al., 2007). In recent years, there has been increasing concern over OWCL due to both a war-related epidemic in Kabul, Afghanistan (Faulde et al., 2006) and numerous cases among U.S. military personnel stationed in the Middle East (CDC, 2004; Willard et al., 2005).

Leishmania parasites are closely related to the causative agents of African trypanosomiasis (African sleeping sickness), Trypanosoma brucei rhodesiense and T. b. gambiense. The diaminopentamide is used as a chemotherapy agent for both leishmaniasis and African trypanosomiasis. Pafuramidine (DB289) (2,5-bis[4-amidinophenyl]furan-bis-O-methylamidino) is the orally active methamidoxime prodrug of the aromatic diamidine DB75 (2,5-bis[4-amidinophenyl]furan). Pafuramidine, which is metabolized to active DB75, is currently undergoing phase III clinical trials for the treatment of human African trypanosomiasis in Angola and the Democratic Republic of Congo.

Dicationsic compounds based on pentamidine exhibit potent antiparasitic activity against a number of organisms (Bell et al., 1991; Lindsay et al., 1991; Lindsay et al., 1994; Blagburn et al., 1998; Patrick et al., 1999; Brendle et al., 2002; Rowland et al., 2003; Mathis et al., 2006; Werbovetz, 2006). Our laboratory has previously focused on antileishmanial activity of novel dications using an Leishmania donovani axenic amastigote model (Brendle et al., 2002; Stephens et al., 2003; Gonzalez et al., 2007). Several dicationsic compounds exhibit improved activity compared to pentamidine against Leishmania species that cause visceral leishmaniasis and New World CL (Bell et al., 1990; Brendle et al., 2002; Rosypal et al., 2007), but their activities against etiologic agents of OWCL have not been examined.

In a recent study, we reported that 2 classes of aromatic dications, furan and reversed amides, have impressive activity against a New World isolate of Leishmania infantum (Rosypal et al., 2007). Reversed amine compounds differ from other furan analogs because the amine is bound to the central aromatic linker via a nitrogen atom rather than a carbon atom. The present research was conducted to evaluate structural analogs of pentamidine for in vitro activity against promastigotes of L. major and L. tropica. This is important because therapeutic agents can exhibit varying efficacies against different Leishmania species and strains (Croft et al., 2006; Rijal et al., 2007). For the first time, we report our findings of dicationic compounds that are considerably more potent in vitro than pentamidine against etiologic agents of OWCL. In addition, we compare the activities of reversed amidines in an axenic amastigote model.

MATERIALS AND METHODS

Cationic compounds

The compounds tested in this study were synthesized in the laboratory of 1 of the authors (D.W.B.) as hydrochloride salts using previously described methods (Das and Boykin, 1977; Boykin et al., 1998), or were prepared by the methods detailed therein. The syntheses of the reversed amidines are reported in Stephens et al. (2001, 2003) or were achieved by the approach described within. Pentamidine dihydrochloride was used as the standard antileishmanial agent for reference and was synthesized in the laboratory of R.R.T. Stock solutions (10 mM) of the compounds were dissolved in sterile water or DMSO and stored at −4 C. Working solutions were freshly diluted to desired concentrations in culture media. The highest concentration of DMSO in drug susceptibility assays was 1% (v/v), which was not harmful to the parasites.

Parasites

Promastigotes of L. major (WHO designation, MHOM/IL/67/Jericho II, ATCC 50122, American Type Culture Collection, Manassas, Virgin-
ia) and *L. tropica* (World Health Organization [WHO] designation, MHOM/SU/74/237, ATCC 50129, American Type Culture Collection, Manassas, Virginia) were grown in 25-cm\(^2\) plastic cell culture flasks in promastigote culture media that consisted of 30% (v/v) fetal bovine serum (Atlanta Biologicals, Atlanta, Georgia) in Grace’s Insect Media (Gibco, Langley, Oklahoma), supplemented with 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 2% (v/v) human urine obtained from a healthy male volunteer. Cell cultures were incubated at 25 °C in a humidified atmosphere. Promastigotes were maintained in logarithmic growth phase by subpassaging every 4–6 days into fresh cell culture media. New cultures were initiated from cryopreserved stock every 8–12 wk.

*Leishmania donovani* (WHO designation, MHOM/SD/62/IS-CL290) was maintained as isolated amastigote-like forms in modified RPMI media (Raz et al., 2007) supplemented with 100 μM 5-fluorodeoxyuridine (BD Biosciences Pharmingen, San Diego, California), 200 μg/ml G418 (ATCC, Manassas, Virginia), and 10% (v/v) fetal bovine serum (Atlanta Biologicals) in Grace’s Insect Media (Gibco, Langley, Oklahoma) every 2–4 wk.

**Leishmania major** and *L. tropica* drug susceptibility assays

The susceptibility of *L. major* and *L. tropica* promastigotes to growth inhibition by cationic compounds was measured by the Alamar Blue assay (Biogen, Cambridge, California) using the methods of Mikus and Steverding (2000) with minor modifications. The Alamar Blue assay measures cell proliferation by monitoring innate metabolic activity of cultured cells with a fluorometric and colorimetric growth indicator.

Drug susceptibility assays for promastigotes were performed in plastic 96-well flat-bottom tissue culture plates using methods described previously for *L. infantum* promastigotes (Rosypal et al., 2007). All experiments were conducted 3 times in triplicate wells. Logarithmically growing promastigotes were suspended in culture media at a concentration of 2 × 10\(^5\) parasites/ml. Promastigotes were dispersed in 100-μl aliquots (2 × 10\(^5\)/well) and cultured with 10-fold serial dilutions of test compounds. Controls consisted of promastigotes in culture media without compound. Cell cultures were incubated at 25 °C in a humidified atmosphere.

For *L. tropica*, after 48 hr of incubation, 10% (v/v) Alamar Blue was added to all wells; plates were subsequently incubated for an additional 24 hr for 72 hr total incubation. For *L. major*, 10% (v/v) Alamar Blue was added after 72 hr to all wells; plates were further incubated for 24 hr for a total of 96 hr incubation. After completing total incubation times for *L. tropica* and *L. major*, fluorescence was measured with a PolarStar fluorescence plate reader (BMG, Durham, North Carolina) using an excitation wavelength of 544 nm and an emission wavelength of 585 nm.

Dose response curves were derived from Alamar Blue fluorescent emission measurements compared to untreated controls and inhibitory concentration 50% (IC\(_{50}\)) was determined for each compound. The IC\(_{50}\) value is the drug concentration that results in 50% inhibition of control cell growth (TD\(_{50}\) value was determined). The TD\(_{50}\) values were used to generate the cytotoxic index, the ratio of mammalian cell cytotoxicity to antileishmanial activity (TD\(_{50}/ IC_{50}\)). Mammalian toxicity values have been previously reported (Rosypal et al., 2007), but were included in the present study for selectivity comparisons by cytotoxic indices.

**RESULTS**

**Antileishmanial activity of pentamidine**

Pentamidine (Figure 1) exerted comparable antiparasitic activity against *L. major* and *L. tropica*. Pentamidine dihydrochloride concentrations of 21.1 μM and 16.4 μM resulted in 50% reduction in promastigote growth for *L. major* and *L. tropica*, respectively. In the axenic amastigote assay, the IC\(_{50}\) value of pentamidine was 3.0 μM, which is comparable to the 3.8 μM cytotoxic dose for L6 cells. This indicates that pentamidine is toxic in general and exhibits little selectivity for *Leishmania* spp. parasites.

**Effects of furan analogs on *L. major* and *L. tropica* promastigotes**

Table I shows IC\(_{50}\) values for activity of 8 diphenyl furan analogs against *L. major* and *L. tropica* promastigotes. Except for 1 (DB154), all compounds in the furan analog series showed some effectiveness against OWCL promastigotes. Five compounds in this class (DB99, DB569, DB103, DB244, DB181) demonstrated antileishmanial activity, which is comparable to furamidine and superior to that of pentamidine. Among the furan analogs, all but 2 compounds (DB154, DB545) were more active than pentamidine against both *Leishmania* species. Compound DB154 was inactive against *L. major* and it was the least active compound against *L. tropica*, with an IC\(_{50}\) value of 89.5 μM. DB545 exhibited IC\(_{50}\) values against OWCL promastigotes comparable to pentamidine. DB569 was the most active compound in this class, with IC\(_{50}\) values of 3.3 μM and 5.8 μM against *L. major* and *L. tropica*, respectively. The inhibitory doses for DB569 exhibit 6.4- and 2.8-fold improved efficacy over pentamidine against *L. major* and *L. tropica* promastigotes, respectively.

The compounds in the present study were tested for toxicity against mammalian cells using rat L6 myoblast cells (Table I). Among the furan analogs, DB103 and DB181 were not cytotoxic (TD\(_{50}\) values >100 μM) against L6 myoblasts, although their antileishmanial activity was only improved approximately 2-fold against OWCL promastigotes. DB545 and DB154, the least active furan analogs, exhibited the greatest toxicity to rat myoblasts, with TD\(_{50}\) values of 0.7 μM and 3.7μM, respective-

---

**Figure 1.** Chemical structure of pentamidine.
Table I. Structures, anti-leishmanial activity, and cytotoxicity results for furan analogs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Leishmania major IC₅₀ [µM]</th>
<th>Leishmania tropica IC₅₀ [µM]</th>
<th>Cytotoxicity TD₅₀ L6 cells [µM]</th>
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<td>&gt;100</td>
<td>89.5</td>
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</table>

* All IC₅₀ and TD₅₀ values are reported in micromolar (µM).
† The IC₅₀ value is the drug concentration that results in 50% reduction of parasite growth compared to untreated controls.
‡ The TD₅₀ value is the toxic dose of compound that results in 50% inhibition of mammalian L6 myoblast cell growth.
ly. DB569 was 6.3-fold less cytotoxic to L6 cells than pentamidine.

**Effects of reversed amidines on *L. major* and *L. tropica* promastigotes**

The series of reversed amidines (Table II) exhibited exceptional antileishmanial activity against promastigotes of both OWCL *Leishmania* species (Table III). All but 1 compound (DB613) in this class show IC₅₀ values below 1 µM. DB746 and DB745 were the most active compounds against *L. major*, with IC₅₀ values of 0.7 nM for both compounds, which is over 25,000 times more active than pentamidine. Against *L. tropica* promastigotes, DB745 and DB766 exhibited the highest antileishmanial activity, with IC₅₀ values of 2.0 nM and 3.7 nM, respectively. In the reversed amidine class, 3 compounds (DB745, DB746, DB766) had IC₅₀ values in the low nM range (less than 20 nM) against both *L. major* and *L. tropica*.

Overall, the high ratio of mammalian cell cytotoxicity to antileishmanial potency (TD₅₀/IC₅₀) among reversed amidines indicated that this compound series is highly selective for *Leishmania* spp. parasites. Cytotoxic indices for *L. major* and *L. tropica* were above 100 for all reversed amidines, except DB613 and DB712. Compounds DB745 and 746 were among the most potent reversed amidines tested in this work and their TD₅₀/IC₅₀ ratios were both above 1,000 for OWCL promastigotes. DB613, which exhibited the lowest antileishmanial activity in this group, demonstrated a TD₅₀ value of 3.8 µM against rat myoblast cells. This value is similar to the IC₅₀ values against OWCL promastigotes and indicates that DB613 is generally toxic and not highly selective for *Leishmania* spp.

**Activity against isolated amastigotes**

The reversed amidines were found to have impressive activity against OWCL promastigotes and they were also tested in our axenic assay using *L. donovani* amastigotelike parasites to determine activity against the intracellular stage. The amastigote screening assay indicated that the reversed amidines possessed excellent antileishmanial activity overall (Table III). With the exception of DB613, which was inactive, all reversed amidines demonstrated IC₅₀ values below 1 µM against isolated amastigotes. Compounds DB745, DB746, and DB766 were among the reversed amidines that displayed the greatest activity against amastigotes, with IC₅₀ values of 0.25 µM, 0.29 µM, and 0.22 µM, respectively. The same compounds exhibited the best activity against OWCL promastigotes.

The TD₅₀ to IC₅₀ ratios determined for isolated amastigotes ranged from 7 to 251, with the exception of DB613. The cytotoxic indices indicate that reversed amidines exhibit selective antileishmanial killing superior to pentamidine, which exhibited a cytotoxic index of 1 for axenic amastigotes.

