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LARVAL DIGENEAN COMMUNITY PARASITIZING THE FRESHWATER SNAIL, CHILINA DOMBEYANA (PULMONATA: CHILINIDAE) IN PATAGONIA, ARGENTINA, WITH SPECIAL REFERENCE TO THE NOTOCOTYLID CATATROPIS CHILINAE

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ABSTRACT: The objective of this research was to describe the structure of larval digenean communities in the freshwater snail Chilina dombeyana in Lake Mascardi, an oligotrophic Andean Patagonian lake. In total, 1,923 snails were collected during 2 sampling periods. Specimens were examined, and 8 digenean species were identified, all with allogenic life cycles. The snail infracomunities nearly always occurred as single-species infections, distributed mainly in the hepatopancreas. The double infections (0.2%) were always prepatent, and involved a schistosome and the notocotyloid Catatropis chilinae. The overall prevalence, the prevalence of C. chilinae, and total species prevalence (without C. chilinae) significantly increased with snail size. Catatropis chilinae was the dominant species in all months and across all snail size classes. Maximum richness (6) was reached in the size class between 16 and 18 mm. Diversity indices, i.e., the Shannon-Wiener Index and the reciprocal of the Simpson Index, increased with snail size only during the second sampling period. No robust evidence of antagonistic interactions was found in the digenean community of C. dombeyana from Lake Mascardi.

Parasite communities in snails are affected by numerous internal and external host factors (Esch et al., 2001). The former can include patterns of recruitment and establishment of the parasites, interactions between parasites species, and behavior, physiology, genetics, and life span of the host (Esch and Fernandez, 1994; Esch et al., 2001). External factors would embrace the behavior of secondary, or definitive, hosts, and seasonal variations of temperature and photoperiod at micro- or macro-scales (Esch and Fernandez, 1994; Esch et al., 2001). Infracommunities of parasites in snails are mainly shaped by interspecific interactions among species (Kuris, 1990; Lafferty et al., 1994), biological features of the definitive hosts, and external factors such as habitat and temporal heterogeneity among snails (Souza, 1990; Fernandez and Esch, 1991a, 1991b; Williams and Esch, 1991; Snyder and Esch, 1993; Sousa, 1993; Sapp and Esch, 1994). At the component community level, the main structuring factors would include variation in the life history of the snail species (Williams and Esch, 1991; Snyder and Esch, 1993), spatial and temporal heterogeneity (Fernandez and Esch, 1991b; Esch and Fernandez, 1994; Sapp and Esch, 1994; Esch et al., 1997), and the distribution of definitive hosts (Esch et al., 1997; Smith, 2001).

Knowledge of digenean ecology in freshwater snails from large, deep oligotrophic lakes is scarce (Keas and Blankespoor, 1997; Väyrynen et al., 2000), particularly in South America, where only 1 seasonal study on the digenean community of Chilina dombeyana in a small, shallow Chilean lake has been done (Olmos and George-Nascimento, 1997). Until now, only taxonomic studies have been attempted for parasites of this snail species in Argentina. Cercariae of Diplometodidae, Strigeidae, Echinostomatidae, and Notocotylidae and their distribution (40°10′–43°15′S, 71°03′–71°48′W) in lakes from the east side of the Andes have been examined (Ostrowski de Núñez and Quaggiotto, 1995; Quaggiotto and Valverde, 1995; Flores and Brugni, 2003).

The objective of the present work was to describe the structure of larval digenean communities in the freshwater snail C. dombeyana in a large, deep Andean Patagonian lake at both the infracomunity and component community levels.

MATERIALS AND METHODS

The study was carried out in Lake Mascardi (41°17′S, 71°38′W), which is located in the Nahuel Huapi National Park, at 796 m above sea level. This glacial lake is oligotrophic, has a surface area of 38 km², and a maximum depth of 218 m. The aquatic vegetation (Schaenoplectus californicus, Potamogeton linguatus, and Myriophyllum quitense) is distributed in patches, and the lake is surrounded by a cold temperate forest dominated by Nothofagus spp. and Austracodrues chilensis. Chilina dombeyana is a pulmonate snail of the monotypic South American Chilinidae (Martín, 2003). Knowledge regarding the taxonomy, ecology, and genetics of this species is scarce, although it is the most representative gastropod species in the Andean Patagonia (Castellanos and Miquel, 1991). It can be found in almost all lakes and rivers (Rumi et al., 2006), living on rocks and aquatic vegetation, and grazing mainly on diatoms (Castellanos and Galillard, 1981; Díaz Vilanueva et al., 2004).

Snails were collected monthly in 2 sampling periods, the first from December 2000 to May 2001, and the second from November 2001 to April 2002 (Table I). The water surface temperature varied between 10 C (May 2001) and 19 C (January–February 2002). Snails were visually located and hand collected along the lake’s edge to a maximum depth of 75 cm. In the laboratory, specimens were held in isolation for 48 hr in individual containers filled with tap water, to detect shedding of cercariae (patent infections). All specimens were then dissected and examined for the presence and location of intramolluscan larval stages (in both prepatent and patent infections). Snail size was measured by shell height.

To describe the recruitment period of Catatropis chilinae, the prepatent infections during the second sampling period were classified in the following stages: (A) with few mother rediae (up to 10), containing daughter rediae or a low number of immature cercariae, distributed around the digestive tube or partially invading the hepatopancreas; (B) with many to countless rediae, containing numerous immature cercariae, and partially or wholly invading the hepatopancreas; and (C) with countless rediae containing many fully developed cercariae, and totally distributed in the hepatopancreas.

The following indices were used to characterize the larval community in C. dombeyana: (1) overall prevalence of infections = the number of parasitized snails/the number of collected snails × 100; (2) prevalence = the number of snails parasitized by 1 species/the number of collected snails × 100; (3) total species prevalence (except C. chilinae) = the number of snails parasitized by any species/the number of collected snails × 100; (4) species richness = the total number of species in a sample; and (5) species dominance, i.e., (pi) = the number of snails parasitized by a particular species/the number of parasitized snails × 100. Two diversity indices were calculated: the exponential of the Shannon-Wiener Index and the reciprocal of the Simpson Index.

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TABLE I. Collection date, sample size, and snail length of Chilina dombeyana from Lake Mascardi.

<table>
<thead>
<tr>
<th>Collection date</th>
<th>Sample size (n)</th>
<th>Range</th>
<th>Snail length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>December 2000</td>
<td>281</td>
<td>5.6</td>
<td>Min 28.64 Max 12.0</td>
</tr>
<tr>
<td>January 2001</td>
<td>152</td>
<td>3.9</td>
<td>Min 26.05 Max 10.7</td>
</tr>
<tr>
<td>February 2001</td>
<td>242</td>
<td>5.89</td>
<td>Min 35.12 Max 13.8</td>
</tr>
<tr>
<td>April 2001</td>
<td>194</td>
<td>8.75</td>
<td>Min 24.34 Max 14.8</td>
</tr>
<tr>
<td>May 2001</td>
<td>182</td>
<td>8.35</td>
<td>Min 32.18 Max 15.1</td>
</tr>
<tr>
<td>November 2001</td>
<td>76</td>
<td>10.06</td>
<td>Min 26.19 Max 15.6</td>
</tr>
<tr>
<td>December 2001</td>
<td>77</td>
<td>8.95</td>
<td>Min 17.95 Max 13.0</td>
</tr>
<tr>
<td>January 2002</td>
<td>164</td>
<td>9.17</td>
<td>Min 19.8 Max 13.5</td>
</tr>
<tr>
<td>February 2002</td>
<td>127</td>
<td>8.8</td>
<td>Min 24.5 Max 15.9</td>
</tr>
<tr>
<td>March 2002</td>
<td>130</td>
<td>10.13</td>
<td>Min 23.73 Max 15.5</td>
</tr>
<tr>
<td>April 2002</td>
<td>85</td>
<td>11.74</td>
<td>Min 25.5 Max 16.1</td>
</tr>
</tbody>
</table>

Shannon-Wiener Index: \( H' = \exp (-\sum p_i \ln p_i) \); and the reciprocal of the Simpson Index: \( 1/\text{Simpson} = 1/2 \pi^2 p_i^2 \) (Fernandez and Esch, 1991b).

A Mann-Whitney U-test was used to compare the mean snail size between sampling periods. A chi-square test for differences in probabilities was used to analyze differences in overall prevalence, prevalence of C. chilinae, and total species prevalence between sampling periods, and between months. A Spearman rank correlation test was used to examine covariation between snail size versus overall prevalence, prevalence, and community indices (Conover, 1980). P-values <0.05 were considered significant.

RESULTS

In total, 1,923 snails were captured (Table I). During the first sampling period, the average snail size was 13.5 ± 4.3 mm (3.9–35.1 mm) and, for the second, 15.2 ± 3.1 mm (8.8–26.2 mm). Snails from the second sampling period were significantly larger (\( U = 279,626.5; P < 0.05 \)).

Four cercariae types (monostome, furcocercous, echinostome, and xiphidiocercaria) were observed, representing 8 digenean species (Table II); all had allogenic life cycles. Five of the 8 species recorded, i.e., C. chilinae, Apatemon sp., Tylodelphys sp., schistosome, and echinocercaria III, were found in both sampling periods (Table III). The remaining species were recorded only in 1 sampling period (Table III).

The infracommunities of C. dombeyana are composed of 2 guilds, i.e., those in the hepatopancreas, and those of the albumin gland (Table II). The hepatopancreas is inhabited by C. chilinae, Apatemon sp., a schistosome, and an echinostome species, and represented 94.5% of the total infections. The albumin gland is parasitized by Tylodelphys sp. and a plagiorchid.

The overall prevalence during the first sampling period was 13.9% (3% patent infections) and 59.2% (12.4% patent infections) during the second. In May 2001, only prepatent infections were observed. The prevalence and the percentage of patent infections for each species in both sampling periods are shown in Table III. Significant differences were detected between sampling periods for overall prevalence (\( \chi^2 = 20.6; P < 0.05 \)), the prevalence of C. chilinae (\( \chi^2 = 20.6; P < 0.05 \)), and total spe-

Table II. Type of cercariae, taxon, intramolluscan stage, site of infection, and infective stage of digeneans occurring in Chilina dombeyana from Lake Mascardi.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Intramolluscan stage</th>
<th>Site of infection</th>
<th>Infective stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furcocercous cercariae</td>
<td>Strigeidaceae</td>
<td>sporocyst</td>
<td>hepatopancreas</td>
</tr>
<tr>
<td>Apatemon sp.</td>
<td>Diplostomidiaceae</td>
<td>sporocyst</td>
<td>albumin gland</td>
</tr>
<tr>
<td>Tylodelphys sp.</td>
<td>Schistosomatidaceae</td>
<td>sporocyst</td>
<td>hepatopancreas</td>
</tr>
<tr>
<td>Schistosome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xiphidiocercaria cercaria</td>
<td>Plagiocirhidaceae</td>
<td>sporocyst</td>
<td>albumin gland</td>
</tr>
<tr>
<td>Plagiocirhidaceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echinostome cercariae</td>
<td>Echinostomatidaceae</td>
<td>redia</td>
<td>hepatopancreas</td>
</tr>
<tr>
<td>Echinocercaria I</td>
<td>Echinostomatidaceae</td>
<td>redia</td>
<td>hepatopancreas</td>
</tr>
<tr>
<td>Echinocercaria II</td>
<td>Echinostomatidaceae</td>
<td>redia</td>
<td>hepatopancreas</td>
</tr>
<tr>
<td>Echinocercaria III</td>
<td>Echinostomatidaceae</td>
<td>redia</td>
<td>hepatopancreas</td>
</tr>
<tr>
<td>Echinocercaria IV</td>
<td>Echinostomatidaceae</td>
<td>redia</td>
<td>hepatopancreas</td>
</tr>
<tr>
<td>Monostome cercariae</td>
<td>Notocotylidaceae</td>
<td>redia</td>
<td>hepatopancreas</td>
</tr>
</tbody>
</table>

Table III. Prevalence and percentage of patent infections for each parasite species in both sampling periods occurring in Chilina dombeyana from Lake Mascardi.

<table>
<thead>
<tr>
<th>Species</th>
<th>Prevalence infections</th>
<th>% patent infections</th>
<th>Prevalence infections</th>
<th>% patent infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apatemon sp.</td>
<td>1.3</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Tylodelphys sp.</td>
<td>1.1</td>
<td>0.4</td>
<td>2.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Schistosome</td>
<td>0.6</td>
<td>0.2</td>
<td>2.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Plagiocirhidae</td>
<td>—</td>
<td>—</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Echinocercaria I</td>
<td>—</td>
<td>—</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Echinocercaria III</td>
<td>—</td>
<td>—</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Echinocercaria IV</td>
<td>0.1</td>
<td>0.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Catatropis chilinae</td>
<td>10.6</td>
<td>1.1</td>
<td>53.4</td>
<td>7.7</td>
</tr>
</tbody>
</table>
cies prevalence ($\chi^2 = 2.8; P < 0.05$). Significant monthly variation was observed in overall prevalence ($\chi^2 = 514.1; P < 0.05$) during both sampling periods. The highest values occurred in November 2001 and March–April 2002, when water temperature reached 12 °C (Fig. 1A). *Catatropis chilinae* exhibited significant monthly variation in prevalence ($\chi^2 = 506.8; P < 0.05$), but no significant monthly variation was observed in total species prevalence ($\chi^2 = 17.2; P < 0.05$). *Apatemon* sp., *Tylodelphys* sp., the schistosome, and the plagiorchiid exhibited different seasonal prevalence patterns, with no trends in either sampling period (Fig. 1C–F). *Catatropis chilinae* was the only species with patent infections during all sampling months, and generally displayed an increase of prevalence from summer to autumn (Fig. 1B). All infection stages were found during the second sampling period. Stage A exhibited a maximum percentage of infection in De-

**FIGURE 1.** Monthly overall prevalence and prevalence of each digenean species of *Chilina dombeyana* from Lake Mascardi in both sampling periods (different Y-axis scales are used).
December 2001 and March 2002. The percentage of infection in stage B decreased from November 2001 to April 2002. The percentage of infection in stage C increased from November 2001 to February 2002 and then decreased (Fig. 2A). Prepatent infections of *Catatropis chilinae* were recorded in snails as small as 10 mm. Stages B and C were found across all snail sizes. Stage A was observed in snails from 10 to 20 mm, but decreased as snail size increased (Fig. 2B).

In both sampling periods, *Chitina dombeyana* became infected when individuals reached 8 mm in size, being first parasitized by *Apatemon* sp. (0.6%) and *Catatropis chilinae* (1.1%). The lowest overall prevalence occurred in snails between 8 and 10 mm in
size (1.2% in the first sampling period; 12.5% in the second sampling period). The highest values were recorded in snails between 22 and 24 mm in size (68.2% in the first sampling period; 93.3% in the second sampling period) (Fig. 3A). Maximum prevalence for each species was recorded at different snail sizes (Fig. 3B–F), e.g., C. chilinae at 22–24 mm (Fig. 3B), and schistosome at 24–26 mm (Fig. 3E). The overall prevalence, the total species prevalence, and the prevalence of C. chilinae were positively correlated with snail size in both sampling periods (Table IV).

No patent double infections were found. Double prepatent infections were observed in only 4 snails (0.2%), with a mean length of 15.1 mm (range 14.2–17.3 mm). These infections occurred in May 2001 (consisting of a sporocyst of an unknown species and rediae of C. chilinae), in December 2001 (sporocysts of a schistosome and rediae of C. chilinae), and in March 2002 (sporocysts of a schistosome and rediae of C. chilinae). In all these double prepatent infections, sporocysts occupied the entire hepatopancreas, and only Stage A of C. chilinae was present.

Catatropis chilinae was the dominant species, occurring with the highest richness value (6) in April 2001. Both diversity indices showed the same trend, with lower values during the second sampling period (Table V).

Catatropis chilinae was the dominant species for all size classes. Species richness showed the same pattern in both sampling periods, increasing gradually with snail size and reaching a maximum (6) in the 16–18 mm class, then declining slightly (3–5) in larger snails (Table VI). The 2 diversity indices showed the same pattern, increasing with snail size during the second sampling period (Table VI). There was a significant correlation between richness and dominance in the first sampling period; and between the diversity indices in the second (Table VII).

**DISCUSSION**

In Lake Mascardi, 28 different species combinations are possible from the 8 recorded species, but only 1 (between sporocysts of schistosome and rediae of C. chilinae) was observed. In this case, sporocysts of the schistosome occupied the entire hepatopancreas, while rediae of C. chilinae were few, appearing to represent a recent infection (Stage A). Double infections and recruitment period of C. chilinae (December and March–April) were recorded at the same time, indicating that the miracidium of schistosome infected earlier. More studies are needed to understand the development and the interactions between these 2 parasite species.

Several explanations have been proposed to characterize the structuring mechanism for infracommunities in snails, i.e., antagonistic interactions (Kuris, 1990; Lafferty et al., 1994), and spatial and temporal heterogeneity (Fernandez and Esch, 1991a; Snyder and Esch, 1993). Antagonistic interactions would occur if the following requirements are satisfied: (1) the presence of a dominance hierarchy; (2) a high overall prevalence of infection; and (3) competition by interference (Kuris, 1990). Lafferty et al. (1994) suggested that a direct measure of competition would be the proportion of snails with double infections. During the 2 sampling periods in Lake Mascardi, there was a low prevalence of species with larger rediae, i.e., echnocercinaria III (rediae size: 544–2,460 μm long) and echinocercaria IV (rediae size: 960–1,536 μm long) and a high prevalence of species with a low hierarchical dominance, i.e., C. chilinae (rediae size: 480–720 μm long). Consequently, a clear dominance hierarchy was not observed; digeneans with larger rediae are dominant over smaller ones, as proposed that it should be by Kuris (1990). A great temporal variation in the overall prevalence was recorded; only a very low percentage of double infections was
FIGURE 3. Overall prevalence and prevalence of each digenean species across size classes of *Chilina dombeyana* from Lake Mascardi in both sampling periods (different Y-axis scales are used).
found, so none of the requirements suggested by Kuris (1990) and Lafferty et al. (1994) was completely satisfied. There is, therefore, no robust evidence to conclude that the infracommunities of digeneans in *C. dombeyana* are structured by antagonistic interactions. In contrast, Olmos and George-Nascimento (1997) indicated this form of interaction as being responsible for structuring infracommunities for the same snail species in Chile.

A major factor controlling the component community of digeneans in freshwater snails is temporal heterogeneity, expressed as seasonal changes in water temperature (Sapp and Esch, 1994; Esch et al., 2001). In Lake Mascaridi, larval digeneans showed monthly variations in their composition and prevalence. This pattern is similar to that observed in many other freshwater snails, such as *Lymnaea stagnalis*, *Lymnaea peregra*, *P. gyrina*, *Helisoma trivolvis*, and *H. anceps* (Williams and Esch, 1991; Snyder and Esch, 1993; Schmidt and Fried, 1997; Yoder and Coggins, 1998; Väyrynen et al., 2000). However, only *C. chilinae* was dominant in *C. dombeyana*, differing from the pattern found in *H. anceps* where 2 dominant species were found (Fernandez and Esch, 1991b). Variations in the use of habitats by definitive hosts may produce differences in the composition and prevalence of digenean communities of snails (Peterson, 2007). These variations were found for *C. dombeyana* in Chile, and have been attributed to the migration of definitive hosts and the increment of activity of resident hosts associated with an increase of temperature (Olmos and George-Nascimento, 1997). In Lake Mascaridi, studies on the identity and ecology of intermediate and definitive hosts are needed to explain the effect of temperature on the larval digenean community.

*Chilina dombeyana* becomes infected with *C. chilinae* and *Apatemon* sp. once it reaches 8 mm in length. The overall prevalence of infections and species richness increased with snail size, reaching maximum values in intermediate-sized specimens. In Chile, only the overall prevalence of infections increased with snail size (Olmos and George-Nascimento, 1997). In Lake Mascaridi, the higher values of overall prevalence during the second sampling period could be attributed to the larger size of *C. dombeyana*. Knowledge of life cycle of this snail is scarce, but data for other species, e.g., *Chilina fluminea* and *Chilina gibbosa* indicate a biannual life cycle (Miquel, 1986; Bosnia et al., 1990). In addition, sample bias could explain the larger snails collected since small specimens (<8 mm) can only be found by looking under stones where snail oviposition occurs. Indirect evidence of a biannual life cycle of *C. dombeyana* is the presence of prepatent infections of stage C of *C. chilinae* in small snails collected in November and December 2001. The 2 diversity indices showed the same trend in each sampling period, but differed between them. *Catatropis chilinae* was the dominant species across all sizes of snails; a similar pattern was found in *P. gyrina* with 1 dominant species and a different one in *H. anceps* and *C. dombeyana* from Chile with 2 dominant species (Fernandez and Esch, 1991b; Snyder and Esch, 1993; Olmos and George-Nascimento, 1997). The species richness pattern in *C. dombeyana* is similar to that observed in *H. anceps*, where maximum values were reached at intermediate sizes (Fernandez and Esch, 1991b), and differs from the one found in Chile, where richness (involving 4 taxa) remains constant across all snail sizes (Olmos and George-Nascimento, 1997).

Sousa (1990) proposed 2 hypotheses to explain the structure of component communities of digeneans in snails. The first hypothesis suggests that interspecific interactions at the level of the infracommunity affect the component community structure when the resource hosts are an ecological constraint, with only a few species hierarchically dominant. His second hypothesis advocates that host resources are always sufficiently available to minimize negative interactions. Under these ecological conditions, the richness and diversity of the component parasite community increase with snail size in Lake Mascaridi, and reach an equilibrium where dominance by a few species does not occur, or is very infrequent. Although species richness in the present study increased with snail size, no robust evidence could be detected for antagonistic interactions. Moreover, the availability of resource hosts changed between sampling periods and only a dominant species was observed. So, the pattern of the component community of *C. dombeyana* from Lake Mascaridi did not fit in any of the hypothetical scenarios proposed by Sousa (1990).

As in Finnish deep lakes (Väyrynen et al., 2000), a significant variation in overall prevalence of larval digenean infections was observed in the present study, but the species com-
TABLE VI. Total richness, dominance, and diversity (Shannon-Wiener and reciprocal Simpson indices) of digeneans across size classes of *Chilina dombeyana* from Lake Mascardi in both sampling periods.

<table>
<thead>
<tr>
<th>Size classes (mm)</th>
<th>Simple size (n)</th>
<th>Total richness</th>
<th>Dominance</th>
<th>Diversity indices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>pi*</td>
<td>Species</td>
</tr>
<tr>
<td>First sampling period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-6</td>
<td>12</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-8</td>
<td>59</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-10</td>
<td>168</td>
<td>2</td>
<td>50.0</td>
<td><em>C. chilinae</em> and <em>Apatemon</em> sp.</td>
</tr>
<tr>
<td>10-12</td>
<td>300</td>
<td>2</td>
<td>54.5</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>12-14</td>
<td>263</td>
<td>3</td>
<td>73.7</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>14-16</td>
<td>182</td>
<td>4</td>
<td>76.9</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>16-18</td>
<td>92</td>
<td>6</td>
<td>52.9</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>18-20</td>
<td>63</td>
<td>4</td>
<td>85.7</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>20-22</td>
<td>56</td>
<td>4</td>
<td>75.0</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>22-24</td>
<td>44</td>
<td>4</td>
<td>86.7</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>24-26</td>
<td>25</td>
<td>3</td>
<td>83.3</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>Second sampling period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-10</td>
<td>8</td>
<td>1</td>
<td>100.0</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>10-12</td>
<td>90</td>
<td>3</td>
<td>93.1</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>12-14</td>
<td>157</td>
<td>3</td>
<td>93.9</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>14-16</td>
<td>171</td>
<td>4</td>
<td>97.3</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>16-18</td>
<td>123</td>
<td>6</td>
<td>89.8</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>18-20</td>
<td>64</td>
<td>5</td>
<td>80.4</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>20-22</td>
<td>22</td>
<td>3</td>
<td>71.4</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>22-24</td>
<td>15</td>
<td>3</td>
<td>78.6</td>
<td><em>C. chilinae</em></td>
</tr>
<tr>
<td>24-26</td>
<td>9</td>
<td>3</td>
<td>50.0</td>
<td><em>C. chilinae</em></td>
</tr>
</tbody>
</table>

* pi*, ([the number of snails parasitized by a particular species] / [the number of parasitized snails]) X 100; N1, Shannon-Wiener index; I/Simpson, reciprocal Simpson index.

Table VII. Correlation between *Chilina dombeyana* snail size and community indices in both sampling periods from Lake Mascardi.

<table>
<thead>
<tr>
<th>Indices</th>
<th>rs *</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>First sampling period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness</td>
<td>0.736</td>
<td>0.010</td>
</tr>
<tr>
<td>Dominance</td>
<td>0.870</td>
<td>0.0001</td>
</tr>
<tr>
<td>I/Simpson</td>
<td>0.077</td>
<td>0.821*</td>
</tr>
<tr>
<td>N1</td>
<td>0.178</td>
<td>0.601*</td>
</tr>
<tr>
<td>Second sampling period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness</td>
<td>0.597</td>
<td>0.052*</td>
</tr>
<tr>
<td>Dominance</td>
<td>-0.05</td>
<td>0.884*</td>
</tr>
<tr>
<td>I/Simpson</td>
<td>0.952</td>
<td>0.0001</td>
</tr>
<tr>
<td>N1</td>
<td>0.934</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* Not significant.

position remained approximately constant during the sampling periods. In *C. dombeyana*, the infection pattern at the infracommunity and component community levels could not be explained by competition. Other factors, like spatial and temporal heterogeneity, snail population dynamics, and life histories traits, should be explored. Host factors seem to be important considering that in a small, shallow pond with 2 pulmonate snail species, the communities at both levels was mainly structured by differences in the host life history (Esch et al., 2002). For example, *H. anceps* has a life span of approximately 12 mo, so the entire component community must re-establish each year since most of the annual snail cohort dies within a period of 6 wk each summer (Esch et al., 2002). In contrast, *C. dombeyana* has a life span longer than 12 mo, so the presence of snails of different sizes during the whole year implies a continuity of host resource for larval digenean recruitment. More information regarding the ecology of definitive and intermediate hosts would also help to understand the flow patterns of larval digeneans in Patagonian freshwater environments.

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**LITERATURE CITED**


PETERSON, N. A. 2007. Seasonal prevalence of *Ribeiroia ondatrae* in one population of *Planorbellia trivolvis* (=*Helisoma trivolvis*), including notes on the larval trematode component community. Comparative Parasitology 74: 312–318.


LIFE HISTORY COST OF TREMATODE INFECTION IN HELISOMA ANCEPS USING MARK–RECAPTURE IN CHARLIE’S POND

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ABSTRACT: Parasitism has the potential to affect key life history traits of an infected host. Perhaps the most studied interactions are in snail–trematode systems, where infection can result in altered growth rates, survival, and/or fecundity of the individual. Positive correlations between host size and parasite prevalence are often attributed to changes in growth rates or mortality, which have been observed in the laboratory. Extending lab-based conclusions to the natural setting is problematic, especially when environmental conditions differ between the laboratory and the field. The present study uses reproduction experiments and mark–recapture methods to directly measure key life history traits of the pulmonate snail Helisoma ancesp in Charlie’s Pond. Based on previous laboratory and field experiments on H. ancesp, we predict a significant reduction in fecundity, but not growth rate or survival, of infected snails. Individual capture histories were analyzed with multistate models to obtain estimates of survival and infection probabilities throughout the year. Recaptured individuals were used to calculate specific growth rates. Trematode infection resulted in complete castration of the host. However, neither survival nor growth rates were found to differ between infected and uninfected individuals. The probability of infection exhibited seasonal variation, but it did not vary with size of the snail. These results suggest that the correlation between host size and trematode prevalence is not due to differential mortality or changes in growth rates. Instead, the infection accumulates in large snails via the growth of smaller, infected individuals.

Trematode larvae commonly castrate their molluscan hosts. The mechanism of castration depends on the specific larval stage infecting the host. For example, rediae mechanically castrate the host by consuming host tissue, including the high-energy fat of the gonads. Sporocysts, lacking a mouth and pharynx, likely castrate the host via chemical inhibition. In particular, Trichobilharzia ocellata stimulates the release of schistosomin, which inhibits oviposition by Lymnaea stagnalis (De Jong-Brink, 1995). Castration could be complete, as in the Helisoma ancesp–Halipegus occidualis system (Crews and Esch, 1986), or partial, as in the mudsnail Hydrobia ventrosa (Kube et al., 2006). The individual cost of castration includes the loss of future reproduction. Fecundity compensation has been proposed as a mechanism by which the host could potentially offset this negative effect (Minchella, 1985; Sorensen and Minchella, 1998). Compensation has been recorded in the T. ocellata–L. stagnalis, Schistosoma mansoni–Biophthalaia glabrata, and S. mansoni–Biophthalaia pfeifferi systems (Sorensen and Minchella, 2001), and it occurs by increasing reproductive effort, i.e., oviposition or maturation, before castration.

Many researchers have noted a positive correlation between prevalence of infection and size of the snail host. This has led some to question whether growth schedules vary between infected and uninfected hosts (Baudoin, 1975; Fernandez and Esch, 1991a; Sorensen and Minchella, 2001). Common responses to infection include gigantism (=the rapid increase in size after infection) and stunting. The growth response by a snail may depend on when it was infected. For example, if infected before reproductive maturity, B. glabrata experiences rapid growth until maturity, and then it grows less rapidly than uninfected individuals (Gerard and Theron, 1997).

Recently, trematode infections have been shown to increase mortality of the snail host. Negative effects on survival have been observed from infections by both sporocysts (Makanga, 1981; Minchella and Loverde, 1981; Zakikhani and Rau, 1999) and rediae (Dreyfuss et al., 1999; Sandland and Minchella, 2003). Physical damage caused by the parasite during feeding and cercariae release, and the energetic cost of an immune response may contribute to, if not cause, the increase in mortality. Reinfection by cercariae has also been implicated in decreasing survival of the snail host (Kuris and Warren, 1980; Sandland and Minchella, 2003).

The difficulties in estimating the true cost of parasitism arise when extending the results derived from laboratory experiments to observations in the field. For example, lab-reared Lymnaea elodes grew faster and laid more eggs than snails raised in the field (Eisenberg, 1966, 1970). If food was supplied in the field, then both growth and fecundity increased. Likewise, H. ancesp in Charlie’s Pond are protein deprived, and they exhibit lower fecundity and growth rates than snails raised in the laboratory (Keas and Esch, 1997). To accurately assess the impact of parasitism, studies should be performed under natural field conditions.

Mark–recapture sampling protocols provide the data required to estimate survival in the field. Moreover, recent advances have expanded the scope of analysis, such that simple estimates of survival and population size are becoming secondary to more complex questions. One advance is the development of multistate models, which permit the estimation of transition probabilities between states (Hestbeck et al., 1991; Lebreton et al., 1992; Brownie et al., 1993; Nichols et al., 1994; Nichols and Kaiser, 1999; Lebreton and Pradel, 2002; White et al., 2006). For example, many researchers have used multistate models to estimate the cost of breeding on survival rates (White et al., 2006), whereas others have calculated dispersal rates between various habitats (Hestbeck et al., 1991). Application of mark–recapture protocols to snail populations has been limited to a few studies (O’Keeffe, 1985; Woollhouse, 1988; Goater et al., 1989; Woollhouse and Chandiwana, 1990; Fernandez and Esch, 1991a; Chlyeh et al., 2002, 2003). One reason for the lack of interest may be the low recapture rate of aquatic snails, as a consequence of their vagility. In Charlie’s Pond, 97% of H.
*H. anceps* were collected within 1 m of the original release site (Fernandez and Esch, 1991b), compared with the 28% of *Physa gyrina* that moved more than 1.5 m away from the site of release in just 2 wk (Snyder and Esch, 1993). Because of its low vagility, *H. anceps* is a suitable candidate for an extensive mark–recapture study to measure both growth rates and survival in the field.

The goal of the present study was to determine how trematode infections affect the life history traits of *H. anceps* as measured in a natural setting. The traits being investigated include survival, fecundity, and growth rates. Mark–recapture methods were used, and the capture histories were then analyzed using multistate models. Based on field (Fernandez and Esch, 1991a) and laboratory estimates (Keas and Esch, 1997), we hypothesize that infection will not alter the observed growth rates or survival probabilities of *H. anceps*. This investigation will also produce estimates for the probability of infection, which is predicted to vary seasonally.

**MATERIALS AND METHODS**

*Helisoma anceps* were collected from 2 sites in Charlie’s Pond, Stokes County, North Carolina. Charlie’s Pond is approximately 1 ha, and it has been described previously (Crews and Esch, 1986; Esch et al., 1997). Beginning in May 2005, snails were collected by random sampling with a dip net for 1 hr each week. Weekly collecting sites alternated between the northwest cove (NW) and east side (ES) of Charlie’s Pond. No collections were made from mid-November 2005 through February 2006. The collection regime resumed in March 2006 and concluded in October 2006.

Snails were transported to the laboratory where they were individually isolated in 55-mm plastic jars. The shell length of each snail was measured to the nearest 0.05 mm, and the jars were examined for presence of cercariae. Each snail was marked with colored enamel paint to signify month, with a letter indicating year and site, and a number. The combination of color, letter, and number represented a unique identifier so that a set of individual capture histories could be obtained for each site within the pond. Snails were returned to their site of capture the following week.

Fecundity was directly measured in the pond using 2 reproduction platforms. Each platform holds 24 plastic jars (Negovetich, 2003); they were placed in the pond 1 wk before the addition of snails. From May to October 2005, reproduction was measured monthly at NW and ES. In 2006, bimonthly measurements were made from March to October. To measure fecundity, 24 snails were collected from NW or ES. A concerted effort was made to collect snails of various sizes. A single snail was placed in each jar, and the platform was submerged to a depth of 5 cm. The following week, the jars were transported to the lab, and the number of eggs and egg masses was recorded. The snails were isolated, measured, and marked in the same manner as individuals that were not placed in the reproduction platform. Fecundity was normalized to eggs/wk for each snail, and mean values from infected and uninfected individuals were compared with nonparametric tests of significance. Randomization tests (10,000 iterations) were performed to determine whether fecundity differs between ES and NW for each month (Manly, 1991).

Differences in mean size of uninfected and infected individuals were investigated. To correct for seasonal variation, an adjusted shell length was calculated for each snail. This was accomplished by extracting the residuals from a linear regression of size as a function of year and month (Sokal and Rohlf, 1995). The seasonally corrected sizes of uninfected, *H. occidualis*-infected, and non-*H. occidualis*-infected snails from NW and ES were compared with nonparametric tests.

Specific growth rates (SGRs) were calculated for each recaptured individual $SGR = [S_i - S_j]/D$, where $S_i$ is size at initial capture, $S_j$ is size at recapture, and $D$ is the number of d between capture). Snails were classified as infected if they were shedding cercariae when initially captured or at recapture. Differences between sites and infection status were investigated by comparing average SGR for each month and size class. Analysis of variance was performed in JMP 4.0.4 (SAS Institute, Cary, North Carolina). If the variances of SGRs were not equal, then a Kruskal–Wallis (KW) test was performed.