**DISCUSSION**

In this work, we determined the antileishmanial effects of dicationic analogs of pentamidine for their activity against *L. major* and *L. tropica* promastigotes and chose the most active compounds for further testing in an axenic *L. donovani* amastigote system for comparison. Of the 18 compounds examined, 16 had greater antileishmanial activity than pentamidine, indicating that many aromatic dications are more active against causative agents of OWCL than the parent compound.

The furan analogs presented in Table II were less active than the reversed amidines; however, 7 of 8 compounds tested in this class exhibited superior antileishmanial activity compared to pentamidine. The 2 compounds that exhibited lower activity than pentamidine against *L. major* and *L. tropica* (DB545 and DB154) were the only cyclic amidine compounds tested in this class. DB154 was the least active compound tested in the present work and it is the only compound with a single toloxylo substitution on the furan ring. Previous research from our laboratory revealed compound DB154 was the least active furan analog against *L. infantum* promastigotes as well (Rosypal et al., 2007). DB569 was the most active compound in this series and it was also the most active compound in this class against *L. infantum* (Rosypal et al., 2007). All other structural modifications on compounds tested in this group resulted in comparable or improved potency compared to pentamidine.

The dications in the reversed amidine series demonstrated exceptional activity against both intracellular and extracellular stages of *Leishmania* spp. Reversed amidine molecules are dicationic compounds with an imino group attached to the anilino nitrogen instead of directly attached to the aryl ring (Stephens et al., 2003). All but 1 (DB613) of the reversed amidines demonstrated 50% inhibitory concentrations below 1 µM against both promastigotes and amastigotetelike forms for all *Leishmania* species tested. *Leishmania tropica* was the most sensitive species to the reversed amidines as evidenced by 50% inhibitory doses, which were all below 100 nM.

Antileishmanial screening assays indicate that the compounds have similar relative efficacy against all *Leishmania* species. In general, amastigotes exhibited higher IC₅₀ values than those determined for promastigotes, regardless of species. All but 1 reversed amidine compound (DB613) showed greatly improved efficacy over pentamidine. The 9 compounds in the class that demonstrate outstanding antileishmanial potency all contain pyridine rings. DB613, however, was inactive against *Leishmania* spp. and it was the only phenyl-substituted reversed amidine. Results from the present study indicate a decrease in activity associated with this particular substitution in the reversed amidine series. Compounds DB712 and DB811 showed the lower antileishmanial activity in this class and these analogs were the only 2 that had −Cl moieties attached to the phenyl rings. All other structural modifications among reversed amidines resulted in at least 250-fold improved efficacy over pentamidine against OWCL promastigotes. Results from the amastigote screening system indicated that 9 of 10 compounds in this class demonstrated a minimum of 4.5-fold greater activity against the clinically relevant parasite stage.

Compounds with selective activity against *Leishmania* spp. parasites are of interest as new chemotherapeutic candidates. The cytotoxic index determined by in vitro testing is a useful indicator of compound selectivity by measuring antileishmanial potency in relation to general cellular toxicity. These diamidines were synthesized with the goal of improving antiparasitic activity while simultaneously decreasing mammalian cytotoxicity in an effort to identify active and safe compounds. Our results show that 8 of 10 reversed amidines had TD₅₀/IC₅₀ ratios >100 against OWCL promastigotes which suggests that they
TABLE II. Chemical structures of reversed amidines.

<table>
<thead>
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<th>Y</th>
<th>Z</th>
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<td>H</td>
<td></td>
</tr>
<tr>
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<td>OC₂H₅</td>
<td>H</td>
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</tr>
<tr>
<td>DB 714</td>
<td>CH₃</td>
<td>H</td>
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<td>DB 814</td>
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<td>SCH₃</td>
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possess potent selective antiparasitic activity, but are generally nontoxic.

In addition to initial toxicity studies, an early step in evaluating activity against intracellular and extracellular parasite stages, which may indicate that the compound has direct or indirect activity against *Leishmania* spp. (Kayser et al., 2001). It is important to test drugs for antileishmanial activity against amastigotes, as this is the stage present in the mammalian host. Our study shows that cations in the reversed amidine series are more potent than the parent drug against the amastigote stage. In a previous study, 3 reversed amidines (DB712, DB746, DB814) were tested for activity against *L. donovani* amastigote-infected macrophages (Stephens et al., 2003). The compounds all had IC	extsubscript{50} values below 1 μM in the macrophage model, and DB712 was over 170-fold more active than pentamidine against infected macrophages (Stephens et al., 2003). Based on results from the present study and previous reports, the remaining reversed amidines should be further tested for antileishmanial activity in an infected macrophage system.

Positively charged amidine groups limit the bioavailability of diamidine compounds (Ansede et al., 2004). Neutral diamidoxime derivatives, however, that are orally administered and are actively converted to the diamidine metabolite have been prepared as prodrugs for a number of protozoan parasites (reviewed by Werbovetz, 2006). Orally bioavailable prodrugs that are metabolically converted to active reversed amidines have not been successfully synthesized to date. It is important to note, however, that CL is a localized disease and a topical formulation applied directly to individual lesions would preclude the requirement for metabolic conversion of an oral prodrug. A topical preparation is an easily administered treatment delivery option for CL cases.

The current increase of war-related OWCL in the Middle East highlights the need for new therapies for CL. There is limited access to effective drugs for CL among Afghan refugees (Ahmad, 2002), and treatment with antimonials in Kabul is not cost-effective (Reithinger and Coleman, 2007). Localized treatment options of cryotherapy or thermotherapy are available for U.S. soldiers in Iraq and Afghanistan, but in some cases systemic or intrasional antimony treatment may be necessary (Willard et al., 2005). The decision to treat OWCL is difficult. Although many uncomplicated cases heal spontaneously, the disease has a long duration and lesion dissemination, secondary infections, and stigmatizing deformities from resultant scars can occur.

In this work, we report the activity of 2 classes of dications inhibiting growth of *Leishmania* spp. parasites that cause OWCL. In general, both classes showed antileishmanial activity, but the reversed amidine series demonstrated exceptional efficacy in comparison to pentamidine. The mode of action of the dications is unknown; however, the lipophilic nature of reversed amidines may improve uptake by macrophage and parasite membranes. Additionally, our study suggests that this compound class is selectively active against *Leishmania* spp. and minimally toxic for mammalian cells. Results from this work provide an initial and rational basis for future development and investigation of novel compounds as potential new drugs for the treatment of OWCL.

### ACKNOWLEDGMENTS

Project support was provided by a grant from the Bill and Melinda Gates Foundation to R.R.T. and the contribution of A.C.R was supported by National Institute of General Medical Sciences GM 00678. A.C.R. is a postdoctoral fellow with the SPIRE (Seeding Postdoctoral Innovators in Research and Education) postdoctoral training program. The authors would like to acknowledge Dr. David S. Lindsay from the Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, for his gift of *L. major* and *L. tropica* cultures. We are grateful to Dr. Reto Brun from the Swiss Tropical Institute, Basel, Switzerland, for providing cytotoxicity data.

### LITERATURE CITED


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**TABLE III. In vitro antileishmanial activities and cell toxicities of reversed amidine dications.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell toxicity</th>
<th>L. major (promastigote)</th>
<th>L. tropica (promastigote)</th>
<th>L. donovani (amastigote)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TD	extsubscript{50} (μM)*</td>
<td>IC	extsubscript{50} (μM)†</td>
<td>Cytotoxic index (TD	extsubscript{50}/IC	extsubscript{50}‡)</td>
<td>IC	extsubscript{50} (μM)†</td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>DB746</td>
<td>7.9</td>
<td>0.0007</td>
<td>12.154</td>
<td>0.0065</td>
</tr>
<tr>
<td>DB745</td>
<td>9.2</td>
<td>0.0007</td>
<td>12.432</td>
<td>0.002</td>
</tr>
<tr>
<td>DB785</td>
<td>8.7</td>
<td>0.0013</td>
<td>6.692</td>
<td>0.05</td>
</tr>
<tr>
<td>DB766</td>
<td>3.0</td>
<td>0.012</td>
<td>250</td>
<td>0.0037</td>
</tr>
<tr>
<td>DB714</td>
<td>3.2</td>
<td>0.022</td>
<td>146</td>
<td>0.016</td>
</tr>
<tr>
<td>DB814</td>
<td>7.3</td>
<td>0.041</td>
<td>178</td>
<td>0.026</td>
</tr>
<tr>
<td>DB786</td>
<td>9.7</td>
<td>0.085</td>
<td>114</td>
<td>0.014</td>
</tr>
<tr>
<td>DB811</td>
<td>128</td>
<td>0.36</td>
<td>356</td>
<td>0.063</td>
</tr>
<tr>
<td>DB712</td>
<td>8.0</td>
<td>0.51</td>
<td>16</td>
<td>0.064</td>
</tr>
<tr>
<td>DB613</td>
<td>3.8</td>
<td>3.2</td>
<td>1</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* The TD	extsubscript{50} value is the toxic dose of compound that results in 50% inhibition of mammalian L6 myoblast growth.
† The IC	extsubcript{50} value is the drug concentration that results in 50% reduction of parasite growth compared to untreated controls.
‡ The cytotoxic index is the ratio of mammalian cell cytotoxicity to anti-leishmanial activity (TD	extsubscript{50}/IC	extsubscript{50}).


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Toxoplasma gondii and Neospora caninum Antibodies in Dogs From Grenada, West Indies

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ABSTRACT: Toxoplasma gondii and Neospora caninum are structurally similar parasites with many common hosts. The prevalence of antibodies to T. gondii and N. caninum was determined in sera from dogs in Grenada, West Indies. Using a modified agglutination test, antibodies to T. gondii were found in 52 (48.5%) of the 107 dogs, with titers of 1:25 in 17, 1:50 in 19, 1:100 in 7, 1:1,600 in 5, and 1:3,200 or higher in 4. Seroprevalence increased with age from 2.2% in dogs <6 mo old to 18.9% in dogs older than 2 yr, indicating postnatal transmission of T. gondii in this population of canines. There was no correlation between the health of the dogs and the seroprevalence or magnitude of the T. gondii titer. Antibodies to N. caninum were determined by the indirect immunofluorescent antibody test (IFAT). Two of the 107 dogs had N. caninum antibodies (IFAT titer 1:100 and 1:400); these dogs had T. gondii titers of 1:1,600 and 1:50, respectively. Results indicate that these 2 structurally similar protozoa are antigenically different. Toxoplasma gondii and N. caninum are related coccidians that can cause fatal infections in dogs (Dubey, Carpenter et al., 1988). There are numerous reports of clinical toxoplasmosis worldwide, and these have been summarized by Dubey (1985) and Dubey and Beattie (1988). After the discovery of N. caninum as a cause of paralysis in dogs, several reports of infections thought to be toxoplasmosis are now considered as neosporosis (Dubey et al., 1989, 2003). Serum-based tests for T. gondii and N. caninum are widely used for serological diagnosis in cattle. To our knowledge, there is no confirmed report of congenital toxoplasmosis in dogs. In the present study, 2 of the dogs (nos. 36 and 37) had high titers of antibodies to T. gondii (IFAT titer of 1:400 and MAT titers of 1:100 and 1:1,600). One of these dogs had a low MAT titer of only 1:25. The MAT is considered specific for T. gondii at a serum dilution of 1:25 or higher based on experimental studies in animals and a validation study in naturally infected pigs (Dubey et al., 1995; Dubey, 1997).

Seroprevalence data in dogs of different ages suggest that the dogs acquired T. gondii infection either by ingesting oocysts or by eating tissues of animals infected with T. gondii. Little is known of the epidemiology of T. gondii infection in Grenada. In a small survey, T. gondii antibodies were found in 35% of 40 cats sampled in 1995, indicating the presence of oocysts shed by these cats (Asthana et al., 2006). Recently, viable T. gondii was isolated from 35 of 102 free-range chickens from Grenada, indicating widespread contamination with oocysts of the soil in rural areas (Dubey et al., 2005). These infected chickens could be a source of infection for dogs. More recently, antibodies to T. gondii were found in only 2 of 238 rats from Grenada and viable parasites were isolated from only 1 of these animals (Dubey et al., 2006). The low prevalence of T. gondii in rodents suggests that they are unlikely to be a source of infection for dogs. Nothing is known of the prevalence of T. gondii in meat consumed by people in Grenada and whether meat scraps can be a source of infection for dogs.

In the present study, 2 of the dogs (nos. 36 and 37) had N. caninum antibodies. Dog no. 36 had a low MAT titer of 1:400 and a T. gondii MAT titer of 1:50, and dog no. 37 had an IFAT N. caninum titer of 1:100 and T. gondii MAT titer of 1:1,600. The low seroprevalence of N. caninum versus high prevalence of T. gondii supports the specificity of the T. gondii and N. caninum serology. Neospora caninum infections in dogs are important clinically and epidemiologically. Neospora caninum causes severe disease in dogs, particularly in neonates, and is considered a primary pathogen. Toxoplasma gondii, on the other hand, is not considered a primary pathogen in dogs, and most fatal infections reported occur in immunosuppressed dogs, usually coinfected with canine distemper virus (Campbell et al., 1955; Capen and Cole, 1966; Dubey, 1985; Dubey et al., 1989, 2003). To our knowledge, there is no confirmed report of congenital toxoplasmosis in dogs in Grenada or elsewhere in the world.

The domestic dog is a definitive host for N. caninum and the oocysts
The hypothesis that these 2 structurally similar organisms are antigenically distinct. Virtually all warm-blooded animals are hosts for *T. gondii*, whereas cattle and deer are thought to be the most important intermediate hosts for *N. caninum* (Dubey et al., 2007). It is likely that the city dogs in the present study did not have access to beef or venison infected with *N. caninum*.

**LITERATURE CITED**


### Table I. General characteristics of the dogs and prevalence of anti-*T. gondii* antibodies as determined by a positive MAT (≥25).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of dogs studied in this category</th>
<th>No. of dogs MAT-positive</th>
<th>% Of dogs seropositive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutered male</td>
<td>5</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>Male</td>
<td>42</td>
<td>14</td>
<td>33.3*</td>
</tr>
<tr>
<td>Spayed female</td>
<td>11</td>
<td>7</td>
<td>63.6</td>
</tr>
<tr>
<td>Female</td>
<td>43</td>
<td>27</td>
<td>62.8*</td>
</tr>
<tr>
<td>TOTAL</td>
<td>101</td>
<td>50</td>
<td>49.5</td>
</tr>
<tr>
<td>Age group (mo)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>10</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>7–12</td>
<td>19</td>
<td>10</td>
<td>52.6</td>
</tr>
<tr>
<td>13–24</td>
<td>28</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>≥25</td>
<td>33</td>
<td>17</td>
<td>51.5</td>
</tr>
<tr>
<td>TOTAL</td>
<td>90</td>
<td>43</td>
<td>47.8</td>
</tr>
<tr>
<td>Residence area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>St. Andrew’s parish</td>
<td>25</td>
<td>12</td>
<td>48.0†</td>
</tr>
<tr>
<td>St. David’s parish</td>
<td>5</td>
<td>4</td>
<td>80.0†</td>
</tr>
<tr>
<td>St. George’s parish</td>
<td>38</td>
<td>20</td>
<td>52.6</td>
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<td>St. John’s parish</td>
<td>15</td>
<td>7</td>
<td>46.7</td>
</tr>
<tr>
<td>St. Mark’s parish</td>
<td>6</td>
<td>0</td>
<td>0†</td>
</tr>
<tr>
<td>St. Patrick’s parish</td>
<td>12</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>Carriacou island</td>
<td>2</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>TOTAL</td>
<td>103</td>
<td>50</td>
<td>48.6</td>
</tr>
<tr>
<td>Health status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>64</td>
<td>30</td>
<td>46.9</td>
</tr>
<tr>
<td>Fair</td>
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<td>15</td>
<td>55.6</td>
</tr>
<tr>
<td>Poor</td>
<td>9</td>
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<td>TOTAL</td>
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<td>Origin</td>
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<tr>
<td>Stray</td>
<td>21</td>
<td>12</td>
<td>57.1</td>
</tr>
<tr>
<td>Pet</td>
<td>86</td>
<td>40</td>
<td>46.5</td>
</tr>
<tr>
<td>TOTAL</td>
<td>107</td>
<td>52</td>
<td>48.6</td>
</tr>
</tbody>
</table>

* The % MAT-positive non-spayed females was significantly higher than the % MAT-positive non-neutered males, at P ≤ 0.01.
† The % MAT-positive dogs from St. Andrew’s and St. David’s parishes were significantly higher than the % MAT-positive dogs from St. Mark’s parish, at P ≤ 0.05. However, results from St. Andrew’s parish were not significantly different from those of St. David’s parish.
Helminths Collected From Imported Pet Murids, with Special Reference to Concomitant Infection of the Golden Hamsters with Three Pinworm Species of the Genus Syphacia (Nematoda: Oxyuridae)

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ABSTRACT: A total of 210 individuals of 13 species belonging to 4 subfamilies of Muridae imported into Japan as pets were examined; 5 species of Syphacia (Nematoda: Oxyuridae), Aspiculuris tetraptera (Nematoda: Heteroxyenematidae), and Rodentoloplos nana (Cestoidea: Hymenolepididae) were collected. Concurrent infection with 3 pinworm species, Syphacia mesocriceti, Syphacia stroma, and Syphacia peromysci, was recorded for the first time in the golden hamster, Mesocricetus auratus. Syphacia mesocriceti was also identified in the desert hamster, Phodopus roborovskii, and S. peromysci was recovered from the fat-tailed gerbil, Pachyuromys duprasi, and the Cairo spiny mouse, Acomys cahirinus. From the pygmy mouse, Mus minutoides, an undetermined species closely resembling Syphacia megaloon and Syphacia obtuorum, both parasitic in Mus sp., was collected. Females of another undetermined Syphacia sp. were observed in the greater Egyptian gerbil, Gerbillus pyramidum. All of the host-Syphacia associations, except S. mesocriceti in the golden hamsters, were recorded for the first time. It is suggested that overlapping breeding situations provided the opportunity for host switching by the pinworms.

Rearing of exotic pet animals has gained worldwide popularity. In Japan, various pet mammals were imported freely from foreign countries until 1 September 2005, when New Notification System for the Importation of Animals was enforced by the Ministry of Health, Labor and Welfare of the Japanese Government. Because quarantine was not previously obligatory for these mammals, there was concern that zoonotic pathogens may have been introduced with them. To determine the prevalence of zoonotic pathogens in various imported rodents, a cooperative survey by veterinary virologists, bacteriologists, parasitologists, and pathologists was conducted from 2003 to 2006. Viral and bacterial agents detected have been reported elsewhere (Une et al., 2004; Maizuwata et al., 2006). Herein, the helminth parasites that were recovered are reported.

Imported murid rodents were purchased from 5 animal brokers in Tokyo. The origin of export countries was not specified in detail, but apparently included the Czech Republic and the Netherlands. The rodents were killed by chloroform inhalation, and their viscera were excised and fixed in 10% neutral-buffered formalin solution. After removing various portions of the visceras for histologic examination and recovery of protozoans, the remaining alimentary canals were subjected to helmintological assessment. The gastrointestinal tracts were cut open and examined using stereomicroscopy for helminths. Parasitic worms collected were preserved in 10% neutral-buffered formalin solution. For light microscopy, parasites were cleared in a glycerol-alcohol solution by evaporating alcohol and then were mounted on glass slides with 50% glycerol solution. For light microscopy, parasites were cleared in a glycerol-alcohol solution by evaporating alcohol and then were mounted on glass slides with 50% aqueous glycerol solution. Intensity data for each species were based on adult-stage worms, as species identification of larval forms was not possible. Eggs excised from gravid females were also observed using a scanning electron microscope (SEM). Voucher specimens were deposited in the National Science Museum, Tokyo, Japan.

A total of 210 individuals of 13 species belonging to 4 subfamilies of Muridae were examined. Five species of Syphacia Seurat, 1916 (Nematoda: Oxyuridae) were collected in 6 murid species from 4 of the 5 dealers (Table I). In addition to Syphacia spp., Aspiculuris tetraptera (Nitzsch, 1821) (Nematod: Heteroxyenematidae) and Rodentoloplos nana (Siebold, 1852) (Cestoidea: Hymenolepididae) were found in the hamsters (Table I). Prevalence and intensity of infections are reported in Table I. Among the 5 Syphacia spp., 4 have not previously been recorded from rodent hosts. Concomitant infection of more than 1 Syphacia spp. was found in 2 groups of the golden hamsters: 1 group was infected with 2 species and the other group was infected with 3 species. Brief comments are made below for each Syphacia sp. collected.

Syphacia mesocriceti Quentin, 1971, was found in 17 of 20 M. auratus, with intensity up to 168 (Table I). The worms were mostly female; the number of males in a host ranged from 1 to 5. This pinworm was also found in P. roborovskii, with low intensity (Table I). Syphacia mesocriceti is characterized by having cephalic papillae projecting dor­sally and ventrally from amphids (Quentin, 1971). It has been classified in the subgenus Crictesoxyuris Hugot, 1988 (Hugot, 1988). Morphology and measurements of the present worms are identical to those of S. mesocriceti reported previously (Quentin, 1971; Dick et al., 1973; Hasegawa, 1981). This species was first observed in a golden hamster in captivity in Alaska (Quentin, 1971). Since then, it has been identified in this host kept as pets and as experimental animals in various parts of the world (Dick et al., 1973; Hasegawa, 1981; Pinto et al., 2001). Phodopus roborovskii is recorded here for the first time as the host of S. mesocriceti.

Syphacia stroma (Linstow, 1884) was recovered from M. auratus, concomitantly with S. mesocriceti in both groups of the golden hamsters examined, with an intensity that ranged from 3 to 99 (Table I). Syphacia stroma has been known as a pinworm of Apodemus spp. in Europe (Quentin, 1971; Tenora and Mészáró, 1975; Genov, 1948). It belongs to the group IX of Quentin (1971), along with Syphacia emiliorum Chabaud et al., 1963. Pinworms of this group are characterized by an oval cephalic plate separated clearly from the cervical region by a groove in which the cephalic papillae are located, along with 4 well-discernible labial papillae and a stout male tail (Quentin, 1971). The characteristics and measurements of the present worms were identical to those of previously described S. stroma, although the males have a somewhat narrower body (1.53–1.75 mm long and 91–115 µm wide at mid-body) and an accessory piece of gubernaculum that lacks faint cuticular creases (Quentin, 1971). The surface features of the eggs are also identical to those of S. stroma (Barus et al., 1979). Mesocricetus auratus is recorded as a new host of S. stroma.

Syphacia peromysci Harkema, 1936, was found in M. auratus, the fat-tailed gerbil, Pachyuromys duprasi, and the Cairo spiny mouse, Acomys cahirinus (Table I). Intensity of infection was highest (>500) in P. duprasi; it ranged from 2 to 5 in M. auratus, and a single female was collected in A. cahirinus (Table I). Syphacia peromysci is a typical member of the subgenus Seuratodoxyuris Hugot, 1888, in having well-developed cervical alae with internal supports and prominent deririts in females, and a short tail and an accessory piece of gubernaculum with cuticular ornamentations in males (Hugot, 1988). It closely resembles Syphacia petrusewiczii Bernard, 1966 and Syphacia rauschi Quentin, 1969 in having a laterally elongated cephalic plate. Syphacia petrusewiczii and S. rauschi are parasitic in voles, Clethrionomyos spp., in the Palaearctic Region and Asia, respectively, whereas S. peromysci is known from the deer mouse, Peromyscus maniculatus, in North America (Bernard, 1966; Quentin, 1969, 1971; Quentin and Kinsella, 1972; Jancév, 1973; Tenora and Mészáros, 1975; Quentin and Gran, 1977; Genov, 1984; Hasegawa et al., 1994). The body dimensions and morphology of the present worms are almost identical to those of S. peromysci (Quentin and Kinsella, 1972). Syphacia minuta Greenberg, 1969, has been described in A. cahirinus in Israel. The present specimens are readily distinguishable from S. minuta by having larger females (3.13–4.11 mm long vs. 1.74–2.85 mm long). Males in the present study have a longer spicule (55–70 /µm long vs. 45–57 /µm long in previous studies), whereas the body length is comparable (0.80–1.04 mm vs. 0.68–1.01 mm) to that described by Greenberg (1969). The present 3 rodents are recorded as new hosts for S. peromysci.