The mark–recapture protocol resulted in individual capture histories that span the entire collecting period. The capture histories consisted of an informational series of 0, A, and B. Each value holds unique information. Zero indicates that the snail was not captured during a specific capture attempt. The letters indicate that the snail was captured and uninfected (A), or captured and infected (B). Although 11 trematode species were identified in 2006 alone, the prevalence, and thus sample size, for most of these infections was too small to adequately estimate their effect on survival. Therefore, trematode infections were pooled into a single infection category. Capture histories grouped by size at initial capture were analyzed with multistate models in Program MARK (White and Burnham, 1999). The general model tested was $\Psi_{ik}$, $\Phi_{ik}$, $\psi_{i}$, $\phi_{i}$. In this model, survival ($\Phi$) and infection ($\Psi$) probabilities vary as a function of size ($s$), time ($t$), and infection status ($i$). We have no reason to suspect that infection status affects recapture probability ($\psi$); thus, the general model allows $\psi$ to vary only with $s$ and $t$. Akaike’s information criterion (AIC) was used for model selection and ranking (Anderson and Burnham, 1999a, 1999b). Parametric bootstrap procedures were used to estimate $\hat{c}$ (estimation of lack-of-fit) (Cooch and White, 2006). This method produced simulated capture histories using parameter estimates from the general model. These capture histories were then used to obtain simulated parameter estimates, including deviance of the model. A new set of capture histories was created for each iteration. One thousand iterations were performed for each size class, and the model deviances were saved. The deviance from the general model was divided by the mean deviance from the simulated models to obtain $\hat{c}$ (Cooch and White, 2006). AIC was divided by $\hat{c}$ to obtain a quasi-likelihood adjust AIC (QAIC), which corrects for excess variation in the models (Cooch and White, 2006). Models with small QAIC are preferred to those with large QAIC. More specifically, differences in QAIC of $\geq 7$ strongly suggest a real difference between the models being compared (Anderson and Burnham, 1999a, 1999b; Cooch and White, 2006).

The POPAN algorithm of Program MARK was used to estimate recruitment (births + immigration) and population size. This method models a super population $N$ that enters the local population with a probability of $b$. The product of the probability $b$ and the super population is the net number of new recruits from either immigration or births during monthly transition $t (\hat{B} = N \cdot b)$. Population size ($\hat{N}$) during month $t$ is derived from estimates of survival, population size, and recruitment in the previous month (Cooch and White, 2006).

**RESULTS**

In total, 910 *H. anceps* were placed in the reproduction platforms. Because fecundity varies from year to year (Negovetich and Esch, 2007), the effect of infection status was assessed by comparing fecundity of only those snails that were isolated in 2006. The analysis included 674 snails, 34 were infected with *H. occidualis* (rediae), and 27 with miscellaneous trematode species (mostly sporocysts). Infection resulted in a significant reduction in egg output (KW: $\chi^2 = 48.3$, 2 df, $P < 0.0001$). Uninfected snails laid an average $\pm$ SE of 13.46 $\pm$ 0.68 eggs/wk compared with 0.56 $\pm$ 0.32 and 0.74 $\pm$ 0.44 eggs/wk for *H. occidualis*-infected and other infected snails, respectively.

Restricting analysis to uninfected snails reveals that monthly mean fecundity decreased throughout the year for both locations within the pond (Fig. 1). Randomization tests detected a significant difference in March ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0003$), and August ($P < 0.0001$). Analysis with POPAN strongly suggests variation in both site and time for the probability to enter the population (AIC = 7,175.8, model likelihood = 1.00, deviance = 7,036.1). For monthly transitions in 2006, recruitment was higher at NW than ES; yet, the
population in ES experienced less fluctuation in snail recruitment than NW (Fig. 2).

The mark–recapture experiment resulted in a total of 4,850 individual capture histories. Of these, 1,114 capture histories represented individuals that were recaptured at least once (Table I). Only a single snail migrated across the pond, and this individual was excluded from analysis. Seasonally corrected mean sizes were significantly different between uninfected, *H. occidualis*-infected, and miscellaneous infected snails (KW: $\chi^2 = 153.1$, 2 df, $P < 0.0001$). Snails infected with *H. occidualis* ($\bar{x} = 9.72 \pm 0.05$ mm [±SED]) were larger than snails infected with other trematode species ($\bar{x} = 9.61 \pm 0.10$ mm), and these were larger than uninfected snails ($\bar{x} = 9.07 \pm 0.02$ mm; Steel-type MC: $P < 0.01$ for all comparisons).
SGR did not differ by site or infection status for almost all size classes examined (Table 3). The single exception when comparing the sites was for the 7-mm size class. SGR for NW was significantly higher than ES (analysis of variance [ANOVA]; $F_{1,131} = 6.9, P < 0.01$). Upon closer inspection, the difference in SGR persisted for uninfected (ANOVA: $F_{1,108} = 7.9, P < 0.006$), but not infected (ANOVA: $F_{1,21} = 0.00003, P > 0.05$), individuals. Seasonally, SGR fluctuated little from March to May, increased to a peak in August, and then decreased until winter (Fig. 4). Uninfected individuals exhibited a higher SGR in August (KW; $\chi^2 = 20.0, 1$ df, $P < 0.0001$). Comparing the sites, significant differences in SGR were observed in August (ANOVA: $F_{1,134} = 6.1, P < 0.015$) and September (KW; $\chi^2 = 19.2, 1$ df, $P < 0.0001$). When examining infection status within these months, SGR was not different in August for both uninfected (ANOVA: $F_{1,98} = 3.5, P > 0.05$) and infected individuals (ANOVA: $F_{1,34} = 0.74, P > 0.05$). However, in September, NW growth rates were significantly higher only for the uninfected snails (KW; $\chi^2 = 13.3, 1$ df, $P < 0.0003$).

The effect of infection on survival was assessed by fitting various models to the set of capture histories from the mark-recapture experiment. To avoid complications of initial size and growth of the snails, analyses were performed by size class. For example, the capture histories of all snails that were 6–6.95 mm at initial capture were analyzed independently from the remaining size classes. After fitting the general model and correcting for overdispersion of the errors, the most likely model that fits the data was $\Phi_1 \phi_1 \Psi_{ij}$ (Table II). For the 10-mm size class, there is some support for a model that allows survival to vary with site and time; yet, the best model was 2.5 times more likely than this second best model. Survival from 1 mo to the next varied seasonally (Fig. 5). Small snails suffered from higher mortality (=low survival probabilities) than larger snails, except in the period during cohort turnover.

Recruitment of trematode infections is reflected in the probability of being infected (Fig. 6). Early in the year, infection probabilities remained under 0.2 for all size classes. Peak infection probabilities occurred from June to August, which corresponds to a large recruitment of trematode infections and new snails at this time. The risk of infection then returned to levels previously observed during spring.

**DISCUSSION**

When comparing fecundity, prominent differences exist between ES and NW. In both locations, fecundity decreases throughout the year, but NW snails lay more eggs/week until cohort turnover in July (Fig. 1). In March, the difference in reproductive output is most related to emergence of snails from the substratum as the water warms after winter. Specifically, more snails per hour were collected in NW than ES during March, which suggests that snails become active earlier in the year at NW than in ES. May was the second month that produced a difference in fecundity. In this month, snails >9 mm were more fecund, and closer inspection reveals that reproductive activity is responsible. The percentage of productively active snails differed for the 9-mm (93% in NW vs. 0% in ES) and >10-mm size classes (96% in NW vs. 43% in ES). When accounting for reproductive activity, snails >10 mm remain more fecund at NW compared with ES. Reproduction is depressed when a snail approaches death (Herrmann and Harman, 1975). Based on size frequency histograms (Fig. 7), cohort turnover at ES is apparent by June, which suggests that those snails were near death in May. Moreover, the NW decrease of fecundity in July corresponded to turnover at that location, which occurred nearly a month after ES.

Alternative explanations for a difference in fecundity between the sites include local productivity and snail size. First, large snails have the capacity to lay more eggs than smaller individuals (Brown, 1985; Brown et al., 1985; Lazaridou-Dimitriadou et al., 1998). The NW snails placed in the reproduction platforms were larger than ES snails in May–July. However, fecundity at the 2 sites was similar in July, which diminishes the likelihood that snail size is the primary cause of the fecundity differences. Second, reproduction is depressed when food supplies are limiting (Brown et al., 1985; Byrne et al., 1989; Lam and Calow, 1989; Wayne, 2001). NW contains more emergent vegetation and leaf litter, surfaces on which periphyton can grow. If productivity is contributing to the differences in fecundity, then NW snails of a specific size class should be consistently more fecund than ES snails of the same size class during a particular month. This was only observed in March for the 8- and 10-mm size classes, and in May for the 9- and 10-mm size classes. The lack of consistency from 1 mo to the next implies that productivity is not the only factor affecting egg production in the Pond. Instead, it may be contributing to the observed differences between the 2 sites.

Inflection severely decreased egg production in *H. aniceps*. For snails infected with rediae of *H. occidentalis*, the mechanism is mechanical castration by direct consumption of the gonads (Crews and Esch, 1986). Of the 27 snails infected with a trematode species other than *H. occidentalis*, 22 individuals were shedding 1 of 2 known sporocid cercariae. Within the snail host, the 2 sporocysts have sporocyst larval stages. Unlike *H. occidentalis*, sporocysts have been known to chemically castrate the host (Cheng et al., 1973; De Jong-Brink et al., 1991; Wayne, 2001). The size at first reproduction was 6 mm, and given that all but 1 snail were larger than 7 mm, it is reasonable to assume that the unknown sporocids also castrate *H. aniceps* in Charlie’s Pond. In fact, 19 of 22 sporocid-infected snails did not lay eggs, and the 3 snails that were productively active produced no more than 10 eggs within a week. In some snail–trematode systems, snails often exhibit fecundity compensation (Sorensen and Minchella, 2001). Specifically, some species increase their reproductive activity between the time of initial exposure to the egg or miracidium, and patency. Fecundity
compensation may be occurring in the Pond, but we feel that it is unlikely given that increases in egg production were not observed in lab studies on *H. aniceps* (Keas and Esch, 1997).

Infected snails are larger than uninfected snails, but this does not imply a difference in growth rate. Much work has been done on the influence of trematode infections on the growth rate of snails. In all of the field studies at Charlie’s Pond, *H. occidualis*-infected *H. aniceps* were larger than uninfected in-

**Figure 3.** Mean specific growth rate (±SE) for the different size classes of snails, examined by (A) infection status and (B) site (ES and NW). Pairs of bars marked with an asterisk were significantly different at the $P \leq 0.05$ level. Only 1 infected snail from the 5-mm size class was recaptured, and that value is excluded from the graph.
A.)

B.)

**FIGURE 4.** Mean specific growth rate (±SE) throughout the year, examined by (A) infection status and (B) site (ES and NW) of the uninfected snails. Pairs of bars marked with an asterisk indicate a significant difference at the $P \leq 0.05$ level.

individually (Crews and Esch, 1986; Fernandez and Esch, 1991c; Williams and Esch, 1991; Negovetich, 2003). Similar associations between prevalence and snail size occur in 68% of marine and 73% of freshwater gastropods (Sorensen and Minchella, 2001). Four possibilities can explain the positive correlation between host size and parasite prevalence, i.e., unequal catchability, differential mortality, variation in susceptibility, and changes in growth rates (Baudoin, 1975).

Catchability describes the likelihood of collecting snails given their infection status. In some systems, the parasite alters the
behavior of the host so that infected individuals are more likely to be consumed by the next host in the parasite’s life cycle (Poulin, 2007). In the context of Baudoin (1975), the parasite may have a size-dependent effect on behavior, such that large, infected individuals will be easier to catch than small, infected individuals. Thus, prevalence would be positively correlated with size of the snail. In Charlie’s Pond, no evidence exists that would suggest that infected snails are easier to collect than uninfected snails. Furthermore, analysis of capture histories indicates that models with capture probabilities that vary with infection status do not fit the data better than the general model (data not shown). Thus, catchability of *H. aniceps* is not influenced by infection status.

There are 2 situations where differential mortality will produce a size difference between uninfected and infected individuals. The first is an increase in mortality of young snails after infection, which seems more common with rediae stages than with sporocysts (Sorensen and Minchella, 2001). If younger snails are more likely to die after infection, then the size distribution of infected snails will be skewed, with a greater quantity of infected snails tending to larger sizes. The second situation is a reduction of mortality of infected individuals. Specifically, if infected snails live longer than uninfected snails, and size is positively correlated with age, then the infected population will be larger, on average, than the uninfected population. In the current study, differential mortality is not apparent. None of the most parsimonious models included infection status as a determinant of survival (Table II). Prepatent infections could have increased mortality, especially in small snails. This increase in mortality would not be detected because the sampling methods only permitted the identification of patent infections. However, laboratory studies have also failed to reveal a

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**Table II.** Summary of the various multistate models tested in Program MARK. Analyses were performed in groups based on the size class of snails at initial capture. Number of parameters and the dispersion parameter (\(c\)) were adjusted to generate the QAIC, the change in QAIC (\(\Delta Q_{AIC}\)), and the quasi-likelihood deviance (\(Q_{Deviance}\)). Survival (\(\Phi\)), catchability (\(\rho\)), and infection transitions (\(\Psi\)) were allowed to vary by site, infection status (inf), and time.

<table>
<thead>
<tr>
<th>Model</th>
<th>QAIC</th>
<th>(\Delta Q_{AIC})</th>
<th>Parameters</th>
<th>(Q_{Deviance})</th>
</tr>
</thead>
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<tr>
<td>6 mm ((c = 2.051))</td>
<td>339.6</td>
<td>0</td>
<td>55</td>
<td>58.6</td>
</tr>
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<td>(\Phi_{site<em>inj</em>time} P_{site<em>inf</em>time})</td>
<td>363.3</td>
<td>23.7</td>
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<td>33.9</td>
<td>69</td>
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<td>441.4</td>
<td>101.8</td>
<td>97</td>
<td>38.2</td>
</tr>
<tr>
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<td>248.4</td>
<td>138</td>
<td>33.6</td>
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<tr>
<td>7 mm ((c = 1.015))</td>
<td>782.8</td>
<td>0</td>
<td>55</td>
<td>177.3</td>
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<tr>
<td>(\Phi_{site<em>time} P_{site</em>inf*time})</td>
<td>808.7</td>
<td>20.5</td>
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<td>(\Phi_{site<em>inf</em>time} P_{site<em>inf</em>time})</td>
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<td>21.5</td>
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<td>(\Phi_{site<em>inf</em>time} P_{site*time})</td>
<td>872.2</td>
<td>84.0</td>
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<td>8 mm ((c = 1.318))</td>
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<td>69</td>
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<td>0</td>
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<td>905.9</td>
<td>215.1</td>
<td>138</td>
<td>136.3</td>
</tr>
</tbody>
</table>
significant difference in survival (Keas and Esch, 1997); so, it seems that mortality of H. aneck in the field is not influenced by infection status.

A positive correlation between prevalence and snail size may have arisen from differences in susceptibility. Two situations would produce the same observation, i.e., equal probability of infection across size classes, or increases of infection probabilities with size (Baudoin, 1975). If the probability of infection is constant across size classes, then infections will accumulate in larger snails. For example, the 9-mm size class includes newly infected 9-mm snails, in addition to individuals that were infected at a smaller size and grew into the size class. In the absence of increased mortality, a plot of prevalence versus size class will produce a straight line with positive slope, where the slope is equal to the probability of infection. Alternatively, the probability of infection could increase with size, in which case prevalence will exhibit a curvilinear increase as a function of size class. The highest prevalence, regardless of trematode species, was observed in June (20%), and it remained above 16% in July and August. To maintain the level of prevalence during cohort turnover, the probability of infection must increase so that young, newly infected individuals replace the dying, in-

Figure 5. Probability to survive (Φ) during the monthly transitions for the 6 size classes of snails.
fected snails (Fig. 6). This corresponds with the timing of peak recruitment and maturation of *H. occidualis* in the green frogs (Goater, 1989; Wetzel and Esch, 1996). There is little evidence to suggest that infection probability varies with size class. With the exception of the July–August and August–September transitions, the 3 largest size classes differ by no more than 12%, and the SE of the estimates overlap, suggesting that infection probabilities are not significantly different. The SE of the 3 smallest size classes also overlap. Thus, the correlation between shell length and prevalence is probably caused by accumulation of infected individuals in the larger size classes.

The final explanation for the correlation between prevalence and size involves growth rates. Sorensen and Minchella (2001) reviewed the literature on snail–trematode life history interactions. They found that enhanced growth rates of infected snails occur more frequently in freshwater systems, whereas stunting is more common in marine systems. Trematode infections can have stage-specific effects on the host, such that infected juveniles might grow faster than uninfected juveniles, whereas infected adults may grow more slowly. Most work has occurred in the laboratory, including experiments on *H. anceps* (Keas and Esch, 1997). Fernandez and Esch (1991c) suggested that
the disparity of conclusions between the field and lab experiments may be due to the abundance of food and constant environmental conditions in the laboratory. Specifically, the cost of parasitic infection on growth rates could be strengthened or diminished depending on the environmental conditions.

Growth rates vary by size class and month. Specifically, small snails grow faster than large snails (Fig. 3), and SGR peaks in August (Fig. 4). The dependence of size on growth rate is well documented for many species, including *H. anceps* (Herrmann and Harman, 1975; Fernandez and Esch, 1991a). The 7-mm size class produced a significant difference of SGR between NW and ES. However, 71% of the uninfected NW snails were initially captured in August–September, the 2 mo with the fastest growth rate, compared with 31% of the ES snails. In contrast, 75% of the infected snails for each site were collected from July to September. Thus, the observed difference in SGR for the 7-mm size class was primarily influenced by seasonal changes in growth rate, and not by variation between the 2 locations within Charlie’s Pond. Similarly, the significant difference in September between the sites is most likely due to the population structure in that month. Snails <8 mm represented 20% of the ES population and 42% of the NW population. Seasonal variation and size class effects suggest that SGR does not differ by site. Therefore, site was ignored when examining the effect of infection on SGR.

August was the only month when SGR of uninfected and infected individuals were significantly different. The distribution of size classes within this month indicates that the infection effect in this case is spurious. Specifically, 59% of the uninfected snails were <8 mm, whereas only 36% of the infected individuals were represented by the fastest growing size classes. Furthermore, no difference in SGR was detected within the size classes examined. This is surprising given the number of host–parasite associations where the growth rate of the host is affected by the trematode (Sorensen and Minchella, 2001). Competing hypotheses explain alterations in growth schedules. The first states that the available energy for the host is contingent on the needs of the parasite (Sousa, 1983). In castrated individuals, the parasite liberates energy for the host that was previously allocated to reproduction. In response, the host exhibits faster growth rates. Alternatively, gigantism could result as a response by the host to increase fitness before castration (Minchella, 1985; Sorensen and Minchella, 1998). Specifically, once infected, the host attains reproductive size and oviposits before castration. Increased growth rates may also delay castration because large hosts maintain viable gonadal tissue longer than smaller snails. In both hypotheses, life history tradeoffs minimize the effect of infection. At Charlie’s Pond, trade-offs in the life history traits of *H. anceps* were not detected. Although the trematode infections castrated the snail host, neither growth rate nor survival probability was altered in comparison with uninfected individuals.

Previous studies have examined key life history traits of *H. anceps* from Charlie’s Pond in the field (Crews and Esch, 1986; Fernandez and Esch, 1991a; Negovetich, 2003) and in the lab (Keas and Esch, 1997). The current study expands on the previous research by comparing 2 locations within Charlie’s Pond and directly estimating life history traits with a mark–recapture experiment. A decrease in fecundity was the only individual cost associated with trematode infection. Castration will negatively impact the snail host by decreasing the population growth rate and reducing the number of genotypes that individual hosts pass on to the next generation. Although individual responses to offset castration were not detected, the population may have responded with changes in the life history strategy, such as a decrease in the age at first reproduction. This could be accomplished via increased growth rates so that reproductive size is reached earlier in life, or it could arise from decreases in size at maturity (Roff, 1992). In Charlie’s Pond, snails became reproductionally mature at 7.5–8.0 mm in mid-1980s (Goater, 1989), but the latest fecundity experiment demonstrated oviposition by 6-mm snails in August and September. The decrease in size at maturity could be an evolutionary response in a system where castration by *H. occidualis* has been occurring in the snail population for nearly 25 yr. The life history estimates derived from the current study are being used to construct a

**Figure 7.** Histogram of shell size (millimeters) for snails collected from ES and NW of Charlie’s Pond throughout the year.
model of the *H. aniceps* population in Charlie’s Pond. A matrix model will quantify the cost of castration at the population level by determining the population growth rate across a range of infection probabilities. Furthermore, the effect of adjustments to the growth rates, survival, and fecundity will be assessed. Thus, mathematical modeling may provide clues as to which life history traits are most likely to offset the cost of parasitic castration.

**LITERATURE CITED**


TEMPORAL VARIATION IN THE HELMINTH PARASITE COMMUNITIES OF THE PACIFIC FAT SLEEPER, DORMITATOR LATIFRONS, FROM TRES PALOS LAGOON, GUERRERO, MEXICO

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ABSTRACT: Temporal variation in the helminth parasite communities of the Pacific fat sleeper, Dormitator latifrons, from Tres Palos Lagoon, Guerrero, Mexico, was studied at the component community and infracommunity levels. In total, 185 host specimens were collected between April 2000 and March 2001. Eight parasite species were identified: Clinostomum complanatum, Echinopisthoe (leopoldinae), Ascocotyle (Phagicola) longa, Pseudoacanthostomum panamense, Saccocoeloides sp., Parvitaenia coellearii, Neoechinorhynchus golvani, and Contracaecum sp. The communities had low numbers of parasite species and diversity, and contained only generalist parasites. Nested (nonrandom) species composition was observed in the infracommunities during all climatic seasons. The variation in nestedness intensity was attributed to a process of sequential colonization by the most common parasite species, because some were more abundant in the dry season, and others were more abundant in the rainy season.

Parasite communities experience temporal structural changes related to seasonal variations in biotic and abiotic environmental factors; these variations can be reflected in species composition and density over time (Zander and Kesting, 1998; Fiorillo and Font, 1999; Zander et al., 1999, 2002; Zander, 2003, 2004, 2005). Most studies designed to examine the seasonal variation in parasite community composition have been carried out in fishes from temperate regions (Mhaisen et al., 1988; Zander and Kesting, 1998; Zander et al., 1999; Klimpel et al., 2003; Zander, 2003; Fellis and Esch, 2004; Zander, 2004; Simková, 2005; Zander, 2005). Many processes have been suggested to influence the seasonal variation in parasite communities in these regions, for example, temperature and other abiotic factors (Eure, 1976; Chubb, 1979; Granath and Esch, 1983); intermediate host abundance (Esch and Fernandez, 1993; Zander and Kesting, 1998; Zander et al., 1999; Fellis and Esch, 2004; Zander, 2004); and changes in host abundance, reproductive and feeding behavior (Mhaisen et al., 1988; Klimpel et al., 2003; Fellis and Esch, 2004; Simková, 2005), and immunity (Kennedy and Hine, 1969; Zander and Kesting, 1998).

There is currently very little research on temporal variation in the parasite communities of tropical or subtropical areas, where temperature changes are not as extreme (Leong, 1986; Fiorillo and Font, 1999; Steinauer and Font, 2003; Vincent and Font, 2003). In these areas, recruitment of helminths might correlate with other factors such as wet–dry seasons, or year-round recruitment (Fiorillo and Font, 1999; Steinauer and Font, 2003).

Research on temporal variation in the parasite communities in Mexico has been done on freshwater cichlids (Salgado-Maldonado, 1993; Pineda-Lopez, 1994; Jiménez, 2003; Vidal-Martínez and Poulin, 2003). These studies have shown high variation in parasite abundance over time in response to alterations generated by the seasonal dry–rain cycle, which affects the parasite species recruitment process (Salgado-Maldonado, 1993), or in response to seasonal increases in host feeding and reproductive activity influenced by seasonal water temperature fluctuations (Jiménez, 2003; Jiménez-García and Vidal-Martínez, 2005). Pineda-Lopez (1994), however, suggested that helminth communities in tropical climates exhibit a stable structure over time.

No studies have been done to date on helminth parasite species composition over time in a tropical brackish-water fish in Mexico. Dormitator latifrons is widely distributed and highly abundant in almost all the Pacific coastal lagoons of Mexico, and is 1 of the most characteristic fish species of the lagoon systems in the state of Guerrero (Yañez-Arancibia and Díaz-González, 1977). The region’s pronounced dry and rainy seasons notably affect Tres Palos Lagoon, leading to questions about whether or not this seasonal variation affects the parasite community of D. latifrons in this tropical, brackish environment. The present study objective was to describe temporal composition and variation in the helminth parasite community of D. latifrons, and to identify possible biotic and abiotic factors responsible for temporal variation.

MATERIALS AND METHODS

Tres Palos Lagoon (16°47′N, 99°39′W) is on the Pacific coast of Mexico, 25 km east of Acapulco (Violante-González, 2006) (Fig. 1). It has a surface area of 55 km² (5,500 ha) and ranges in depth from 0.5 to 8 m. Constant urban waste discharge into the lagoon by means of the Sabana River causes it to be eutrophic (Violante-González, 2006). As a result, primary productivity is very high (80–106 μg/L·1 chlorophyll-a concentration), particularly during the rainy season (Banderas and González, 2000). The region experiences 2 distinct climatic seasons, a rainy period from June to November (precipitation ≈ 430 mm), and a dry time from December to May (precipitation ≈ 100 mm) (Violante-González, 2006). In the present study, the surface water temperature (measured at 0.5-m depth) and salinity were measured monthly with a YSI model 33, at San Pedro las Playas, where D. latifrons specimens were collected.

In total, 185 adult D. latifrons specimens were collected with the use of gill nets between April 2000 and March 2001. The 1-yr sampling period was divided into 4 3-mo periods: April–June, July–September, October–December, and January–March. The number of specimens examined per sampling period ranged from 46 to 47 fish (Table 1). Fish sex and total length were recorded. A complete necropsy was done on all hosts, including all organs. Internal and external metazoan parasites collected from the hosts were processed according to Lamothe-Argumedo (1997). Voucher specimens of all taxa were deposited in the National Helmint Collection, Institute of Biology, National Autonomous University of Mexico, Mexico City (CNHE: 4901, 4903, 4904, 4906, 4911, 4912, 4917, 4920). A species-richness sampling effort curve was applied to determine if sample size was sufficient to recover the highest possible proportion of parasite species, and thus to characterize ade-
State of Guerrero, Mexico

Acapulco City

Pacific Ocean

FIGURE 1. Location of Tres Palos Lagoon in the state of Guerrero, Mexico. (Insets: temperatures and salinity recorded during a 1-yr sampling period.)

quately the component communities (Aho et al., 1991). Cumulative species curves were plotted per sampling period, and the observed values fitted to the Clench model to assess any asymptotic trend (Clench, 1979).

Autogenic parasite species were defined as those that reach maturity in aquatic hosts and thus have a limited ability to colonize new locations. Allogenic species were those with birds or mammals as definitive hosts and whose natural migrations favor dispersion, giving them a wide geographic distribution (Esch et al., 1988). Active transmission was defined as movement of a parasite to a host by its own means, whereas passive transmission was defined as movement of a parasite from 1 host to another without energy expenditure by the parasite (Bush et al., 2003). The host-range concept (Rohde, 2005) was used to classify the parasite species as specialists or generalists. According to this concept, a specialist parasite species is one that has a marked affinity for a specific host family, genus, or species, whereas a generalist is one that parasitizes a number of families. An Olmstead-Tukey association test (Sokal and Rohlf, 1998) was applied to classify the parasite species based on infection parameters of prevalence and abundance: dominant (abundant and frequent), common (low abundance but frequent), rare (low abundance and low frequency), and indicators (abundant but low frequency). Numerically dominant species were those with more than 40% prevalence and a mean abundance of 10 or more parasites per fish, rare species were those with prevalence below 5% and mean abundance below 5 parasites per fish, and indicator species were those with mean abundance >10 parasites and prevalence <5%.

Variation in species composition over time was described with the use of prevalence (percent infected host) and mean abundance (mean number of parasites per examined fish), calculated and applied according to Bush et al. (1997) for each helminth species per sampling period. To determine possible differences in infection parameters between sampling periods, G-tests were used for prevalence and a 1-way ANCOVA for abundance, with total length as a covariate to control the influence of host size. Normality was determined with the Kolmogorov-Smirnov test with the use of Lilliefors’ approach (Sokal and Rohlf, 1998). When important deviations from normality were found, the data were transformed to Naperian logarithms (ln[X + 1]). The significance of all statistical analyses was established at $P = 0.05$, unless stated otherwise.

Analyses were made at the component community and infracommunity levels (Holmes and Price, 1986); that is, we considered all the helminth parasites in all the hosts collected per sampling period and all the helminth parasites in an individual fish. The component community parameters included total number of parasite species, total number of individual parasites, the Shannon-Wiener index ($H$) as a measure of diversity, species evenness (equitability) (Krebs, 1999), and the Berger-Parker index ($BPI$) as a measure of numerical dominance (Magurran, 1991). Component community predictability was determined by measuring quantitative and qualitative similarities between sampling periods. The Jaccard index was used for qualitative analysis and the percentage of similarity (PS) index for quantitative analysis (Krebs, 1999). Kruskal-Wallis tests were used to determine significant differences in the component community parameters between sampling periods at this level. Correlations were made with the use of the Spearman range coefficient ($r_s$) (Krebs, 1999). The infracommunities were described by the mean number of helminth parasite species per fish, mean number of parasite individuals per fish, and the mean value of the Brillouin
### Table I. Prevalence (upper value) and mean abundance (lower value) ± standard deviation of helminth parasites of the fat sleeper, *Dormitator latifrons*, from Tres Palos Lagoon, Guerrero, Mexico. Colonization strategy: Au = autogenic species, Al = allogenic species. Distribution: Fw = freshwater; Mw = marine water; Bw = brackish water.

<table>
<thead>
<tr>
<th>Sampling periods</th>
<th>Dry season</th>
<th>Rainy season</th>
<th>Dry season</th>
<th>Au/Al</th>
<th>Microhabitat</th>
<th>Transmission form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>April–June 2000</td>
<td>July–September</td>
<td>October–December</td>
<td>January–March 01</td>
<td>Fu/Mv/Bw</td>
<td></td>
</tr>
<tr>
<td>Number of hosts</td>
<td>46</td>
<td>47</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Mean total length (cm)</td>
<td>21.46 ± 1.97</td>
<td>22.96 ± 1.73</td>
<td>20.79 ± 1.80</td>
<td>21.90 ± 1.64</td>
<td>20.79 ± 1.80</td>
<td>21.90 ± 1.64</td>
</tr>
</tbody>
</table>

**Digenea (adult)**

<table>
<thead>
<tr>
<th>Species</th>
<th>Dry season</th>
<th>Rainy season</th>
<th>Dry season</th>
<th>Au/Al</th>
<th>Microhabitat</th>
<th>Transmission form</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccocoeloides</em> sp. Fw</td>
<td>11.1</td>
<td>32.4</td>
<td>50.0*</td>
<td>58.3*</td>
<td>Au</td>
<td>Intestine</td>
</tr>
<tr>
<td>0.2 ± 0.8</td>
<td>1.3 ± 5.2</td>
<td>9.9 ± 22.1*</td>
<td>4.6 ± 8.1</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

**Digenea (larvae)**

<table>
<thead>
<tr>
<th>Species</th>
<th>Dry season</th>
<th>Rainy season</th>
<th>Dry season</th>
<th>Au/Al</th>
<th>Microhabitat</th>
<th>Transmission form</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ascocotyle (Phagicola) longa</em> Fw</td>
<td>8.3</td>
<td>16.7</td>
<td>54.4*</td>
<td></td>
<td>Al</td>
<td>Heart</td>
</tr>
<tr>
<td>0.2 ± 1.5</td>
<td>21.6</td>
<td>8.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Clinostomum complanatum** Fw

<table>
<thead>
<tr>
<th>Species</th>
<th>Dry season</th>
<th>Rainy season</th>
<th>Dry season</th>
<th>Au/Al</th>
<th>Microhabitat</th>
<th>Transmission form</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 ± 2.9</td>
<td>5.3 ± 54.9</td>
<td>0.1 ± 0.6</td>
<td>2.1 ± 18.7</td>
<td></td>
<td>Al</td>
<td>Liver, mesentery</td>
</tr>
<tr>
<td>75.0*</td>
<td>27.0</td>
<td>25.0</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Echinochasmus leopoldinae** Fw

<table>
<thead>
<tr>
<th>Species</th>
<th>Dry season</th>
<th>Rainy season</th>
<th>Dry season</th>
<th>Au/Al</th>
<th>Microhabitat</th>
<th>Transmission form</th>
</tr>
</thead>
<tbody>
<tr>
<td>315.1 ± 371.4*</td>
<td>2.5 ± 6.0</td>
<td>2.1 ± 6.7</td>
<td>172.2 ± 408.9*</td>
<td></td>
<td>Al</td>
<td>Gills</td>
</tr>
<tr>
<td>47.2*</td>
<td>16.2</td>
<td>22.2</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Pseudoacanthostomum panamense** Mw, Bw

<table>
<thead>
<tr>
<th>Species</th>
<th>Dry season</th>
<th>Rainy season</th>
<th>Dry season</th>
<th>Au/Al</th>
<th>Microhabitat</th>
<th>Transmission form</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2 ± 6.3</td>
<td>1.2 ± 4.3</td>
<td>1.1 ± 5.0</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

**Cestoda (larvae)**

<table>
<thead>
<tr>
<th>Species</th>
<th>Dry season</th>
<th>Rainy season</th>
<th>Dry season</th>
<th>Au/Al</th>
<th>Microhabitat</th>
<th>Transmission form</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Parvitena cochlearia</em> Fw</td>
<td>13.9</td>
<td>8.1</td>
<td>55.6*</td>
<td>30.6</td>
<td>Al</td>
<td>Liver</td>
</tr>
<tr>
<td>3.3 ± 38.7</td>
<td>0.4 ± 0.6</td>
<td>17.3 ± 54.8*</td>
<td>3.6 ± 23.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Acanthocephala (adult)**

<table>
<thead>
<tr>
<th>Species</th>
<th>Dry season</th>
<th>Rainy season</th>
<th>Dry season</th>
<th>Au/Al</th>
<th>Microhabitat</th>
<th>Transmission form</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Neoehichinorhynchus golvani</em> Fw</td>
<td>100</td>
<td>94.6</td>
<td>83.3</td>
<td>97.2</td>
<td>Au</td>
<td>Intestine</td>
</tr>
<tr>
<td>52.0 ± 54.4*</td>
<td>21.4 ± 40.4</td>
<td>19.1 ± 20.0</td>
<td>39.6 ± 41.5*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Nematoda (larvae)**

<table>
<thead>
<tr>
<th>Species</th>
<th>Dry season</th>
<th>Rainy season</th>
<th>Dry season</th>
<th>Au/Al</th>
<th>Microhabitat</th>
<th>Transmission form</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Contracaecum</em> sp. Fw</td>
<td>5.6</td>
<td>10.8</td>
<td>11.1</td>
<td>5.6</td>
<td>Al</td>
<td>Liver, mesentery</td>
</tr>
<tr>
<td>0.5 ± 10.6</td>
<td>0.2 ± 0.8</td>
<td>0.3 ± 1.4</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05.
diversity index ($H'$) per fish. The host specimens were pooled into 3 size classes based on total length, i.e., $<19$ cm, $\geq 20$ and $<22$ cm, and $\geq 23$ cm. A 1-way ANCOVA was used to determine significant differences in infracommunity parameters between sampling periods.