Syphacia sp. 1 was recorded from the pygmy mouse M. minutoides, with low intensity (Table I). The present species is included in group VIII of Quentin (1971) as having a well-developed cephalic vesicle, a

laterally elongated cephalic plate, and a facial mask that includes cephalic papillae and amphids that are laterally placed. Among the members of this group, S. megaloon Quentin, 1966, and S. ohtaaorum Hasegawa, 1991, resemble the present worms by having a stout body and a short tail in females (Quentin, 1966; Hasegawa, 1991). Syphacia megaloon was originally described from M. minutoides, the same host species seen in the present worms, and Mus setulosus of Zaire (Congo). However, the worms in the present study are distinguished from S. megaloon by having more eggs (about 100 vs. 20–30) of much smaller size (123–132 × 35–42 μm vs. 150 × 60 μm) as described by Quentin (1966, 1971). The present males are differentiated from S. ohtaaorum by having a spicule constricted in the proximal 114 position, whereas the present females are thinner than those of M. minutoides, (104–110 × 25–30 μm vs. 70–116 × 30–38 μm; Quentin, 1971).

Syphacia spp. nematodes are usually host-specific and have a tendency to co-evolve with their hosts since the environmental phase in their monoxenous life cycle is much reduced or absent (Hugot, 1988). Consequently, mixed infections in the present golden hamsters with 3 Syphacia spp. are of special interest. Syphacia meosoriceti has been known as being host-specific for golden hamsters, although it has not been reported in the feral hamster. Syphacia stroma is parasitic in Apodemus spp. in the Palaearctic region, and S. peromysci has been recorded only in North America (Hugot, 1988); thus, they were certainly acquired by hamsters under captive conditions. Likewise, the fat-tailed gerbil may have become infected with S. peromysci during captivity because this rodent is endemic to the north Sahara region. Apparently, pet breeders’ facilities provide suitable conditions for host capture by pinworms, which may seldom occur under natural conditions.

Pinto et al. (2001) reported mixed infections of golden hamsters with 2 pinworm species, S. mesosriceti and Syphacia criceti Quentin, 1969. However, the identification of the latter pinworm is questionable. In S. criceti, the tail of the male narrows suddenly and its length is almost twice that of the spicule (Quentin, 1971), whereas the male referred to as S. criceti sensu by Pinto et al. (2001) has a gradually tapered tail and the length is far greater than twice the length of the spicule. These features for S. criceti Pinto et al., 2001, are similar to those of S. stroma collected in the present survey. The shape of gubernaculum, as depicted by Pinto et al. (2001), is also identical to that of the present S. stroma.

### Table I. Prevalence of helminth parasites in pet murids imported into Japan.

<table>
<thead>
<tr>
<th>Rodent host</th>
<th>No. hosts examined</th>
<th>Date of necropsy</th>
<th>Helminths found</th>
<th>% Prevalence and intensity (range [mean])</th>
<th>NSMT accession No.</th>
<th>Dealer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lagurus lagurus</td>
<td>9 (6M, 3F*)</td>
<td>15 IV 2006</td>
<td>—†</td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Mesocricetus auratus</td>
<td>10 (6M, 4F)</td>
<td>10 VI 2006</td>
<td>Syphacia mesosriceti</td>
<td>70 (1–10 [6.2])</td>
<td>As 3093</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Syphacia stroma</td>
<td>80 (3–99 [19.9])</td>
<td>As 3095</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Syphacia peromysci</td>
<td>80 (2–5 [3.4])</td>
<td>As 3096</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rodontelepis nana</td>
<td>40 (1–6 [3.0])</td>
<td>PI 5611</td>
<td></td>
</tr>
<tr>
<td>Phodopus roborovskii</td>
<td>10 (5M, 5F)</td>
<td>10 VI 2006</td>
<td>S. mesosriceti</td>
<td>100 (7–168 [86.6])</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. stroma</td>
<td>10 (47 [47])</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R. nana</td>
<td>50 (2–13 [8.2])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phodopus sungorus</td>
<td>10 (7M, 3F)</td>
<td>10 VI 2006</td>
<td>S. mesosriceti</td>
<td>30 (1–4 [2.0])</td>
<td>As 3094</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aspicularis tetraperta</td>
<td>30 (1 [1])</td>
<td>As 3495</td>
<td></td>
</tr>
<tr>
<td>Mus minutoides</td>
<td>10 (3M, 7F)</td>
<td>10 VI 2006</td>
<td>A. tetraptera</td>
<td>90 (1–82 [14.4])</td>
<td>As 3496</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A. tetraptera</td>
<td>60 (1–227 [22.5])</td>
<td>PI 5612</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R. nana</td>
<td>100 (2–157 [48.5])</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R. nana</td>
<td>40 (1–30 [8.8])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acomys cahirinus</td>
<td>20 (12M, 8F)</td>
<td>11 X 2004</td>
<td>S. peromysci</td>
<td>15 (1 [1])</td>
<td>As 3098</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A. tetraptera</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acomys russatus</td>
<td>9 (6M, 3F)</td>
<td>2 IV 2005</td>
<td>—</td>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Lemniscomys barbarus</td>
<td>13 (6M, 7F)</td>
<td>2 IV 2005</td>
<td>—</td>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Mus minutoides</td>
<td>11 (8M, 3F)</td>
<td>2 VII 2005</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. setulosus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gerbillinae</td>
<td>20 (15M, 5F)</td>
<td>11 X 2004</td>
<td>Syphacia sp. 1</td>
<td>60 (1–7 [3.2])</td>
<td>As 3099</td>
<td>E</td>
</tr>
<tr>
<td>Gerbillus pyramidal</td>
<td>10 (7M, 3F)</td>
<td>4 VI 2005</td>
<td>Syphacia sp. 2</td>
<td>60 (1–8 [2.4])</td>
<td>As 3100</td>
<td>A</td>
</tr>
<tr>
<td>Meriones tristriatus</td>
<td>4 (4M)</td>
<td>11 X 2004</td>
<td></td>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Pachyuromys duprasi</td>
<td>5 (5F)</td>
<td>13 VII 2003</td>
<td>—</td>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>8 (7M)</td>
<td>2 IV 2005</td>
<td>—</td>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>10 (6M, 4F)</td>
<td>4 VI 2005</td>
<td>S. peromysci</td>
<td>50 (1–34 [11.2])</td>
<td>As 3097</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>10 (5M, 5F)</td>
<td>15 IV 2006</td>
<td>S. peromysci</td>
<td>90 (6– &gt;500 [ &gt;100])</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Psammomys obesus</td>
<td>11 (1M, 10F)</td>
<td>2 IV 2005</td>
<td>—</td>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Sceketamyx calurus</td>
<td>10 (4M, 6F)</td>
<td>4 VI 2005</td>
<td>—</td>
<td></td>
<td></td>
<td>A</td>
</tr>
</tbody>
</table>

* M, male; F, female.
† Negative.
Therefore, it is strongly suggested that S. stroma infection of golden hamsters is global.

Syphacia sp. 1 from M. minutoides is surmised to have been maintained in this host from its original capture in Africa, because it resembles S. megaloon and S. ohtaorum, both parasitic in Mus spp. Syphacia megaloon is distributed in Africa and S. ohtaorum is distributed in Japan, Inner Mongolia, China, and Nepal (Hasegawa, 1991; H. Hasegawa, unpubl. obs.). Presumably, this pinworm lineage has co-evolved with Mus spp. and is dispersed in a wide geographic range from the Far East to Africa. It is necessary to determine the intraspecific variations that might have been formed during dispersal. For this purpose, DNA sequence analysis may be advantageous. For example, GREENBERG, GENOV, DICK, T. A., J. C. QUENTIN, AND R. S. FREEMAN, 1973. Redescription of Syphacia obvelata (Rudolphi, 1802), another pinworm of Mus spp. If a similar analysis is applied to all of the present species, the geographic and host origins of Syphacia spp. in the pet murids may be elucidated.

Thanks are rendered to A. Yasuda and H. Kawabata, Institute of Scientific Research, Oita University, for their kind support with SEM observation. This study was partially supported financially by grants-in-aid from JSPS 19580355 and 20570090.

LITERATURE CITED


Strategies for the Storage of *Ancylostoma caninum* Third-Stage Larvae


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**Abstract:** Although cryopreservation protocols for storage of hookworm larvae have been described, the circumstances under which the technique is necessary to ensure larval survival are not well defined. The motility of infective-stage larvae (as judged by observation) and their ability to migrate through canine skin in vitro were measured over a 7-mo period in worms held at room temperature and worms that had been cryopreserved at the start of the experiment. Cryopreserved worms showed motility and migration proportions of 45.6–48.0% and 26.8–34.0%, respectively, throughout the experiment, compared with percentages of 92.7 and 84.1%, respectively, in the original fresh worms. Larvae held at room temperature showed a gradual decrease in motility and migration ability over the experimental period. Motility and migratory ability of cryopreserved larvae was only significantly higher (P < 0.01) than room temperature-stored larvae from 4 and 5 mo onward, respectively.

Studies involving the canine hookworm, *Ancylostoma caninum*, and other hookworms, often require a means of preserving important strains of the parasite over a period of months or even years. Infective third-stage larvae (L3) of *A. caninum* do not develop further without contacting a suitable host (Hawdon and Schad, 1990), making this life stage the obvious choice for storage. Although L3s of certain nematode species, such as *Haemonchus contortus*, can be stored for at least 9 mo at 5 C (Troell et al., 2006), temperatures below 15 C are lethal to *A. caninum* L3 (Levine, 1968). For this reason, cryopreservation in liquid nitrogen is preferred over refrigeration as a storage method for *A. caninum* larvae, and successful protocols for this have been published (Miller and Cunningham, 1965; Kelly et al., 1976). Significant attrition, however, occurs with freezing and thawing, such that even the most effective protocols described result in at least a 40% reduction in the number of viable larvae after thawing. In addition, the use of liquid nitrogen requires facilities and equipment that may not be available to all investigators. We, therefore, undertook a study to compare cryopreservation with room temperature storage of larvae to determine time intervals for which each technique may be the most appropriate storage option. Assessment of room temperature storage included determination of whether monthly changes of storage medium offered benefit over storage without medium changes.

Third-stage *A. caninum* larvae were obtained by vermiculite coprophagy (Cassai, 1999) of pooled feces from stray dogs in Brisbane, Australia. After harvest, L3s were purified by the Baermann technique (Kassai, 1999) and placed in phosphate-buffered saline (PBS) ( Gibco, Invitrogen Corporation, Melbourne, Australia) at a concentration of 1,000 larvae/ml until use. All larvae were used for experiments within 2 wk of harvest.

In a preliminary experiment, we examined whether it was necessary to exsheath the *A. caninum* L3 before cryopreservation. Observation of motility in larvae defrosted 3 days after cryopreservation indicated that the proportion of exsheathed larvae with normal motility was 49.3% (95% confidence interval [CI] 45.0–53.6), compared with mean motility of 5.7% (95% CI 3.2–8.3) among nonexsheathed worms (P < 0.001). Similarly, 25% (95% CI 18.1–32.8) of the exsheathed worms were able to migrate through dog skin, compared with a complete absence of migration for the exsheathed worms (P < 0.001). In the subsequent experiment, cryopreservation was performed using exsheathed worms.

Cryopreservation was carried out using a protocol that combined approaches used in previous studies (Miller and Cunningham, 1965; Kelly et al., 1976; Duarte et al., 2003). Glycerol (Miller and Cunningham, 1965), dimethyl sulfoxide (DMSO) (Kelly et al., 1976) have been used separately as cryoprotectants; however, we used both in combination, carried in an RPMI 1640 medium base (Duarte et al., 2003). Larvae (140,000) were exsheathed by gently bubbling 40% CO₂ into the PBS/larvae suspension for 3 hr. The PBS/larvae suspension was held in a water bath at 37 C during the exsheathing process. Mass exsheathing was confirmed by microscopic examination of a small aliquot. After exsheathment, larvae were pelleted, and the supernatant was removed. The pellet was then suspended in cryoprotectant (10% glycerol in RPMI 1640 medium [Gibco] + 10% DMSO [Sigma-Aldrich, St. Louis, Missouri]) to a total volume of 7 ml in a 10-ml flat-bottomed plastic container ( Sarstedt, Nürnberg Germany), and the solution was incubated for 20 hr at 26 C (Miller and Cunningham, 1965). The solution was then gently vortexed and aliquoted into 1-ml cryovials, each containing approximately 20,000 larvae. All vials were slow-frozen in a −80 C freezer for 24 hr (Duarte et al., 2003), and then they were transferred immediately to liquid nitrogen for storage. At monthly intervals, 1 aliquot was thawed in a 40 C water bath for 10 min (Kelly et al., 1976; Duarte et al., 2003). Larvae were then pelleted and resuspended in 20 ml of RPMI 1640 medium. A representative 2-ml sample, containing approximately 2,000 larvae, was taken for viability assessment.

Fifty thousand larvae designated for room temperature storage were placed in PBS with 100 μl/ml amphotericin B ( Gibco, Invitrogen Corporation) and 10 μl/ml penicillin-streptomycin (Sigma-Aldrich) at a concentration of 1,000 larvae/ml. This pool of larvae was divided into two 25-ml aliquots, both of which were placed into sterile (150-mm-diameter tissue culture flasks (Nunclon, Rochester, New York) and kept in darkness at a constant 22 C. For 1 aliquot only, monthly media changes were made. This was done by pelleting larvae in 50-ml centrifuge tubes, washing once in PBS, and resuspending larvae in 25 ml of fresh storage medium. All centrifuge steps were undertaken for 10 min at 1,500 g.

Monthly viability assessments were undertaken by taking 0.5 ml from the carpet of larvae at the bottom of each flask, yielding approximately 2,000 larvae. These larvae were then suspended in PBS to a concentration of 1,000 larvae/ml. Larval viability was assessed using 2 parameters, motility and migratory ability. To assess motility, a representative 120-μl sample of larval suspension was placed on a microscope slide, and 100 larvae were individually categorized as being either motile (displaying typical smooth sinusoidal motion) or nonmotile. This process was repeated 3 times for each storage condition at each time point. The migration assay apparatus was constructed by stretching shaved dog skin over 30-mm tubing, which was subsequently fitted tightly into the neck of a 50-ml tube filled with PBS. Skin was harvested from the abdomen of freshly killed dogs, and all assessments at each time point were performed on the same day. Contact between the skin and PBS. Larvae (approximately 500 as determined by counting 3 representative 10-μl aliquots) were then gently pipetted onto the exposed surface of the skin. After 2 hr, the tubing and skin were removed from the 50-ml tubes, which were subsequently centrifuged for 10 min at 1,000 g and reduced to a volume of 5 ml. Numbers of larvae in ten 0.1-ml aliquots were counted from each tube, and the average was extrapolated to estimate the total number of larvae that had successfully migrated through the skin. Proportions of larvae that migrated were assessed using 3 subsets of larvae for each storage condition at each time point.

Graphing of data was performed using the software package Prism (GraphPad Software, San Diego, California), and statistical analyses performed using the statistical package Stata version 9.2 (Stata Corporation, College Station, Texas). All statistical testing was undertaken with the individual larva as the unit of analysis. This approach assumes that outcomes (motile and migrated) were not clustered within storage condition at each time point, i.e., that within a storage condition at each time point, individual larvae were statistically independent and that there were no ‘flask effect,’ i.e., no clustering of outcome by flask. The assumption of no flask effect is supported by the observation that proportions of cryopreserved larvae that were motile and proportions that migrated did not deviate substantially from a smooth trend line over the 7-mo period, even though different cryo-vials were thawed for assessment at each time point. Although motility and migration were always measured in 3 subsets of larvae, to ensure consistency in experimental
temperature storage is presented below with reference only to room temperature larvae being migratory were also significantly higher than cryopreserved larvae at 5-, 6-, and 7-mo time points did cryopreservation result in significantly higher motility and migration raising the possibility that long-term viability may have been due to this regular stimulation resulting in the nonfeeding life stage exhausting its finite energy stores more rapidly. The ability of A. caninum larvae to migrate through canine skin in the in vitro migration assay is likely to be an indicator of their in vivo infectivity, and it has been used to assess the efficacy of candidate hookworm vaccines (Bethony et al., 2005; Fujisawa et al., 2007). However, variation in dog skin used in migration assessments is likely to be particularly important, and although care was taken in this study to ensure that, at each time point, similar skin was used for each storage condition, differences in individual skin samples, even from the same dog, might explain the variation in migration seen between the 2 groups of room temperature larvae after only 1 mo. Further work is required to allow a better understanding of the factors influencing in vitro migration of A. caninum larvae. These experiments were approved by the University of Queensland’s Animal Ethics Committee, approval S/133/05.

MATERIALS AND METHODS


Effects of High Pressure Processing on Toxoplasma gondii Oocysts on Raspberries

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ABSTRACT: Oocysts are the environmentally resistant life stage of Toxoplasma gondii. Humans can become infected by accidentally ingesting the oocysts in water or from contaminated produce. Severe disease can occur in immunocompromised individuals, and nonimmune pregnant women can infect their offspring. Chronic infection is associated with decreased mental functions, vision and hearing problems, and some mental disorders such as schizophrenia. High pressure processing (HPP) is a commercial method used to treat food to eliminate pathogens. Treatment of produce to eliminate viable T. gondii oocysts would provide a means to protect consumers. The present study was done to better define the effects of HPP on oocysts placed on raspberries. Raspberries were chosen because they are a known source of a related human intestinal parasite, Cyclospora cayetanensis. Raspberries were inoculated with ~10^6 oocysts of the VEG strain of T. gondii for 20 hr prior to HPP. Individual raspberries were exposed to 500 MPa, 400 MPa, 340 MPa, 300 MPa, 270 MPa, 250 MPa, 200 MPa, 100 MPa, or no MPa treatment for 60 sec in a commercial HPP unit (1 MPa = 10 atm = 147 psi). Treatment of raspberries with 340 MPa for 60 sec was needed to render oocysts spot inoculated on the raspberries noninfectious for mice. Treatment of raspberries with 200 MPa or less for 60 sec was not effective in rendering oocysts noninfectious for mice.

Toxoplasma gondii is a protozoan parasite that infects humans and most other warm-blooded animals. Humans become infected by ingesting meat containing tissue cysts or by ingesting oocysts in the environment. It is estimated that there are 1,500,000 cases of toxoplasmosis in the United States each year and about 15% of those infected develop clinical disease (Jones, Kruiszon-Moran et al., 2001). Congenital toxoplasmosis has long been recognized for the devastating effects it can have on the infected fetus (Jones, Lopez et al., 2001). The relative importance of meat or oocysts as a source of human infection in the United States is unknown. The importance of chronic T. gondii infection on human health was manifested in the AIDS epidemic with the numerous cases of toxoplasmonic encephalitis due to reactivated infection. The association of chronic T. gondii infection and behavioral changes has come to light in recent years and been strengthened by many studies in humans, mice, and rats (Holliman, 1997; Webster, 2003). Most notably, the association of chronic T. gondii infection and schizophrenia has gained attention (see Torrey and Yolken, 2003).

High pressure processing (HPP) has been shown to be an effective nonthermal means of eliminating nonsome forming bacteria from a variety of food products (Flick, 2003). The shelf life of the products is extended and the sensory features of the food are not, or are only minimally, affected by HPP (Ananth et al., 1998; San Martin et al., 2002). Other advantages of HPP over traditional thermal processing include reduced processing times; minimal heat damage problems; retention of size and multiple ice-phase forms; and minimal undesirable functional changes in food during pressure-shift freezing due to reduced crystal size and multiple ice-phase forms; and minimal undesirable functional alterations (Tewari et al., 1999). Oocysts of the VEG strain (Dubey et al., 1996) of T. gondii were obtained from the Animal Parasitic Diseases Laboratory (APDL), Beltsville, Maryland. Oocysts were treated with 10% bleach solution for 10 min and then the bleach was washed off by centrifugation in sterile Hanks’ balanced salt solution without calcium or magnesium (HBSS). Oocysts were counted in a hemocytometer and diluted in HBSS to a concentration of 5 X 10^5 per ml. Raspberries were obtained from a local grocery store but were grown in Mexico (distributed by Global Berry Science and Technology, Kent, Washington) with 7XS-6000 intensifier pump and maximum operating pressure of 600 MPa. The HPP unit was installed and operated at Virginia Polytechnic Institute and State University’s Department of Food Science and Technology. All samples were exposed for 60 sec. Table I describes the pressures and exposure times examined in the HPP study.

The HPP-treated raspberries were processed individually as follows. A 60-sec HPP was removed from its HPP treatment bag and placed in a stomacher bag containing 10 ml of HBSS and stomached for 90 sec at normal speed (Seward Lab Blender Stomacher 80, Seward Limited, Worthing, West Sussex, UK). The raspberry suspension was filtered through cheesecloth to separate oocysts from mash and seeds. The filtrate was concentrated by centrifugation and resuspended in 1.5 ml of HBSS and placed in 2 ml screw-cap tubes. Tubes were placed in sealable bags and sealed. Samples were shipped to the APDL on cold packs by overnight express courier to bioassay for viable T. gondii. Negative controls consisted of raspberries that were not inoculated with T. gondii oocysts and not placed under HPP. Positive controls consisted of raspberries that were spot inoculated with 5 X 10^4 per ml T. gondii oocysts and not placed under HPP.

Mice were orally fed using a mouse-feeding needle. During the study, impression smears were made from the livers or lungs of any mice that died, and were examined unstained by light microscopy for tachyzoites. Forty-five days postinoculation (PI), all surviving mice were bled from the retro-orbital plexus. The serum was collected and examined for antibodies to T. gondii in a modified direct agglutination assay (MAT) (Dubey and Desmonts, 1988). The mice were killed on day 57 PI and brain squashes of all mice were examined for tissue cysts, irrespective of serologic data (Dubey and Beattie, 1988). Mice were considered negative if they had a negative MAT and no tissue cysts were seen in their brains. Mice were considered positive when T. gondii was demonstrable in tissues.

None of the mice inoculated with the mixture of noninoculated raspberries in HBSS (negative controls) became infected with T. gondii (Table I). All mice inoculated with the mixture of spot-inoculated non-pressure-treated raspberries (positive control) in HBSS became infected (Table I). Treatment of raspberries with 340 MPa (1 MPa = 10 atm = 147 psi) for 60 sec was needed to render oocysts spot inoculated on the raspberries noninfectious for mice (Table I). Treatment of raspberries with 200 MPa or less for 60 sec was not effective in rendering oocysts noninfectious for mice (Table I). Liddle (personal communication) and others (Dubey et al., 1996) have shown that pressures of greater than 200 MPa kill 8-wk-old Trichinella spiralis larvae. Gamble et al. (1998) determined 55 to 60 MPa did not kill all T. spiralis larvae in pork tenderloin or diarrhaphm. Treatment at 200 MPa for 10 min at temperatures between 0 and 15 C kills Anisakis simplex larvae with a lack of motility being used as an indicator of larval death (Molina-Garcia and Sanz, 2002). Dong et al. (2003) found the times and pressures required to kill 100% of A. simplex larvae in king salmon and arrowtooth flounder were 30–60 sec at 441 MPa, 90–180 sec at 276 MPa, and 180 sec at 207 MPa. Rosypal et al. (2007) have demonstrated that unembryonated Ascaris suum eggs fail to develop after exposure to pressures of 241 MPa or more for 60 sec, or in eggs exposed to 276 MPa for 10–30 sec. Shiffk et al. (2000) examined the effects of 550 MPa on Cryptosporidium parvum oocysts in apple juice and orange juice and demonstrated that oocysts survive exposure to 550 MPa for 30 min.
juice. They determined that a 60 sec exposure at 550 MPa was 100% effective in decreasing infectivity of oocysts for cell cultures. The effects of HPP on the infectivity of *C. parvum* oocysts recovered from experimentally exposed HPP-treated oysters for neonatal mice were examined by Collins et al. (2005). They found that a dose of 550 MPa at 180 sec of holding time produced the maximum decrease in infectivity of *C. parvum*-positive mouse pups (93.3%) (Collins et al., 2005). Jordan et al. (2005) examined the effects of HPP treatment of *Encephalitozoon cuniculi* spores on subsequent development in vitro. Spores treated with between 200 and 275 MPa showed reduction of infectivity for host cells and, after treatment of 345 MPa or more, spores were unable to infect host cells (Jordan et al., 2005).

**Toxoplasma gondii** oocysts in HBSS or distilled water treated with HPP for 60 sec at 550 MPa, 480 MPa, 400 MPa, or 340 MPa were rendered noninfectious for mice (Lindsay et al., 2005). No structural alterations occurred in these oocysts. Oocysts in HBSS or distilled water treated for 60 sec using HPP at 100 MPa, 140 MPa, 200 MPa, or 270 MPa for 60 sec were infectious for mice (Lindsay et al., 2005). Results of the present study using *T. gondii* oocysts recovered from HPP-treated raspberries is similar to those reported on oocysts treated in HBSS; alterations occurred in these oocysts.