Nestdedness of parasite infracommunities between sample periods was tested with the use of the "nestdedness temperature calculator" (Atmar and Patterson, 1995). This calculator considers a random matrix in terms of disorder, or entropy. The relationship between entropy and heat is used to quantify a randomness matrix in terms of temperature, with 0° representing minimum entropy (perfect nestedness) and 100° representing maximum entropy (randomness). This is done by building a presence–absence matrix of parasite species in each infracommunity for each sample. The nestedness temperature calculator packs the observed data into the matrix to concentrate presences in the upper left-hand corner as much as possible, while preserving the primary information (species-per-site data) within the matrix. The packed matrix is then compared with a maximally nested equivalent to identify unexpected presences and absences. For each observed matrix a $T^c$ value is calculated where $0^c$ represents a perfectly nested matrix and 100° a completely random matrix. The significance of a derived $T^c$ was estimated with a $t$-test, with the use of a normal distribution of 500 Monte Carlo simulated $T^c$ values. As a complement to this analysis, the infracommunity index (ICI) (Zander, 2004), which describes the frequency of double and multiple infections by a single parasite species in a distinct host, was calculated as follows: $ICI = (M_j/N_j) \times I_j$, where $I_j$ is the mean of parasite species in host $j$ (mean infracommunity); $M_j$ is the ratio (numbers) of multiple infected host $j$ with parasite $i$ and other parasites; $N_j$ is the ratio (numbers) of infected host $j$.

RESULTS

Abiotic factors

Water temperature (measured at 0.5-m depth) varied only slightly between sampling periods, ranging from 25.9°C (April 2000) to 28.4°C (January 2001) (Fig. 1). Salinity also varied minimally, ranging from 3.0 (November 2000) to 5.8 ppm (May 2000), indicating an oligohaline environment (Fig. 1).

Species composition

Eight helminth parasite species were identified in the 185 D. latifrons specimens collected during the 1-yr study period (April 2000 to March 2001), including 5 digeneans, 1 cestode, 1 acanthocephalan, and 1 nematode (Table I). Six parasite species were found as larvae in different organs, such as the heart, liver, mesentery, muscles, and gills (see Table I). In contrast, only 1 adult digenean (Saccocoeloides sp.) and an adult acanthocephalan (N. golvari), both intestinal parasites, were recovered. Five of the species found in the larval stage were classified as allogenic, because they mature in ichthyophagous aquatic birds living in the lagoon. Autogenic species included the 2 species collected as adults, as well as the metacercariae P. panamense (Table I). All the helminth parasites were classified as generalists because they have been recorded in other hosts from different families living in the same lagoon (data not shown; see Violante-González, 2006).

Seasonal variation in parasite species

No significant difference was observed between parasite species prevalence and mean abundance parameters for male and female hosts ($P > 0.05$); therefore, both sexes were pooled for subsequent statistical analyses. The prevalence of at least 3 parasite species differed significantly between sampling periods (Table I). Higher infection percentages were observed for E. leopoldinae in the 2 dry seasons (April–June, 75%; January–March, 54%) ($G = 37.48, P < 0.05$), for P. cochlearii in October–December (55%) ($G = 50.27, P < 0.05$), and for Saccocoeloides sp. in 2 contiguous periods (October–December 2000 and January–March 2001) ($G = 34.55, P < 0.05$). Five of the parasite species had variable mean abundance over time (Table I): E. leopoldinae and N. golvari had the highest mean abundance in April–June and January–March (1-way ANCOVA $F = 35.63; F = 9.41, P < 0.05$) (both dry seasons); only P. panamense was more abundant in April–June (1-way ANCOVA $F = 7.81, P < 0.05$); and Saccocoeloides sp. and P. cochlearii had higher mean abundance values in October–December (rainy season) (1-way ANCOVA $F = 4.78; F = 8.53, P < 0.05$, respectively).

Component community

Table II shows the descriptors of the helminth component communities of D. latifrons through the sampling periods. All examined D. latifrons specimens were infected, with 9.7% parasitized by only 1 species, and 90.3% having mixed infections. Of the total number of host specimens, 33.8% were infected with 3 parasite species, and 14.5% had up to 4 parasite species. Based on cumulative species curves, the sample sizes used were sufficient to recover all dominant parasite species, and the number of missing rare species in some sampling periods was minimal (1–2 species) (data not shown).

Between 6 and 8 parasite species were collected in each sampling, though the number did not vary significantly between periods (Kruskal–Wallis $H = 4.91, P > 0.05$). Total number of individual parasites ranged from 1,342 (July–September) to 13,493 (April–June), and varied significantly between periods (Kruskal–Wallis $H = 9.03, P < 0.05$), with the highest total number of individuals collected during April–June (dry season) (Table II). The digenean E. leopoldinae numerically dominated during the 2 dry season periods, whereas the acanthocephalan N. golvari dominated in the 2 rainy periods (Table II). The Shannon–Wiener diversity index values ranged from 0.77 to 1.77 (Table II). Qualitative similarity within each sampling period ranged from 0.72 in July–September to 0.96 in April–June, and quantitative similarity ranged from 33.31 (October–December) to 61.39% (January–March). The only significant difference in community similarity was observed between periods at the quantitative level (Kruskal–Wallis $H = 9.12, P < 0.05$), and was higher in the 2 dry season sampling periods (Table II).

Infracommunities

The mean number of parasite species ranged from 2.1 ± 1.0 (July–September) to 2.8 ± 1.1 (April–June period) and the mean number of parasite individuals ranged from 36.5 ± 48.9 (July–September) to 375.3 ± 360.3 (April–June period) (Table II). Brillouin diversity index values varied from 0.53 ± 0.39 (April–June) to 0.69 ± 0.40 (October–December).

All hosts were adults, though body size differed significantly between sampling periods (1-way ANOVA $F = 7.97, P < 0.05$). The largest hosts were collected in July–September (22.96 ± 1.73), and the smallest in January–March (21.46 ± 1.97). Overall, host length was positively correlated with mean number of individual parasites ($r = 0.368, P < 0.05, n = 185$). Size class analysis indicated that the lowest values for mean number of parasite species and individual parasites were ob-
served in the smallest host-size class in all sampling periods (Fig. 2).

Mean number of parasite species was generally higher in the April–June (dry) and January–March (dry) periods (1-way ANCOVA $F = 4.37, P < 0.01$), and mean number of individuals was also significantly higher during these periods (ANCOVA $F = 22.84, P < 0.0001$). No significant differences were observed in the mean Brillouin diversity index values between sampling periods (1-way ANCOVA $F = 1.29, P > 0.05$).

**Testing for nestedness**

Table III shows the values of nestedness for each sampling period in Tres Palos Lagoon. Nestedness occurred during all 4 periods, but only its intensity (lowest value $T^*$) was highest in July–September (rainy season) ($T^* = 15.82^\circ$). Species composition remained almost unchanged in all 4 periods, although the rank assigned to each species inside the packed component community matrix did vary between periods. Only the acanthocephalan *N. golvani* was top ranked in all 4 periods, and this was also the only parasite species considered dominant in all 4 sampling periods (Table III).

The infracommunity index values (ICI) indicated that 4 parasite species had a higher number of double or multiple co-occurrences with other parasite species (ICI > 0.20) in all 4 periods, although this only occurred consistently with *N. golvani*. This parasite species exhibited the highest number of co-occurrences in July–September (ICI = 0.448), when the maximum nestedness value was observed (Table III).

**DISCUSSION**

The results indicate that even though species composition, richness, and diversity of the parasite communities were rather similar throughout the study period, some parasite species presented temporal changes in their infection levels. Two biotic factors were considered as possible causes of the temporal variations observed, i.e., alterations in the recruitment processes of some allogenic parasite species as result of environmental changes originated by the dry and rainy seasons that influence the availability of intermediary hosts, and changes in the feeding and reproductive behavior of *D. Zatifrons* that influence the recruitment of intestinal autogenic species. The temporal variation pattern observed depends, therefore, on how the parasite invades the host (Chubb, 1979), i.e., its form of transmission.

Three of the 8 metazoan parasite species identified in *D. latifrons* are new geographical records for Tres Palos Lagoon, Guerrero, Mexico, i.e., *E. leopoldinae*, *A. (P.) longa*, and *P. cochlearii*. The remaining 5 have been reported previously from this location (Garrido-Olvera et al., 2004). Of the identified species, only 3 were autogenic (mature in aquatic hosts); i.e., *Saccococceloides* sp. and *N. golvani* mature in *D. latifrons*, and *P. panamense* matures in *Hexanematichthys guatemalenensis*. The other 5 species all mature in the aquatic birds, *Casmerodius albus* (great egret) and *Phalacrocorax olivaceus* (Neotropic cormorant), that inhabit Tres Palos Lagoon (Violante-González, 2006) and were thus classified as allogenic, i.e., *C. complanatum*, *E. leopoldinae*, *A. (P.) longa*, *P. cochlearii*, and *Contracaecum* sp.

Five of the parasite species were identified exclusively as larval stages, indicating that *D. latifrons* mainly acts as an in-
termediate host to helminth parasite species in Tres Palos Lagoon (Zander et al., 1999). All 8 identified parasite species were generalists. The digeneans were clearly the numerically dominant group, representing 62.5% of all helminth species in the D. latifrons component community. High digenean abundance in coastal lagoons may be linked to the high temperatures and high productivity of eutrophic systems. These factors favor development of large herbivorous snails and crustacean populations, which act as intermediate hosts for parasites such as digeneans and acanthocephalans (Valtonen et al., 1997; Zander et al., 1999). Moreover, the shallow depth of coastal lagoons and the detritophagous habits of D. latifrons (Yañez-Arancibia and Díaz-González, 1977) place it in vicinity of the snails that act as primary intermediate hosts for digenean species.

These results coincide with others reported for eutrophic estuarine systems in other latitudes, in which generalist parasites (mainly digeneans) dominate and specialists are a minor element of the parasite communities (Zander, 1998; Zander and Reimer, 2002).

Species composition of the D. latifrons component community exhibited a clear freshwater influence as 6 of the 8 identified species are of freshwater origin. This may be linked to the lagoon’s oligohaline condition (4.30 ± 0.85%), the result of its greater freshwater contribution from rainfall and its temporally limited connection with the sea during the rainy season (Violante-González, 2006). A similar situation has been reported for low-salinity (0.5–3.5%), temperate brackish environments in which marine parasite species represented only 12.69% of total recovered species from 31 host fish species, and freshwater parasite species dominated community composition (Valtonen et al., 2001).

At least 2 of the most abundant species (E. leopoldinae and N. golvani) exhibited clear temporal variation in mean abundance between climatic seasons, with both species reaching their highest mean abundance during the dry season. Currently, data for Tres Palos Lagoon are insufficient to make a definitive explanation of this variation. In the case of E. leopoldinae, however, its higher mean abundance may be due to greater recruitment of infective stages ( cercariae) during this period (Moravec et al., 2002). Tres Palos Lagoon experiences relative environmental stability during the dry season (December–May) (Violante-González, 2006). This can favor the transmission of parasites such as E. leopoldinae, through higher availability of intermediate hosts (snails), as well as of other actively transmitted digeneans such as C. complanatum and P. panamense, which both had high mean abundance during at least 1 dry season (Table I).

In contrast, during the rainy season (July to November) environmental conditions in the lagoon become unstable in response to the addition of high volumes of organic matter transported by the contiguous Sabana River and other tributaries. This causes a high mortality of benthonic invertebrates, such as snails, which are buried by silt (Violante-González, 2006). However, the addition of organic matter to the lagoon also enhances productivity during the rainy season (Banderas and González, 2000), favoring the transmission process of other helminths that use crustaceans as primary hosts, such as the allogenic cestode P. cochlearii, which was most abundant during the most intense rainy period (October–December, Table I).

A similar dynamic was reported by Salgado-Maldonado (1993) in a study of the parasite communities of the cichlid Cichlasoma urophthalmus in Celestun Lagoon in southeast Mexico. Although the transmission process was continuous year-round, it registered a drastic fall in the early rainy season. This dynamic may explain, therefore, the temporal variation among allogenic parasite species, but is not applicable to intestinal autogenic species.

In this sense, the higher abundance of the acanthocephalan N. golvani in the dry season may be linked to higher levels of feeding activity by D. latifrons during this period. During its July–October (rainy season) reproductive period (data not shown), D. latifrons exhibits reduced feeding activity, which may explain the lower N. golvani mean abundance recorded during this season (Table I). In particular, the ostracods, crustaceans considered to be the first intermediate host of the acanthocephalan N. golvani (Salgado-Maldonado, 1993), were scarce in the stomachs of D. latifrons during the rainy season.

Temporal variation in infection levels of the acanthocephalan Neoechinorhynchus agilis was also related to reproductive aspects of its host Liza abu (Mhaisen et al., 1988). Indeed, many studies have shown that seasonal variation in the diet and amount of food ingested by hosts is clearly linked to parasite population dynamics and structure of parasite communities (Esch et al., 1988; Zander and Kesting, 1998; Fiorillo and Font, 1992)
Abiotic factors, such as temperature and salinity, were not highly variable during the study period, and were therefore not considered important in determining helminth parasite community structure. This also coincides with Zander and Kesting (1998), who reported that salinity was not an important abiotic factor for the presence of fish parasite species in the brackish Baltic Sea, whereas the degree of eutrophication appeared to have more influence.

The parasite communities in this lagoon had low numbers of species and diversity at both studied levels (component and infracommmunity. Table II), a scenario that is also typical in many parasite communities of gobies (Gobiidae: Teleostei) in eutrophic brackish water in temperate latitudes (Zander et al., 1999).

A possible explanation for the low richness of parasite species in this host may be related to the low-complexity food-web structure in this lagoon; food-web structure is known to affect parasite species richness and diversity in many freshwater systems (Carney and Dick, 2000; Marcogliese, 2001). Zooplankton are not very diverse, and the macrobenthos, including molluscs, are scarce, being represented by just 6 species (Stuardo and Villarroel, 1976). The impoverished biodiversity in this system clearly restricts intermediate host availability in the food web, reducing the opportunity for transmission of higher numbers of parasite species (Carney and Dick, 2000; Marcogliese, 2001).

At the component community level, both community composition and number of parasite species were similar throughout the study period. Total number of parasite individuals, however, exhibited clear seasonal variation, being significantly higher in both dry seasons (Table II). This was attributed to the higher abundance of *E. leopoldinae* and *N. golvani* during these periods. The high qualitative similarity between sampling periods at this level (0.72–0.96), suggests that the community has high predictability year-round. The higher qualitative similarity resulted from the constant presence of a group of 4 abundant and frequent species, i.e., *E. leopoldinae*, *Saccocoeloides* sp., *P. cochlearii*, and *N. golvani*.

The positive significant correlation of mean number of parasite individuals with total host length, and the analysis considering the 3 size classes, indicate that larger hosts harbored more parasite individuals and more parasite species than smaller ones (Fig. 2). This is attributed to the facts that larger, i.e., older, fish (1) offer larger target areas for parasites transmitted by cercariae, (2) ingest larger quantities of food, and (3) have had more time to accumulate parasites than smaller, i.e., younger, fish (Zander and Kesting, 1998; Fiorillo and Font, 1999; Poulin, 2000; Bush et al., 2003; Fellis and Esch, 2004; Zander, 2004).

Although host size may have a significant effect on infracommmunity structuring, it did not affect the present results. For example, the largest hosts were collected in July–September (rainy season), but the mean number of parasite species and individuals was lower during this period than in the dry season (Table II). When the influence of host size was removed statistically from the analyses on temporal variation between climatic seasons, the nature of the observed temporal patterns in the parasite infections dynamics infers a biological effect, and is
not a result of changes in host demographics (Fiorillo and Font, 1999).

The presence of nestedness in all the sampling periods suggests that a certain degree of structure was apparent in infracomunity species composition over time; i.e., a certain order existed in the species composition. This means that the parasite species with high prevalence were present in all infracomunities class (rich or poor), whereas the rare ones were mainly in infracomunities rich in species (Poulin and Valtonen, 2001).

Several processes have been suggested as generators of nestedness within infracomunities, including passive sampling and sequential colonization of parasite species (Carney and Dick, 2000). The passive sampling hypothesis, based on probability, predicts that common species will occur in many hosts and rare species in fewer hosts, producing a nested pattern. The nestedness observed here may have been caused by the high stability in species composition, which is reflected in the high qualitative similarity values (0.72–0.96, Table II). However, because some of the dominant parasite species occurred in a higher number of double or multiple co-occurrences with other parasite species (CI > 0.20, Table III) and were more abundant in different seasons, a sequential colonization process may explain the variation in nestedness intensity observed during the study period.

Nestedness can, therefore, be considered a common feature for the parasite infracomunity composition of D. laitifrons in Tres Palos Lagoon, which coincides with reports for other fish species from other locations (Carney and Dick, 2000; Poulin and Valtonen, 2001; Valtonen et al., 2001; Vidal-Martinez and Poulin, 2003; Norton et al., 2004). Thus, temporal variation in the recruitment process of some of the main parasite species can be considered as an important influence on nestedness intensity.

Clearly, more research is needed on temporal variation in the parasite communities of this and other hosts over longer periods (Zander, 2005) to understand better the mechanisms that influence variation in eutrophic tropical brackish water parasite communities.

ACKNOWLEDGMENTS

The authors wish to thank the students of the Marine Ecology Academic Unit, UAG, for their help in the field and laboratory. We are also grateful to Guillermo Salgado-Maldonado and David Osorio-Sarabia for their assistance in identifying some of the parasite species. We thank 2 anonymous reviewers whose extensive and thoughtful comments substantially improved the manuscript. We also appreciate the valuable suggestions and comments of Dr. Cameron Goater. This research was financed by the Sistema de Investigación Benito Juarez (SIBEJ).

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A NEW MITE SUPERFAMILY CLOACAROIDEA AND ITS POSITION WITHIN THE PROSTIGMATA (ACARIFORMES)

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ABSTRACT: The external morphology of 2 closely related mite families, Cloacaridae and Epimyodicidae, comprising highly specialized endoparasites of vertebrates, is analyzed. These mites exhibit strong regression of many structures ancestrally present in other Prostigmata as a consequence of their endoparasitic mode of life. The relationships of these 2 families with other taxa in the infraorder Eleutherengona are still not clear. Our reinterpretation of the chelicerae as unfused precludes inclusion of this lineage in the Cheyletoidea as proposed previously. A new superfamily, Cloacaroidea superfam. nov., incertae sedis, within the infraorder Eleutherengona is established for these 2 families, and their host–parasite relationships are briefly discussed. A new cloacarid species, Caminacarus dawsoni n. sp., from Graptemys pseudogeographica (Testudines: Emydidae) from the United States is also described.

Cloacaridae was established by Camin et al. (1967) for Cloacarus Camin, 1967, with 2 species, Cloacarus faini Camin and Singer, 1967, and Cloacarus beeri Camin and Oliver, 1967, collected from the cloacas of the North American turtles Chelydra serpentina (Linnaeus, 1758) (Chelydridae) and Chrysemys picta (Schneider, 1783) (Emydidae), respectively. These mites show strongly reduced external morphological features that are probably related to their endoparasitic mode of life. The following year, Fain (1968) described 3 more cloacarid genera, i.e., Caminacarus Fain, 1968, containing 6 species, the monobasic Enyduracarus Fain, 1968, and Theodoracarus Fain, 1968. All specimens were collected from the cloacas of turtles from various regions and belong to both extant turtle suborders, Cryptodira and Pleurodira (Gaffney and Meylan, 1988). Only 2 papers containing descriptions of new cloacarids from turtles have been subsequently published, i.e., 2 species of Caminacarus and the monobasic Chelonacarus Pence and Wright, 1998, were described by Pence and Casto (1975) and Pence and Wright (1998), respectively. Fain et al. (1982) significantly extended the diagnosis of the Cloacaridae when they transferred Epimyodex Fain and Orts, 1969, from the Demodicidae to the Cloacaridae and established the monobasic Epimyodicinae. Epimyodex includes 4 species of mites inhabiting the subcuctaneous tissues of small mammals (Fain and Orts, 1969; Fain et al., 1982; Fain and Bochkov, 2001). Finally, Fain and Smiley (1989) described a new cloacarid genus and species, Pneumophagus babonis Fain and Smiley, 1989, from the lungs of the great horned owl Bubo virginianus (Gmelin, 1788) (Strigidae: Strigiformes). This species is morphologically distant from all other cloacarids and was placed by Fain and Smiley (1989) in the monobasic Pneumophaginae. Bochkov (2002) elevated the Epimyodicinae to the familial rank, but stressed the sister relationship between Cloacaridae and Epimyodicidae.

According to the traditional point of view, the Cloacaridae (and also its sister family; see Bochkov, 2002) has been referred to the Cheyletoidea (Camin et al., 1967; Fain, 1968; Kethley, 1970, 1982; Krantz, 1978; Bochkov, 2002). Camin et al. (1967) included Cloacaridae in the Cheyletoidea based primarily on the dorsal position of the male aedeagus and the fused genital and anal openings in these mites. In fact, the fused anal–genital opening characterizes many phylogenetically distant eleutherengone taxa. The dorsal position of the aedeagus also is not an exclusive character of the parasitic cheyletoids as was believed by Camin et al. (1967), but is widely distributed among parasitic Eleutherengona, i.e., Myobiidae, some Pterygosomatidae, Heterostigmata, and Tetranychoida. In addition, mites of the Epimyodicidae have clearly separated chelicerae, whereas in all cheyletoids, the chelicerae are fused into a stylophone (Kethley, 1970, 1982; Krantz, 1978; Bochkov, 2002).

In this paper, we will clarify the position of the Cloacaridae–Epimyodicidae lineage within the Prostigmata based on comparative analysis of their external morphology. The host–parasite relationships of these mites are briefly reviewed, and a new species from G. pseudogeographica from the United States is described.

MATERIALS AND METHODS

The external morphology of mites belonging to 5 of the 7 genera known in the Cloacaridae and Epimyodicidae was studied (Table I). The specimens examined are housed in the Institut royal des Sciences naturelles de Belgique, Brussels, Belgium (IRSNB); Natural History Museum, University of Kansas, Lawrence, Kansas (NRMKU); The Acarology Laboratory, Ohio State University, Columbus, Ohio (OSAL); University of Michigan Museum of Zoology, Ann Arbor, Michigan (UMMZ); National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM); and the Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia (ZISP). Data for the monotypic Chelonacarus and Theodoracarus were derived from the original descriptions (Fain, 1968; Pence and Wright, 1998). Drawings were made with a phase contrast Zeiss microscope with a camera lucida. In the description, all measurements are given in μm. The composition of the acarine infraorder Eleutherengona follows the tree diagram, suggesting relationships among higher taxa of Prostigmata presented by Kethley (in Norton et al., 1993).

DESCRIPTION

The external morphology of Cloacaridae and Epimyodicidae

Gnathosoma: In cloacarids and epimyodicids, gnathosoma positioned ventrally. In Epimyodicidae, subcapitulum well developed (Fig. 1C) and gnathosoma positioned parallel to idiosomal axis. Posterior margin of subcapitulum more heavily sclerotized compared with the remainder of gnathosoma. Palps strongly reduced; each represented by short, articulated segment bearing 2–3 claw-like setae terminally. Chelicerae very small, separated from each other and devoid of movable and, probably, fixed digits. Basal parts of chelicerae bulb-like, each with narrow, anteriorly directed projection dentate apically. Subcapitular setae absent. In Cloacarinae, gnathosoma strongly reduced, oriented perpendicular to idiosomal axis, retracted into idiosoma, and surrounded ventrally by idiosomal wall (Fig. 1A). Remnants of posterior subcapitulum repre-
Table 1. Materials examined (for *Caminacarus dawsoni*: see taxonomic summary under the species description).

<table>
<thead>
<tr>
<th>Mite species</th>
<th>Specimens examined</th>
<th>Host species and family</th>
<th>Locality and collector</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Emyduracarus</em> sp. 1</td>
<td>4 ♂ (UMMZ)</td>
<td><em>Chelodemia longicollis</em> (Shaw) (Chelidae)</td>
<td>AUSTRALIA: New South Wales, Armidale, Rockvale Rd., 6.VII.1982, M. Georgi</td>
</tr>
<tr>
<td><em>Emyduracarus</em> sp. 2</td>
<td>2 ♂ (UMMZ)</td>
<td><em>Chelodinia expansa</em> Gray (Chelidae)</td>
<td>AUSTRALIA: Queensland, Gracemere Lagoon, IV.1982, M. Georgi</td>
</tr>
<tr>
<td><em>Pneumophagus bubonis</em> Fain and Smiley, 1989</td>
<td>♀ holotype, 1♂ paratype (USNM), 1♀ paratype (ZISP)</td>
<td><em>Bubo virginianus</em> (Gmelin) (Strigidae)</td>
<td>U.S.: Michigan, East Lansing, 1.XII.1986, T. Schillhorn van Veen</td>
</tr>
<tr>
<td><em>Epimyodex talpae</em> Fain and Orts, 1969</td>
<td>1♀, 1♂ paratypes (ZISP)</td>
<td><em>Talpa europaea</em> L. (Talpidae)</td>
<td>BELGIUM: Walloon Reg., Lille, 30.VI.1968, A. Fain</td>
</tr>
<tr>
<td><em>Epimyodex microti</em> Fain et al., 1982</td>
<td>1♀, 1♂, 1 nymph paratype (ZISP)</td>
<td><em>Microtus arvalis</em> (Pallas)</td>
<td>NETHERLANDS: Nijmegen, 1.1971, F. S. Lukoschus</td>
</tr>
</tbody>
</table>

In males of *P. bubonis* (Pneumophaginae), gnathosoma completely absent; in females, gnathosoma similar to that of Cloacarinae (Fig. 1B). Gnathosomal appendages smooth and partly covered by a pair of idiosomal membranes. Gnathosoma of pneumophagines distinguished from cloacarine gnathosoma by absence of posterior remnants of subcapitulum (subcapitular ring) and internal median apodemes.

Idiosoma: In Cloacaridae, idiosoma elliptical and distinctly flattened dorso-ventrally; in Epimyodicidae, less flattened. In Epimyodicidae, the anterior margin of idiosoma attenuated into epistome or gnathosomal hood. Idiosomal cuticle generally soft. Propodonotal shield present and

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Figure 1. Female gnathosoma. (A) *C. dawsoni* n. sp. (B) *P. bubonis*. (C) *Epimyodex talpae*. Abbreviations: a.m., antero-median sclerite; ch., chelicera; i.a., internal median apodeme of gnathosoma; p., palp; s.p., sigmoid piece.
occupies most of the propodonotal surface, distinctly ornamented in Cloacaridae, and weakly sclerotized and devoid of ornamentation in Pneumophagidae. In Epimyodicidae, shield weakly sclerotized and devoid of ornamentation, with the exception of some ring-like structures in *Epimyodes microti* Fain et al., 1982, and *Epimyodes sorics* Fain and Bochkov, 2001. In live *Cloacarus*, pair of subintegmental red pigment spots observed on propodonotal shield (Camin et al., 1967). Spots not pigmented in ethanol-preserved specimens; in males, spots clearly marked by distinct round membranous areas, but rather indistinct in females and immatures. Same areas also present in *Emyduracarus*.

Opisthosoma well developed in Epimyodicidae and longer than metapodosoma; opisthosoma of Cloacaridae, especially Cloacarinae, distinctly shorter than metapodosoma (Fig. 5). Opisthosoma indistinctly striated in Epimyodicidae; opisthosomal striations absent in Cloacaridae. In females of both families, anal and genital openings fused to each other, forming a single opening (Fig. 5A). Among cloacarine genera, this opening situated ventrally in *Emyduracarus*, terminally in *Chelonacarus* and *Cloacarus*, and dorsally in *Caminacarus* and *Theodoracarus*. In females of latter 2 genera, short sclerotized fold (genital sclerite) situated immediately above the genital-anal opening. In females of *Chelonacarus* and *Cloacarus*, lateral walls of the genital-anal opening elongated and modified into a pair of short, finger-like projections (Fig. 4B). In *Chelonacarus*, the genital-anal opening covered ventrally by a pair of triangular sclerotized folds. In Pneumophaginae and Epimyodicidae, the genital-anal opening situated dorsally and terminally, respectively, and devoid of any projections and folds. In known males of both families, the aedeagus present and the genital opening situated dorsally on the anterior part of the body.

Coxae of cloacarids and epimyodicids completely fused with the ventral surface of the idiosoma; only their anterior margins represented by distinct coxal apodemes (Fig. 5B). Coxal apodemes very elongated, longer than half the idiosomal width. In adult Epimyodicidae and Cloacaridae, coxal apodemes I fused, forming sterno. Coxal apodemes I separated from each other in immature Epimyodicidae. In Cloacarinae, sternum with internal median keel, at least in the anterior half. Keel continues anteriorly as median apodeme with bifurcating apex. Apices fused anteriorly with subcapitular remnants and associated with sclerites, interpreted here as sigmoid pieces (= capillary apophyses) (Fig. 1A). Keel sigmoid pieces absent in Epimyodicidae. Sigmoid pieces also absent in *Pneumogamus*, but in females, anterior parts of coxal apodemes I fused with anterior part of subcapitular remnants; in males, gnathosoma entirely absent. In *Theodoracarus* (Cloacaridae), apodemes II fused with apodemes III, and apodemes III fused in turn with apodemes IV on each side of the body, closing coxal fields II and III. In *Chelonacarus*, apodemes II fused to each other by their distal ends. In other cloacarids, distal ends of coxal apodemes II–IV are free, but close situated to each other in apodemal pairs II and III. In Epimyodicidae, distal ends of the legs are free; in *Caminacarus and Theodoracarus*, coxal apodemes IV broadly rounded.

In Cloacaridae and Epimyodicidae, proximal parts of coxal apodemes I–IV with 2 condyles at trochanteral articulation. Dorsal condyle shifted onto ventral side of idiosoma due to coxal flattening in these mites. Ventral condyles distinctly smaller than dorsal condyles (Fig. 4A). Distance between the proximal ends and ventral condyles of coxal apodemes short; proximal apodemal apices well recognizable in Epimyodicidae. In cloacarines, the apices of the distal ends not discernible within sclerotization of ventro-marginal frame bordering the lateral margins of the idiosoma. In *Caminacarus*, *Cloacarus*, and *Chelonacarus*, distance between proximal ends of coxal apodemes and ventral condyles short; in *Emyduracarus and Theodoracarus*, this distance distinctly longer and the proximal ends directed anteriorly. In *Pneumogamus*, proximal ends of coxal apodemes directed anteriorly and fused with proximal parts of next anterior apodemes, forming sclerotized framework marking lateral margins of idiosoma.

In mites of both families, idiosomal setae strongly reduced in size and number. One to four pairs of setal alveoli present on propodonotum; opisthosoma bearing 1–3 pairs of setae. In females of Epimyodicidae, maximum number of propodonotal setal alveoli 4 pairs, probably vi, ve, sc, and 4G; in *Cloacarus* 4 pairs, 2 pairs in *Chelonacarus*; and in *E. sorics*. In *E. microti*, 3 pairs of propodonotal alveoli present, with setae sce probably reduced; in *Epimyodes crociareus* Fain and Lukoschus, and *Rosmalen*, 1982, only 1 pair of propodonotal alveoli, probably representing setae vi, present. Female opisthosoma of epimyodicids bearing pair of well-developed terminal papillae with setae flankning anal-genital opening. Setation of epimyodicids in males more reduced than in females. One pair of propodonotal alveoli, probably representing setae sce, observable on idiosoma. In cloacarines, female propodonotum with alveoli of 2 pairs (Chelonacarus, Cloacarus, and Emyduracarus) or only 1 pair (Caminacaris and Theodoracarus) of setae. Propodonotal alveoli not observed in *Emyduracarus australis* Fain, 1968, the only described species in this genus. Two pairs of propodonotal alveoli in an undescribed species of *Emyduracarus from Australian turtle, Chelonia longicollis* (see Table 1); otherwise, very close to previous species. Alveoli probably present in *E. australis*, but indistinct because of extensive ornamentation of propodonotal shield. Opisthosomal setation of cloacarine females richer than in epimyodicids bearing 2–3 pairs of setae. In females of *Chelonacarus* and *Cloacarus*, 1 pair of fleshy setae situated on lobes of anal–genital opening, and 2 other pairs of fleshy setae situated dorsally and ventro-laterally on posterior opisthosomal margin, respectively. In most females of *Caminacaris and Theodoracarus*, 1–2 pairs of indistinct, papilliform setae situated on posterior end of opisthosoma, dorsally and terminally, respectively, and 1 pair of well-developed, fleshy papilliform setae, similar to those in epimyodicids, situated on coxal fields IV. In females of *Emyduracarus*, opisthosoma with 2 pairs of papilliform setae, 1 pair terminal and 1 pair flankning, ventrally situated at anal–genital opening. Males known only in 3 cloacarine genera, i.e., *Chelonacarus, Cloacarus, and Theodoracarus*. Se­tation of propodonotum similar to that observed in females; opisthosoma with single pair of terminal, fleshy setae. In females of *Pneumogamus*, propodonotum with 1 pair of alveoli, opisthosoma with 3 pairs of short, spine-like setae situated dorsally, terminally, and ventrally. Pair of alveoli at anal–genital opening dorsally, in males, only 1 pair of short, spine-like setae present, situated dorsally on opisthosoma.

**Legs**: Legs I–IV arising ventro-laterally and relatively slender in Epimyodicidae, consisting of 5 freely articulated, weakly sclerotized segments. Trochanters of all legs distinctly larger than other leg segments. Condyles of leg segments indistinct. Trochanters, femora, and genua of all legs without setae. All tibiae with 1 dorsal and 1 ventral spine-like setae. Tarsi of all legs devoid of empodium, but with a pair of distinctly curved, simple claws and 3 spine-like setae: 1 dorsal, 1 paraxial, and 1 antaxial. In addition, *E. microti* and *E. sorics* with apical microseta on all tarsi.

All cloacarid legs with 3 articulated segments, very short; rudimentary segment inserted proximal to apical segment. Two basal leg segments devoid of setae. Trochanters of cloacarid largest leg segments. Posterior margins of all trochanters with large triangular projection articulated with respective coxal apodemes. In most cloacarids, reduced tibiae with 1 spine-like seta, but 2 setae in *Emyduracarus* tibiae (Fig. 4G); devoid of setae in *Pneumogamus*, except for 1 seta on tibia I (Figs. 3A–C). Tarsi broadly rounded in cloacarines (Figs. 2A–D, 3A–D); tarsus II with 9 setae (Fig. 3A–D); tarsus III with 7 setae (Figs. 3A–D). Claws absent on all tarsi in Cloacaridae. In Cloacarinae, tarsi I and III each with 8 setae (in *Caminacaris chrysemys* Pence and Casto, 1975, tarsi III with 9 setae); tarsi II, 9 setae; and tarsi IV, 7 setae. Short globose solenidion on tarsi I–III ventro-terminally. Tarsi I–IV bearing 5, 9, 12, 12 setae, respectively, in *Pneumogamus*; solenidion absent on all tarsi.

**Immature stages**: Only larval and 1 nymphal stage known in *Epimyodicidae*. Larval gnathosoma indistinct and probably absent or strongly reduced. Coxal apodemes I separated medially. Anal opening and idiosomal setae absent. Legs I–III consisting of 3 freely articulated segments, tibiotarsus, femurogenou, and trochanter. Pretarsal claws absent, and tibiotarsi I and II with 5 spine-like setae, tibiotarsi III–IV with 4 spine-like setae. Nymph differs from larva by presence of legs IV and gnathosoma resembling that of adults. Larva of Cloacaridae unknown. Nymphs known for only 4 species, i.e., *Caminacaris pelomedusa* Fain, 1968, *C. fanti, C. beeri* (known from nymphs only), and *E. australis* (Camin et al., 1967; Fain, 1968).