**Table 1. Protocol and results of high pressure processing treatment on the infectivity of Toxoplasma gondii** oocysts on raspberries for mice.

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>Exposure time (sec)</th>
<th>No. mice inoculated</th>
<th>No. mice positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>60</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>400</td>
<td>60</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>340</td>
<td>60</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>60</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>270</td>
<td>60</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>250</td>
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<td>2</td>
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<td>100</td>
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<tr>
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</tr>
<tr>
<td>Positive control</td>
<td>None</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

This study was financially supported in part by an Animal Health and Disease grant to DSL and GJE. We thank Laura S. Douglas for her help with HPP treatment operations.

**LITERATURE CITED**


Strongyloides stercoralis Hyperinfection in an Immunocompetent Patient with Extreme Eosinophilia

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ABSTRACT: This report examines an unusual case of Strongyloides stercoralis hyperinfection in a 63-year-old man. The patient had a history of vitamin B deficiency, on and off diarrhea, and clinical pellagra for a decade and a half. There was also evidence of extreme eosinophilia. The patient did not have any associated illness suggestive of immunosuppression. Treatment with ivermectin resulted in remarkable clinical improvement and reversion of eosinophil count to normal.

Strongyloides stercoralis is a widespread, soil-transmitted intestinal nematode affecting a hundred million people in 70 countries, most commonly in tropical and subtropical areas (Siddiqui and Berk, 2001; Concha et al., 2005). Internationally, sub-Saharan Africa, southern and southeastern Asia, Central America, and South America, and parts of eastern Europe are considered endemic areas of Strongyloides spp. infection.

Human infection by Strongyloides spp. occurs when filariariform larvae penetrate the intact skin and enter the venous microcirculation via the lymphatics. The organism then travels to the lungs, penetrates the alveoli, and moves up the bronchus, where it is swallowed, moving down the esophagus to the intestine. In the duodenum and upper jejunum, the larvae mature into adult females that produce eggs. These eggs are shed in the stool as rhabditiform larvae. Although most rhabditiform larvae are excreted, some return to being filariform larvae and begin an autoinfective cycle by penetrating colonic mucosa or perianal skin, leading to chronic infection (Concha et al., 2005).

In half of all cases, chronically infected individuals are asymptomatic. In an immunocompetent person, the disease is generally asymptomatic. However, strongyloidiasis has the potential to cause serious life-threatening disease in an immunocompromised patient.

A 63-year-old male patient was admitted to a neurology hospital in Baroda, India, with complaints of acute abdominal pain, diarrhea, headache, fever, chills, dizziness, generalized weakness, loss of appetite, loss of sleep, nausea, and knee pain. The patient had history of gait imbalance and skin discoloration over distal extremities. His Rhomberg’s test was positive.

The patient was originally from Anand, Gujarat, India, where he had lived continuously for >30 yr. The patient was nondiabetic, and he was receiving treatment from a neurophysician for his complaints of diarrhea, pellagra, gait ataxia, and general weakness. The patient was diagnosed to have subacute combined deficiency with recurrent hospitalization from 1999 and 2002 for i.v. vitamin therapy. Again, in 2006, the patient presented with complaints of gait ataxia, pellagra, and diarrhea, for which he was treated with antibiotics and vitamins. He had lost about 5 to 6 kg over the past 5 yr.

Hematological abnormalities included total WBC count 31.3 \( \times 10^9 \) \( \mu l \), with an absolute eosinophil count 24.2 \( \times 10^3/\mu l \), hemoglobin of 10.0 g%, erythrocyte sedimentation rate of 45 mm at 1 hr., and a platelet count 3.66 \( \times 10^5/\mu l \). A peripheral smear revealed hypochromia, macrocytosis, and mild polychromasia.

Other blood measures indicated normal plasma glucose level, normal renal function, and normal serum alanine aminotransferase and aspartate aminotransferase. Electrolyte abnormalities included borderline hypokalemia. An HIV (I and II) antibody test was negative. His earlier reports showed severe vitamin B deficiency.

A stool examination done on the day of admission revealed pus cells and was strongly positive for occult blood. A repeat stool examination was performed the next day. Lugol-stained portions of an unconcentrated stool specimen fixed with formalin immediately after defection showed abundant rhabditiform and filariform larvae of S. stercoralis (Fig. 1). The rhabditiform (Fig. 2) larvae were 220 to 260 \( \mu m \) in length and could be identified by their typical esophageal structure, with a club-shaped anterior portion, a postmedian constriction, and a posterior bulb. The delicate filariform larvae (Fig. 3) measured 540 to 570 \( \mu m \), with the esophagus half the length of the body.

The simultaneous presence of numerous rhabditiform and a few filariform larvae in the unconcentrated stool examination indicated an autoinfective state and hyperinfection. The patient was placed on 12 mg ivermectin per day for 7 days. The eosinophil count started to decline almost immediately and returned to 10% with total white blood cell (WBC) count of 8.2 \( \times 10^3/\mu l \) by seventh day. A repeat stool examination did not show any parasites, even after concentration. The patient showed remarkable improvement clinically over 10 days, and he was then discharged.

Although half of the chronically infected cases of S. stercoralis are asymptomatic, it is important to detect latent infections before administering chemotherapy or before the onset of immunosuppression. Strongyloidiasis is difficult to diagnose because the adult parasite load is low and the larval output is irregular. Results of a single stool examination by use of conventional techniques fail to detect larvae in up to 70% of the cases. Several immunodiagnostic assays have been found ineffective in detecting disseminated infections, but they show extensive cross-reactivity with hookworms, filarial worms, and schistosomes. The present case raises the need to develop specific and sensitive diagnostic test for early diagnosis of S. stercoralis infection even in patients with no clinical signs of immunosuppression, to avoid long suffering and the peril of serious, life-threatening hyperinfection.

In cases of immunosuppression, S. stercoralis can overwhelm its host by extensive tissue invasion, a life-threatening condition known as hyperinfection. Severe infection can occur in which the filariform larvae disseminate widely, leading to fatal sepsis, meningitis, and acute respiratory distress syndrome (Ghoshal et al., 2002). Moreover, some studies have proposed that S. stercoralis infection is a risk factor for biliary tract cancer (Hirata et al., 2007).

In the present study, the combination of acute abdominal pain, diarrhea, headache, fever, chills, dizziness, generalized weakness, loss of appetite, loss of sleep, and nausea, with extreme eosinophilia, was suggestive of worm infestation. The simultaneous presence of rhabditiform and filariform larvae of S. stercoralis was a risk factor for hyperinfection even in patients with no clinical signs of immunosuppression, to avoid long suffering and the peril of serious, life-threatening hyperinfection.

Because the patient was suffering from pellagra for more than a decade, in the absence of any associated illness suggestive of immunosuppression, long-standing vitamin B deficiency looks to be the only predisposing factor for hyperinfection in this case. Some workers have examined the effect of vitamin B deficiency on the immune system. A significant decrease in the absolute number of CD8+ cells, suppressed natural killer cell activity in vitamin B12-deficient patients, and antitumor effects of vitamin B12 have been reported by Tamura et al. (1999). They also suggested that these abnormalities could be partially restored by methyl-B12 treatment.

It is important to note that the patient’s robust allergic immune response against the worm infestation led to marked eosinophilia. The extent of eosinophilia is generally proportional to the intensity of worm infestation. T-helper 2 cytokine interleukin-4 stimulates the production of immunoglobulin E, which on contact with parasite antigen, stimulates mast cell degranulation and goblet cell mucin secretion (Siddiqui and Berk, 2001). The stimulation of gut motility and the release of mucus inhibit worm attachment and tissue invasion. Experimental studies have demonstrated that eosinophils play an important role in protection against S. stercoralis, but the mechanism regulating eosinophils are not known (Mir et al., 2006). Extreme eosinophilia, in the present situation, seems to have occurred to counter the hyperinfection.

In immunocompromised individuals, blood counts performed during hyperinfection may show eosinophilia, but more often they show a suppressed eosinophil count (Keiser and Nutman, 2004). Individuals who have increased peripheral eosinophilia during hyperinfection seem to have a better prognosis (Jamil and Hilton, 1992). High eosinophil count
seems to play an important role in restricting the hyperinfection from wide dissemination, because the person in the present study did not have any respiratory symptoms, and his liver and renal function tests were within normal limits. The patient was successfully treated with the antihelminthic drug ivermectin. Moreover, the eosinophil count steadily returned to normal within 10 days, and the patient improved remarkably well.

The present study suggests the protective role of eosinophils along with the humoral and cellular mechanism in combating the *S. stercoralis* hyperinfection and emphasizes the requirement of more detailed study on the effect of vitamin B deficiency on immune response in relation to tissue nematodes infections.

We thank Dr. K. R. Buch for help in providing clinical history of this patient.

**LITERATURE CITED**


Experimental Toxoplasma gondii Infection in Striped Skunk (Mephitis mephitis)

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ABSTRACT: Twenty-three striped skunks (Mephitis mephitis) without demonstrable antibodies in 1:25 serum dilution in the modified agglutination test (MAT) were fed sporulated Toxoplasma gondii oocysts (9 skunks) or tissue cysts (10 skunks), and 4 skunks (controls) were not fed T. gondii. Skunks were bled before feeding T. gondii, 10 and 23-25 days postinoculation (PI). All 9 seronegative skunks fed oocysts died of acute toxoplasmosis between 7 and 19 days PI; T. gondii tachyzoites were found in histological sections of many tissues. One of the 10 skunks fed tissue cysts and of the 4 controls also died of acute toxoplasmosis days 19 and 20 PI; these animals probably became infected by ingestion of unexcysted oocysts passed in feces of skunks fed oocysts that were housed in the same room that skunks fed tissue cysts were housed. The remaining 9 skunks fed tissue cysts and the 3 controls developed only a mild illness and were killed in good health on days 23-25 PI. Antibodies to T. gondii were not found in 1:25 serum dilution of any of the 19 of 23 skunks that were alive on day 10 PI; 12 of 13 skunks had antibodies (MAT 1:80 or higher) on the day they were killed. Antibodies were not found in 1 skunk. Results indicate that skunks can develop IgG antibodies to T. gondii within 3 wk PI, and primary toxoplasmosis can be fatal in skunks.

Toxoplasma gondii infections are widely prevalent in humans and other animals worldwide (Dubey and Beattie, 1988). The striped skunk (Mephitis mephitis) is an omnivore and widely distributed in North America. The prevalence of T. gondii in omnivores collected in urban areas reflects the presence of oocysts in the environment and tissue cysts in food items for these animals (Mitchell et al., 2004). Antibodies to T. gondii were found in skunks surveyed from Canada and the United States (Schowalter et al., 1980; Dubey et al., 1995; Smith and Frenkel, 1995; Hill et al., 1998; Mitchell et al., 2006; Hwang et al., 2007); however, antibodies to T. gondii were not found in 1:25 serum dilution of any of the 19 of 23 skunks that were alive on day 10 PI; 12 of 13 skunks had antibodies (MAT 1:80 or higher) on the day they were killed. Antibodies were not found in 1 skunk. Results indicate that skunks can develop IgG antibodies to T. gondii within 3 wk PI, and primary toxoplasmosis can be fatal in skunks.

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likely that feces passed into adjoining enclosures and were ingested via contaminated food and water or grooming behavior. One skunk fed tissue cysts died of acute toxoplasmosis, and the lesions were the same as those in animals fed oocysts; this skunk also was probably accidentally contaminated with oocysts. Although housing conditions were not ideal to prevent cross-contamination, there is no doubt that skunks died from acute toxoplasmosis. In retrospect, it would have more informative to use a high and a low dose of oocysts, but there was no prior experiment in skunks to use as a guideline. The number of tissue cysts fed to skunks was unknown, but is likely to have been more than 100,000 bradyzoites, considering that there were numerous large tissue cysts in the brains of mice and a tissue cyst may contain hundreds of bradyzoites (Dubey et al., 1998). Quantification of tissue cysts and bradyzoites in mouse brains is not accurate because size of the tissue cysts and the number of enclosed bradyzoites vary a great deal (Dubey et al., 1998). In the present study, there was no histologic evidence of immunosuppression and viral inclusion bodies, indicating that skunks died of primary toxoplasmosis.