**Remarks**

Most authors following Camin et al. (1967) interpreted the gnathosomal appendages of cloacarids as the palps (Fain, 1968; Pence and Casto, 1968; Pence and Wright, 1998; Bochkov, 2002). Camin et al. (1967) depicted 2 antagonistic muscles inserted on the gnathosomal appendages of cloacarids. In Prostigmata, both the palps and the che­liceræ bear the insertions of 2 antagonistic muscles (Evans, 1992);
therefore, this character is not useful in clarifying the homology of the appendages. Camin et al. (1967) also mentioned some structural similarity between the gnathosomal appendages of Cloacaridae and the palps of Myobiidae, i.e., "... an apparent tendency in the Myobiidae toward reduction of the pedipalps and the hypertrophy of the apical projections of their terminal segments. The fang-like structures of the cloaca mite gnathosoma probably are extremely hypertrophied homologues of the palpal projections of the Myobiidae." Subsequently, Bochkov and Ląbrzycka (2003) studied the external morphology of the gnathosoma in Myobia spp. von Heyden, 1826, in detail, using both scanning electron

Figure 2. C. dawsoni n. sp, legs of female. (A–D) Tibia and tarsus of legs I–IV dorsally. (E, F) Legs I and II ventrally. (G, H) Tibia and tarsus of legs III and IV ventrally. Abbreviations: f.g., femorogenu; p.t., projection of trochanter; ta., tarsus; ti., tibia; tr., trochanter.

Figure 3. Details of cloacarid legs. (A–C) P. bubonis, female. (A) Femorogenu-tarsus I ventrally. (B) Femorogenu-tarsus II dorsally. (C) Leg II ventrally. (D–G) Caminacarus chrysemys, female. (D) Tibia and tarsus III dorsally. (E) Same ventrally. (F) Tarsus IV dorsally. (G) Same ventrally. Scale bars: 25 μm (A–C); 50 μm (D–G).
microscopy and light microscope examination. It appeared that myobiid mites have strongly reduced, 2-segmented palps, with each palpal segment bearing a seta. The basal segment, not the terminal segment as Camin et al. (1967) believed, bears the terminal hook-like projection and covers dorsally the small apical segment of the palp. We did not observe any actual similarities between these gnathosomal appendages in representatives of Cloacaridae and Myobiidae. The myobiid palps are 2-segmented and strongly reduced, with a strongly curved hook-like projection, and weakly sclerotized. In contrast, the appendages of the cloacarid gnathosoma are without any vestiges of segmentation, more or less straight, relatively large, and heavily sclerotized. They are situated very close to each other and possess a complicated basal structure. These appendages are very similar to the chelicerae in mites of the Epimyodicidae. They also resemble the chelicerae of some other Pros-tigmata, e.g., Pterygosomatidae, in possessing separate cheliceral bases devoid of setae and movable digits. Thus, we regard the gnathosomal appendages of cloacarid mites as the chelicerae, which have lost their digits.

Most authors (Camin et al., 1967; Fain, 1968; Bochkov, 2002) interpret the rudimentary segment of cloacarid legs as the tibia and the next more basal segment as a completely fused femorogeni. This homology is questionable. The apical segment, bearing numerous setae, could represent a completely fused tibia and tarsus, and the reduced segment, the genu, partially fused with the femur. Here, we follow the traditional view, hoping that future morphological studies will clarify this situation.

Most authors (Camin et al., 1967; Fain, 1968; Pence and Wright, 1998) have homologized a pair of the long spines, situated posteroventrally on the cloacarine tarsi, with pretarsal claws (Figs. 2E–H, 3E, G, 4D, G). However, according to Fain et al. (1982), the true tarsal claws are absent in cloacarids. We agree with the latter authors that the ventral spine-like structures on the cloacarine tarsi are actually strongly elongated setae and that the true claws, as well as the pretarsi, are absent in these mites. The “setal” hypothesis is supported by the following direct and indirect evidence. These structures are straight spines and quite comparable in form with many other tarsal setae, i.e., their bases are not different from the bases of the other tarsal setae, and there are no internal structures associated with these spines. Finally, in Pneumophagus, which possesses the maximal set of tarsal setae, these spines

**Figure 4.** Details of Cloacaridae. (A–D) Cloacarus faini, female. (A) Joint of trochanter and coxa II. (B) Vulvar walls. (C) Tibia and tarsus I dorsally. (D) Same ventrally. (E–G) Emyduracarus sp. 1. (E) Chelicera, lateral view. (F) Tarsus I dorsally. (G) Leg I ventrally. Abbreviations: d.c., dorsal condyle; g.c., glenoid cavity of trochanter; v.c., ventral condyle.
are arranged in a row with the other setae and are not distinguishable from them (Figs. 3A-C). We suggest that the elongation and ventral position of these setae are cloacarine adaptations for moving in the subdermal tissues of their hosts.

Camin et al. (1967) treated nymphs of *C. faini* as protonymphs and those of *C. beeri* as deutonymphs strictly because nymphs of the former species were distinctly smaller than nymphs of the latter species. We think that the difference in size between nymphs of these 2 species could actually represent simple interspecific variability. Thus, in both families, only 1 nymphal stage has been conclusively demonstrated at present. Nymphs of *Cloacarus*, which were available for our study, differ from females only in the absence of 1 pair of opisthonotal setae flanking the anal-genital opening.

**Caminacarus dawsoni** n. sp. (Figs. 1A, 2, 5)

Female (holotype): Body 470 long (448–470 in 9 paratypes), 300 wide (280–320). Subcapitular ring distinctly sclerotized, closed in posterior part. Chelicerae densely dentate, 65 long (60–65) (Fig. 1A). Propodonotal shield strongly reticulate, 350 long (320–365). Anterior margin of propodonotal shield deeply concave. Lateral elongation of propodonotal shield distinctly developed, 55 long (50–60). Posterior elongation of propodonotal shield reaching genital sclerite. Genital sclerite distinctly sclerotized, with convex posterior margin. Anal-genital opening about 35 long, with moderately sclerotized walls (Fig SA). Sternum 85 long (85–90). Length of coxal apodemes II–IV: II 150 (145–155), III 145(140–150), and IV 140 (140–150). Two pairs of fleshy opisthosal setae present, 1 pair of very short, almost indistinct, setae terminally and 1 pair of well-developed papilliform setae 9 long (9–11) ventrally on coxal fields IV (Fig 5B). Legs I–IV, excluding posterior projection of trochanters, 75–90 long. Setation of tibiotarsi I–IV, as in Figure 2. Tarsi III with 8 setae and 1 solenidion. Ventro-posterior spines of tarsi I–IV about 18 long.

Male and immature instars unknown.

**Taxonomic summary**

**Type host:** *G. pseudogeographica* (Gray, 1831) (Testudines: Emydidae).

**Type locality:** Ithaca, Tompkins Co., New York, 6 February 1979 (captive animal obtained from a biological supply company), mites collected by B. M. O’Connor. The natural distribution of this host species ranges throughout the Mississippi–Missouri River drainages and their western tributaries (Iverson, 1992).

**Site:** Cloaca.

**Type specimens:** Female holotype (BMOC 79-0206-001, 1) and 6 female paratypes (UMMZ), 1 female paratype each (OSAL, USNM, ZISP).

**Etymology:** The new species is named for Dr. William R. Dawson, director emeritus of the University of Michigan Museum of Zoology, in recognition of his contributions in the field of physiological ecology.

**Remarks**

This new species is similar to other North American species described from turtles of the Emidae, i.e., *Caminacarus deirochelys* Fain, 1968, *C. chrysemys* Pence and Casto, 1975, and *Caminacarus terrapene* Pence and Casto, 1975, in possessing lateral projections of the propodonotal shield. It differs from all of these species by the larger chelicerae, 60–65 long (42 long in *C. deirochelys*; 40–45 long in *C. chrysemys*, 38 long in *C. terrapene*). In addition, the new species differs by the subcapitular ring being closed posteriorly (open in *C. deirochelys* and *C. terrapene*), by the distinctly developed lateral elongation of the propodonotal shield (very short in *C. deirochelys*), by the posterior prolongation of the propodonotal shield fused with the genital sclerite (not fused in *C. chrysemys* and *C. terrapene*), and by the presence of 8 setae on tarsi III (9 setae in *C. chrysemys*, Fig. 3D–G).
DISCUSSION

Bochkov (2002) listed 8 unique synapomorphies that are shared between the Cloacaridae and Epimyodicidae. However, 2 of these are incorrect. The absence of solenidia and of II is not a synapomorphy, because these solenidia are present in the Cloacarinae. The presence of so-called “anal papillae,” which are actually the fleshy opisthosomal setae, is a dubious synapomorphy, because these setae are absent in some species. According to the results of the present morphological analysis, the Cloacaridae and Epimyodicidae share 7 apparent synapomorphies. (1) The gnathosoma is shifted ventrally. In most cheyletoid mites the gnathosoma is situated terminally. It is positioned subterminally only in some specialized free-living or parasitic genera of the Cheyletidae (e.g., Chelonotus Berlese, 1893, Samsinakia Volgin, 1965). (2) The chelicerae are devoid of digits and setae. In all eleutherengones, the movable digits of the chelicerae are distinctly developed and typically styliform, and the chelicerae are devoid of setae in all representatives of the cohort Raphignathae. (3) The subcapitular setation is completely lost. In most eleutherengones, subcoxal setae el of the gnathosoma and subcapitular setae m are present, except in some Heterostigmata (Lindquist, 1986). (4) The coxae are completely fused with the idiosoma. In all eleutherengones, the coxal fields are discernible ventrally. (5) The coxal apodemes are longer than half of the body width. In all eleutherengones, the coxal apodemes are distinctly shorter than half of the idiosomal width. (6) In adults, coxal apodemes I are fused into a sternum. In most cheyletoides, coxal apodemes I are separated from each other; however, they are fused to each other in some Heterostigmata (Lindquist, 1986). (7) The female genital and anal openings are completely fused to each other. In most eleutherengone females, these openings are situated close to each other. In most species of the Raphignathoidea and the Pterygosomatidae, they are almost, but not completely, fused and covered by a pair of common folds. Only in Demodicidae, Psorergatidae, and more derived Heterostigmata, which are phylogenetically distant from these mites, the anal and genital openings are completely fused, and in representatives of the last group, this opening functions in insemination, but not oviposition, which occurs from a larger opening immediately behind legs IV (Lindquist, 1986). In addition to these characters, there are some other features shared by these 2 families. In males, an aedeagus is present and situated dorsally, and the anal–genital opening opens in the anterior half of the idiosoma. The last character, however, occurs in other, unrelated, parasitic taxa of eleutherengone mites. In all Myobiidae, many higher Taronemina and Tetranychoidae, and even in some Pterygosomatidae, the male anal–genital opening is more or less displaced dorsally. For example, even within the Cheyletidae, dorsal migration of the aedeagus has originated repeatedly in several taxa that independently adapted to parasitism (Bochkov and Fain, 2001; Bochkov, 2004). The idiosomal setation in mites of the Cloacaridae–Epimyodicidae lineage is strongly reduced and represented by only 1–4 pairs of proprodonotal alveoli and by 1–4 pairs of fleshy opisthosomal setae. The trochanters, femora, and genua of all of the legs are devoid of setae (in Demodicidae, the leg setae are completely absent, but a solenidion is present on tarsi I and II), and most setae of the tibiae and tarsi are modified into spines. Finally, it is quite possible that their life cycle is similar and includes only a single nymphal stage in the female line (in cheyletoids, there are 2 nymphal stages in females).

The relationships of these 2 families with other progestigmatic mites are not clear. These mites are strongly modified for an endoparasitic mode of life and have lost many morphological structures present in other Prostigmata. For this reason, the clarification of their phylogenetic position with methods of cladistic analysis, entirely based on morphological data, seems ill advised because the many similar regressive traits shared with other parasitic Prostigmata are of dubious homology and would overwhelm other characters in such an analysis. Given this situation, we can provide mainly indirect arguments about the phylogenetic position of the Cloacaridae–Epimyodicidae lineage. The gnathosomal appendages of cloacarids interpreted by Camin et al. (1967) as “palps” are here reinterpreted as distinctly separated chelicerae. This feature of these mites obviously prevents their inclusion in the Cheyletoidea, because all cheyletoid families have completely fused chelicerae with stylet-like movable cheliceral digits (Kethely, 1970, 1982; Krantz, 1978; Bochkov, 2002). The 2 “cheyletoid” characters mentioned by Camin et al. (1967), i.e., (1) the fusion of the anal and genital openings and (2) the dorsal position of the aedeagus in males, indicate the affinity of these 2 families with the infradorder Eleutherengona, but are not helpful in clarifying their relationships to other lineages within the infraorder. We establish for these 2 families a new superfamily Cloacaroidea Camin, Moss, Oliver, and Singer, 1967 superfam. nov., which we regard as incertae sedis within the infraorder Eleutherengona. Synapomorphies characterizing this new superfamily are mostly regressive and could possibly have arisen independently as adaptations to intradermal parasitism. On the other hand, we have not observed any characters that contradict a sister relationship between Cloacaridae and Epimyodicidae; therefore, these 2 families should be considered as members of 1 superfamily until evidence against monophyly of this superfamily is discovered.

Our analysis of the external morphology provides additional data confirming the limits and relationships of the cloacaroid families and subfamilies. All synapomorphies characterizing cloacaroid families and subfamilies are listed in Table II. The monophyly of the Epimyodicidae is supported by 6 synapomorphies: (1) in adults and nymphs, the palps are reduced to a single articulated segment; (2) in larvae, the gnathosoma is indistinct (and probably absent); (3) in females, the opisthosoma bears a single pair of papilla-like setae; (4) the absence of an anal opening in immature instars; (5) the absence of solenidia on all of the leg tarsi (the solenidia of the tarsi are also absent in Pneumaphagiae); and (6) the legs of immature stages consist of 3 articulated segments and are devoid of claws.

The monophyly of the Cloacaridae is strongly supported by 10 synapomorphies: (1) the gnathosoma is inserted perpendicularly to the idiosomal axis; (2) the subcapitulum is strongly reduced; (3) the palps are completely lost; (4) the opisthosoma is shortened and distinctly shorter than the metapodosoma; (5) idiosomal striaion is absent; (6) there is a sclerotized ventromarginal frame bordering the lateral margins of the idiosoma; (7) the legs are inserted laterally and are strongly shortened and thickened; (8) the femora and genua of all of the legs are completely fused; (9) the leg trochanters have distinct basal projec-
tion bearing glenoid cavities; and (10) the pretarsal claws are absent.

A morphological comparison of *Pneumophagus* with other cloacarids supports its taxonomic position as a subfamily distinct from other representatives of the family. The Cloacarinae is characterized by 2 synapomorphies: (1) the remnants of the subcapitulum are joined with the sternum via the antero-median sclerite, and (2) the setal alveoli flanking the anal-genital opening are absent.

The monobasic Pneumophaginae is characterized by 6 autapomorphies: (1) the gnathosoma of the male is absent; (2) branches of the sternum surround the female gnathosoma; (3) the coxal apodemes are fused to each other proximally, forming a ventral idiosomal frame; (4) tibiae II–IV are devoid of setae; (5) solenidia are absent on all leg tarsi; and (6) tarsi II–IV are flattened laterally, and most of their setae are arranged in a row.

The host associations of cloacaroid mites are still an intriguing problem. Mites of this superfamily show a high level of host specificity; however, cloacaroid families and subfamilies are associated with phylogenetically distant lineages of vertebrates i.e., turtles, mammals, and birds. Mites of the Cloacarinae are associated exclusively with turtles. Most of the known cloacarine species are known only from females collected from the turtle cloaca. The possible explanation of this phenomenon is that most males, immature instars, and virgin females inhabit the subcutaneous tissues of their hosts, whereas the inseminated females migrate to the cloaca and concentrate there. Females and the male of *Theodoracarus testudinis* Fain, 1968, were collected only from subcutaneous tissues (Fain, 1968). These mites probably infest their hosts exclusively via venereal transmission (Camin et al., 1967; Pence and Wright, 1998), and this could be the primary reason for such female concentration in the host cloaca; however, arrhenotoky also could not be excluded, at least in some species (Pence and Casto, 1975). Cloacarines are known from turtles of the Cheloniidae, Chelydridae, Emydidae, Testudinidae, Trionychidae (Cryptodira), Chelidae, and Pelomedusidae (Pleurodira) (Pence and Wright, 1998). Considering the wide distribution of these mites among turtle taxa, their extreme specialization to an endoparasitic mode of life, the high specificity to turtle hosts, and venereal transmission from host to host, the relationship between cloacarines and turtles seems ancient, as hypothesized by most previous authors (Camim et al., 1967; Pence and Casto, 1975; Pence and Wright, 1998; Bochkov, 2002). In this case, the common ancestor of cloacarines may have become associated with turtles before the late Triassic, the assumed time of Pleurodira–Cryptodira divergence (Gaffney and Meylan, 1988).

The record of *P. bubonis* from the lungs of a single specimen of *B. virginianus* by Fain and Smiley (1989) is still enigmatic. As discussed above, these mites, which do exhibit a typical cloacarid morphology, are clearly distinguished from representatives of the Cloacarinae, and their placement into the separate Pneumophaginae has strong morphological support. The distinctions between pneumophagines and cloacarines are stronger than are these between cloacarines parasitizing the 2 chelonian suborders Pleurodira and Cryptodira. Such morphological distinctions suggest a long history of pneumophagine parasitism on non-turtle hosts. We believe, therefore, that this record is not likely to be a result of a host switch from some as-yet unexamined turtle species that happened within the lifespan of
that individual bird. On the other hand, the knowledge of the diversity and host distribution of pneumophagines is so far limited to this single record and we, therefore, cannot hypothesize whether this parasitism has an ancient origin with birds inheriting cloacarids from their common ancestor with turtles or, and which seems much more probable, it is a result of an ancient host shift following predation by some birds on live or dead turtles.

All 4 currently known species of the Epimyodicidae are endoparasites in the subcutaneous tissues of voles (Rodentia: Cricetidae), moles, and shrews (Eulipotyphla: Soricidae) (Fain and Orts, 1969; Fain et al., 1982; Fain and Bochkov, 2001). As in the case with cloacarines, these mites possibly disperse via the urogenital system. It seems likely that they are more widely distributed and may parasitize a variety of small mammals. However, their small size, poor sclerotization, and endoparasitic mode of life make them difficult to observe and collect. The 3 alternative hypotheses for the origin of parasitic associations among cloacaroid mites and their vertebrate hosts are depicted in Figure 6. According to the first hypothesis developed by Bochkov (2002), the sister relationship between Cloacaridae and Epimyodicidae could reflect parasitism by their common ancestor on archaic amniotes before the divergence of the Synapsida (ancestors of mammals) and Diapsida (ancestors of turtles and birds) (Fig. 6A). In this case, the more archaic features of the Epimyodicidae could be explained by the early divergence of their synapsid hosts from the common amniotic stock. On the other hand, the ancestors of epimyodicids, retaining some features of their free-living ancestors, could have switched from diapsids onto synapsids considerably later (hypothesis 2, Fig. 6B). Finally, the probability that synapomorphies uniting Cloacaridae and Epimyodicidae are actually convergences cannot be completely excluded. In this case, these 2 families would represent independent origins of intradermal parasitism in turtles (with probable secondary switching to birds) and mammals (hypothesis 3, Fig. 6C). To date, the last hypothesis has no morphological support, and the data provided above suggest to us a monophyletic origin of intradermal parasitism in cloacaroid mites (hypotheses 1 and 2, Figs. 6A, B).

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FIGURE 6. Alternative hypotheses for the origin of host-parasite associations among cloacaroid mites and vertebrates. (A–C) Hypotheses 1–3, respectively. See text for explanations.
sity provided the host specimen from which the junior author collected the specimens of the new species. This research was supported by a grant from the U.S. National Science Foundation DEB-0118766 (PEET) (BMOC).

LITERATURE CITED


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MULTIPLE PATERNITY IN IXODES RICINUS (ACARI: IXODIDAE), ASSESSED BY MICROSATellite MARKERS

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ABSTRACT: This investigation examines multiple paternity in *Ixodes ricinus* (Acari: Ixodidae). Previous studies have shown that multiple mating occurs in this tick, but this is the first evaluation of multiple paternity. Three family groups were examined by a panel of polymorphic microsatellite loci; all ticks were bred from wild-collected engorged females with a copulating male attached. For most larvae, the attached males could be excluded as possible sire, and in the 3 tested families, at least 2 of 3 females mated successfully with more than 1 male. This finding suggests that multiple paternity is a common reproductive strategy in *I. ricinus*, which may have consequences for the ticks’ dispersal success by increasing the genetic diversity in broods from single females colonizing new sites.

Mating patterns have received much attention in the study of the reproductive strategies of arthropods (e.g., Barnard, 2004; Alcock, 2005). Multiple paternity will increase the offspring’s genetic diversity. This may have a positive effect on founding success by reducing the risk of local, stochastic extinction by inbreeding and by improving tolerance to environmental variability (e.g., Saccheri et al., 1998; Frankham et al., 2004). In Europe, *Ixodes ricinus* show clear signs of increasing abundance and expansion along the northern part of its range (Lindgren and Gustafson, 2001), and increasing attention has been paid to this species, notably as a vector of the pathogenic bacteria *Borrelia burgdorferi* s.l. and tick-borne encephalitis (Randaloph, 2001). Several studies exist on the mating patterns of ixodid ticks (Bouman et al., 1999; Kiszewski et al., 2001; Zemek et al., 2002), but multiple paternity in *I. ricinus* has not been studied specifically. In an observational study of *I. ricinus*, repeated mating was observed both in males (maximum 4 times) and females (maximum 2 times) (Graf, 1978). Multiple mating is known to occur in many genera of hard and soft ticks (Oliver, 1974), also in *Ixodes scapularis* (Yuval and Spielman, 1990) and *Ixodes uriae* (McCoy and Tirard, 2002). However, multiple mating does not necessarily mean multiple paternity. A more detailed study of the related species *I. uriae* (McCoy and Tirard, 2002) showed multiple paternity in 2 of 7 family groups tested by use of microsatellite analyses. To test whether this is also the case in *I. ricinus*, we analyzed 3 family groups, i.e., the female, the last mating male, and a selected number of hatching offspring for a panel of polymorphic microsatellite loci.

MATERIALS AND METHODS

Three fully engorged female *I. ricinus* with a copulating male attached were collected from cows on the island of Hille in West Agder, Norway. After completing copulation in a glass vial, the males were removed and stored in 70% ethanol. The engorged females were incubated at room temperature until oviposition, and the eggs were incubated further until hatching of larvae took place. When the larvae emerged, they and the female were also transferred to 70% ethanol. Fifteen larvae in 2 family groups and 18 larvae in 1 group were examined. In using larvae instead of eggs, we assumed all larvae to be diploids, i.e., no homozygosity due to unfertilized eggs (see McCoy and Tirard, 2002).

The specimens were crushed using the tip of a glass rod and DNA was isolated using DNeasy® Tissue kit (QIAGEN, Valencia, California). DNA was amplified in polymerase chain reaction (PCR) with 10 different *I. ricinus*-specific microsatellite primers (IRN-4, IRN-7, IRN-8, IRN-12, IRN-14, IRN-15, IRN-17, IRN-30, IRN-31, and IRN-34; Røed et al., 2006). In our analysis of paternity, we used only the 3 loci where both the mother and the attached male were in most cases scored as heterozygote, i.e., IRN-14, IRN-15, and IRN-34. The forward primers were end-labeled with fluorescence, and the PCR was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California). Each PCR contained 10-μl reaction mixtures, 20–40 ng of genomic template DNA, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP, and 0.5 U of Taq polymerase (QIAGEN). Thermocycling parameters after denaturation at 94°C for 5 min were 30 cycles of 95°C for 1 min, annealing temperature (cf. Røed et al., 2006) for 30 sec, followed by extension at 72°C for 1 min. The last polymerization step was extended to 10 min. PCR products were added to buffer containing formamide and 5-carboxytetramethylrhodamine-labeled standard (Applied Biosystems), and electrophoresed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

In contrast to the microsatellites used by McCoy and Tirard (2000, 2002) in *I. uriae*, microsatellites of *I. ricinus* are often associated with genotypes deviating from Hardy–Weinberg equilibrium by heterozygote deficiency and cases of nonamplified products (cf. de Meedt et al., 2002; Røed et al., 2006). Such genotypic errors are usually related to null alleles (e.g., Dakin and Avise, 2004) and allelic dropout (e.g., Wattier et al., 1998), both of which may introduce substantial errors into empirical assessments of species mating systems by scoring individuals erroneously as homozygotes (Blovien, 2003; Dakin and Avise, 2004). Therefore, it is necessary to account for the possibility that some alleles may be present without being scored and that some apparent homozygotes may be due to null alleles. We adopted this conservative approach to avoid overestimating multiple paternity, although it could lead to an underestimation.

RESULTS

In all families, there were several loci for which the mother and her larvae were scored as different homozygotes (Table I). This suggests the frequent presence of null or nonamplified alleles. In all cases where the mothers were scored as heterozygous, 1 of the maternal alleles was detected in all larvae (except for some larvae where no product was amplified at certain loci). For family 1, there were 4 different alleles in IRN-14 of which 1 did not occur in the copulating pair. This is consistent with 1 additional sire being involved. A previous male with IRN-14 104/108, IRN15 101/112, and IRN-34 114/116 could have sired all the offspring. Regardless, the attached male could be excluded as possible sire for 5 of the 15 larvae (Table I). In family 2, at least 2 sires were involved, as there were 3 nonmaternal alleles both in IRN-14 and IRN-15. The attached male could not have sired any of the tested offspring. Three larvae showed no amplified allele in locus IRN-14, whereas the mother was...
Table I. Alleles scored in 3 polymorphic microsatellite loci in 3 family groups of *I. ricinus*. Alleles excluding the attached male as sire are marked in bold.

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</tr>
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</tr>
<tr>
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<td>148</td>
<td>83</td>
</tr>
<tr>
<td>Larva 3.14</td>
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<td>83</td>
</tr>
<tr>
<td>Larva 3.15</td>
<td>108</td>
<td>128</td>
<td>83</td>
</tr>
</tbody>
</table>

heterozygous. This inconsistency may be due to low amplifiability. Some microsatellites may be more difficult than others to amplify. Furthermore, in tick larvae there are very small amounts of DNA. In family 3, 6 different alleles of IRN-14 were noted in the larvae. Even if there were a nonamplified maternal allele in the mother, at least 3 fathers would be necessary to sire this brood.

**DISCUSSION**

Even though we analyzed a limited number of larvae in each family group and applied a highly conservative criterion for accepting additional fathers, we have shown that at least 2 of 3 females studied mated successfully with more than 1 male. Seemingly, the copulating males contributed little, if anything, to the genes of the larvae tested. As *I. ricinus*, females lay about 2,000 eggs (Randolph, 1998), several more successful conceptions might have taken place in addition to those observed in our analyzed material. In a similar study of *I. uriae*, McCoy and Tirard (2002) found multiple paternity (in 2 of 7 family groups), and they discussed this in relation to climate variability of its habitat and adaptation to host responses. The latter argument seems particularly relevant for *I. ricinus*, which can parasitize virtually any mammal and bird living in their habitat. However, the fact that 2 species with a different biology show multiple paternity suggests that this may be a more general characteristic of ticks, not necessarily explained by special adaptations in each species. This view is supported by the fact that multiple mating is common in Ixodidae (Oliver, 1974), although data on actual multiple paternity are lacking. Genetic studies of family groups of other tick species would be needed to determine whether *I. uriae* and *I. ricinus* are unique in this respect.

In its northern distribution range, such as in Norway, *I. ricinus* are accidentally found in new locations, probably brought there by birds, because birds are known to carry ticks (e.g., Mehl et al., 1984). Multiple paternity, as found by McCoy and Tirard (2002) and the present study, may have considerable consequences for the ability of engorged females to colonize new sites, and for improving the overall fitness by producing offspring with variable genotypes (McCoy and Tirard, 2002). With multiple paternity, 1, or very few, females could be in a position to produce offspring with sufficient genetic diversity to form a founder population.

Multiple mating may also be important in increasing the likelihood of the sexual transmission of pathogens between ticks, a mechanism that has been experimentally demonstrated for both tick-borne encephalitis virus (Chunikhin et al., 1983) and *Borrelia* sp. (Alekseev et al., 1999). Furthermore, the spotted fever group, Rickettsiae, has been detected in immature spheromatozoa of *I. ricinus* (Hayes et al., 1980), suggesting that this class of pathogens may also be transmitted sexually. Because *I. ricinus* copulates several times, this may partly explain the much higher prevalence of tick-borne encephalitis virus found in engorged ticks than in specimens collected by flagging in endemic areas (Asokliene, 2004; Suss et al., 2004).

Thus, multiple mating may be of importance to the probability of a tick acting as a disease vector. If multiple paternity improves the ability of ticks to colonize new habitats and to
resist the immune responses of hosts, it will indirectly be important for disease dynamics.

ACKNOWLEDGMENTS

We thank Liv Midthjell for skilful assistance in genotyping, and 2 anonymous referees who provided valuable comments on this manuscript. Thanks also to Roche for economic support.

LITERATURE CITED

FIVE NEW SPECIES OF RHINONYSSIDAE (MESOSTIGMATA) AND ONE NEW SPECIES OF DERMANYSSUS (MESOSTIGMATA: DERMANYSSIDAE) FROM BIRDS OF ALBERTA AND MANITOBA, CANADA

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ABSTRACT: Three major lineages of mites are parasitic in the nasal passages of birds, i.e., Rhinonyssidae (Mesostigmata), Ereynetidae (Prostigmata), Cytoditidae, and Turbinoptidae (Astigmata). The most diverse family of avian nasal mites is Rhinonyssidae, which include obligate hematothophagous endoparasites of nonratite birds worldwide. Nasal mites have been surveyed extensively in the United States, yet there has never been a Canadian survey. There are only 4 published, and 3 unpublished, rhinonyssid species records from birds in Canada. While surveying the nasal mites associated with birds of Alberta and Manitoba (western Canada), 1 new species of Dermanyssus and 5 new species of Rhinonyssidae were recovered. Herein, I describe and illustrate Dermanyssus diphyes n. sp., Ptilonyssus calvaria n. sp., P. nivalis n. sp., P. pinicola n. sp., P. plesiotypicus n. sp., and Stemostoma setifer n. sp.

Birds are host to a broad diversity of symbiotic animals. Mites are among the most diverse groups of these symbionts, with at least 40 families and approximately 3,000 described species known from avian hosts (Proctor and Owens, 2000). The superfamily Dermanysoidea contains most of the bird-associated Mesostigmata (Radovsky, 1994). Within the Dermanyssoidae, species of Dermanyssidae, Rhinonyssidae, Laelapidae, and Macronyssidae parasitize birds in North America. Species of Dermanyssidae Kolenati, 1859, are obligate hematothophagous ectoparasites on birds and mammals. Members of Dermanyssus Dugés, 1834 are ectoparasites of birds worldwide, with generally broad host ranges (Moss, 1978). For example, Dermanyssus gallinae (De Geer, 1778) has been recorded from at least 30 host species, and D. hirundinis (Hermann, 1804) from at least 14 (Moss, 1978). With the exception of D. gallinae, Dermanyssus species are predominately parasites of cavity-nesting birds, such as flickers (Picidae) and swallows (Hirundinidae) (Moss, 1978). Most species are nidicolous ectoparasites, briefly feeding on the host at night and returning to the nest and associated substratum during the day (Baker et al., 1956). A few species, including D. grochovskae Zemskaya, 1961 and D. quintus Vitzthum, 1921, spend most of their life cycle on the host (Moss, 1978).

Rhinonyssids are obligate hematothophagous endoparasites in the nasal passages of nonratite birds worldwide. Rhinonyssid genera vary in their degree of host specificity, with some genera being restricted to single host families, and others found in hosts from different orders (Pence, 1973). In North America, passeriform, caprimulgiform, falconiform, and apodiform host species are parasitized by Ptilonyssus. species. Sternostoma species parasitize passeriform, piciform, and charadriiform birds, while Rhinonyssus species infect anseriforms, podicipediforms, and charadriiforms. Ciconiiform and columbiform birds are parasitized by Taniminysus species. Owls (Strigiformes) are infected by Rhinociclus species; generally, each Rhinociclus species occurs in a different species of owl. Birds of the Gallinidae are parasitized by Gallinynysus species, while Larinyssus species are associated with gulls and terns (Laridae). Rhinonyssids are sluggish mites, which occur predominately in the nasal passages, although some species invade the tracheal tissues, lungs, and body cavity (Porter and Strandtmann, 1952; Krantz, 1978). Generally, rhinonyssids are not considered to cause significant pathology to their hosts, with the exception of Sternostoma tracheacolum Lawrence, 1948, which invades the lower respiratory tract, lungs, and air sacs of a host (Stephan et al., 1950).

Nasal mites have been surveyed in many geographic locations worldwide, including Taiwan, Australia, and Guatemala, and Louisiana and Texas in the United States (Hyland, 1963; Mao and Ku, 1965; Domrow, 1969; Pence, 1973; Spicer, 1984, 1987). However, there has never been a Canadian survey. Canadian records include only 4 published species records, i.e., Rhinonyssus sp. from the guillemot, Uria aalge (Ballard and Ring, 1979), Sternostoma tracheacolum from the red-winged blackbird, Agelaius phoeniceus (Hood and Welch, 1980), Ptilonyssus japaibensis Castro, 1948 from the chipping sparrow, Spizella passerina (Pence, 1975), and Ptilonyssus sairae Castro, 1948 from the chipping sparrow, S. passerina (George, 1961). In addition to these reports, there are 3 unpublished species records from specimens deposited in the Canadian National Collection of Insects and Arachnids (CNCI&A) in Ottawa, i.e., Ptilonyssus bombicillae Fain, 1972 from the Bohemian waxwing, Bombycilla garrulus, Rhinonyssus convexitris Trouessart, 1894 from the red knot, Calidris canutus, and Sternostoma boydi Strandmann, 1951 from the ruddy turnstone, Arenaria interpres. While surveying the nasal mites associated with birds of Alberta and Manitoba, I collected 1 new species of Dermanyssus and 5 new species of Rhinonyssidae. Herein I describe and illustrate these 6 new species.

MATERIALS AND METHODS

The laboratory of Heather Proctor at the University of Alberta had a collection of approximately 700 bird carcasses from Alberta, largely from the contributions of the Alberta Fish & Wildlife Forensic Laboratory, the Royal Alberta Museum, waterfowl hunters, and colleagues at the University of Alberta, Edmonton, Alberta, Canada. Collection data were sparse for many of these specimens, and for some I can say only that the birds were collected somewhere in Alberta. Host taxonomy and authorities follow Clements (1991-1996) provided by Andrew and McAllan (1998), selecting the “Clements 1991–1996” taxonomy option in Nomina version 1.0. Bird bodies were maintained at −20°C until processing. Frozen birds were first thawed and then washed using the following method. The bird was placed in a suitably sized container, ranging from 4 to 18 L, with a drop of dish detergent, enough 95% ethanol to soak the plumage of the bird, and enough water to submerge it. The sealed container was then shaken vigorously for 5 min. Partic-
ularly large birds were washed in a basin and thoroughly massaged while in the solution. Each bird was then removed from the container and rinsed thoroughly over a Fisher Scientific 53-μm mesh filter; large birds were rinsed over a washing basin. The washing liquid was filtered, and the container and lid were rinsed thoroughly over the same 53-μm filter. The material remaining in the filter was stored in 30-ml snap cap and scintillation vials in 95% ethanol.

Mites were also collected from some individual birds by dissecting the host’s nasal cavities under a laminar flow exhaust hood. The host was decapitated, and the head was secured in a table top drill press vice. Depending on bird size, a scalpel, molybdenum steel scissors, or molybdenum steel bone shears to sagittally section the head and expose the nasal cavities. The dissected halves were placed in appropriately sized vials and stored at −20°C until inspection. For inspection, the dissected heads were placed in a glass dish with 80% ethanol and the tissues examined using a dissecting stereomicroscope.

Nasal mite samples were received from Dr. Terry Galloway’s lab at the University of Manitoba, Winnipeg, Manitoba. Galloway’s lab performed nasal flushings on Manotoban birds using orthodontic syringes, 15 ml for larger birds and 3 ml for smaller birds. A solution of warm water and mild soap was flushed through each nostril, back out the mouth and into a Petri dish. Occasionally nasal mites were also collected in whole-body washings of birds. Body-washing methods in the Manitoba lab were similar to those described above, except that ethanol was not added to the washing solution, and the washing solution was filtered through a 0.9-μm filter.

Washings and dissections were examined using Leica MZ16 and MZ6 dissecting microscopes at 20–25× magnification. Mites were removed from ethanol and cleared in 85% lactic acid for 1–24 hr, depending on the degree of original opacity. Mites were mounted in a polyvinyl alcohol medium (6371A, BioQuip Products, Rancho Dominguez, California). Slides were cured on a slide warmer at about 40°C for 3–4 days. Slide-mounted specimens were examined with a Leica DMLB compound microscope with differential interference contrast (DIC) at 400× magnification. Species-level identifications were made using keys (Pence, 1975; Moss, 1978) and species descriptions from the primary literature.

Initial drawings were made with pencil on paper using a camera lucida. These were later merged in Adobe Photoshop CS v8.0, and redrawn in Adobe Illustrator CS v11.0 (Adobe Systems Inc., San Jose, California) using an Intuos 2 graphics tablet from Wacom Co. (Saitama, Japan). Leg chaetotaxy is based on the system proposed by Evans (1963) and Evans and Till (1965). Idiosomal chaetotaxy is based upon the system proposed by Lindquist and Evans (1965). Palp chaetotaxy is listed from basal to apical segments. Descriptions are based upon the holotype, and paratypes if present. All measurements are in micrometres (μm) and are in the form “holotype (range for paratypes, mean).” In cases where there was no variation, only a single value is presented. Habitats scale bars are 250 μm, all other scale bars are 25 μm. The following designations, adapted from Fain and Hyland (1962), are used in the species descriptions as illustrated (Figs. 1–6) and are arranged in the order used in the descriptions: LB, length of body including palps; WID, width of idiosoma; LPS, length of podosomal shield; WPS, width of podosomal shield; LOS, length of opisthosomal shield; WOS, width of opisthosomal shield; LpS, length of pygidial shield; WpS, width of pygidial shield; LID, length of dorsal shield; WDSM, width of dorsal shield maximum; WDSm, width of dorsal shield minimum; LSS, length of sternal shield; WSS, width of sternal shield; LGS, length of genital shield; WΩ, width of genital shield; LGSs, length of sternogenital shield; WSGs, width of sternogenital shield; LVS, length of ventral shield; WVS, width of ventral shield; LAS, length of anal shield; WAS, width of anal shield; LP, length of peritreme; LG, length of gnathosoma, ventral view, including palps; WG, width of gnathosoma; LCH, length of chelicera; WCH, width of chelicera; LLeg, length of leg, including coxa, excluding ambulacrum (LLeg I to LLeg IV).

Abbreviations for depositories are CNCIA & (Canadian National Collection of Insects and Arachnida, Ottawa, Ontario), UASM (University of Alberta E. H. Strickland Entomological Museum, Edmonton, Alberta), JBWME (J. B. Wallis Museum of Entomology, University of Manitoba, Winnipeg, Manitoba), and ZIN (Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia).

DESCRIPTIONS

**Dermanyssus diphyes n. sp.**

*(Figs. 7–8)*

**Diagnosis:** Medium-sized mites with characteristics of *Dermanyssus*, with undivided dorsal shield. Characters distinguishing this species include dorsal shield shape and dimensions, presence of prominent pair of humeral pores on dorsal shield, presence of j4 setae, absence of pores on posterior dorsal shield, absence of setae st4, genital shield constricted anteriorly and expanded posteriorly with rounded terminus, number of ventral opisthosomal setae (16 to 20 pairs), and peritreme extending beyond middle of coxa II.


**Dorsum:** Dorsal shield well sclerotized and occupying most of dorsal idiosoma, bearing 11 pairs of setae (j2, j4–6, j1, j3, j4, z2, z4, z5, s4), 2 pairs of small pores, and prominent pair of humeral pores on dorsal shield posterolateral to setae j4. Dorsal shield setae j4 unpaired in holotype and 2 paratypes, paired in 2 paratypes. Scutal setae unequal length, anteriormost pair j2 longest at 48 long, anterolateral setae z2, z4, z5, s4, 30 long; remaining scutal setae j4–6, j1, j3, j4 ranging from 15 to 20 long. Dorsal shield anterior margin straight, widest medially at level with humeral pores, tapering distally to truncate posterior margin. Soft cuticle of dorsum bears 1 pair of short sharp-tipped setae (j1) 18 long and 19 pairs of long sharp-tipped barbed setae ranging from 40 to 75 long.

**Venter:** Ventral setae sharp-tipped. Sternal shield lightly sclerotized, bearing 2 pairs of long setae (st1, st2), 1 pair of long setae (st3) off shield in integument. Sternal setae, st1–3, lengths 53, 50, and 58, respectively. Metasternal setae (st4) absent. Genital shield with 1 pair of short setae (gen1) and 1 pair of pores. Genital shield widest anteriorly, constricted medially, expanded posteriorly with rounded terminus. Anal shield well sclerotized, with short paranal and postanal setae, paranal setae level with anal opening, paranal and postanal setae equal length. Ventral opisthosoma with 16 to 20 pairs of long predominately barbed setae ranging from 38 to 63 long. Peritreme extends beyond middle of coxa II.


**Legs:** Claws of legs I–IV strongly curved. Leg chaetotaxy as in Table 1.

**Male, nymphs, larva:** Unknown.

**Taxonomic summary**

**Type host:** *Turdus migratorius* Linnaeus, 1766, American robin.

**Type locality and collection date:** Four mites from Edmonton, Alberta (53°34’N, 113°28’W), 17 July 2005; coll. D. Walter.

**Additional locality and collection date:** Four mites from Millet, Alberta (53°05’52”N, 113°28’22”W), 13 May 1996.

**Types:** ♀ holotype, ♂ paratype CNCIA&A Nos. 23510, 23511. Two ♀ paratypes UASM Nos. 80557, 80558. One ♀ paratype in possession of author.

**Etymology:** *diphyes* (a Greek masculine adjective) means “of double nature, 2-fold.” This name reflects the fact that this species possesses characteristics of 2 different groups of *Dermanyssus* species, with prominent humeral pores on the dorsal shield and relatively long peritremes.

**Remarks**

*Dermanyssus diphyes* n. sp. is most similar to *D. alaudae* (Schrank, 1781), described from *Alauda arvensis*, *D. brevis* Ewing, 1936, described from *Eremophila alpestris* merrielli from Oregon, and *D. bir­itus* Moss and Radovsky, 1967, described from *Colaptus auratus* cafer from Oregon. *Dermanyssus diphyes* n. sp. is distinguished from these species in several ways. The dorsal shield length ranges from 603 to

705 and maximum width ranges 305 to 388, while for D. brevis the dorsal shield length is 506 and maximum width is 286. The dorsal shield anterior margin is straight in the new species, widest medially at the level of the humeral pores, and tapers distally to a truncate posterior margin (Fig. 7); in D. alaudae and D. hirsutus, the dorsal shield tapers only slightly distally to a rounded posterior margin. Prominent pair of humeral pores are located on the dorsal shield posterolateral setae s4 of the new species, while in D. hirsutus the humeral pores are located on the dorsal shield, but they are very small and barely noticeable. Setae j4 is on the dorsal shield of D. diphyes n. sp.; D. alaudae setae j4 is absent. No pores are on the dorsal shield posterior margin of the new species; D. hirsutus has a pair of pores on the dorsal shield posterior margin. In D. diphyes n. sp., the metasternal setae, st4, is absent, while D. hirsutus has metasternal setae. The genital shield is widest anteriorly, constricted medially, expanded posteriorly to a rounded terminus in the new species, whereas the genital shield of D. alaudae and D. brevis genital shield is slightly wider anteriorly, and tapers slightly to a rounded terminus without expanding distally. The anal shield length of D. diphyes n. sp. ranges 123–148, and the anal shield width ranges 113–143. In D. brevis, the anal shield is 116 long and 105 wide. In the new species, the ventral opisthosoma has 16–20 pairs of long predominately barbed setae; D. hirsutus has ventral opisthosomal hypertrichy with at least 24 pairs of setae. In D. diphyes n. sp., the peritreme extends anteriorly beyond the middle of coxa II; the D. alaudae peritreme extends only to middle of coxa III, while the D. brevis peritreme reaches just posterior to the middle of coxa III, and the D. hirsutus peritreme extends to the middle of coxa II.

Ptilonyssus calvaria n. sp. (Figs. 9–16, 43–44)

Diagnosis: Large mites with podosomal and pygidial shields. Eight pairs of setae on podosomal shield with distinctively trilobed posterior margin, 5 pairs of mesolateral setae of approximately equal length, paranal and postanal setae of unequal length, cribrum not extending to...
posterior end of anal shield, and relatively large hyp1 setae with round-
ed tips distinguish this species from closely related species.

Female (based on holotype and 4 paratypes): LB 880 (730–875,
(113–123, 118). WSS 80 (65–75, 73). LGS 103 (103–120, 111). WGS
35 (34–36, 35). LLeg I 268 (263–300, 278). LLeg II 208 (188–213,

Dorsum: Dorsal setae sharp-tipped. Podosomal and pygidial shields
sclerotized with areas of muscle insertions as illustrated (Fig. 9). Po-
dosomal shield with 8 pairs of minute setae (j2–5, x2–5). Pygidial shield
with 1 pair of short setae (J4) and 1 pair of pores. Podosomal shield

Figure 7. Dermanyssus diphyes n. sp. female dorsum.
FIGURE 8. *Dermanyssus diphyes* n. sp. female venter.
Table I. *Dermanyssus diphyes* n. sp. female leg chaetotaxy.

<table>
<thead>
<tr>
<th>Leg segment</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
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</tr>
<tr>
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<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>Tibia</td>
<td>2-2</td>
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</tbody>
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anterior margin slightly invaginated medially, anterolateral corners rounded, laterally excavated level with coxa III, widest posteriorly, distinctly trilobed posterior margin. Mesosomal shieldlets present. Pygidial shield small, wider than long, rounded anteriorly, slightly invaginated posteriorly. Five pairs of setae (s5, s6, r3, r5, r6) in integument adjacent to podosomal shield, all approximately equal length. Subposterior setae (j6) just off posterior margin of podosomal shield, half size of longest mesolateral setae (s5–6). Eight pairs short setae (J1–3, Z1–3, R1, R2) in dorsal opisthosoma. Stigmata dorsolateral, at level of coxa III.

**Venter:** Ventral setae sharp-tipped. Sternal shield lightly sclerotized with distinct margins, 1 pair of setae (s1) on shield, 2 pairs of setae (s2, s3) off shield. Genital shield with 1 pair of short setae (gen1), pair of pores in integument off shield. Anal shield distinct, distally tapering paranal and postanal setae, paranal setae level with anal opening, paranal and postanal setae unequal length. Cribrum in narrow band, not extending to posterior of anal shield. Seven pairs of setae (JV1–4, ZV2, ZV3, UR1) in ventral opisthosoma, and pair of pores of lateral. Anal shield variable, anterior margin straight, widest medially, posterior margin slightly invaginated. Five pairs of setae (s5, s6, r3, r5, r6) in integument next to podosomal shield. Subposterior setae (j6) just off posterior margin of podosomal shield. Five pairs of setae (Z1–3, R1, R2) on dorsal opisthosoma. Stigmata dorsolateral, at level of coxa III.


**Dorsum:** Dorsal setae sharp-tipped. Podosomal and opisthosomal shield lightly sclerotized, areas of muscle insertions were not observed. Podosomal shield with 8 pairs of minute setae (j2–5, z2–5). Opisthosomal shield with 4 pairs of setae (3 pairs short setae (J1–3), 1 pair very long barbed setae (j4, 30 long). Podosomal shield anterior margin slightly invaginated medially, anterolateral corners rounded, laterally excavated level with coxa III, widest posteriorly, distinctly trilobed posterior margin. Opisthosomal shield variable, anterior margin straight, sides parallel, posterior margin inverted. Five pairs of setae (s5, s6, r5, r6) in integument next to podosomal shield. Subposterior setae (j6) just off posterior margin of podosomal shield. Five pairs of setae (Z1–3, R1, R2) on dorsal opisthosoma. Stigmata dorsolateral, at level of coxa III.

**Venter:** Ventral setae sharp-tipped. Sternal shield absent. Three pairs of short sternal setae (sl–3) present. Genital shield absent, very small genital setae (gen1) present. Anal shield lightly sclerotized, bearing paranal and postanal setae, paranal setae level with anal opening, paranal and postanal unequal length. Cribrum in narrow band, does not extend to posterior of anal shield. Ventral opisthosoma with 6 pairs of setae (JV1–4, ZV2, ZV3).

**Gnathosoma:** Sharp-tipped subcapitular setae, 3 pairs hyposomal setae (hyp1 relatively long blunt-tipped, hyp2 short blunt-tipped, hyp3 relatively long sharp-tipped), 9 deutosternal teeth. Chaetotaxy of palps: 1-2-4-12. Tibia-tarsus sensory pit with 7 pairs of short setae. Chelicerae widest proximally, tapering slightly to relatively large digits, with short and slender spermatadactyl attached (Fig. 44).

**Legs:** Claws of legs I–IV strongly curved. Chaetotaxy of legs: Coxa 2-2-2-1. Trochanter 4-4-4-5. Femur 9-7-4-5. Genus 5-5-6-3. Tibia 7-7-6-5. Tarsus 22-17-17-17. Tarsus II–IV ventral subapical setal pair long strong spikes.


**Dorsum:** Dorsal setae sharp-tipped. Podosomal and opisthosomal shield lightly sclerotized, areas of muscle insertions not observed. Podosomal shield with 8 pairs of minute sharp-tipped setae (j2–5, z2–5). Pygidial shield of variable shape, always wider than long, with 1 pair of very long barbed sharp-tipped setae (J4), 27 long. Podosomal shield anterior margin slightly invaginated medially, anterolateral corners rounded, laterally excavated level with coxa III, widest posteriorly, distinctly trilobed posterior margin. Mesosomal shieldlets present. Five pairs of short blunt-tipped setae (s5, s6, r3, r5, r6) in integument alongside podosomal shield. Subposterior setae (j6) just off posterior margin of podosomal shield. Eight pairs of short blunt-tipped setae (J1–4, Z1–3, R1, R2) on dorsal opisthosoma. Stigmata dorsolateral, at level of coxa III.

**Venter:** Sternal setae absent. Three pairs of short blunt-tipped sternal setae (sl–3) present. Pair of genital pores located in similar location as in adult females. Anal shield lightly sclerotized, with very short blunt-tipped paranal and postanal setae, paranal setae level with anal opening, paranal and postanal setae equal length. Cribrum extends to posterior of anal shield. Five pairs of blunt-tipped setae (JIV1–4, ZV2) on ventral opisthosoma.

Figure 15. *Ptilonyssus calvaria* n. sp. male dorsum.
Figure 16. *Ptilonyssus calvaria* n. sp. male venter.
**Lecture:** Claws of legs 1–IV are strongly curved. Chaetotaxy of legs: Cox a 2-2-2-1. Trochanter 4-4-4-4. Femur 8-7-5-4. Genu 6-6-6-3. Tibia 7-7-7-6. Tarsus 22-17-17. Tarsus II-IV ventral subapical setal pair short blunt-tipped setae.

**Larva:** Unknown.

**Taxonomic summary**

Type host: Spizella passerina Bechstein, 1798, chipping sparrow.

Type locality and collection date: Seven mites from Winnipeg, Manitoba (49°'54"N, 97°'08"W), 19 May 2004; coll. T. Galloway and D. Holder.

Addition locality and collection dates: Five mites from Winnipeg, Manitoba (49°'54"N, 97°'08"W), 26 May 2005; coll. T. Galloway and D. Holder. One mite from 3 September 2004; same collectors.

Six mites from Millet, Alberta (53°'05"22"N, 113°'28"22"W), 21 May 1999; coll. PMA.

Types: \( \tilde{\varphi} \) holotype, \( \tilde{\varphi} \) paratype CNCI&A Nos. 23513, 23514. Two \( \varphi \) paratypes JBWME Nos. 0003053, 0003054. Two \( \varphi \) paratypes ZIN No. 9988. Two \( \varphi \) paratypes, allotype \( \delta \) UASM Nos. 80561-80563. Remaining vouchers in possession of author.

**Etymology:** calvaria (Latin feminine noun) means “skull.” The shortened cranium gives the anal shield a skull-like appearance.

**Remarks**

Ptilonyssus calvaria n. sp. is most similar to P. sairae Castro, 1948, described from Tangara seledon from Brazil, P. japubensis Castro, 1948, described from Ramphocelus carbo centralis from Brazil, and P. ludovicianus Cerny, 1969, described from Pheucticus ludovicianus in Cuba. Ptilonyssus sairae, P. japubensis, and P. ludovicianus are all members of the “sairae” species complex, and the morphological differences among them are minimal. As a result of the tenuous species boundaries between these species, I performed the differential diagnosis based upon the original species descriptions. Ptilonyssus calvaria n. sp. is distinguished from these species by the following. The new species has 8 pairs of setae on podosomal shield; P. japubensis and P. ludovicianus have 7 pairs of setae. The new species has a podosomal shield posterior margin that is distinctly trilobed, while the podosomal shield posterior margins of P. sairae and P. ludovicianus are not distinctly trilobed; they are straight. Five pairs of mesolateral setae that are of approximately equal length in the new species; P. sairae and P. ludovicianus have 5 pairs of mesolateral setae too, but at least 2 pairs are twice as long as the other mesolateral setae, whereas P. japubensis has 4 pairs of mesolateral setae, and at least 2 pairs are twice as long as the other mesolateral setae. Paranal and postanal setae unequal length in P. calvaria n. sp.; in P. sairae, P. japubensis, and P. ludovicianus, the paranal and postanal setae are of approximately equal length. In P. calvaria n. sp., the cranium is in a narrow band, not extending to posterior of anal shield, while in P. sairae, P. japubensis, and P. ludovicianus, the cranium extends to posterior of anal shield. Pence and Casto (1976) reported that P. sairae from the white-throated sparrow, Zonotrichia albicollis, had the cranium restricted to a narrow band; however, in the “sairae” species complex, mites examined from Z. albicollis from Manitoba had a cranium that extended to the posterior of the anal shield. Three pairs of hypostomal setae, hyp1 and hyp2 are blunt-tipped, and hyp3 are sharp-tipped in P. calvaria n. sp.; P. sairae and P. japubensis have 3 pairs of sharp-tipped hypostomal setae, but the original species description of P. ludovicianus does not mention the hypostomal setae or provide illustrations.

**Ptilonyssus nivalis n. sp.**

(Figs. 17–22, 45)

**Diagnosis:** Medium-sized mites with podosomal and opisthosomal shields. Three pairs of mesolateral setae, 6 pairs of dorsal opisthosomal setae, opisthosomal shield shape, 2 pairs of hypostomal setae, 7 pairs of ventral opisthosomal setae, and sternal shield pattern distinguish this species from closely related species.


**Dorsum:** Podosomal and opisthosomal shields lightly sclerotized with areas of muscle insertions (Fig. 17). Podosomal shield with 9 pairs of setae (8 pairs very short blunt-tipped, 1 pair short blunt-tipped). Sub-podosomal setae on posterior margin of podosomal shield much shorter than mesosomal setae. Opisthosomal shield with 4 pairs of setae (3 pairs very short blunt-tipped [J1–3], 1 pair short blunt-tipped [J4]), 3 pairs of pores. Podosomal shield rounded anteriorly, widest medially, laterally excavated at level of coxa III, trilobed posterior margin. Mesosomal shieldlets present. Opisthosomal shield anterior margin straight, widest anteriorly, laterally excavated posteriorly, tapering to bilobed invaginated posterior margin. Three pairs of short setae in integument along with podosomal shield, all approximately equal length. Six pairs of short blunt-tipped setae (Z1–4, R1, R2) in integument adjacent to opisthosomal shield. Dorsal opisthosomal setae R2 unpaired in holotype (left R2 absent), and 1 paratype (right R2 absent). Setae R1 and R2 unequal in 1 paratype (left R1, and right R2 absent). Stigmata dorso-lateral, at level of coxa III.

**Venter:** Sternal shield with distinct margins and scaling pattern resulting from horizontal striations. Three pairs of short blunt-tipped setae (S1–3) on shield, short blunt-tipped metasomal setae (4d) in integument posterior of shield. Genital shield narrow, slightly sclerotized with 1 pair of short blunt-tipped setae (gen1), pair of pores in integument off shield. Anal shield with moderately sclerotized lateral margins, distally tapering blunt-tipped paranal and postanal setae, paranal setae anterior to anal opening, and postanal and postanal setae unequal length. Seven pairs of short blunt-tipped setae (ZV1–4, ZV1–3) on ventral opisthosoma. Ventral opisthosomal setae ZV1 unpaired in holotype, paired in paratypes.

**Gnathosoma:** Very short blunt-tipped subcapitular setae, 2 pairs hypostomal setae (hyp2–3), 6 deutosternal teeth. Chaetotaxy of palps: 0-1-2-11. Tibia-tarsus sensory area with 4 very short setae. Chelicerae widest proximally, tapering distally, with small sharp-tipped moveable and fixed digits (Fig. 4).

**Legs:** Claws of legs I–IV strongly curved. Chaetotaxy of legs: Cox a 2-2-2-1. Trochanter 4-4-4-4. Femur 9-8-5-5. Genu 6-6-6-5. Tibia 6-6-5-6. Tarsus 19-15-15-16. Tarsus IV ventral subapical setal pair long with 1 as a strong spike and the other a filamentous spike; tarsus II–III subapical setae long strong spikes.

**Male, nymphs, larva:** Unknown.

**Taxonomic summary**

Type host: Plectrophenax nivalis Linnaeus, 1758, snow bunting.

Type locality and collection date: Three mites from Oak Hammock Marsh, Manitoba (50°1’N, 97°0’W), 1 December 2002.

Types: \( \tilde{\varphi} \) holotype, \( \varphi \) paratype CNCl&A Nos. 23516, 23517. One \( \varphi \) paratype JBWME No. 0003056.

**Etymology:** nivalis, derived from the specific epithet of the Snow Bunting, Plectrophenax nivalis (nix, nivis, a Latin feminine noun).

**Remarks**

Ptilonyssus nivalis n. sp. is most similar to P. emberizae Fain, 1956, described from Emberiza flaviventeris from Ruanda-Urundi, and P. fringillae Fain and Sixl, 1971, described from Fringilla coelebs from Austria. Ptilonyssus nivalis n. sp. has 3 pairs of mesosomal setae, P. emberizae and P. fringillae have 4 pairs. The new species has 6 pairs of dorsal opisthosomal setae, whereas P. emberizae has 4 pairs and P. fringillae has 5. The opisthosomal shield is laterally excavated posteriorly and the posterior margin is invaginated; in P. emberizae, the opisthosomal shield lateral margins are less excavated and the posterior margin is rounded. There are 2 pairs of hypostomal setae in P. nivalis n. sp., while P. fringillae has 3 pairs. Seven pairs of ventral opisthosomal setae are present in the new species; P. emberizae and P. fringillae have 6 pairs of setae. Sternal shield with distinct scaling pattern in P. nivalis n. sp., while P. fringillae has a poorly developed scaling pattern.

**Ptilonyssus pinicola n. sp.**

(Figs. 23–30, 46–47)

**Diagnosis:** Large mites with podosomal and opisthosomal shields. Relatively long subposterior setae on posterior margin of podosomal
Figure 29. *Ptilonyssus pinicola* n. sp. male dorsum.
FIGURE 30. *Ptilonyssus pinicola* n. sp. male venter.
shield, presence and form of vestigial tritosternum, paranodal and postanal setae unequal length, 7 pairs of ventral opisthosomal setae, form of hypostomal setae, and differences in leg chaetotaxy distinguish this species from closely related species. 


**Dorsum**: Podosomal and pygidial shields sclerotized with areas of muscle insertions (Fig. 23). Podosomal shield with 7 pairs of setae (4 pairs short sharp-tipped, 3 pairs long filamentous tipped). Podosomal shield in holotype bearing 13 setae, 1 mesolateral setae unpaired; in paratypes all podosomal shield setae are paired. Setae on anterolateral and posterolateral corners of podosomal shield much longer than other dorsal idiosomal setae. Subposterior setae on posterior margin of podosomal shield longer than mesolateral setae. Opisthosomal shield with 4 pairs of setae (3 pairs short filamentous-tipped [J1–3], 1 pair short conical pointed [J4]). Opisthosomal shield rounded anteriorly, widest medially, without lateral excavations, trilobed posteriorly. Opisthosomal shield anterior margin medially straight with anterolateral projections, widest medially tapering to rounded posterior. Four pairs of short blunt-tipped setae in integument alongside podosomal shield, all approximately equal length. Six pairs of short filamentous-tipped setae (Z1–4, R1, R2) in integument alongside opisthosomal shield. Stigmata dorsolateral, at level of coxa III.

**Venter**: Very short blunt-tipped vestigial tritosternum level with coxa I (length 3.13 µm, width 2.23 µm). Sternal shield with distinct margins, with 3 pairs of sharp-tipped setae (st1–3) and 2 pairs of pores. Short sharp-tipped metasternal setae (st4) in integument posterior to shield. Lightly sclerotized genial seta with 1 pair short sharp-tipped setae (gen1), 1 pair of pores in integument off shield. Anal shield with distally tapering sharp-tipped paranal and postanal setae, paranal setae level with anal opening, paranal and postanal setae unequal length. Seven pairs short-tipped setae (Jv1–4, Zv1–3) on ventral opisthosoma. Ventral opisthosomal setae Zv1 unpaired in holotype, paired in paratypes.

**Gnathosoma**: Short blunt-tipped subcapitular setae, 3 pairs blunt-tipped hypostomal setae (hyp1–3), 6 deutostomal teeth. Chaetotaxy of palps: 0-3-3-14. Tibia-tarsus sensory area with 8 short setae. Chelicerae widest proximally, tapering distally, with small sharp-tipped movable and fixed digits (Fig. 46).


**Prototypus** (based on 2 specimens): LB 675 and 585. WID 350 and 370. LPS 180 and 173. WPS 165 and 163. LpS 43. WpS 80. LSS 100 and 103. WSS 73 and 70. LAS 73. WAS 54 and 58. LG 88 and 85. LG 75 and 80. LCH 66. WCH 16 and 18. LLeg I 200 and 185. LLeg II 153 and 158. LLeg III 163 and 165. LLeg IV 188 and 190.

**Dorsum**: Podosomal and pygidial shields lightly sclerotized, areas of muscle insertions not observed. Podosomal shield with 6 pairs of setae. Setae on anterolateral and posterolateral corners of shield much longer than other dorsal idiosomal setae, except setae J4. Pygidial shield of variable shape, always wider than long, with 1 pair of very long, barbed, sharp-tipped setae (J4); 41 long. Podosomal shield anterior margin either rounded or with a short medial projection, widest medially, slightly trilobed posterior margin. Mesosomal shieldlets present. Five pairs of setae (2 pairs sharp-tipped, 3 pairs blunt-tipped), 1 pair of pores in integument alongside podosomal shield. Eight pairs of short filamentous-tipped setae (J1–3, Z1–3, R1, R2) on dorsal opisthosoma. Stigmata dorsolateral, at level of coxa III.

**Venter**: Sternal shield lightly sclerotized, with 3 pairs of setae (st1, st2 sharp-tipped, st3 filamentous tipped) and 2 pairs of pores. Pair of genital pores in similar location as in adult females. Anal shield bearing filamentous-tipped paranal and postanal setae, paranal setae level with anal opening, paranal and postanal setae unequal length. Five pairs of short filamentous-tipped setae (Jv1–4, Yv2) on ventral opisthosoma.

**Gnathosoma**: Short blunt-tipped subcapitular setae, 3 pairs blunt-tipped hypostomal setae (hyp1–3, where hyp1 larger distally inflated bulblike setae), 7 deutosternal teeth. Palp chaetotaxy could not be reliably determined due to specimen condition. Chelicerae widest proximally, tapering distally, with small sharp-tipped moveable and fixed digits. 


**Dontonymph, larva**: Unknown.

### Taxonomic summary

**Type host**: *Pinicola enucleator* Linnaeus, 1758, pine grosbeak.

**Type locality and collection date**: Four mites from Coyote Lake Nature Sanctuary, Alberta (53°16'06"N, 113°32'06"W), 5 December 2002; coll. PMA.

**Additional localities and collection dates**: One mite from Barrhead, Alberta (54°07'23"N, 114°24'07"W), 8 December 1996; coll. PMA. Nine mites from Millet, Alberta (53°05'52"N, 113°28'22"W), 14 February 1995; same collector. Eight mites from Barrhead, Alberta (54°07'23"N, 114°24'07"W); same collector. Five mites from Pinawa, Manitoba (50°09'N, 5°33'W), 12 February 2005; coll. T. Gaylor and C. Dugal.

**Types**: holotype CNCi&A No. 23515. One ♀ paratype JBWM 0003055. One ♂ paratype ZIN No. 9989. One ♀ paratype, allotype ♂ UASM Nos. 80564, 80565. Remaining vouchers in possession of author.

**Etymology**: *pinicola*, derived from the generic name of the Pine Grosbeak, *Pinicola* (Latin feminine noun).

### Remarks

*Pilonyssus pinicola* n. sp. is most similar to *P. plesiopicus* n. sp., described herein from *Carpodacus purpureus* from Alberta, and *C. carduelis* Fain, 1962, described from *Carduelis cannabina* from Belgium.
The body length of *P. pinicola* n. sp. ranges from 790 to 960; in *P. plesiotypicus*, the body length ranges from 590 to 775 and is 643 in *P. carduelis*. Subterminal setae on the posterior margin of the podosomal shield are much longer than the anal setae in the new species; in *P. carduelis*, the subterminal setae are not longer than mesosomatic setae. In *P. plesiotypicus*, there is a very short blunt-tipped ventral trigonomark level with coxa I, while in *P. plesiotypicus*, the ventral tristernum is short and sharply pointed, and *P. carduelis* lacks a ventral tristernum. In the new species, paranal and postanal setae are unequal in length and tapered distally; in *P. plesiotypicus*, the paranal and postanal setae are constricted proximally and unequal in length, while in *P. carduelis*, the paranal and postanal setae taper distally and are equal length. Seven pairs of ventral opisthosomal setae are present in *P. pinicola* n. sp.; *P. plesiotypicus* has 8 pairs, and *P. carduelis* has 6 pairs. The new species has a blunt-tipped peglike hypostomal seta; *P. plesiotypicus* has blunt-tipped setae, where hyp1 is bulbike and distally inflated, and *P. carduelis* has blunt-tipped peg-like setae similar to *P. pinicola* except they are relatively shorter in *P. carduelis*. In the new species, coxa I setae are blunt-tipped pegs; *P. carduelis* has 1 blunt-tipped peg seta and 1 sharp-tipped spike seta on coxa I. Five setae are on femur IV and 3 setae on genu IV in the new species; *P. plesiotypicus* has 6 setae on femur IV and 2 setae on genu IV.

*Ptilonyssus plesiotypicus* n. sp. (Figs. 31–36, 48)

**Diagnosis:** Medium-sized mites with podosomal and opisthosomal shields. Relatively long setae on posterolateral corners of podosomal shield, relatively long subterminal setae on posterior margin of podosomal shield, 6 pairs of dorsal opisthosomal setae, presence of ventral tristernum, anal setae constrict proximally with long filamentous tips, paranal and postanal setae unequal length, 8 pairs of ventral opisthosomal setae, 3 pairs of large distally inflated hypostomal setae, and differences in leg chaetotaxy distinguish this species from closely related species.


**Male, nympha, larva:** Unknown.

**Taxonomic summary**

*Type host:* Cardopacus purpureus Gmelin, 1789, purple finch.

*Type locality and collection date:* Five mites from Alberta, 4 May 1996; coll. PMA (Provincial Museum of Alberta)

**Types:** ♀ holotype CNCI&No. 23512. Two ♀ paratypes UASM Nos. 80559, 80560. Two ♂ paratypes in possession of author.

**Etymology:** *plesiopic* (latinized Greek masculine adjective), in the cladistic meaning ‘primitive character.’ Named for the presence of a plesiomorphic character, a ventral tristernum.

**Remarks**

*Ptilonyssus plesiotypicus* n. sp. is most similar to *Ptilonyssus pinicola* n. sp., described herein from Piniocola enucleator from Alberta, *P. carduelis* Fair, 1962, described from Carduelis cannabina from Belgium, and *P. melissae* Spicer, 1977, described from *Cardopacus purpureus* from Texas. *Ptilonyssus plesiotypicus* n. sp. has a body length ranging from 590 to 775; the body length of *P. pinicola* ranges from 790 to 960, and *P. melissae* body length ranges from 561 to 668. The new species has a relatively longer setae on the posterolateral corners of podosomal shield; *P. melissae* has much shorter setae on the posterolateral corners. There are subterminal setae on the posterior margin of podosomal shield that are much longer than mesosomal setae in the new species; in both *P. carduelis* and *P. melissae*, subterminal setae are not longer than mesosomal setae. *Ptilonyssus plesiotypicus* n. sp. has 6 pairs of setae in the integument next to the opisthosomal shield; *P. melissae* has 5 pairs. The new species has a short sharply pointed ventral tristernum level with coxa I; the ventral tristernum of *P. pinicola* is very short and blunt-tipped, while both *P. carduelis* and *P. melissae* lack a ventral tristernum. The paranal and postanal setae constrict proximally (within one third of length from base) forming long filamentous tips in the new species; in *P. pinicola*, *P. carduelis*, and *P. melissae*, the anal setae taper distally. The paranal setae are level with the anal opening, and longer than the postanal setae in the new species, while in *P. carduelis* the paranal and postanal setae are equal length, and in *P. melissae* the paranal setae are anterior to the anal opening and no longer than the postanal seta. The new species has 8 pairs of ventral opisthosomal setae; *P. pinicola* and *P. melissae* have 7 pairs, and *P. carduelis* has 6 pairs. Three pairs of blunt-tipped hypostomal setae, where hyp1 is bulbike and distally inflated are present in *P. plesiotypicus* n. sp.; *P. pinicola* and *P. carduelis* have 3 pairs of hyposomal setae, but hyp1 is not bulbike or distally inflated, and *P. melissae* has 1 pair of hyposomal setae and does not appear to be distally inflated and bulbike. Coxa I setae are blunt-tipped pegs in the new species; *P. carduelis* has 1 blunt-tipped peg seta and 1 sharp-tipped spike seta on coxa I, and *P. melissae* coxa I setae are sharp-tipped spikes. Six setae are on femur IV and 2 setae on genu IV in the new species, whereas *P. pinicola* has 5 setae on femur IV and 3 setae on genu IV.