The results of the present study indicate that skunks can develop IgG antibodies within 4 wk PI. None of the skunks had antibodies at 10 day PI, but became seropositive by days 23–25 PI. The MAT detects only IgG antibodies because the mercaptoethanol used in the test destroys IgM-like substances that interfere with the specificity of the test (Dumont and Remington, 1980). In the present study, IgG antibodies were not found 10 days PI in the 6 skunks that died 14–20 days PI, and we did not examine these sera for IgM antibodies. These results should be considered in the diagnosis of acute toxoplasmosis in skunks or other animals.

Bradyzoites and tissue cysts are integral parts in the life cycle of T. gondii (Dubey and Frenkel, 1976). In mice orally inoculated with T. gondii oocysts or tissue cysts, bradyzoites were detected immunohistochromically at 5 days PI in the intestine and at 8 days PI in the brain; these BAG 1 positive organisms were mostly singles until the second week PI (Dubey, 1997; Dubey et al., 1997). Results of the present study in skunks confirm the earlier results obtained with mice. However, unlike mice, more tissue cysts were formed in the myocardium than the brain of skunks.

We would like to thank Drs. O. C. H. Kwok, N. Sundar, and G. V. Velmurugan for their help in this study. This research was supported in part by the Delta Waterfowl Research Foundation.

### Table I. Experimental toxoplasmosis in skunks.

<table>
<thead>
<tr>
<th>Skunk no.</th>
<th>T. gondii stage fed</th>
<th>10 days</th>
<th>23–25 days</th>
<th>Day died or killed*</th>
<th>Histologic observations</th>
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<tbody>
<tr>
<td>4023</td>
<td>Oocysts</td>
<td>&lt;20</td>
<td>NS (Died)</td>
<td>D19</td>
<td>Many tissues†</td>
</tr>
<tr>
<td>7430</td>
<td>Oocysts</td>
<td>&lt;20</td>
<td>NS (Died)</td>
<td>D9</td>
<td>Many tissues</td>
</tr>
<tr>
<td>7865</td>
<td>Oocysts</td>
<td>&lt;20</td>
<td>NS (Died)</td>
<td>D10</td>
<td>Many tissues</td>
</tr>
<tr>
<td>7290</td>
<td>Oocysts</td>
<td>&lt;20</td>
<td>NS (Died)</td>
<td>D14</td>
<td>Many tissues</td>
</tr>
<tr>
<td>3944</td>
<td>Oocysts</td>
<td>Died</td>
<td>NS (Died)</td>
<td>D9</td>
<td>Many tissues</td>
</tr>
<tr>
<td>9043</td>
<td>Oocysts</td>
<td>Died</td>
<td>NS (Died)</td>
<td>D8</td>
<td>Many tissues</td>
</tr>
<tr>
<td>7831</td>
<td>Oocysts</td>
<td>Died</td>
<td>NS (Died)</td>
<td>D8</td>
<td>Many tissues</td>
</tr>
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<td>8071</td>
<td>Oocysts</td>
<td>&lt;20</td>
<td>NS (Died)</td>
<td>D14</td>
<td>Many tissues</td>
</tr>
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<td>&lt;20</td>
<td>K24</td>
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<tr>
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<td>&gt;2,560</td>
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<tr>
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<td>Tissue cysts</td>
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<td>1,280</td>
<td>K23</td>
<td>H(l), T(l), S(l)</td>
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<tr>
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<td>Control</td>
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<td>&lt;20</td>
<td>640</td>
<td>K25</td>
<td>H(l), S(l), T(l)</td>
</tr>
</tbody>
</table>

* B = brain, D = died, H = heart, K = killed, NS = no sample, S = skeletal muscle, T = tongue, I = lesions, t = T. gondii seen.
† Acute toxoplasmosis.

### Table II. Bradyzoite formation in tissues of skunks fed T. gondii.

<table>
<thead>
<tr>
<th>Skunk no.</th>
<th>Day of death*</th>
<th>Brain</th>
<th>Heart</th>
<th>Intestine</th>
<th>Liver</th>
<th>Lung</th>
<th>Muscle</th>
<th>Tongue</th>
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<td>+</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>3944</td>
<td>D9</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>D19</td>
<td>+</td>
<td>+</td>
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<tr>
<td>8375</td>
<td>K24</td>
<td>–</td>
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<tr>
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<td>NS</td>
<td>NS</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* D = died, K = killed.
† Minus sign (−) = bradyzoites not seen in sections; plus sign (+) = bradyzoites seen in sections.
LITERATURE CITED


Prevalence of Antibodies to *Toxoplasma gondii* in Wolverines From Nunavut, Canada

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**ABSTRACT:** The prevalence of antibodies to *Toxoplasma gondii* was determined in blood and tissue exudates recovered from the spleens of 41 wolverines (*Gulo gulo*) collected in Nunavut, Canada, using a modified agglutination test (MAT). Antibodies to *T. gondii* were found in 17 (41.5%) of the 41 wolverines with MAT titers of 1:25 in 1, 1:50 in 4, 1:100 in 5, 1:200 in 6, and 1:400 in 1. This is the first report of antibodies to *T. gondii* in wolverines, and the results indicate that exposure is common.

Infection with *Toxoplasma gondii* is common in humans, domestic animals, and wildlife worldwide, including Canada (Dubey and Beattie, 1988; Philippa et al., 2004). The wolverine (*Gulo gulo*) is the largest member of the weasel family and is found in circumpolar regions throughout North America and Eurasia. We report the prevalence of antibodies to *T. gondii* in wolverines for the first time from this host.

Carcasses of 41 wolverines were collected from hunters and trappers in Kugluktuk, Nunavut, Canada, from November 2006 until April 2007 (Fig. 1). Sex, approximate age (juvenile or adult), waypoint coordinates, and the straight-line distance from the tree line to where each wolverine was harvested were recorded. All carcasses were frozen until May 2007 when a partial necropsy was performed. Spleen and other tissues were collected and frozen, at −20 °C or with dry ice, until they were shipped to Oklahoma State University (OSU; Stillwater, Oklahoma) in August 2007. At OSU, blood and tissue exudates were collected from the spleen of each wolverine and frozen. The samples were then shipped to the Animal Parasitic Diseases Laboratory in Beltsville, Maryland, where serology was performed using 2-fold dilutions from 1:25 to 1:400 with the modified agglutination test (MAT) described by Dubey and Desmonds (1987). Even though there is no information on the sensitivity and specificity of the MAT for detecting antibodies to *T. gondii* in wolverines, based on previous studies (Dubey et al., 1995; Dubey, 1997) a titer of 1:25 was considered indicative for *T. gondii* exposure. Chi-square tests (Sokal and Rohlf, 1997) were used to compare the prevalence of *T. gondii* antibodies between sex and age classes of wolverines. The Mann-Whitney *U*-test (Sokal and Rohlf, 1997) was used to compare the distances to the tree line of wolverines that tested positive for

![Map of Nunavut with sample locations](image)

**FIGURE 1.** Location in Nunavut where wolverines were collected. Sample designations in large font (e.g., KU-13) indicate wolverines that tested positive for antibodies to *T. gondii*. Sample designations in small font (e.g., KU-14) tested negative.
Antibodies to T. gondii were found in 17 (41.5%) of the 41 of the wolverines with titers ranging from 1:25 to 1:400 (Table I). Prevalence of T. gondii exposure (Table I) did not differ significantly between sex and age of wolverines (χ² = 1.284, df = 1, P = 0.257 and χ² = 0.451, df = 1, P = 0.502, respectively). In a previous study, Philippa et al. (2004) did not detect antibodies to T. gondii in the sera from 20 wolverines collected from British Columbia; however, the results of the present study suggest that exposure to T. gondii is common for wolverines in the Kitikmeot region of Nunavut. It is unclear why the results differ between the 2 studies.

Toxoplasma gondii infections may occur through ingestion of sporulated oocysts. Although cougars (Puma concolor) are occasionally observed in the Northwest Territories (Gau et al., 2001), Canadian lynx (Lynx canadensis) are likely the only wild felid that would overlap the range of the 41 wolverines in the present study and most likely responsible for shedding oocysts into the environment (Labelle et al., 2001). Studies conducted on Canadian lynx reported 44.0% of 106 from Quebec (Zarnke et al., 2004) had antibodies to T. gondii. Canadian lynx generally prefer to stay below the tree line but are occasionally observed and harvested by Kugluktuk hunters and trappers.

Wolverines could also become infected with T. gondii through ingestion of bradyzoites encysted within the tissues of prey. While wolverines are generalists with regard to feeding, scavenging on large mammal carrion (especially ungulates) has been shown to determine wolverine distribution, survival, and reproductive success (Banci, 1994). Antibodies to T. gondii have been found in several wild ungulates of the Arctic and sub-Arctic of North America, including caribou (Rangifer tarandus) from Alaska (Zarnke et al., 2000), the Northwest Territories and Nunavut (Kutz et al., 2001), and Quebec (McDonald et al., 1990); bison (Bison bison) from Alaska (Zarnke et al., 2000); Dall sheep (Ovis dalli) from Alaska (Zarnke et al., 2000); and muskoxen (Ovibos moschatus) in the Northwest Territories (Kutz et al., 2000). Virus T. gondii was isolated from cougars in British Columbia (Aramini et al., 1998; Dubey et al., 2008), raccoons (Procyon lotor), a striped skunk (Mephitis mephitis), and domestic cats (Felis domesticus) from Maniota, and a black bear (Ursus americanus) from Kuujjuaq, Canada (Dubey et al., 2008).

Thirty-eight (92.7%) of the 41 of the wolverines in the present study were harvested above the tree line (Fig. 1). The average distance (m) ± SE from the tree line for wolverines with antibodies to T. gondii was 60 ± 15 compared to 90 ± 15 for those without antibodies, although a significant difference was not detected (T = 295.0, P = 0.104). Home ranges of wolverines in North America vary from 100 to 900 km² and are dependant on sex of the animal and the abundance and distribution of preferred prey (Banci, 1992). Adult male wolverines from the BC interior (McDonald et al., 2001). Wolverines from the Kitikmeot region of Nunavut was harvested approximately 205 km from the tree line. Although the distance from the tree line to where KU-25 was harvested was one of the greatest in the present study, it was well within the maximum displacement distance of 326 km previously documented for wolverines in the present study area (Mulders, 2001). Based on their dispersal characteristics, large home range sizes, and proximity to the tree line, it is conceivable that wolverines encounter T. gondii in their environment. Close proximity to the tree line has been shown to be an important variable for exposure to T. gondii in other Arctic mammals. For example, the seroprevalence of T. gondii antibodies was lower for island populations (farther from the tree line) of both caribou (Kutz et al., 2001) and muskoxen (Kutz et al., 2000) than for mainland populations.

Even though the prevalence of antibodies to T. gondii in wolverines from Nunavut was relatively high, clinical disease in this mustelid is unknown. Additionally, since harvesting wolverines (and other fur-bearing animals) has traditional and economic importance to native Inuit communities and that skinning of animals for fur has been identified as a significant risk factor for T. gondii exposure (McDonald et al., 1990), studies examining the occurrence and prevalence of live bradyzoites in wolverine tissues will be required to determine public health risks.

The present study was funded in part by the Center for Veterinary Health Science, OSU, and the Department of Environment, Government of Nunavut. We thank the Kugluktuk Hunters’ and Trappers’ Organizational support and participation with the wolverine carcass collection program, O. C. H. Kwok for running the serologic tests, and Jennifer Jane Garrett for reviewing an earlier version of the present paper.

LITERATURE CITED


Occurrence of Neospora caninum Antibodies in Capybaras (Hydrochaeris hydrochaeris) From São Paulo State, Brazil

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ABSTRACT: Capybara (Hydrochaeris hydrochaeris) is a large rodent distributed throughout tropical America. Antibodies to Neospora caninum in 213 feral capybaras from 11 counties of the State of São Paulo, Brazil, were assessed using the indirect immunofluorescent antibody test (titer ≥1:25) and found in 20 (9.4%), with titers of 1:25 in 4, 1:50 in 7, and 1:100 in 9 animals. This is the first report of occurrence of N. caninum antibodies in capybaras.

Neospora caninum is one of the most important causes of abortion in dairy cattle worldwide (Dubey, 2003). Cattle become infected transplacentally or by the ingestion of oocysts that have been shed by dogs and coyotes (McCullister et al., 1998; Gondim et al., 2004). Although antibodies to N. caninum have been reported in a variety of domestic and wild animals, infected animals that serve as sources of N. caninum infection for dogs and coyotes are unknown (Dubey et al., 2007). Rodents and other small mammals are considered as a potential source of N. caninum infection for dogs and coyotes. Neospora caninum antibodies have been reported in rats (Rattus spp.) and mouse mice (Mus musculus) from Grenada, West Indies, and the United States (Jenkins et al., 2007). Neospora caninum DNA was found in tissues of rats and/ or mice from Italy (Ferroglio et al., 2007), the United Kingdom (Hughes et al., 2006), Taiwan (Huang et al., 2004), and Grenada, West Indies (Jenkins et al., 2007).