**Sternostoma setifer n. sp.** (Figs. 37–42, 49)

**Diagnosis:** Medium-sized mites with podosomal and opisthosomal shields. Three pairs of setae on opisthosomal shield, 4 pairs of dorsal podosomal setae, pair of genital shield, paranal setae on anal shield, comparatively long postoralateral palp genu seta, 1 pair of hyposomal setae, form of apical ventral setal pair on tarsus I, form of ventral, ventrolateral and apical setae on tarsus II–IV distinguish this species from closely related species.

**Female (based on holotype and 1 paratype):** LB 535 (575, 555), WID 268 (35, 296), LPS 160 (165, 163), WPS 180 LOP 135. WOS 143 (145, 144), LSS 104 (105, 104), WSS 89 (84, 86), LGS 100. WGS 70 (73, 71). LAS 60 (58, 59), WAS 53 (45, 49), LG 78, WG 75 (65, 70). LCH 64 (60, 62). WCH 20 (19, 19). LLeg I 190 (205, 198). LLeg II 140 (158, 149). LLeg III 163 (175, 169). LLeg IV 200 (203, 201).
Figures 40–42. *Sternostoma setifer* n. sp. female. (40) Venter. (41) Venter of tarsus I. (42) Venter of tarsus IV. Unlabeled scale bars 25 μm.
Figures 43-49. Chelicerae. (43) Ptilonyssus calvaria n. sp. female; (44) P. calvaria n. sp. male; (45) P. nivalis n. sp. female; (46) P. pinicola n. sp. female; (47) P. pinicola n. sp. male; (48) P. plesiotypicus n. sp. female; (49) Sternostoma setifer n. sp. female. Unlabeled scale bars 25 μm.
Dorsum: Podosomal and opisthosomal shields lightly sclerotized with areas of muscle insertions as illustrated (Fig. 37). Podosomal shield with 8 pairs and opisthosomal shield with 3 pairs of short sharp-tipped setae. Podosomal shield triangular, rounded anteriorly, widest posteriorly, posterior margin straight. Opisthosomal shield widest anteriorly, with slight lateral excavation. Four pairs of short sharp-tipped setae in integument alongside opisthosomal shield. Stigmata dorsolateral, at level to podosomal shield posterior margin.

Venter: Sternal shield lightly sclerotized with distinct margins and 3 pairs of blunt-tipped setae (st1-3) on shield. Genital shield broad, lightly sclerotized with pair of short sharp-tipped genital setae (gen1) in integument off shield. Anal shield with heavily sclerotized lateral margins; cirrum and blunt-tipped paranal setae on dorsum posterior to anal opening. Three pairs of short sharp-tipped setae on ventral opisthosoma.


Male, nymph, larva: Unknown.

Taxonomic summary

Type host: Empidonax minimus Baird & Baird, 1843, least flycatcher. Type locality and collection date: Two mites from Delta Marsh, Manitoba (98°23′W, 50°11′N), 11 June 2001; coll. T. Galloway and D. Hold­er. Types: 1 holotype CNCI&A No. 23518. One 1 paratype JBWME No. 0003057.

Etymology: setifer (Latin masculine adjective), meaning “bearer of setae.”

Remarks

Sternostoma setifer n. sp. is most similar to S. sayornis Ponce and Casto, 1975 described from Sayornis nigricans from Texas, S. pencei Spicer, 1984, described from Empidonax flavescens from Guatemala, S. darlingi Spicer, 1984, described from Mitrephanes phaeocerus from Guatemala, and S. hedonophilum Fain and Aitken, 1969 described from Platyvinchus saturatus from Brazil. Sternostoma setifer n. sp. has 3 pairs of setae on the opisthosomal shield; S. darlingi has 2 pairs of setae on the shield. Four pairs of dorsal opisthosomal setae are present in the new species; S. sayornis has 6 pairs, and S. hedonophilum has 1 pair. Stigmata are not surrounded with sclerotized cuticle in S. setifer n. sp.; S. hedonophilum stigmata are surrounded with hardened cuticle. In S. setifer n. sp., there is a genital setal pair off shield in the integument; S. sayornis has no genital setae, while S. pencei, S. darlingi, and S. hedonophilum have genital setae on shield. Paranal setae on the anal shield of the new species; S. sayornis, S. pencei, and S. darlingi have no anal setae. In the new species, postero-lateral palp genu seta are as long or longer than palp genu, whereas in S. sayornis, S. pencei, S. darlingi, and S. hedonophilum, the postero-lateral palp genu seta is clearly shorter than palp genu. The new species has 1 pair of hypostomal setae; S. sayornis and S. pencei have no hypostomal setae, S. darlingi has 2 pairs, and S. hedonophilum has 3 pairs. In S. setifer n. sp., there are no subcapitular setae, while S. hedonophilum has subcapitular setae. The tarsus I apical ventral setal pair of S. setifer n. sp. has a truncate tip; S. sayornis has a setal pair with serrated tips and a single spine at one side, while the S. hedonophilum setal pair have a bifurcate tip. The tarsus I-IV ventral and ventrolateral setae are relatively long (9.4 μm), with cylindrical blunt-tipped setae in the new species; in S. sayornis and S. hedonophilum, the ventral and ventrolateral setae are relatively short and taper distally, and in S. pencei and S. darlingi, the ventral and ventrolateral setae are relatively short, but have the same cylindrical form.

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LITERATURE CITED


GEORGE, J. E. 1961. The nasal mites of the genus Ptilonyssus (Acarina:


A NEW GLOSSIPHONIID LEECH FROM CATEMACO LAKE, VERACRUZ, MÉXICO

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ABSTRACT: Haementeria acuecueyetzin n. sp. from Catemaco Lake, Veracruz, Mexico, is described based on the examination of 6 specimens. This new hematophagous leech species resembles other members of the genus in the number and position of the eyespots, number of compact salivary glands, and in the presence of 2 pairs of spheroidal mycetomes, but it is distinguished from the other species by having 6 rows of longitudinal smooth white papillae in the dorsal surface and numerous tubercles in dorsal and ventral surfaces. This new species represents the third species of Haementeria in the Northern Hemisphere of the Americas.

Haementeria de Filippi, 1849 includes approximately 10 species that are blood feeders on vertebrates. Species of the genus are common in South America, but there are only 2 representatives in North America, i.e., Haementeria officinalis de Filippi, 1849 and H. lopezi Oceguera-Figueroa, 2006. This genus comprises species with 1 or 2 compact salivary glands connected to the base of the proboscis and 2 pairs of spheroidal symbiont-bearing organs called mycetomes (Ringuet, 1976, 1985). Members of this group have been variously used in studies of neurophysiology (De-Miguel et al., 2001), salivary anticoagulant properties (Salzet, 2001), and coevolutionary patterns with associated bacteria (Perkins et al., 2005).

MATERIALS AND METHODS

One specimen of the species described herein was sent to me in March 2005. This leech was removed from the leg of a high school student in Catemaco Lake, Veracruz, Mexico. A scientific expedition to the same locality was conducted in September 2005 to obtain additional specimens. Leeches were collected by immersing legs into the water in the edges of the lake, waiting 1 min, and then examining for leeches attached to skin. Six leeches were obtained before blood ingestion; they were placed in plastic containers and transported to the laboratory. Specimens were relaxed with gradual addition of ethanol and fixed with 4% formalin. The posterior sucker of 1 specimen was fixed in 100% ethanol. One leech was compressed between 2 glass slides, stained with a mixture of Ehrlich hematoxylin-Mayer’s paracarmine, and mounted on a slide. Dissections were accomplished using stereoscopic microscopy. Photographs of whole specimens were taken using a Sony Cyber-shot DSC-H5. Photographs of internal structures were taken using a Nikon SMZ-U stereomicroscope with a SPOT-RT (Diagnostic Instruments, Inc., Sterling Heights, Michigan) digital camera. Drawings were made by superposition of vector-art on placed images in an Adobe Illustrator (Adobe Systems, San Jose, California). Specimens were deposited in the Colección Nacional de Helminitos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México and the American Museum of Natural History, New York, New York (AMNH).

DESCRIPTION

Haementeria acuecueyetzin n. sp. (Figs. 1–5)

External morphology (on the basis of 5 specimens): Brownish, largest specimen 58 mm long, 10.7 mm wide. Complete somite triannulate, a1, a2, and a3 dorsally subdivided, but only a1 and a3 subdivided ventrally. Somites I–II unisegmented. Somites III–IV bisegmented. Somites V–XXIV triannulate. Somites XXV–XXVII bisegmented. Dorsum rough with 6 longitudinal rows smooth white papillae along the body, 1 pair paramedial, 1 pair lateral, and 1 pair submarginal. The white papillae occur on a2 of each somite, typically spherical, but in some cases bar-shaped. All annuli with numerous (>20) tubercles, not forming obvious longitudinal rows. Each tubercle covered with minute papillae. Additionally, small papillae in space not occupied by tubercles and smooth papillae (Fig. 1). Well-defined dark longitudinal mid-dorsal line starts immediately anterior to eyespots continuing to end of body, partially interrupted on a2 of each somite anteriorly. Some specimens with 2 additional indistinct longitudinal rows, 1 on each side of the mid-dorsal line (Fig. 2). Cephalic somites with 4–6 white spots on a2 arranged metamerically. One pair of eyespots separated by less than the diameter of an eyespot, located on first annulus of biaxially somite III. Ventral surface lighter than dorsum, fully covered with small tubercles (Fig. 3). Male gonopore conspicuous, between XI–XII. Female gonopore on XII between a2/(b5+b6). Two annuli between gonopores. Oral sucker somewhat triangular, mouth pore subterminal in anterior border. Anus located on dorsal surface, at XXVII. Caudal sucker directed ventrally in relaxed specimens, thinner than posterior part of body, with small tubercles covered with minute papillae and radial white lines on dorsal surface. Internal morphology (on the basis of 2 dissected specimens and 1 permanent slide): Proboscis in membranesh tongue extending posteriorly to somite XIV/XV, forming loop, returning anteriorly and connecting with esophagus at XII/XIII. Two pairs compact salivary glands discharge into base of proboscis; largest pair is located from somite X to XII/XIII connecting anteriorly to base of proboscis via strong ducts; second pair of compact salivary glands folded in somite XIII, each provided with a peduncle connecting the distal region with parenchyma. Esophagus extending anteriorly to somite XII/XIII, forming loop, returning posteriorly to crop. Two pairs spheroidal mycetomes located lateroventrally in somite XI. Each mycetome connecting independently via thick ductules to esophagus (Fig. 4). Crop with 7 pairs branched caeca from XIII to XIX; last pair forming postcaeca. Intestine with 4 simple caeca in somites XX–XXIII. Atrial cornua well developed. Highly coiled ejaculatory ducts immediately posterior to well developed atrial cornua. Six pairs of spherical intersegmental testisacs from XIII/XIV to XVIII/XIX. Ovisacs short and simple, forming a ring around the ventral nerve cord and reaching somite XIII b6 (Fig. 5).

Taxonomic summary

Type host: Unknown. This species has been observed on cattle and horses, and sometimes on humans, but a spectrum of vertebrate hosts is likely.

Type locality: Catemaco Lake, Veracruz State, Mexico. Near “Colonia La Victoria” 18°22′33.4″N, 95°06′34.4″W. Altitude 335 m.

Type material: Holotype, CNHE 6036; Paratypes CNHE (3 specimens) 6037, 6038, AMNH (2 specimens) 5428 Annelida. Collected by A. Oceguera-Figueroa, 9 September 2005. All specimens collected in the same locality.

Etymology: Acuecueyetzin means “leech” in the Nahualt language, and derives from the conjunction of the words a = atl = water; cueeya = cueyatl in plural = frogs and tzintli = diminutive; that is, small water frogs.

Remarks

According to the diagnosis of Haementeria elaborated by Ringuet (1976, 1985), members of this group have 1 pair of eyespots, 1 or 2
FIGURE 1. Dorsal view of the posterior sucker and somites of *Haementeria acuecueyetzin* n. sp. (smp) smooth papillae; (t) tubercles; Small papillae not shown.
pairs of compact salivary glands, and 2 pairs of spheroidal mycetomes and triannulate somites with annulus a1 and a3 subdivided ventrally, giving the appearance of quinquannulate somites in the ventral surface. These characteristics are consistent with those of the new species.

Two species of *Haementeria* have been reported in Mexico, i.e., *H. officinalis* (Caballero, 1930, 1932, 1940, 1941; Oka, 1932; Ringuelet, 1976, 1981, 1985; Sawyer, 1986; Lamothe-Argumedo et al., 1997) and *H. lopesi* (see Oceguera-Figueroa, 2006). *Haementeria acuecueyetzin* n. sp. can be easily distinguished from *H. officinalis* because the former has 6 longitudinal dorsal rows of smooth white papillae, which *H. officinalis* lacks. Additionally, *H. officinalis* lacks tubercles on the ventral surface. *Haementeria lopesi* has undivided dorsal annuli with almost flat white papillae in contrast to the subdivided dorsal annuli and conspicuous papillae and tubercles of *H. acuecueyetzin* n. sp.

The greatest diversity of *Haementeria* is found in South America, with 8 species having been described from the area (Ringuelet, 1985). Unlike *H. acuecueyetzin* n. sp., *Haementeria ghilianii* de Filippi, 1849 from Brazil and French Guyana lacks subdivided dorsal annuli, and it may reach almost 200 mm. *Haementeria molestia* (Cordero, 1934), from Brazil and Uruguay, has only 1 annulus between the gonopores and 5 longitudinal rows of dorsal papillae. Like *H. acuecueyetzin* n. sp., *Haementeria paraguayensis* (Weber, 1915) exhibits ventral tubercles, but it differs in these tubercles being arranged in 4 to 6 longitudinal rows. Moreover, *H. paraguayensis* is unusual among species of the genus in having only a single pair of compact salivary glands discharging into the base of the proboscis. Adult *Haementeria eichhorniae* Ringuelet, 1978 measure 15 mm, much smaller than *H. acuecueyetzin* n. sp., and they have 5 longitudinal rows of dorsal papillae, whereas *H. acuecueyetzin* n. sp. exhibits 6 rows and lacks a mid-dorsal row.

Other species in the genus differ from *H. acuecueyetzin* n. sp. by having a mid-dorsal row of papillae; these include *Haementeria lutzi* (Pinto, 1920) with 7 longitudinal rows and *Haementeria vizottoi* (Castro, 1971) with 5 rows. *Haementeria depressa* (Blanchard, 1849) is reported to have 4 or 5 rows, either of which is fewer than those described here for *H. acuecueyetzin* n. sp. None of the foregoing has tubercles in the ventral surface. *Haementeria tuberculifera* (Grube, 1871) lacks a mid-dorsal row of papillae, and it has 6 longitudinal rows of dorsal papillae like *H. acuecueyetzin* n. sp., but it clearly lacks tubercles on the ventral surface.

**DISCUSSION**

Early studies on the geographic distribution of *Haementeria* hypothesized that a unique taxon dispersed from South America to North America after the formation of the Panamanian Isthmus. With the recognition of *Haementeria acuecueyetzin* n. sp., the third species of the genus described for the Northern Hemisphere, a more complicated pattern is emerging. Whether Mexican species form a monophyletic group with post-dispersal speciation, or each species represents an individual dispersal event from the Southern Hemisphere needs to be clarified in
Figure 4. Base of the proboscis of *H. acuecuyetzin* n. sp. from the dorsal perspective. Somites are numbered with roman numbers and correspond with individual ganglia. pr, proboscis; bpr, base of the proboscis; my, mycetomes; csg, compact salivary glands; es, esophagus.
FIGURE 5. Male and female reproductive systems of *H. acuecueyetzín* n. sp. from the dorsal perspective. Somites are numbered with roman numbers and correspond with individual ganglia. ov, ovisacs; ac, atrial cornua; ed, ejaculatory ducts.
order to improve our understanding of biogeographic history of the Mesoamerican transitional zone.

ACKNOWLEDGMENTS

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LITERATURE CITED


IMMUNOSTIMULATING COMPLEXES INCORPORATING EIMERIA TENELLA ANTIGENS AND PLANT SAPONINS AS EFFECTIVE DELIVERY SYSTEM FOR COCCIDIA VACCINE IMMUNIZATION


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ABSTRACT: Immunostimulating complexes (ISCOMs) are unique, multimolecular structures formed by encapsulating antigens, lipids, and triterpene saponins of plant origin, and an effective delivery system for various kinds of antigens. The uses of ISCOMs formulated with saponins from plants collected in Kazakhstan, with antigens from the poultry coccidian parasite Eimeria tenella, were evaluated for their potential use in developing a vaccine for control of avian coccidiosis. Saponins isolated from the plants Aesculus hippocastanum and Glycyrrhiza glabra were partially purified by HPLC. The saponin fractions obtained from HPLC were evaluated for toxicity in chickens and chicken embryos. The HPLC saponin fractions with the least toxicity, compared to a commercial saponin Quil A, were used to assemble ISCOMs. When chicks were immunized with ISCOMs prepared with saponins from Kazakhstan plants and E. tenella antigens, and then challenged with E. tenella oocysts, significant protection was conveyed compared to immunization with antigen alone. The results of this study indicate that ISCOMs formulated with saponins isolated from plants indigenous to Kazakhstan are an effective antigen delivery system which may be successfully used, with low toxicity, for preparation of highly immunogenic coccidie vaccine.

Avian coccidiosis, an intestinal disease caused by intracellular coccidian protozoan parasites (Eimeria spp.), is estimated to cost the worldwide poultry industry over $1 billion annually through feed loss and lower bird productivity. The disease has been primarily controlled by use of anti-coccidial compounds that are mixed in the feed, but extensive use of these compounds over the past 40 yr has resulted in the inevitable development of drug resistance by these parasites (Yadav and Gupta, 2001; Allen and Fetterer, 2002; Williams, 2006). Many studies on the control of avian coccidiosis have centered on elicitation of protective immune response to parasite infection by development, delivery, and effective use of live, attenuated, or subunit recombinant parasite vaccines (Danforth et al., 1989, 1997; Jenkins, 1998; Dalloul and Lillehoj, 2005). Immunostimulating complexes (ISCOMs) are one of the most successful delivery systems for various kinds of antigens of microbial, parasite, or viral origin. ISCOMs are a unique, multi-molecular structure formed by encapsulating antigens, lipids, and triterpene saponins of plant origin (Kensil, 1996; Morein and Abasugra, 2004). Previous studies have shown that ISCOMs incorporating Quil A, a saponin isolated from the bark of the South American tree Quillaja saponaria, or purified triterpene saponin QS-21 isolated from Quil A by HPLC fractionation, initiate a wide range of antigen-specific immune responses. Some of these include humoral and CD4/CD8 cell-mediated responses, stimulation of IL2 and IFN-gamma production, and mucosal immune response through subcutaneous, intranasal, and oral routes of immunization (Kensil, 1996; Agrawal et al., 2003; Morein and Abasugra, 2004). Various saponins, with complex-forming and immunostimulating activity similar to Quil A saponin, have been isolated from Polygala senega, a plant indigenous to Canada (Estrada et al., 2000).

It is proposed that ISCOMs made from plant saponins may be uniquely suitable for vaccine development because of their ability to elicit an effective, protective, low-toxicity immune response to coccidial antigens in birds. The purpose of the present research is the construction and validation of an effective ISCOMs delivery system for isolated Eimeria spp. antigens.

MATERIALS AND METHODS

Hosts and parasites

Chickens (Titan broilers) were purchased from local hatcheries. Feed and fresh water were given ad libitum. Before any experimental procedures were initiated, chickens were allowed to acclimate for 1 day. Chickens with a negative antibody titer for E. tenella antigens were transferred to separate housing for experimental procedures. Chickens used in all experiments were handled according to the guidelines of the Institutional Animal Care and Use Committee, Institute of Virology and Microbiology, Almaty, Kazakhstan.

Eimeria tenella (strain AK-1) were single oocyst-derived lines isolated from naturally infected chickens in Almaty, Kazakhstan, and were maintained at the Institute of Microbiology and Virology, Almaty, Kazakhstan by serial passage in 10- to 14-day-old chicks. Oocysts were produced and maintained by routine passage in 2-wk-old Titan broilers with an infection dose of 125,000 oocysts/chicken. Oocysts were collected from infected bird ceca, cleaned, sporulated, and excysted as previously described (Tomley, 1997; Fetterer and Barfield, 2003).

Eimeria tenella antigens

Crude E. tenella antigens were prepared by treating the coccidia suspension (a mixture of sporozoites, sporocytes, and oocysts) with 5% MESK, a novel non-ionic dialyzable detergent consisting of sugars and fatty acids (Berezin et al., 1988). After detergent treatment for 30 min at 4 °C, the mixture was centrifuged at 10,000 g for 15 min at 4 °C. Supernatant fluid was retained for preparation of ISCOMs as described below.

Saponin isolation

Saponins used in ISCOMs formation and immunological experiments were isolated from the plants Aesculus hippocastanum and Glycyrrhiza glabra, collected in mountainous areas of southeastern Kazakhstan. Crude saponins were obtained from roots and seeds by 95% ethanol extraction and then partially purified from low-weight substances by...
extensive dialysis against phosphate-buffered saline. Plant extracts were lyophilized and fractionated by HPLC. Elution was performed with a linear gradient from a 0.1% trifluoroacetic acid (TFA) in water to an 80% acetonitrile (ACN) in water with 0.1% TFA. HPLC experiments were developed on a Waters (Milford, Massachusetts) chromatography system. Either an analytical column (Jupiter 5u C4 300A, 250 x 4.6 mm; Phenomenex, Torrance, California) or a semi-preparative column (Jupiter 5u C4 300A, 250 x 10.0 mm; Phenomenex) was used for separations. Absorbance was monitored at 208 nm and 353 nm using a photodiode array detector (Waters model 762). Aliquots (1 ml) were collected and pooled, as needed. Pooled fractions were dried under vacuum and stored at −20°C.

Toxicity studies

Toxicity of saponins was studied on 1-day-old chicks (broiler cross “Titan”) and 9-day-old chick embryos. Crude saponin preparations and pooled HPLC fractions were tested in animals by administration of 0.2 ml intranasally in chickens or by inoculation into allantoic cavities of 9-day-old chick embryos. Toxicity of each saponin preparation was examined in a dose of 40–1,000 μg per animal. Toxicity of samples was determined by counts of dead animals or dead embryos 72 hr after inoculation of saponins, in each concentration. Hemolytic activity of saponin preparations was determined by measuring the release of hemoglobin from chicken erythrocytes (Kaler et al., 1986). Saponins were added in a 0.1 ml volume to 2 ml of 2% suspension of chicken erythrocytes, for a final concentration of 0.125 mg/ml, and incubated for 30 min at 37°C. After incubation with periodic shaking, the mixture was centrifuged at 1,000 g for 10 min. Released hemoglobin in supernatant was measured by spectroscopy at 412 nm. All solutions were prepared in isotonic buffer with 1% phenolred and negatively stained with 3% uranil acetate in a boiling water bath for 3 min (Laemmli, 1970). Protein concentration was determined by Bradford method using Coomassie blue stain. Optical density was measured at 595 nm (Bradford, 1976); 60–70 μg mixed proteins of E. tenella were loaded onto the gel. Commercial preparations of purified saponin Quil A (Super Fos Biotech, Fredrickson, Denmark) were also used for ISCOMs formation and for immunological experiments for saponins from Kazakhstan plants, in the same manner described above. The preparations of plant saponins were free from proteins that may interfere with E. tenella antigens.

Challenge experiments

Two-wk-old chickens (7 birds per group) were immunized intranasally with various vaccine preparations containing E. tenella antigens. Two wk after immunization, all groups except the non-immunized, uninfected control group, were infected with live E. tenella at a dose of 50,000 oocysts per bird by oral gavage. Six days after infection, chickens were weighed and killed by cervical dislocation and the number of oocysts in the ceca counted.

Statistical analysis

The results are expressed as the mean ± SD. Statistical significance (P < 0.05) was determined using Student’s 2-tailed t-test. Results are expressed as the mean ± SD of individual responses.

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**Figure 1.** HPLC fractionation of saponins extracted from Aesculus hippocastanum and Glycyrrhiza glabra. Saponins were separated by reverse-phase chromatography using a linear gradient. The optical density at 208 nm (solid line) and 353 nm (dashed line) was measured. Aesculus hippocastanum HPLC fraction nos. 5–8 were pooled as HPLC fraction no. 1; fraction nos. 9–12 were pooled as HPLC fraction no. 2; fraction nos. 24–27 were pooled as HPLC fraction no. 3; fraction nos. 29–32 were pooled as HPLC fraction no. 4; and fraction nos. 33–36 were pooled as HPLC fraction no. 5. Glycyrrhiza glabra HPLC fraction nos. 6–8 were pooled as HPLC fraction no. 1; fraction nos. 10–13 were pooled as HPLC fraction no. 2; fraction nos. 15–18 were pooled as HPLC fraction no. 3; fraction nos. 20–23 were pooled as HPLC fraction no. 4; and fraction nos. 25–30 were pooled as HPLC fraction no. 5.
hemolytic activity compared to extracts or fractions from *A. hippocastanum*. Assay, extracts and HPLC fractions from *G. glabra* collected in Kazakhstan had lower hemolytic activity than commercial preparations of Quil A (Fig. 3). Similar to the toxicity of the extract and the HPLC fractions of *G. glabra*, their toxicity was determined. The results of studies in chickens compared with Quil A (Fig. 2). However, the saponins from both plant species were generally of lower toxicity when compared with Quil A saponin, and HPLC fractions from both plants had lower hemolytic activity than corresponding extract.

**RESULTS**

**Saponin isolation**

The results of HPLC fractionation of saponin extracts prepared from *A. hippocastanum* and *G. glabra* plant tissues are presented in Figure 1. Numerous peaks at 208 nm, which is the maximum absorbance for saponins, were observed. A lesser number of peaks were observed at 353 nm (maximum absorbance for flavonoids and carotenoids). HPLC fractions containing saponins were pooled, as indicated in Figure 1.

**Saponin toxicity**

Plant extracts and pooled HPLC fractions were collected and their toxicity was determined. The results of studies in chickens and chick embryos demonstrated that saponins isolated from both plant species were generally of lower toxicity when compared with Quil A (Fig. 2). However, the saponins from both the extract and the HPLC fractions of *G. galabra* had the lowest toxicity when compared to Quil A. For both *A. hippocastanum* and *G. glabra*, the HPLC fractions had lower toxicity than crude plant extracts.

Saponin preparations isolated from the 2 plant species collected in Kazakhstan had lower hemolytic activity than commercial preparations of Quil A (Fig. 3). Similar to the toxicity assay, extracts and HPLC fractions from *G. galabra* had lower hemolytic activity compared to extracts or fractions from *A. hippocastanum*, and HPLC fractions from both plants had lower hemolytic activity than corresponding extract.

**ISCOMs structure**

The results of electrophoresis analysis showed that an extract of *E. tenella* antigens possessed a number of proteins with molecular weights from 160 kDa to 18kDa (Fig. 4). ISCOMs assembled with isolated *E. tenella* antigens and saponins purified by HPLC fractionation from *A. hippocastanum* (HPLC fraction no. 4) and *G. glabra* (HPLC fraction no. 2) were studied by electron microscopy. ISCOMs incorporating saponins isolated from native plants demonstrated the characteristic cage-like structure, about 40–60 nm in size (Figs. 5A, B). ISCOMs assembled with Quil A saponin exhibited similar particles, about 50 nm in size, when examined by electron microscopy (Fig. 5C).

**Challenge experiments**

Birds immunized with ISCOMs induced a greater level of protection against challenge with live *E. tenella* than with parasite antigens alone (Fig. 6). Immunization with ISCOMs containing Quil A or *G. glabra* saponins prevented daily weight decrease (Fig. 6A) and stimulated a decreased number of *E. tenella* oocysts in the ceca of immunized chickens (Fig. 6B).

**DISCUSSION**

There remains a need for treatments of low toxicity, treatments that do not rely on live parasites for a vaccine to protect...
FIGURE 4. Electrophoretic analysis of *E. tenella* crude antigens isolated by MESK detergent extraction. (A) *Eimeria tenella* cell extract without MESK. (B) MESK isolated antigens. (C) Molecular weight markers. A mixture of purified *E. tenella* sporozoites, sporocysts, and oocysts was used for extraction of coccidian antigens.

FIGURE 5. Electron microscopy study of complexes formed by saponins of different origin. (A) Saponins isolated from *G. glabra* (HPLC fraction no. 2). (B) Saponins isolated from *A. hippocastanum* (HPLC fraction no. 4). (C) Quil A.

chickens against economic losses due to avian coccidiosis. However, subunit vaccines, based on natural antigens isolated from the parasite or recombinant antigens, have been shown in birds to stimulate only partial protection against parasite chal-

FIGURE 6. Protective activity of ISCOMs containing *E. tenella* antigens and *G. glabra* saponins. (A) Average daily chicken weight increase after *E. tenella* infection with a dose of 50,000 oocysts per chicken. (B) Cecal oocysts after immunization with ISCOMs: (a) non-immune infected chickens; (b) infected chickens immunized with isolated *Eimeria* antigens; (c) infected chickens immunized with ISCOMs incorporating Quil A; (d) infected chickens immunized with ISCOMs incorporating *G. glabra* saponins (HPLC fraction no. 2); (e) infected chickens immunized with ISCOMs incorporating *A. hippocastanum* saponins (HPLC fraction no. 4); (f) non-immune, uninfected chickens. The results are expressed as the mean ± SD of individual responses. Values are means for 7 chickens per group.
challenge (Murray et al., 1986; Danforth et al., 1989; Crane et al., 1991; Jenkins, 1998). This emphasizes the need for improvement in the method to elicit a protective immune response.

To effectively deliver parasite antigens, ISCOMs should be assembled with saponins that will physically support the ISCOMs structure, while demonstrating low toxicity. The ISCOMs should also stimulate host immune responses and, most importantly, they must protect the host against challenge infection. Our current results indicate that ISCOMs made with saponins from native plants that incorporate E. tenella antigens meet the above criteria.

Our current results demonstrate that partially purified saponins from the 2 plant species collected in Kazakhstan can be used to form the ISCOM structure. In addition, the partially purified saponins collected in pooled HPLC fractions have a significantly lower toxicity, as measured by both reduction in mortality and hemolytic activity when compared to unfraccionated Quil A, making them quite suitable for ISCOM construction. Of the 2 plants studied, the extract and fractions of G. glabra appear to have the lowest toxicity and therefore, may be the best saponin candidate for ISCOM construction.

Immunization of chickens with ISCOMs, prepared as described, offered protection to challenge infection, suggesting that ISCOMs can be the basis of an antigen delivery system for a coccidial vaccine. In addition, the protection observed with ISCOMs was superior to that observed with antigen alone, which is indicative of the immunostimulatory effect of ISCOMs. The birds were protected against 2 major effects of a coccidial infection, i.e., a decrease in weight gain and in oocyst output. There are a number of other response parameters of coccidial infection, including malabsorption, anemia, oxidative stress, and antibody responses, that were not measured in the current study. These parameters will be monitored in future, larger-scale trials of ISCOM preparations. However, preliminary results suggest that ISCOMs elevate antibody levels in chickens and induce cytokine production in mice (V. Berezin, unpublished).

Realistically, a coccidia vaccine will consist of 1 or more recombinant proteins rather than crude E. tenella proteins, as used in the current study. Our continuing goal is to evaluate recombinant proteins in the ISCOM delivery system in order to develop a practical coccidia vaccine.

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LITERATURE CITED


SCISTOSOMA HAEMATOBIUM (EGYPTIAN STRAIN): RATE OF DEVELOPMENT AND EFFECT OF PRAZIQUANTEL TREATMENT

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ABSTRACT: This study investigates the development of the Egyptian strain of Schistosoma haematobium and the resultant immunohistopathology and biochemical changes in organs affected. In addition, the response of different developmental stages of *S. haematobium* worms to praziquantel (PZQ) was examined. Schistosoma haematobium-infected hamsters were classified into 4 groups and were treated at day 35, 55, 75, and 95 postinfection (PI), respectively. Each group was subdivided into 3 subgroups. Two of them were treated orally with PZQ (300 mg/kg or 500 mg/kg divided equally on 2 consecutive days), and the third group was left without treatment. Treated groups were killed 20 days posttreatment. Infection with *S. haematobium* became patent 73 days PI; tissue egg load and worm fecundity were higher at 95 days and maximal 115 days PI, with an oogram pattern comparable to that in *Schistosoma mansoni* infection. In the liver, small cellular granulomas were observed 75 days PI, with predominance of CD+ T-cell phenotypes. In the urinary bladder, only submucosal focal Brun’s-­formation and angiogenesis without typical granulomas were observed. Ninety-five and 115 days PI, confluent granulomata with multiple eggs in the center were observed in the liver and urinary bladder, with a preponderance of CD+ positive T cells in the liver and hyperplasia of the urinary bladder epithelium with cystitis cystica and papillae formation. One hundred percent worm eradication was recorded with the higher dose of PZQ in animals treated 75 and 95 days PI. In conclusion, in spite of the long prepatent period of the Egyptian strain of *S. haematobium*, sensitivity to PZQ was recorded soon after infection. Granulomata were similar to those of *S. mansoni* in the liver and urinary bladders, but they were confluent with multiple eggs in the centers, hyperplasia of the urinary bladder urothelium with cystitis cystica, papillae, and Brun’s-­formation predictive of malignant changes with no hepatocyte dysplasia.

Urinary schistosomiasis represents a significant health burden for many developing countries (El-Khoby et al., 2000). The prevalence of *S. haematobium* in some areas of Africa has reached 70–90% (Cunin et al., 2003; Wagatsuma et al., 2003). In Upper Egypt, urinary schistosomiasis is considered to be 1 of the most important endemic diseases (El-Khoby et al., 2000). Possible consequences of *S. haematobium* infection include haematuria, dysuria, nutritional deficiencies, lesions of the bladder, kidney failure, elevated risk of bladder cancer, and growth retardation in children. Accordingly, estimates for morbidity and mortality in affected populations are high (WHO Expert Committee, 2002; Van Der Warf et al., 2003). Accumulation of parasite eggs in host tissues and the consequent egg-induced inflammatory responses lead to both acute and chronic damage of the urinary tract (Smith and Christie, 1986). Several factors influence the pathology induced by *S. haematobium* infection, e.g., the rate of egg deposition in tissues versus the rate of egg destruction, the intensity of infection, and the site of egg deposition (Cheever et al., 1975; Nash et al., 1982). A variety of different mechanisms have been implicated in the regulation of schistosome egg pathology. These include suppressor CD+ T cells (Colley, 1976; Chensue et al., 1993); cross-regulation by cytokines produced by Th1 or Th2 cells (Pearce et al., 1991; Flores Villanueva et al., 1996), and the development of anti-idiotypic antibodies (Montesano et al., 1997). All the above regulatory pathways involve the participation of T-lymphocytes, acting either directly or indirectly, through their helper function for antibody synthesis, or the host’s effective response to eggs.

Praziquantel (PZQ) will certainly remain the drug of choice over the next several years (Fenwick et al., 2003). Unfortunately, during the last decade there has been mounting evidence for the development of resistance to praziquantel in some schistosome populations (Ismail et al., 1996; Guisse et al., 1997; Stelma et al., 1997; William, Botros et al., 2001; William, Sabra et al., 2001; Danso-Appiah and De Vlas, 2002). A critical aspect in the assessment of PZQ efficacy is its activity against the different parasite development stages of different schistosoma species. Experimental laboratory studies showed that the activity of PZQ is stage dependent. Botros et al. (2005) reported that *S. haematobium* worms obtained at different times after infection and exposed in vitro to PZQ were refractory to low drug concentrations between 4 and 6 wk, but were clearly affected at higher concentrations and at latter times. The present work examines the parasitological criteria expressing *S. haematobium* development and the resultant immunohistopathology and biochemical changes in organs likely to be affected. The response of different developmental stages of *S. haematobium* worms to PZQ was examined in vivo by conducting the treatment at different time intervals after infection.

MATERIALS AND METHODS

Animals

Golden hamsters (*Mesocricetus auratus*), weighing 80–100 g, bred in the animal house of Theodor Bilharz Research Institute (TBDI), were used. Animals were maintained on a standard commercial pellet diet under suitable conditions of 12-hr light-dark periods.

Infection of animals

Six- to 8-wk-old, male golden Syrian hamsters were individually infected with 400 cercariae of *S. haematobium* (Egyptian strain). The Egyptian strain of *S. haematobium* has been collected from Abu Rawash, Giza Governorate (Upper Egypt), kept at Lowell University, Lowell, Massachusetts, for 10 yr, and then brought back to the Schistosome Biology Supply Center, TBRI. The strain has now been passed for more than 20 yr in hamsters and Balinus truncatus. Pooled cercariae used for infection were obtained from batches of at least 20 infected B. truncatus snails. Hamsters were infected with the use of abdominal skin exposure. Each animal was weighed and anesthetized by intramuscular injection with 0.5-g sodium thiopental diluted 1:10 in physiological saline. Anesthetized animals were placed prone with their bellies in contact with water in glass watch dishes into which the specified number of cercariae had been pipetted. They were left for a period of at least 20 min (Liang et al., 1987).
Experimental design

Five weeks PI, living infected hamsters were divided into 4 main groups according to the time of treatment, i.e., 35, 55, 75, and 95 days PI. Each of these groups was subdivided into 3 subgroups; 2 of them were treated orally with PZQ in a dose of 300 and 500 mg/kg divided equally on 2 consecutive days, and the third was left without treatment as controls. Treated groups were killed 20 days posttreatment, i.e., 55, 75, 95, and 115 days PI in parallel with their respective infected, but untreated, controls.

Parasitological assessments

To recover worms for subsequent counting and determination of sex, hepatic and mesenteric vessels of animals were perfused according to Duvall and DeWitt (1967), with no resort to general anesthesia. To examine worm distribution hepatic worms were separated from mesenteric ones by ligating the portal vein close to the duodenum. 

The number of ova per gram of liver or intestinal tissue (tissue egg load) was counted according to the methods of Cheever (1968), in which a piece of large intestinal or hepatic tissue was weighed before KOH digestion. The number of eggs/g of liver or intestine was calculated. Female fecundity was calculated as the number of total tissue egg counts (liver and intestine) divided by the total number of females for each individual hamster, and the final value was then divided by the number of days starting from the first day of egg deposition. The percentage of different egg developmental stages (oogram pattern) was studied according to the method of Pellegrino et al. (1962), in which eggs at different stages of maturity were identified (from I to IV) according to the size of the embryo and were counted. In addition, mature eggs containing fully developed miracidium and dead eggs (granular, dark, and semitransparent) were also counted in 3 fragments of large intestine (close to the rectum) and the mean number of each stage was calculated.

Histological examination

Liver and urinary bladder specimens were fixed in 10% formalin, processed to paraffin blocks, sectioned (4 μm thick) and stained with H&E and Masson trichrome. With the use of light microscopy, sections were examined for the presence of hepatic granulomas and associated histological changes. The rest of the livers and urinary bladders were employed for studying the proportional distribution of CD4+ and CD8+ positive T-lymphocytes with the use of the standard avidin–biotin immunoperoxidase method.

Immunohistochemical procedure for CD4+ and CD8+ antibodies

A standard avidin–biotin immunoperoxidase technique (Hsu and Raine, 1981) was used. Paraffin sections were dewaxed in xylene and hydrated in descending grades of ethanol. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide and 100% methanol for 20 min. Antigen retrieval was performed by microwaving the sections in citrate buffer (pH 6.0) for 15 min at 700 W. Sections were incubated overnight at 4°C with the primary anti-CD4+ and anti-CD8+ antibodies (Santa Cruz Biotechnology, Santa Cruz, California). Both antibodies were diluted 1:50 in PBS and incubated for 24 hr at 4°C. Sections were incubated for 15 min with biotinylated secondary antimouse antibody and then with avidin–biotin complex (ABC) horse-radish peroxidase solution (DAKO, Copenhagen, Denmark). After incubation for 10 min, the peroxidase reaction was developed with 0.01% hydrogen peroxide in 0.05% diaminobenzidine tetrahydrochloride. Sections were counterstained with Meyer's hematoxylin and dehydrated in ascending grades of ethyl alcohol prior to mounting. Liver and urinary bladder sections with voluntary omission of the primary antibody and its replacement by PBS served as negative controls for CD4+ and CD8+. Positive controls were sections of lymph node specimens.

With the use of the ocular grid method, a Zeiss standard light microscope equipped with an ocular grid reticle in the eyepiece and standard objective lenses was used (Konstantinidou et al., 1996). Lymphocytes with brownish positive staining of the cytoplasm (CD4+, CD8+) within the squares of the ocular reticle were counted at 400× magnification. An average of 100–300 cells within 10 fields was counted/section.

Enzyme assessments

Serum alanine aminotransferase (ALT), total protein, albumin, and urea were assayed according to the methods of Reitman and Frankel (1957), Weichselbaum (1946), Doumas et al. (1971), and Patton and Crouch (1977), respectively.

Statistical analysis

Results were analyzed with the use SPSS software, version 9.0. Values were expressed as means ± SE. Means of groups were compared with the use of an unpaired t-test. For comparison of more than 2 groups, an ANOVA test was used. Data were considered significant at P < 0.05.

RESULTS

Schistosoma haematobium development

By 35 and 55 days PI, 10–11% of the invading cercariae had developed into worms (41.25 ± 9.90 and 45.17 ± 10.37, respectively), with no eggs detected. Maximum worm load was observed 75 days PI (120.00 ± 7.61). Mean worm counts recorded 95 and 115 days PI (85.25 ± 7.61 and 73.60 ± 9.66, respectively) were significantly (P < 0.01) less than that recorded 75 days PI, with significantly higher tissue egg loads and female fecundity. Pilot examination of a limited number of animals starting 60 days PI revealed the presence of eggs 73 days PI. The fecundity of female worms was higher at 95 and 115 days PI (4,334.15 ± 603.59 and 5,150.66 ± 1,797.82, respectively), when compared to that recorded 75 days PI (114.8 ± 45.60), and was maximal at 115 days PI. In infected untreated hamsters, worms in couples were 25–33%, whereas they ranged from 0 to 1% in most of the PZQ-treated groups. Portalmesenteric S. haematobium worms in infected hamsters were more than 2-fold the number of hepatic worms, with a preponderance of males over females. At times of treatment (95 and 115 days PI) when worm eradication ranged from 97 to 99%, residual worms were recovered mainly from the hepatic compartment. Like S. mansoni eggs, S. haematobium eggs at different developmental stages were observed, where 61–76% of the eggs were immature, 15–30% were mature, and only 6–9% were dead in infected untreated hamsters examined 75, 95, and 115 days PI. On the contrary, in PZQ-treated hamsters there was complete disappearance of immature and mature eggs, with 100% dead eggs (Tables I, II).

Response of different parasite developmental stages to PZQ

Praziquantel in doses of 300 or 500 mg/kg given to 35-day S. haematobium–infected hamsters reduced worm burden by 68 and 72%, respectively (P < 0.01), with higher significant (P < 0.001) worm reduction in those treated 55, 75, and 95 days PI, and with almost complete eradication of worms in animals receiving the higher dose of PZQ (Table I). Administration of PZQ to 75- and 95-day S. haematobium–infected hamsters significantly reduced (P < 0.001) the hepatic and intestinal tissue egg loads in hamsters treated 75 days PI and killed 95 days PI, and only the intestinal tissue egg load (P < 0.05) in those treat-
TABLE I. Effect of praziquantel treatment administered at different time intervals postinfection of hamsters with *Schistosoma haematobium* (400 cercariae/hamster) on worm distribution, sex, and load 20 days after treatment.*

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Time of sacrifice (days postinfection)</th>
<th>PZQ dose (mg/kg)</th>
<th>Total hepatic</th>
<th>Total portomesenteric</th>
<th>Total males</th>
<th>Total females</th>
<th>Total couples</th>
<th>Total worms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected control</td>
<td>35</td>
<td>11.8 ± 5.91</td>
<td>29.45 ± 5.23</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>41.25 ± 9.97†</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>18.57 ± 5.7</td>
<td>26.60 ± 5.03</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>45.17 ± 10.37†</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>38.50 ± 4.85</td>
<td>81.50 ± 6.82</td>
<td>74.67 ± 5.48</td>
<td>45.33 ± 4.58</td>
<td>39.33 ± 3.86</td>
<td>ND</td>
<td>120.00 ± 7.61</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>31.65 ± 4.26</td>
<td>53.60 ± 4.1</td>
<td>63.65 ± 7.18</td>
<td>21.60 ± 2.18</td>
<td>19.40 ± 2.01‡</td>
<td>ND</td>
<td>85.25 ± 7.61‡</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>20.60 ± 3.62</td>
<td>53.00 ± 7.4</td>
<td>51.85 ± 8.33‡</td>
<td>21.75 ± 3.12‡</td>
<td>18.25 ± 2.66‡</td>
<td>ND</td>
<td>73.60 ± 9.66‡</td>
</tr>
<tr>
<td>Infected PZQ-treated</td>
<td>55</td>
<td>6.6 ± 2.25</td>
<td>7.90 ± 4.75</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>14.50 ± 9.17 (68)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>8.67 ± 1.65</td>
<td>3.90 ± 1.01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>12.60 ± 2.38 (72)</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>1.13 ± 0.4</td>
<td>1.67 ± 0.71</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.80 ± 0.66‡ (98)</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>0.33 ± 0.21</td>
<td>0.27 ± 0.17</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.60 ± 0.24# (100)</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>1.43 ± 0.54</td>
<td>0.17 ± 0.17</td>
<td>1.10 ± 0.40#</td>
<td>0.50 ± 0.34#</td>
<td>0.17 ± 0.17#</td>
<td>ND</td>
<td>1.60 ± 0.68# (98)</td>
</tr>
<tr>
<td></td>
<td>115</td>
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<td>0.00#</td>
<td>0.00#</td>
<td>0.00#</td>
<td>0.00#</td>
<td>0.75 ± 0.20#</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>0.00#</td>
<td>0.00#</td>
<td>0.00#</td>
<td>0.00#</td>
<td>0.00#</td>
<td>0.20 ± 0.20#</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>1.00 ± 0.32</td>
<td>0.40 ± 0.20#</td>
<td>0.20 ± 0.20#</td>
<td>0.20 ± 0.20#</td>
<td>ND</td>
<td>0.60 ± 0.25#</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Results are presented as means ± SE. Number of hamsters/group = 6. Numbers in parentheses indicate the percentage worm reduction from the corresponding infected untreated control. ND: Sex not differentiated.

† Significant difference versus control values recorded 75 days postinfection at *P* < 0.001.
‡ Significant difference versus control values recorded 75 days postinfection at *P* < 0.01.
§ Significant difference versus control values recorded 75 days postinfection at *P* < 0.001.
|| Significant difference from the corresponding infected untreated control at *P* < 0.01.
# Significant difference from the corresponding infected untreated control at *P* < 0.001.
ed 95 days PI and killed 115 days PI (Table II). Complete disappearance of immature egg stages with a decrease in mature eggs and an increase in dead eggs were observed in hamsters treated 75 and 95 days PI and killed 95 and 115 days PI. Dead eggs were 100% in hamsters treated with 500 mg/kg PZQ (Table II).

**Immunohistological findings**

*Schistosoma haematobium*-infected, untreated hamsters revealed no granuloma 35 and 55 days PI in the liver or 35, 55, and 75 days PI in urinary bladder sections, with no lymphocytes positive for anti-CD4⁺ or CD8⁺ antibodies. Only simple inflammatory cellular collections (eosinophils and macrophages) with focal cloudy swelling and hydropic degeneration were seen in the livers. Bile duct proliferations and angiogenesis in portal tracts with small cellular granuloma were recorded 75 days PI. Ninety-five and 115 days PI, hepatic single or confluent granuloma with multiple adherent eggs in the center were observed (Fig. 1A). At that time, granuloma were cellular, fibrocellular, and fibrous. At 115 days PI, they were mainly situated in the prevascular tissues and were formed of large histiocytes and epithelioid cells with a rim of lymphocytes in the outer zone on a background of fine fibrillar network with a thicker outer cuff of collagen bundles. Granuloma were similar to those of *S. mansoni*, but with multiple eggs in the center and prevalence of CD4⁺ over CD8⁺ T cells at 75 and 95 days PI that was only significant (*P < 0.05*) 75 days PI (Fig. 2A, B). CD8⁺ T-positive cells were more prevalent (*P < 0.05*) as compared with CD4⁺ T-positive cells at 115 days PI. Urinary bladder urothelium showed hyperplasia, focal ulceration, and papilloid formation 55 days PI. Focal Brunn’s-nest formation without ova deposition or granuloma formation, either in the lamina propria or muscle layer, were seen 75 days PI. Only infiltrates of scarce eosinophils and lymphocytes with vascular angiogenesis were observed. Single or confluent granuloma with multiple adherent eggs in the center in addition to chronic inflammatory reaction, including cystitis cystica, urothelial hyperplasia, Brunn’s nests, and papillae formation (Fig. 1B) were seen 95 and 115 days PI at the lamina propria of the urinary bladder (Table III).

**Immunohistological changes in PZQ-treated hamsters**

*Schistosoma haematobium*-infected hamsters treated 55 days PI with PZQ showed a decrease in the number of chronic inflammatory cells and CD4⁺ positive cells in the liver (*P < 0.05*; Table III), whereas those treated 75 and 95 days PI, showed a few small hepatic granuloma in 4/6 and 6/6 PZQ-treated hamsters, respectively, with presence of amalgamated dead eggs surrounded by pigment macrophages (Fig. 1C). CD4⁺ positive cells (*P < 0.01*) were significantly higher in the group treated 75 days PI with 300 mg/kg PZQ and significantly less (*P < 0.01*) in the group receiving PZQ in doses of 300 mg/kg or 500 mg/kg 95 days PI and killed 20 days posttreatment, when compared to their corresponding untreated infected controls (Table III). Bladder sections showed decreased number and size of granuloma. Hyperplasia of the lining epithelium in all the examined specimens with cystitis cystica, Brunn’s nests, and papillae formation with increased angiogenesis in the lamina propria was comparable to that in infected untreated controls in all PZQ-treated groups. (Fig. 1D).

**Biochemical changes in PZQ-treated hamsters**

*Schistosoma haematobium* infection significantly reduced the serum levels of total proteins and albumin 95 (*P < 0.05*) and 115 days (*P < 0.05, P < 0.01*, respectively) PI when compared to normal values. Levels were restored to normal (*P < 0.05*) upon treatment with PZQ in a dose of 300 or 500 mg/kg. Serum ALAT was significantly increased from 35 days to 95 days PI and then declined 115 days PI. At the same time, serum urea was significantly elevated at most of the observation periods starting from 55 days PI. The elevation in both ALAT and urea was normalized in hamsters treated with 500 mg/kg PZQ.

**DISCUSSION**

In this work, 10–11% of infecting cercariae developed into worms 35 and 55 days PI, respectively, although their sex could
not be differentiated. By day 75, they were differentiated and the actual number of worms was easily quantified. Smith et al. (1976) reported a scattered distribution of *Schistosoma haematobium* worms between 4 and 8 wk PI. Botros et al. (2005) reported low worm recovery early after *S. haematobium* infection that was attributed to the slow development of *S. haematobium* female worms after pairing and the length of time it takes to migrate from the lung to portomesenteric sites. In the present study, fewer worms were recorded 95 and 115 days PI. Sadun et al. (1970) reported fewer worms in *S. haematobium*-infected chimpanzees with a longer duration of infection.

Like *S. mansoni*, portomesenteric worms exceeded the hepatic with a preponderance of males over females. Pellegrino and Katz (1969) reported 20% recovery of worms from the liver, with the remaining worms retrieved from the portomesenteric veins in *S. haematobium* infection. Sex ratio in favor of males between 9 and 20 wk PI was also recorded (Imbert-Establet et al., 1992). In animals infected with Sudanese (Smith et al., 1976) and Ghana strains (Burden and Ubelaker, 1981) of *S. haematobium*, eggs were found to first appear 60 and 65 days PI, respectively. Guided by these data, killing of 1 or 2 infected hamsters at days 60, 65, 70, and 73 days PI revealed that eggs started to appear 73 days PI for the Egyptian strain of *S. haematobium*. Different developmental times with consequent variable times for egg production are probably the underlying reason for such differences between strains. Smith et al. (1976) reported an equivalent rate of development to that for *S. mansoni* in the mouse up to the stage when pairing occurs. They
added that subsequent to pairing (day 31), somatic growth of *S. haematobium* continues, but genital organ development is arrested for almost a month and egg production occurs by day 65. This is contrary to female *S. mansoni* worms, in which fully mature, egg-producing worms were recorded on day 35 after pairing on day 28 (Clegg, 1965). The very long time lag between pairing and full sexual maturation is a remarkable feature of *S. haematobium* (Smith et al., 1976). In the present study, although the number of worms diminishes with time, tissue egg loads and fecundity were higher. This increase in egg load could be due to either an increase in egg production, or a decrease in egg destruction, or both. Cheever et al. (1977) reported longer persistence of *S. haematobium* eggs after the death of worms. Agnew et al. (1988) reported egg laying 9.5 wk after *S. haematobium* infection, and that eggs accumulate in the tissues throughout the period of infection. Imbert-Establet et al. (1992) recorded a high number of eggs in the liver from the 12th wk with eggs in the bladder 20 wk PI with *S. haematobium*. They attributed the high count of ova in the intestinal tissue to better pairing among schistosomes in the mesenteric tributaries (Ghalieb et al., 1979).

In the present work, granulomata were not detected in liver and urinary bladder up to 55 days PI. Only a few collections of inflammatory cells were seen in between hepatocytes, which could be a response to worms recruiting inflammatory cells to sites of toxins emitted by them. At 75 days PI, small cellular granulomata typical of *S. mansoni*, but with multiple eggs in the center, were observed in liver sections. Warren and Domingo (1970) reported that granuloma formation around *S. mansoni* and *S. haematobium* eggs is similar, quantitatively and qualitatively. On the other hand, Hussein et al. (2005) reported heterogeneity of cell populations between *S. haematobium* and *S. mansoni* granulomas, suggesting different tissue reactions to the deposited ova. Livers showed increase in the number of granulomata around amalgamated eggs 95 and 115 PI, which was in agreement with the findings of Ebeid et al. (2005). In *S. mansoni* infection (Hernandez et al., 1999; Fallon et al., 2000), the granulomatous response to schistosome eggs is a CD4⁺ T-cell dependent immunopathologic response. In the present

**TABLE III.** Effect of praziquantel (PZQ) administered at different time intervals postinfection of hamsters with *Schistosoma haematobium* (400 cercariae/hamster) on immunopathological changes 20 days after treatment.*

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>PZQ dose (mg/kg)</th>
<th>Time of sacrifice (days postinfection)</th>
<th>Mean % of positive cells ± SE</th>
<th>Liver</th>
<th>Urinary bladder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD⁺ cells</td>
<td>CD⁺⁺ cells</td>
<td>CD⁺ cells</td>
</tr>
<tr>
<td>Infected</td>
<td>—</td>
<td>75</td>
<td>68.33 ± 2.10†</td>
<td>29.16 ± 3.20</td>
<td>14.33 ± 1.49</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>95</td>
<td>40.10 ± 2.88</td>
<td>31.50 ± 1.72</td>
<td>12.66 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>115</td>
<td>33.50 ± 4.30</td>
<td>50.33 ± 3.78†</td>
<td>13.33 ± 0.88</td>
</tr>
<tr>
<td>Infected</td>
<td>300</td>
<td>75</td>
<td>51.16 ± 3.11‡</td>
<td>31.66 ± 4.50</td>
<td>12.50 ± 0.76</td>
</tr>
<tr>
<td>PZQ-treated</td>
<td>500</td>
<td>75</td>
<td>53.50 ± 4.70‡</td>
<td>35.66 ± 4.54</td>
<td>14.50 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>95</td>
<td>50.00 ± 2.59§</td>
<td>36.50 ± 1.26</td>
<td>12.66 ± 0.66</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>95</td>
<td>45.00 ± 2.58</td>
<td>33.33 ± 2.47</td>
<td>11.50 ± 1.89</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>115</td>
<td>27.50 ± 2.10§</td>
<td>58.00 ± 1.87</td>
<td>12.66 ± 0.66</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>115</td>
<td>25.80 ± 1.32§</td>
<td>48.50 ± 4.75</td>
<td>12.66 ± 0.66</td>
</tr>
</tbody>
</table>

* Results are presented as means ± SE. Number of hamsters/group = 6.
† Significant difference of CD⁺⁺ versus CD⁺⁺, or CD⁺⁺ versus CD⁺⁺ in the same infected group at *P* < 0.05.
‡ Significant difference versus the corresponding infected untreated control at *P* < 0.05.
§ Significant difference versus the corresponding infected untreated control at *P* < 0.001.
study, examination of T-cell phenotypes in granulomas of *S. haematobium*-infected animals revealed predominant CD4+ T cells in the acute phase of granuloma formation (75 days PI); this was followed by a decrease in the number of CD4+ T cells with time and an increase in the number of CD8+ T cells (115 days PI). Nmorsi et al. (2005) reported that the granulomatous reaction in urinary schistosomiasis is CD4+ T-cell dependent and that CD8+ T cells were found to be cytotoxic to parasites and are activated by CD4+ T cells.

In the urinary bladders, granulomata started to appear 95 days PI around multiple central eggs; there was also urothelial hyperplasia with Brunn’s nests, cystitis cystica, and papillae formation 115 days PI. Vuong et al. (1996) reported that in *S. haematobium*-infected rodents, 7 of 10 urinary bladders revealed granulomata with or without chronic cystitis, fibrosis, polyp formation, and urothelial changes. Ghoneim (2002) reported urothelial hyperplasia with cystitis cystica in the bladders of Egyptian patients infected with *S. haematobium*.

PZQ in divided doses of 300 or 500 mg/kg, on 2 consecutive days, reduced worm burden by >95% regardless of the time of treatment (55, 75, and 95 days PI), with the complete disappearance of immature ova, a higher percentage of dead ova, and a greater reduction in hepatic and intestinal tissue egg loads. These findings were evidently observed in the groups treated with 500 mg/kg PZQ rather than 300 mg/kg. In these groups, worm reduction was almost 100%, with complete disappearance of immature and mature eggs and 100% dead eggs. Meanwhile, residual worms recovered from animals revealing highest worm eradication were recorded from the hepatic compartment. Ebeid et al. (2001) reported complete parasitological cure (100% worm reduction) 2 and 4 wk after treatment of hamsters with PZQ in a total dose of 200 mg/kg divided on 2 days. Guirguis (2003) reported a 98–99% worm reduction following treatment of *S. haematobium*-infected hamsters with PZQ in a dose of 500 mg/kg for 2 consecutive days. Data show that *S. haematobium* worms are more sensitive to PZQ than *S. mansoni*. Pica-Mattoccia and Cioli (2004) reported that *S. haematobium* worms were twice as sensitive to the drug as the same sex of *S. mansoni*. Mott et al. (1985) demonstrated that PZQ reduced the clinical signs due to *S. haematobium* infection with high cure rates, or reduction of egg excretion, or both. Marked resolution of inflammatory cellular reactions in the liver and urinary bladder of *S. haematobium*-infected hamsters was observed in animals treated with PZQ 75, 95, and 115 days PI. This was in agreement with the results of Webb et al. (1981). At the same time, significant reduction in the percentage of hepatic CD4+ T cells was observed in animals treated with PZQ (300 mg/kg or 500 mg/kg) 95 days PI and killed 115 days PI. In animals treated with PZQ, granulomata appeared small in size, but hyperplasia of the urinary bladder epithelium with cystitis cystica, Brunn’s nests, and papillae formation were still recorded in all specimens examined. Like *S. mansoni*, *S. haematobium* infection caused significant elevation in serum levels of ALAT in all infected groups (Ahmed and Gad, 1995; Ebeid et al., 2001). With a longer duration of infection, *S. haematobium* infection produced hypoalbuminemia. Reduction in total serum protein and albumin concentration has been reported in *S. mansoni*-infected mice (Abdel-Ghaffar, 2004). This reduction could be a consequence of impaired protein synthesis in the liver, utilization of amino acids by schistosomes (Abdel-Ghaffar, 2004), and/or increased leakage of albumin through the intestinal mucosa (El-Raziky et al., 1985). Elevation in serum urea of *S. haematobium*-infected hamsters was noticed at most of the observation periods. Cheever et al. (1977) reported concentration of 61% of *S. haematobium* eggs in the genitourinary tract. Sadun et al. (1970) reported increased serum urea in *S. haematobium*-infected chimpanzees. They attributed this increase to enhanced catabolism of tissue proteins and nucleic acids, leading to elevated blood urea with disturbed renal function (Nelson and Cox, 2000). Moreover, immune complex deposition as a result of schistosomal infection in the glomeruli may lead to glomerulonephritis (Andrade and Abreu, 1971). Treatment of infected hamsters with PZQ normalized these enzymes. Similar findings were recorded by Ebeid et al. (2001).

Unlike other geographical strains of *S. haematobium* infection, the Egyptian strain needed a longer time to become patent (73 days PI), yet sensitivity to PZQ was noticed soon after infection. At 95 and 115 days PI, granulomata in the livers and urinary bladders were very similar to those of *S. mansoni*, but they were confluent, surrounding from 3 to 5 eggs. *Schistosoma haematobium* eggs stimulated CD4+ T-cell–mediated granulomatous response in the livers similar to that of *S. mansoni*. Hyperplasia of the epithelium, with cystitis cystica and papillae formation, was observed in the urinary bladders at most of the observation periods, although these were more evident 115 days PI. Maximal parasitological cure was recorded with the higher dose of PZQ and was clearly evident in the groups treated from 75 days PI onwards. Moreover, PZQ normalized the biochemical changes with fewer histological changes in the liver, but not the urinary bladder, which may be considered a potential for subsequent malignant transformation in this organ.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


SCHISTOSOMA JAPONICUM: PROTECTIVE IMMUNITY INDUCED BY SCHISTOSOMULUM-DERIVED CELLS IN A MOUSE MODEL

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ABSTRACT: We previously reported that immunization with intact live cells from schistosomula of Schistosoma japonicum (S.j) partially protected the Kunming strain of mice from challenge infection. In the present work, 2 immune protective experiments were designed to further validate the protective effect induced by this type of vaccine and to optimize the immunization protocol, including the number of inoculations and parasite stages from which immunogenic cells were derived. Three antigens derived from 18-day-old postinfection live (LLC) and dead (DLC) larval worm cells and from dead 42-day-old postinfection adult worm cells (DAC) were used as immunogens. Our results demonstrate that live cells from 18-day-old worms are capable of inducing significant protection in mice using a murine-Sj challenge model as shown by reduction rates of worm recoveries and egg burdens. The development of adult worms was stunted. A Thl-biased immune response was reflected in the protected groups as evidenced by the ratio of IgG2a/IgG1. A 38-kDa polypeptide was recognized by sera from LLC immunized animals. We demonstrate that live parasite cells are a source of novel protective antigens that can be exploited for vaccine development.

Schistosomiasis is a debilitating, chronic, widespread disease with a major health impact in endemic countries. More than 600 million people are at risk and about 200 million actually infected in 74 countries in Africa, Middle East, South America, and southeast Asia, with 280,000 annual deaths due to schistosomiasis (WHO, 2002; Chi, 1996). Fifty years ago, China was one of the most afflicted countries for schistosomiasis; the disease covered 12 provinces along the Yangtze River with about 100 million people at risk of infection. Aggressive programs have controlled schistosomiasis in many areas, but the disease is still endemic in Hu Nan, Hu Bei, Jiang Xi, An Hui, and Jiang Su, and the mountain areas of Si Chuan and Yun Nan provinces. Today, it is estimated that 820,000 people and several hundred thousand domestic animals are infected (Office of Endemic Diseases Control—China, 1998). Of particular concern has been the recent increase in prevalence of schistosomiasis in the flood plains of Yangtze River and Dong Ting Lake. Moreover, the massive Three Gorges Dam across the Yangtze River in southern China, soon to be completed, has the potential to significantly increase Schistosoma japonicum (S.j) transmission and introduce the infection into unaffected areas (Ross et al., 2001). The availability of praziquantel (PZQ) has rapidly made chemotherapy the cornerstone of control, leading to dramatic reduction of morbidity in endemic areas. However, not only are the costs of diagnosis and the delivery of treatment high, but reinfection occurs rapidly and treatment must be repeated at frequent intervals at the risk for creating drug resistance (Ismail, Botros et al., 1996). Despite the fact there is not yet clear-cut evidence for the existence of a PZQ-resistant schistosome strain, decreased susceptibility to the drug has been observed in several countries, although not as yet in China (Fallon et al., 1995; Stelma et al., 1995; Ismail, Metwally et al., 1996). Therefore, effective application of an antischistosome vaccine is important, especially in the areas where domestic animals serve as reservoir hosts and are responsible for spreading S. japonicum (McManus et al., 2004).

Induction of specific immunity against schistosomes by prior exposure to ultraviolet-attenuated cercariae has been demonstrated in rodents, pigs, and water buffaloes (McManus and Bartley, 2004). Although it is neither ethical nor practical to use such a vaccine in humans (Waine et al., 1993), such induction, together with the encouraging results obtained with defined antigens, suggests that development of a vaccine is achievable (McManus, 1999). However, in spite of considerable time and effort spent on testing the parasite antigens and having identified more than 100 antigens, so far, none (including 6 selected schistosoma proteins: PMY, GST, IrV5, membrane-associated antigen, TPI, and FABP) has fulfilled the standard of consistently engendering 40% protection as requested by WHO (1996), except for schistosome antioxidant enzymes (Shalaby et al., 2003). Even in the most successful individual cases, the protection levels obtained were not close to those achieved using attenuated vaccines (Bergquist, 1998). These results are perhaps explained by the fact that the schistosome parasite is such an antigenically complex organism that the immune responses induced by single antigen vaccination may not be strong enough to combat the challenge infection. In contrast, the live attenuated vaccines induce immune responses against many antigenic components, several of which may contribute to vaccine efficacy. This suggests that development of a multivalent antigen vaccine, including worm-derived cells or cultured cells in vitro, may be a way forward.

The immunostimulatory capacity of several cells renders them attractive candidates to modulate immunity against pathogens, including viruses, bacteria, fungi, eukaryotic parasites, and tumors (Pedersen et al., 1987; Vermorken et al., 1999; Cantabu et al., 2000; Gustavson et al., 2002; Bozza et al., 2004). Previous reports regarding research on cell-type vaccines have been focused mainly on induction of immunity against tumors or microorganisms. For example, to date, active specific immunotherapy (ASI) with autologous tumor cell vaccine has been successfully applied to melanoma and renal cell carcinomas with 4 randomized clinical trials reporting overall survival or relapse-free survival benefit for patients treated with tumor cell lysates (Sondak et al., 2002) or irradiated tumor cells (Hoever et al., 1993; Vermorken et al., 1999).
As to research of cell-type vaccines against schistosomiasis, studies have examined the potential protective efficiency induced by PIII loaded macrophages (Gustavson et al., 2002). However, the possibility of schistosome worm-derived cells or in vitro cultured worm cells serving as a vaccine against the disease has not received attention. In preliminary work, our group demonstrated that immunization of mice with live larval worm cells from *S. japonicum* could eliciting effective protection against challenge infection (Zeng et al., 2004).

Here, we report the results of our recent efforts to further examine the immunoprotection in mice afforded by live and dead cells derived from *S. japonicum*. Our results showed that vaccination of mice with LLC or DLC significantly reduced adult worm burden and fecundity after challenged with *S. japonicum* cercariae by utilization of Kunming murine strain as an experimental model, suggesting that this vaccine, which is effective in reducing transmission and disease, could be a novel source of candidate vaccine antigens.

**MATERIALS AND METHODS**

**Parasites and animals**

The Chinese Hunan strain of *S. japonicum* was used in the present study. Cercariae were shed from field-collected *Oncomelania h. hupensis* snails provided by the Institute of Prevention and Controlling of Schistosomiasis, Hunan Province, People’s Republic of China. Six-week-old female Kunming mice (18–20 g), an outbred mouse strain widely used as an experimental animal model in China (Zhang et al., 2001), and New Zealand white hybrid rabbits were purchased from Centre Southern University Animal Unit (Changsha City, Hunan 410078).

**Preparation of cells and cell fragments**

Eighteen-day-old larval worms and 42-day-old adult *S. japonicum* were recovered from infected rabbits by perfusion of mesenteric blood vessels and washed with sterile saline containing antibiotics (1,000 IU/ml penicillin G and 1,000 μg/ml streptomycin) and 10 IU/ml heparin to thoroughly remove host contaminants. The worms were minced to fragments on a 300-mesh screen. Then, the worm fragments were digested in 10 volumes of calcium- and magnesium-free D-Hank’s solution supplemented with 0.25% trypsin (Invitrogen, Carlsbad, California) and 0.02% EDTA for 30 min at 4°C. Supernatants containing cells were collected and digestion was terminated with Hank’s solution (containing Ca²⁺ and Mg²⁺). After centrifugation at 2,000 rpm at 4°C for 5 min, the supernatants were removed. The sedimentary cell pellets were resuspended in 10 volumes of sterile D-Hank’s solution. All the above manipulations were performed under aseptic conditions.

After cell enumeration with Neubauer’s cell counting plate, cell viability was determined by trypan blue exclusion. Approximately 4 × 10⁷ cells were obtained from an 18-day-old worm and approximately 1 × 10⁵ cells were obtained from a 42-day-old worm. The cell suspensions were adjusted to different final concentrations with D-Hank’s and antibiotics, were present.

Vaccine administration

To compare protective efficacy of different vaccination formulations, 2 experiments were performed, i.e., 4 inoculations to compare the protective efficacy induced by LLC, DLC, and DAC antigens, and series of 1-3 inoculations to evaluate the effect of the number of vaccinations on the protective efficacy induced by LLC. In Experiment I, 4 groups of mice were each injected subcutaneously at separate sites along the back with 3 × 10⁶ cells/0.1 ml/mouse for the initial dose, 2.5 × 10⁶ cells/0.1 ml/mouse for the second dose, and 1 × 10⁶ cells/0.1 ml/mouse for the last 2 booster doses, each at 2-wk intervals. The cells were derived from the same aliquot of live cells for each vaccination. Control groups of mice were each injected with 0.1 ml D-Hank’s solution. All groups of mice were vaccinated at weeks 0, 2, 4, and 6.