Capybara (Hydrochaeris hydrochaeris) is a large herbivorous rodent distributed widely in tropical America, and its meat is consumed by humans in many countries. We previously reported seroprevalence of antibodies to Toxoplasma gondii in 149 capybaras from Brazil (Cañón-Franco et al., 2003). The objective of the present paper is to report seroprevalence of N. caninum in capybaras, the first time from this host.

In total, sera of 213 capybaras from 11 counties were tested (Table I). Of these, 149 sera were those previously tested for T. gondii antibodies, and 64 were additional animals. Blood samples were collected from jugular or brachial veins, stored for 6 to 8 hr at approximately 4 C, and centrifuged: sera were stored at -20 C. The indirect immunofluorescent antibody test (IFAT) was used to detect antibodies to N. caninum with a cutoff value of 1:25 (Camargo, 1974; Dubey et al., 1988). The positive sera were serially diluted 2-fold up to the maximum titer for the reaction. Anti-capybara conjugate prepared in sheep and labeled with fluorescein isothiocyanate was used in a 1:200 dilution after standardization as previously reported (Cañón-Franco et al., 2003). Positive and negative sera were obtained by screening the sera using IFAT at the dilution of 1:16, 1:32, and 1:64. A serum titer of ≥64 was considered positive and a titer of ≥16 as negative. Positive and negative controls were added to each slide.

Antibodies to N. caninum were detected in 20 (9.4%) of 213 capybaras, with titers of 1:25 in 4 (1.9%), 1:50 in 7 (3.3%), and 1:100 in 9 (4.2%). Seropositive capybaras were detected in 7 of the 11 counties, with an occurrence that ranged from 0.0% in Andradina, Paulínia, and São João da Boa Vista, and Valparaiso, to 30.8% in Ribeirão Preto (Table I). Results of the present study suggest that infected capybaras may be a source of N. caninum infection for wild canids in Brazil (Cañón-Franco et al., 2003).

Seroprevalence for N. caninum was much lower (9.4%) compared with 69% seropositivity for T. gondii in capybaras from the same state (Cañón-Franco et al., 2003), indicating specificity of the tests used. This is the first report of the presence of antibodies to N. caninum in capybaras.

The authors thank FAPESP for the fellowship to A. M. A. Ragozo and CNPq for the fellowship to S. M. Gennari. We also thank Zoonosis Control Center, Prefeitura do Município de São Paulo, for providing capybara conjugate.

LITERATURE CITED


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TABLE I. Seroprevalence against N. caninum (IFAT ≥ 25) in capybaras from different counties in Sao Paulo State, Brazil.

<table>
<thead>
<tr>
<th>County</th>
<th>Samples</th>
<th>Positive</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Andradina</td>
<td>10</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Campinas</td>
<td>61</td>
<td>7</td>
<td>11.5</td>
</tr>
<tr>
<td>Cordeirópolis</td>
<td>9</td>
<td>2</td>
<td>22.2</td>
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<tr>
<td>Cosmorama</td>
<td>15</td>
<td>1</td>
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<td>Monte Alegre do Sul</td>
<td>32</td>
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</tr>
<tr>
<td>Paulínia</td>
<td>68</td>
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<td>0.0</td>
</tr>
<tr>
<td>Pirassununga</td>
<td>17</td>
<td>1</td>
<td>5.9</td>
</tr>
<tr>
<td>Ribeirão Preto</td>
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<td>4</td>
<td>30.8</td>
</tr>
<tr>
<td>São João da Boa Vista</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>São Paulo</td>
<td>40</td>
<td>4</td>
<td>10.0</td>
</tr>
<tr>
<td>Valparaiso</td>
<td>7</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>213</td>
<td>20</td>
<td>9.4</td>
</tr>
</tbody>
</table>
Suppression of Th2 Cytokines Reduces Tick-Transmitted *Borrelia burgdorferi* Load in Mice

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**ABSTRACT:** Previous work has indicated that both *Borrelia burgdorferi* and the process of tick feeding (saliva) modulate the host immune response. Molecules have been identified in tick saliva that effect T cell proliferation by binding to specific cytokines, thereby promoting a Th2 cytokine response that does not afford protection against tick-transmitted *B. burgdorferi* in mice. Moreover, reconstitution of a Th1-biased T cell response prior to spirochete challenge effectively neutralizes tick modulation of host immunity and affords protection against tick transmission of spirochetes. The current studies were undertaken to determine the effect of neutralizing specific Th2 cytokines prior to tick feeding and subsequent transmission of *B. burgdorferi*. The results indicate that suppression of both IL-4 and IL-5 prior to the feeding of *B. burgdorferi*-infected ticks significantly decreased spirochete load in target organs such as joint, bladder, heart, and skin of the Lyme disease-susceptible host.

Lyme disease, caused by the bacterium *Borrelia burgdorferi*, is the most common vector-borne disease reported in the United States (CDC, 2002). *Borrelia burgdorferi* is transmitted by 2 Ixodid tick species, *Ixodes scapularis* in the northeastern and midwestern states, and *Ixodes pacificus* in the western United States (Pierson and Sinsky, 1988; Clover and Lane, 1995). Disease presentation ranges from an acute dermatological lesion, erythema migrans, to development of chronic disease manifested as arthritis, myocarditis, and lesions of the nervous system (Steere, 1989; Dennis, 1995). Ixodid tick feeding and concomitant injection of tick saliva induces numerous physiological changes, both locally and systemically, within the mammalian host (Ribeiro, 1987). Tick saliva alters vascular flow and permeability, induces a local anesthetic reaction, and promotes blood pooling due to injected prostaglandins and anticoagulant factors (Ribeiro et al., 1985). More importantly, factors in tick saliva modulate both the innate immune system and specific acquired responses to heterologous antigens (Wikel, 1999).

Previous studies have documented that tick feeding promotes an inappropriate Th2-mediated response while actively suppressing cellular immunity (Brossard and Wikel, 1997; Schoeler et al., 1999), which occurs to a greater extent in the Lyme disease-susceptible host (Zeidner et al., 1997). Previous work has also demonstrated that I. scapularis tick feeding synergizes with *B. burgdorferi* to suppress Th1 immune responses while promoting humoral immunity (Zeidner et al., 1997). Reconstitution of Th1-associated cytokines at the time of tick feeding induced resistance to subsequent tick-transmitted infection (Zeidner et al., 1996). Since Th1-associated cytokines are associated with anti-tick immunity, as well as immunity to tick-transmitted *B. burgdorferi* infection, the present studies were conducted to determine whether anticytokine treatment could effectively control spirochete transmission in the susceptible murine host.

For cytokine neutralization studies, 6 C3H/HeJ mice received 3 intraperitoneal injections of 1 mg of purified antimurine IL-4 antibody (clone 11B11) (ATCC, Manassas, Virginia) or 1 of an IgG2a isotype control antibody (Invitrogen, Carlsbad, California) in 100 µl of PBS at days -7, -3, and 0 (the day of challenge) with 5 infected nymphal ticks (CDC, Fort Collins, Colorado). Fourteen days after ticks were fed to repletion, mice were necropsied and tissues were harvested for measurement of spirochete load. This experiment was repeated a second time (n = 12 mice total). Similarly, IL-5 knockout mice (n = 5) (C57BL/6;IL-5<sup>−/−</sup>, Jackson Laboratories, Bar Harbor, Maine) were challenged with 5 B31-infected nymphal ticks (CDC, Fort Collins, Colorado) and each tissues were harvested for DNA analysis 14 days after ticks fed to repletion. These mice produce no IL-5 and demonstrate no defects in cytotoxic T cell responses (Kopf et al., 1996). Wild-type C57BL/6 mice challenged with infected ticks served as controls. Significant differences in the mean numbers of spirochetes per mg of tissue (skin, bladder, heart, and joint) were determined by Student’s t-test, and
The role of saliva in tick-borne disease transmission has been the subject of considerable research. Salivary gland extracts have been shown to inhibit killing of the pathogen, Anaplasma phagocytophila, feeding. This indicates a statistically significant difference in the mammalian host response to tick saliva, which appears inappropriate in terms of generating the immunity required for subsequent containment of tick-transmitted bacterial diseases like Borrelia burgdorferi. The mammalian host response to tick saliva is strongly polarized towards an inappropriate and nonprotective response, and awareness that inhibiting the host cellular response noted here has been altered is vital to those considering the development of alternative anti-tick vaccines used to protect against tick-borne diseases. Further studies are needed to determine the specific factors in tick saliva that promote strong polarization of the mammalian host immune response toward an inappropriate and nonprotective response, and awareness that inhibiting the host cellular response noted here has been altered is vital to those considering the development of alternative anti-tick vaccines used to protect against tick-borne diseases.

**LITERATURE CITED**


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SYSTEMATICS-PHYLOGENETICS
A Review of Species in the Genus Rhopalias (Rudolphi, 1819). TERRY R. HAVENKOST and SCOTT L. GARDNER .................................................. 716
A New Species of Eimeria (Apicomplexa: Eimeriidae) From the Marbled Salamander, Ambystoma opacum (Caudata: Ambystomatidae), From Northern Louisiana. CHRIS T. MCALLISTER and STEVE J. UPTON ........................................... 727
Molecular Characteristics of Camallanus Spp. (Spirurida: Camallanidae) in Fishes From China Based on ITS rDNA Sequences. SHAN GONG WU, GUI TANG WANG, BING WEN XI, DIAN GAO, and PIN NIE ................................................................. 731
Description of a New Species of Myxobolus (Myxozoa: Myxobolidae) Based on Morphological and Molecular Data. YUANJUN ZHAO, CHUNYAN SUN, MICHAEL L. KENT, JUNLIN DENG, and CHRISTOPHER M. WHIPPS ........................................... 737

THERAPEUTICS-DIAGNOSTICS
Inhibition by Dications of In Vitro Growth of Leishmania major and Leishmania tropica: Causative Agents of Old World Cutaneous Leishmaniasis. ALEXA C. ROSYPAL, KARL A. WERBOVETZ, MANAR SALEH, CHAD E. STEPHENS, ARVIND KUMAR, DAVID W. BOYKIN, JAMES E. HALL, and RICHARD R. TIDWELL ........................................... 743

RESEARCH NOTES
Toxoplasma gondii and Neospora caninum Antibodies in Dogs From Grenada, West Indies. J. P. DUBEY, D. STONE, O. C. H. KWOK, and R. N. SHARMA .......................................................... 750
Helminths Collected From Imported Pet Murids, with Special Reference to Concomitant Infection of the Golden Hamsters with Three Pinworm Species of the Genus Syphacia (Nematoda: Oxyuridae). HIDEO HASEGAWA, HIROSHI SATO, ERI IWAKIRI, YATSUKAHO IKEDA, and YUMI UNE .......................................................... 752
Effects of High Pressure Processing on Toxoplasma gondii Oocysts on Raspberries. DAVID S. LINDSAY, DANIEL HOLLMAN, GEORGE J. FLICK, DAVID G. GOODWIN, SHEILA M. MITCHELL, and J. P. DUBEY .......................................................... 757
Strongyloides stercoralis Hyperinfection in an Immunocompetent Patient With Extreme Eosinophilia. A. MARATHE and V. DATE .......................................................... 759
Experimental Toxoplasma gondii Infection in Striped Skunk (Mephitis mephitis). TRAVIS QUIRK and J. P. DUBEY .......................................................... 761
Prevalence of Antibodies to Toxoplasma gondii in Wolverines From Nunavut, Canada. MASON V. REICHHARD, LUIGI TORRETTI, JASON M. GARVÓN, and J. P. DUBEY .......................................................... 764
Suppression of Th2 Cytokines Reduces Tick-Transmitted Borrelia burgdorferi Load in Mice. NORDIN S. SEIDNER, BRADLEY S. SCHNEIDER, JEREMIAH S. RUTHERFORD, and MARC C. DOLAN .......................................................... 767

COVER CAPTION: Rhopalias coronatus. Entire specimen. Scale bar = 0.1 mm. Figure 1 from Haverkost and Gardner 94: 716-726.