The procedure for Experiment II was identical to that of Experiment I except a series of 1–3 inoculations (V1, vaccination once; V2, vaccination twice; and V3, vaccination 3 times) only with LLC, live larval cells 2.5 × 10⁶/0.1, dose of immunization per time, 12 mice per group, was carried out.

**Challenge infection**

In both experiments, 30 ± 1 S. japonicum cercariae were applied to the shaved abdomen of anesthetized mice by the cover slip method (Harn et al., 1984) 1 wk after the last booster dose in Experiment I, and 2 wk (V2 and V3 groups) or 4 wk (V1 group) after the final booster dose in Experiment II.

**Collection of serum and worm, egg count, and hepatic egg granuloma measurements**

Serum samples were taken at weeks 0, 3, 5, 7, and 13 for Experiment I and at weeks 0, 6, 8, 10, and 12 for Experiment II by bleeding from the tail (during immunization) or eye vein (just prior to being killed). Sera were aliquoted and stored at –20°C until use. Mice were killed at 42 days or 45 days PI for Experiment I and Experiment II, respectively. Afterward, perfusion was performed to determine worm burden as previously described (Duvall et al., 1967). At the end of the perfusion, mouse livers were removed for monitoring egg granulomas and processed for egg counts according to modified procedures (Yang et al., 1995). Briefly, 1 g of liver from each mouse in same group were placed into 20 ml of 4% potassium hydroxide at 37°C on a rocking platform for 16 hr and the number of eggs in three 200-μl aliquots were then counted using light microscopy and expressed as LEPG. The ratio “number of eggs/number of female worms” was calculated and expressed as EPF in order to obtain individual fecundity of the parasites. A fragment of ventral median lobe from each mouse liver in the 3 vaccinated and control groups in Experiment II were collected, fixed in 10% methanol, dehydrated, and paraffin embedded. Ten-μm thick sections were stained with haematoxylin–eosin (HE) for microscopic observation of egg granulomas. In an attempt to insure some level of uniformity, only those granulomas in mouse liver tissue slices with a single well-defined egg nidus in which an egg shell was visible, as well as a portion of the miracidium inside, were measured. For evaluation of egg granuloma size, the areas represented by squares under both egg granulomas and eggs were measured with Motic Images Advanced 3.2 image analysis system (Motic China Group Co., LTD, Xianmen, PRC). Results are expressed as the average area square (μm² ± SD) calculated from 30–50 granulomas per group of mice. A granuloma square was calculated with the formula: Granuloma square (μm²) = square of egg granuloma - square of egg.

The level of protection was expressed as a percentage based on the reduction in worm recovery, eggs per g of liver (LEPG), or liver eggs per female worm (EPF) of the vaccinated group compared to the control group. The reduction of worm number, LEPG and EPE in vaccinated groups was calculated by using the following formula:

\[
\text{Reduction} = \frac{(\text{Average number in control group} - \text{Average number in experimental group})}{\text{(Average number in control group)}} \times 100\%
\]

Male and female worms were separated morphologically and measured to assess effects on worm (length) development.

**Antibody response**

Antibody responses to *S. japonicum* cell-derived soluble antigens were determined in mice by ELISA. In all ELISA procedures, LLC sonicate was used to measure anti-LLC and anti-DLC antibody, and...
DAC sonicate to measure anti-DAC antibody. Approximately 1.5 × 10⁶ larval worm cells or adult worm cells were suspended in distilled water and subjected to 3 freeze-thaw cycles, followed by 7 × 50 sec cycle sonication on ice and centrifugation at 10,000 g for 30 min at 4°C. Two supernatants were collected and protein concentrations were measured by BCA reagent (Pierce Chemicals, Rockford, Illinois) and used as soluble antigens in the assays. The concentrations for all reagents were optimized in a series of checkerboard titrations prior to the ELISA. Conventional ELISAs were carried out in both experiments. Briefly, microtiter plates (Nunc, Copenhagen, Denmark) coated with 1 μg/well cell-derived soluble antigens in 100 μl 0.05 M, pH 9.6, carbonate-bicarbonate buffer were blocked, washed, and incubated with a 100-μl pooled mouse sera at serial 2-fold dilutions from 1/100 to 1/1,280 in duplicate wells for 1 hr at 37°C. Total bound antibodies were detected after incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG + A + M (Bio-Rad, Richmond, California) for 1 hr at 37°C. Optical density at 450 nm (OD) was read versus a PBS blank with an automated microtiter plate reader (ELX800, Bio Tek Instruments, Inc., Winooski, Vermont).

IgG subclass ELISAs were normalized as previously described (Boyle et al., 1997). Briefly, microtiter plates were coated with 1 μg/well of either purified IgG1 or IgG2a mouse protein (Bio-Rad) over-night at 4°C and then incubated with serial dilutions of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies to IgG1 or IgG2a. The dilutions of each anti-mouse IgG subclass antibody conjugates that gave identical OD values in the ELISA were chosen (1/4,000 for IgG1 and 1/5,000 for IgG2a) for use in assays of sera from vaccinated mice. Since IgG2a and IgG1 have been used as indicators of the induction of Th1 and Th2 responses, respectively, the IgG2a/IgG1 ratio was used as an indicator of Th1 or Th2 biased responses induced by vaccines (Coffman et al., 1988).

All optical density (OD) values ≥2.1 times the amount obtained with a reference negative serum were considered as significant (Zhu et al., 2004).

**Western blot**

SDS-PAGE was performed on 18-day-old PI schistosomula soluble antigen and 42-day-old adult worm soluble antigen, which were prepared as previously described (Dunne et al., 1994), as well as 18-day-old schistosomula cell sonicated and 42-day-old adult worm cell sonicated by a modified protocol of methods described by Laemmli (1970). For Western blot, about 10 μg/lane of larval total cell protein and 30 μg/lane of larval soluble protein resolved by SDS-PAGE were transferred from the unstained gels onto nitrocellulose membranes (PVDF Amersham Biosciences, Bucks, England) using the modified system described by Towbin et al. (1979). In brief, transfer was performed electrophoretically in a Minigel Transfer Unit (Bio-Rad) at 100 V for 4 hr. Five-mm strips of the nitrocellulose membranes were cut and saturated against non-specific binding with 5% skim milk in PBST. Then, the strips were overlaid with different serum samples diluted 1/100 and incubated for 1 hr at ambient temperature with constant rocking. Optimum concentrations for all immunoblot reagents had been determined in a series of preceding studies (citations) by titration using negative and positive control sera. After 3 five-minute washes with TBS, strips were incubated for 1 hr at room temperature with 2 ml of a 1/4,000 dilution of an HRP-conjugated goat anti-mouse IgG (H + L) (Bio-Rad). After 3 additional rinses, bound HRP activity was visualized by incubation of the strips with a 3,3-diaminobenzidine (DAB; Sigma, St. Louis, Missouri) substrate. The reaction was stopped by distilled water. The results were analyzed by using coelectrophoresed marker protein as a standard.

**Statistical analysis**

Data were expressed as mean ± standard deviation and analyzed statistically by Student’s 2-tailed t-test, using the computer software package SPSS version 11.0 for platform version (SPSS Inc., Chicago, Illinois) with the level of significance set at P < 0.05.

**RESULTS**

**Morphology type of LLC**

As shown in Figure 1, live cells from 18-day-old larval worms (LLC) used in experiments were mainly round, oval, or irregular granular cells, with a smooth surface and approximately 4~15 μm in diameter. Trypan blue exclusion showed 90% viability for each cell preparation.
Protection from *S. japonicum* challenge infection induced by LLC

In Experiment I, mice vaccinated with LLC and DLC revealed significant reduction in worm recovery, while LEPG and EPF compared with the control group (*P* < 0.01). Interestingly, significant differences in reduction of worm recovery, LEPG, and EPF were also found between mice given LLC or DLC and mice given DAC (*P* < 0.01), but no significant difference was found between DAC and the control group (*P* > 0.05) (Table I).

In Experiment II, the percentage of worm- and liver egg-recovery and EPF was significantly reduced in mice immunized with LLC once (V1), twice (V2), and 3 times (V3), compared with control mice (*P* < 0.01). In addition, significant differences were also observed between V2 and V1 and between V3 and V1, respectively, as shown in Table II. However, this difference was not found between V2 and V3 (*P* > 0.05), except for EPF (*P* < 0.01). Compared with worms from control mice, a stunted developmental phenotype was evident in worms isolated from vaccinated mice at day 45 PI (Table III). The mean sizes of worm bodies from V2 mice and V3 mice were smaller than those from the control group (*P* < 0.05), but there was no significant difference among vaccinated groups (*P* > 0.05).

**Effects of vaccination on egg granuloma formation**

Using a Motic Images Advanced 3.2 image analysis system (Motic Instruments), the differences in hepatic granuloma sizes among the experiment groups in Experiment II was compared.

**Table I. Efficacy of vaccination with parasite cells (Experiment 1).**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. mice</th>
<th>No. worms (mean ± SD)</th>
<th>% Worm burden reduction</th>
<th>LEPG* (×10³)</th>
<th>% LEPG reduction rate</th>
<th>No. female EPF* reduction (×10³)</th>
<th>% EPF reduction rate EPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co*</td>
<td>10</td>
<td>15.25 ± 4.52</td>
<td>—</td>
<td>22.68 ± 10.23</td>
<td>51</td>
<td>9.47</td>
<td>—</td>
</tr>
<tr>
<td>LLC*</td>
<td>18</td>
<td>5.33 ± 3.51</td>
<td>65.1</td>
<td>5.34 ± 3.45</td>
<td>76.5</td>
<td>4.47</td>
<td>52.8</td>
</tr>
<tr>
<td>DLC*</td>
<td>13</td>
<td>6.92 ± 3.88</td>
<td>54.5</td>
<td>7.69 ± 3.57</td>
<td>66.9</td>
<td>5.73</td>
<td>39.5</td>
</tr>
<tr>
<td>DAC*</td>
<td>11</td>
<td>15.91 ± 4.72%</td>
<td>-2.6</td>
<td>25.73 ± 11.32</td>
<td>-10.3</td>
<td>10.67</td>
<td>-12.7</td>
</tr>
</tbody>
</table>

* Co, control group; LLC, live larval cell immunization group; DLC, dead larval cells immunization group; DAC, dead adult worm cells immunization group; LEPG, number of eggs per gram liver; EPF, number of liver eggs per female (mean LEPG per group × total liver weight per group ÷ female worms per group).† Compared with control group, *P* < 0.01.‡ Compared with LLC group, *P* < 0.01.§ Compared with DAC group, *P* < 0.01.¶ Number of female worms represented the number of all female worms from all mice per group.

The results are shown in Figure 2 and Table IV. Maximal granuloma formation occurred in control mice (Fig. 2A). However, when mice were immunized with LLC, granuloma formation in these animals was markedly reduced, as evidenced by reduction in granuloma size. Interestingly, the number of immunizations correlated with the rate of reduction, with the smallest average area being observed in the V3 group and followed by the V2 and V1 groups, respectively. The mean granuloma areas are given in Table IV. Mice vaccinated with LLC 3 times, twice, and once had mean granuloma areas of 95.40 μm² ± 64.94, 129.40 μm² ± 85.27, and 161.50 μm² ± 42.64, respectively, significantly smaller than those of control mice (1,476 μm² ± 431.92). Furthermore, significant differences were also found among the 3 immunization groups.

**Antibody response of mice vaccinated with schistosomula cell vaccine**

As shown in Figure 3, the curves of total antibody levels as determined by ELISA revealed a steady increase after each vaccination. The highest sera titer of 1:3,200 was obtained in LLC group, followed by DAC and DLC group with titer of 1:400. Six weeks after challenge infection, a persistent increase of total antibody titers were seen with the highest 1:6,400 in LLC, followed by DAC at 1:3,200 and DLC at 1:1,600. Dead worm cells induced lower antibody levels than live cells in vaccinated animals.

**Table II. Efficacy of vaccination with live cells from 18-day schistosomes (Experiment 2).**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. mice</th>
<th>No. worms (mean ± SD)</th>
<th>% Worm burden reduction</th>
<th>LEPG* (×10³)</th>
<th>% LEPG reduction rate</th>
<th>No. female EPF* reduction (×10³)</th>
<th>% EPF reduction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co*</td>
<td>7</td>
<td>15.30 ± 5.79</td>
<td>—</td>
<td>21.10 ± 7.94</td>
<td>36</td>
<td>16.53</td>
<td>—</td>
</tr>
<tr>
<td>V1*</td>
<td>7</td>
<td>10.00 ± 4.32†</td>
<td>34.7</td>
<td>15.45 ± 6.65</td>
<td>26.8</td>
<td>9.41†</td>
<td>43.07</td>
</tr>
<tr>
<td>V2*</td>
<td>6</td>
<td>5.43 ± 3.00‡</td>
<td>64.5</td>
<td>7.13 ± 4.12‡</td>
<td>66.2</td>
<td>8.32‡</td>
<td>49.67</td>
</tr>
<tr>
<td>V3*</td>
<td>6</td>
<td>5.80 ± 3.77‡</td>
<td>62.1</td>
<td>7.15 ± 3.96‡</td>
<td>66.1</td>
<td>6.68§</td>
<td>59.59</td>
</tr>
</tbody>
</table>

* Co, control group; V1, mice vaccinated once; V2, mice vaccinated twice; V3, mice vaccinated thrice; LEPG, number of liver eggs per gram; EPF, number of liver eggs per female (mean LEPG per group × total liver weight per group ÷ female worms per group).† Compared with control group, *P* < 0.01.‡ Compared with V1 group, *P* < 0.01.§ Compared with V1 or V2 group, *P* < 0.01.¶ Number of female worms represented the number of all female worms from all mice per group.
TABLE III. Worm size at 45-day postinfection (Experiment 2). Worms were recovered by hepatic and mesenteric perfusion. After enumeration, paired worms were separated and intact male and female schistosomes were measured.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. worms</th>
<th>Length of worm (mm) (mean ± SD)</th>
<th>% Reduction rate of worm size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Co*</td>
<td>56</td>
<td>36</td>
<td>10.27 ± 1.60</td>
</tr>
<tr>
<td>VI*</td>
<td>28</td>
<td>28</td>
<td>8.61 ± 2.15†</td>
</tr>
<tr>
<td>V2*</td>
<td>20</td>
<td>20</td>
<td>7.63 ± 1.10‡</td>
</tr>
<tr>
<td>V3*</td>
<td>18</td>
<td>16</td>
<td>7.77 ± 0.72†§</td>
</tr>
</tbody>
</table>

* Co, control; VI, vaccination once; V2, vaccination twice; V3, vaccination 3 times.
† Compared with Co, P < 0.01.
‡ Compared with Co, P > 0.05.
§ Compared with VI, P < 0.01.
‖ Compared with V2, P > 0.05.

Control mice reached 1:3,200 at 6 wk PI. The highest antibody titer (1:6,400) was obtained with pooled sera from the mice immunized with LLC 3 times at 4 wk postchallenge infection. Apparently, the number of the immunizations has an effect on the total antibody titer (Fig. 4).

We also compared IgG1 and IgG2a antibody responses in vaccinated and control groups. The ratios of IgG2a/IgG1 were much higher (>1.5) in both LLC and DLC groups compared with the control and DAC groups (<1) at time of challenge and host death (Table V). Interestingly, the ratio of IgG subclasses showed a slight reduction after challenge. Furthermore, the ratios of IgG2a/IgG1 were much higher (>2) in mice vaccinated with LLC 2 and 3 times from Experiment II at all time points as shown in Table VI.

**Immunoblot analysis**

Western blot results (Fig. 5) analyzed with the BandScan Gel Image analysis system (Glyko Inc., Novato, California) showed that the antiserum against schistosomula worm soluble antigen yielded 6 bands (64, 52, 47, 35, 30, 19, and 15 kDa) (lane 2) and the antiserum against schistosomula-derived cells yielded 7 bands (64, 52, 47, 38, 35, 30, 19 and 15 kDa) (lane 3). Among them, an approximately 38-kDa band in larval total cell sonicate recognized by antiserum against LLC was immunodominant.

**DISCUSSION**

In a preliminary study, we attempted to identify the effects of vaccination with worm homogenate and crude cells from different developmental stages, i.e., cells and homogenates from 42-, 30-, 18-, and 12-day-old worm bodies, and from both sexes. We found that live cells from *S. japonicum* schistosomula without adjuvant could induce more significant protective immunity against *S. japonicum* challenge infection than fractions of the entire schistosomulum in a mouse challenge model (Zeng et al., 2004). To further verify the effect of this cell-type vaccine, we designed 2 experiments using live and dead schistosome-derived cells from different developmental stages. First, the mice immunized with LLC or DLC induced significant protection to a challenge infection. Second, the optimal protective efficacy was from immunization of mice with LLC twice, which induced a 65.1% worm burden reduction, a 76.5% liver egg reduction, a 93.5% reduction in the egg granuloma area, and a 26.5% stunting of worm size compared to controls. Most importantly, the protective levels induced in mice immunized with LLC and DLC are similar to those induced by immunization with X-irradiated *S. japonicum* cercaria (Zhang et al., 1999).

However, no protection was induced by DAC vaccination (adult worm-derived dead cells). How is it that significant protection against *S. japonicum* challenge can be induced by LLC and DLC, but not by DAC? It is hard to answer this question without knowing the mechanism of immune responses induced by the vaccine.

Previous reports have demonstrated protection induced by attenuated cercariae, synthetic polypeptides, recombinant antigens, or DNA vaccines are associated with a predominantly Th1 type immune response (Hota-Mitchell et al., 1999; Wilson et al., 1999; Eberl et al., 2000; Ayash-Rashkovsky et al., 2001). In the present study, sera from mice immunized with LLC and DLC exhibited higher ratios of IgG2a/IgG1 (Tables V, VI).
Generally, an IgG2a/IgG1 ratio of >1.0 reflects a predominantly Th1 type immune response, whereas an IgG2a/IgG1 ratio of <1.0 is an indicator of mostly a Th2 type immune response (Haddad et al., 1998). It is evident that LLC and DLC derived from liver-stage schistosomula are able to elicit a predominantly Th1 type immune response, as seen in animals immunized with soluble and released antigens obtained from lung-stage larvae (Mountford and Harrop, 1998), which protected mice from challenge infection in an animal model. The fact that vaccination with DLC, but not with DAC, induced significant protection against challenge infection in mice in our study led us to propose that the parasite stage from which cells were derived is an important factor for inducing protection. Although DAC elevated the absolute level of IgG2a compared with the control group, protection following S. japonicum challenge was not achieved (Table V). This result is consistent with the findings from other studies using cocktail DNA vaccines against S. japonicum and S. mansoni (Wilson et al., 1999; Da’Dara et al., 2003; Siddiqui et al., 2003, 2005). This might imply that protective immunity induced by this type of vaccine is more closely related to the IgG1/IgG2a balance than to the absolute level of each subset.

Schistosomula-derived cells may possess antigens that are able to induce a stage-specific cellular immune response that is crucial for protective immunity in animals. This hypothesis is supported by the fact that an effective vaccination against schistosomes induced simultaneously both humoral and cell-mediated immunity (Jankovic et al., 1999). More recently, data from double-cytokine knockout mice suggest that establishment of a robust cellular and humoral response is probably the key to generating maximal immunity to schistosomes (Wynn and Hoffman, 2000).

Furthermore, although ratios of antibody isotypes may be important in eliciting immunity at the time of challenge, there is a whole body of literature demonstrating that site-specific immunity may be important, such as that in the skin and lungs (Coulson and Wilson, 1988; Mountford and Trottein, 2004; Ganley-Leal et al., 2005). The actual site of immune elimination of parasites in our model is yet to be determined.

### Table IV. Hepatic granuloma size in mice immunized with LLC (Experiment 2). The size of each granuloma surrounding each mature egg was measured with the aid of a Motic Images Advanced 3.2 analysis unit. Results are expressed as the average of the area (mean ± S.D. mm²) calculated from 30 to 50 granulomas per group of mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. measurements</th>
<th>Mean area of hepatic granulomas</th>
<th>% Reduction rate compared with control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co*</td>
<td>50</td>
<td>1476.00 ± 431.92</td>
<td>—</td>
</tr>
<tr>
<td>V1*</td>
<td>38</td>
<td>161.50 ± 42.64</td>
<td>89.06</td>
</tr>
<tr>
<td>V2*</td>
<td>45</td>
<td>129.40 ± 85.27†</td>
<td>91.23</td>
</tr>
<tr>
<td>V3*</td>
<td>30</td>
<td>95.40 ± 64.94†‡</td>
<td>93.54</td>
</tr>
</tbody>
</table>

* Co, control; V1, vaccination once; V2, vaccination twice; V3, vaccination 3 times.
† V3 or V2 compared with V1, P < 0.01.
‡ V3 compared with V2 group, P < 0.05.
The size of each granuloma surrounding each mature egg was measured with the aid of a Motic Images Advanced 3.2 analysis unit. Results are expressed as the average of the area (mean ± S.D. mm²) calculated from 30 to 50 granulomas per group of mice.

Why could juvenile, but not adult, worm cells induce a protective Th1 type immune response? One reason may be that adult worms, unlike larvae from the lung-stage and liver-stage, are not considered as an ideal immune target. A previous study (Mountford and Harrop, 1998) have demonstrated that the main target of the immune response is the lung stage and skin stage and followed by liver stage larvae. Further, it is the irradiated larval stage that induces protective immunity (Dean et al., 1987). As LLC and DLC antigens are larval antigens, we consider that a property of the cells from the larval stage is the ability to engender a protective immune response. Curwen et al. (2004) reported that there are a significant number of common components and specific antigens among skin-stage juvenile, liver-stage juvenile, and adult worms. Fitzpatrick et al. (2005) also demonstrated that there are a myriad of differences...
in gene expression by the 3 mammalian stages, which may explain the differences between larval and adult cells.

As shown in Figure 3, DLC induced a lower titer of antibodies than LLC. We conjecture that devitalized ingredients during the process of repeated freeze-thaw cycles resulted in DLC being a weaker immunogen than LLC, and even DAC.

The only reported antigens to consistently show greater than 40% protection are antioxidant enzymes, such as *S. mansoni* Cu/Zn superoxide dismutase and *S. mansoni* glutathione-S-peroxidase. These antioxidant enzymes exhibit the highest level of expression in adult worms at the host-parasite interface (Mei et al., 1996; LoVerde, 1998; LoVerde et al., 2004). The antioxidant enzymes that are thought to protect adult worms from reactive oxygen species-derived damage are important vaccine candidates (Shalaby et al., 2003; LoVerde et al., 2004). In future studies, we will evaluate the immune effect induced by live adult worm cells.

There was only a slight difference in the level of protection between groups vaccinated with LLC and DLC. We assume that the vitality of cells was a less important factor than the parasite stage from which cells were derived. Further, mice vaccinated twice obtained the highest protection. Subsequent vaccinations did not confer additional protection even though the antibody titer was higher. It seems that protection induced by immunization of cell vaccine was not injection number-dependent in our experiment. This result is in agreement with comments by MacManus and Bartley (2004) in their review. However, from the results shown in Table IV, the impact of the vaccine on egg granuloma formation seems to be immunization-number dependent. Therefore, the optimum vaccination formula will have to await further experimentation.

Generally, induction of immunity in vaccinated mice has been shown to closely correlate with down-regulation of cell-mediated immune response (Gustavson et al., 2002). Thus, vaccinated mice displayed a decrease in the granulomatous hypersensitivity from a challenge infection against eggs trapped in the livers. The areas occupied by egg granulomas in livers of mice immunized with LLC and DLC were significantly less than in mice immunized with DAC or D-Hank’s. This result has the benefit of reducing morbidity due to smaller granuloma size (Wilson et al., 2007).

By enumerating LEPG and EPF, we also demonstrated that this novel cell-type vaccine possessed an anti-fecundity effect. From SDS-PAGE, we could find that total larval cell extract possessed far less electrophoresed polypeptide bands on acrylamide resolving gel than worm antigens (data not shown). In the lanes of cell proteins, abundant small molecular-weight proteins could be found at the bottom of SDS-polyacrylamide gels. The action of these small molecular weight proteins awaits further study. In western blotting, a 38-kDa polypeptide in cell antigens was strongly recognized by antiserum from mice immunized with living larval cell, but not by infected sera, suggesting that this polypeptide may be one of the dominant protective molecules existing in LLC. A similar sized molecule from SEA (soluble egg antigen) has been identified as a diagnostic antigen (Zhou et al., 2000). The protective potential of this molecule will be determined in future experiments.

In this study, we further verified that the cells derived from *S. japonicum* schistosomula induced similar protection as that observed in radiation-attenuated vaccination in a mouse model of *S. mansoni*. Although there is a large gap between employing the cells obtained from live schistosomes as a vaccine in an experimental model and the practical application as a human vaccine, our results are significant since they demonstrate that live cells are just as effective as irradiated cercariae in stimulating a high level of protective immunity. With a reductionist approach of purifying the cell types to identify those important in stimulating protective immunity, molecular approaches may

### Table V. Ratio of IgG2a/IgG1 antibody subclasses in mice (Experiment 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>LLC*</th>
<th>Ratio</th>
<th>DLC*</th>
<th>Ratio</th>
<th>DAC*</th>
<th>Ratio</th>
<th>Co*</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCS*</td>
<td>0.30/0.12</td>
<td>2.5</td>
<td>0.37/0.14</td>
<td>2.64</td>
<td>0.33/1.0</td>
<td>0.33</td>
<td>0.17/0.34</td>
<td>0.5</td>
</tr>
<tr>
<td>PSS*</td>
<td>1.91/0.89</td>
<td>2.15</td>
<td>1.40/0.80</td>
<td>1.75</td>
<td>0.40/0.59</td>
<td>0.68</td>
<td>0.58/0.70</td>
<td>0.83</td>
</tr>
</tbody>
</table>

* PCS, sera collected prior challenge diluted at 1:100; PSS, sera collected prior sacrifice diluted at 1:100; LLC, live larval cell immunization group; DLC, larval cell piece immunization group; DAC, dead adult worm cell immunization group; Co, control group.

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**Table VI. Ratio of IgG2a/IgG1 antibody subclasses in mice (Experiment 2).**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample 2*</th>
<th>Sample 3†</th>
<th>Sample 4‡</th>
<th>Sample 5§</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>0.38/0.25</td>
<td>1.52</td>
<td>0.65/0.38</td>
<td>1.71</td>
</tr>
<tr>
<td>V2</td>
<td>0.48/0.23</td>
<td>2.09</td>
<td>0.71/0.33</td>
<td>2.15</td>
</tr>
<tr>
<td>V3</td>
<td>0.48/0.21</td>
<td>2.28</td>
<td>1.04/0.37</td>
<td>2.81</td>
</tr>
<tr>
<td>Co</td>
<td>0.03/0.08</td>
<td>0.38</td>
<td>0.27/0.36</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* Sera taken just prior to challenge.
† Sera taken 2 wk postchallenge.
‡ Sera taken 4wk postchallenge.
§ Sera taken just prior to sacrifice.
∥ V1, vaccination once group; V2, vaccination twice group; V3, vaccination 3 times group; Co, control group.
identify novel effective antigen candidates. Furthermore, our study sheds light on developing a novel “consensus” vaccine against all 3 human pathogens (S. japonicum, Schistosomiasis haematobium, and S. mansoni) and the results encourage future vaccination trials with the establishment of in vitro cultivation of S. japonicum cell lines that would facilitate our study on vaccine development.

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LITERATURE CITED


**HUMORAL ANTIBODY RESPONSE OF THE TILAPIA OREOCROMIS NILOTICUS AGAINST CICHLIDOGYRUS SPP. (MONOGENEA)**

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**ABSTRACT:** The humoral immune response of the tilapia Oreochromis niloticus was evaluated using a direct ELISA. Serum was tested from fish infected with Cichlidogyrus spp. (Monogenea) and from fish injected intraperitoneally with the Cichlidogyrus spp. antigenic extract, i.e., 150 μL of the Cichlidogyrus spp. saline extract diluted in Freund’s complete adjuvant (FCA) (1:1) were inoculated intraperitoneally at day 0, followed by 2 dosages of 50 μL of the same Cichlidogyrus spp. saline extract diluted in Freund’s incomplete adjuvant (FIA) (1:1) at weeks 2 and 4, respectively. The humoral response was also evaluated by the double immunodiffusion test (DID) and by serum protein and total immunoglobulin (Ig) determinations. The IgM OD values in the hyperimmune fish were significantly higher than in the infected and uninfected fish groups. In the DID test, a precipitation (antigen–antibody) band was observed between the Cichlidogyrus spp. saline extract and hyperimmune sera, but not with the other groups. Increases in serum protein concentration and total Igss were observed in the immunized fish at weeks 2 and 10 postinjection. Results from this study suggest that tilapia is capable of producing an induced humoral immune response against an antigenic extract of Cichlidogyrus spp.

Tilapia, Oreochromis niloticus, is cultured widely around the world, with a strong established market demand. For example, in 2004 alone, the United States expended $350 million to import tilapia products (Fitzsimmons and Pantoja, 2005).

Tilapia farms can be infested with monogeneans such as Cichlidogyrus spp. These are ectoparasitic monogeneans that attach to the fish gills, producing cichlidogyriasis. Some epizootic outbreaks of cichlidogyriasis have been associated with crowding and poor water quality conditions, which increase the extent of parasite transmission (Khalil, 1971; Paperna, 1980; Bondad-Reantaso and Arthur, 1990; Jiménez-García et al., 2001). These outbreaks can be concurrent with secondary infections from opportunistic pathogens because of mechanical damage in the lamellae produced by the special posterior-positioned attachment organs of the monogenean flukes.

However, even when intensity of infection is high, there are no records of economic loss due to ill effects of infection by monogenean infections among cichlids in Africa or Israel. Only Cichlidogyrus sclerosus has been reported to harm cultured Oreochromis spp. in southeast Asia (Khalil, 1971; Kabata, 1985). Thus, there are only sporadic reports linked to tilapia mortalities, and those have been restricted to only descriptive records of C. sclerosus (Khalil, 1971; Bondad-Reantaso and Arthur, 1990).

Little is known about the immune response of tilapia against monogenean infections. In this regard, the implementation of serological tests would help in the presumptive diagnosis of cichlidogyriasis. Some studies in temperate latitudes have shown that teleost fish are able to generate a specific immune response against gill monogeneans (Vladimorov, 1971; Buchmann, 1993; Buchmann and Bresciani, 1998; Jaso-Friedmann and Evans, 1999; Mazzanti et al., 1999; Jaso-Friedmann et al., 2000; Taylor et al., 2001).

The present study is focused on using a direct ELISA, serum protein determination, and the double immunodiffusion method (DID) to evaluate the immune humoral response of tilapia against Cichlidogyrus spp., all of which methods are known to be useful in this sort of work (Williams and Hoole, 1992).

**MATERIALS AND METHODS**

**Experimental protocol**

All tilapia (O. niloticus) used in this study were from the CINVESTAV-IPN Unidad Mérida aquaculture facilities, Mérida, Mexico. Before the experiments, the fish were acclimatized for 15 days at 25 ± 3°C in 500-L fiberglass tanks with constant aeration and fed with commercial pellets (40% protein). The fish were divided into 5 groups. Group 1 consisted of 45 tilapia (mean length: 21 ± 1 cm; mean weight: 196 ± 41 g) infected with Cichlidogyrus spp. (mean abundance: 135 ± 166 parasites/fish; prevalence 100%). Group 2 consisted of 26 tilapia (mean length: 19 ± 2 cm; mean weight: 145 ± 44 g) that had been previously infected (mean abundance: 40 ± 42 parasites/fish; prevalence 98%). Fish from this group received a treatment of 2 mL of formalin in water for 40 min to eliminate Cichlidogyrus spp. 4 mo before starting the experiment. These fish were kept in quarantine to allow their immune systems to recover from the influence of formalin (Ellis, 1989). At necropsy, none of the fish from this group was infected with monogeneans.

Groups 3–5 (21 fish; 24 ± 3 cm; 361 ± 144 g) were used for the immunizations. Group 3 consisted of 7 tilapia injected with Cichlidogyrus spp. saline extract (see below for preparation) in Freund’s adjuvant (complete and incomplete); this group was referred to as hyperimmune sera (HS). Group 4 consisted of 7 tilapia injected only with adjuvant; this group was referred to as Freund’s adjuvant sera (FAS). Group 5 consisted of 7 tilapia injected with PBS and used as controls; this group was referred to as the saline solution fish (SS) (Table I).

**Antigen preparation**

To prepare the Cichlidogyrus spp. saline extract, 7,000 monogeneans (~1.6 g) were isolated from the gills of infected fish, placed in Eppendorf tubes, and washed twice with sterile and cold phosphate-buffered saline (PBS; 0.02 M phosphate, 0.15 M NaCl, pH 7.2) to eliminate any host tissues. The parasites were then centrifuged 3 times at 13,000 g for approximately 5 min; 0.8 g of the concentrate was suspended in 2.5 mL of PBS and macerated manually in the Eppendorf tube. Tissue disruption was stimulated by adding 2 mL of liquid N2 and 30 μL of protease K (20 μg/μL). The homogenate was then centrifuged twice at 14,000 g for 30 min. The supernatant (protein extract) was stored at −70°C and subsequently used in direct ELISA and DID tests, as well as for fish immunization to obtain the hyperimmune sera.

The protocol to obtain the antiserum included 3 immunizations every 2 wk (weeks 0, 2, and 4), for 12 wk. Fish from Group 3 (HS) were injected intraperitoneally at day 0 with 150 μL of the Cichlidogyrus spp. saline extract (1.0 μg/μL) in FCA (1:1); 2 and 4 wk later, they were injected with 50 μL of the same parasitic extract in FIA (1:1), following the protocol described by Harlow and Lane (1988). Fish from Group 4 (FAS) were injected intraperitoneally first with FCA (150 μL) and then received 10 July 2007; revised 27 July 2007. 23 August 2007; accepted 23 August 2007.

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with FIA (50 μl), while fish from Group 5 (SS) were injected 3 times with 150 μl of sterile PBS.

All fish were bled before killing. Blood samples (~1–5 ml) were taken via caudal puncture using a disposable plastic syringe with a 22-G needle. Blood samples from Groups 3, 4, and 5 were taken 2, 4, 6, and 8 wk before the first inoculation (time 0) and at weeks 2, 3, 5, and 8 after the last inoculation (Table I). In all cases, the blood was left to coagulate for 30 min in Eppendorf tubes at room temperature. After that, samples were centrifuged at 1,500 g for 10 min. Each sample was then placed in new Eppendorf tubes and stored at −20 C.

### Enzyme-linked immunosorbent assay (ELISA)

A direct ELISA was used to detect specific IgM. Briefly, ELISA plates (Immulon 4HBX; Dynex Technologies Ltd., Billingham, U.K.) were coated overnight (O/N) at 4 C with 100 μl of a 1:80 dilution of the Cichlidogyrus spp. antigen extract in 0.05 M carbonate buffer (1.16 μg/ml; pH 9.6). The next day, plates were washed 3 times for 5 min with low salt wash buffer (LSWB), (0.3 M NaCl, 0.05% Tween 20; pH 7.3), then blocked with 250 μl of BSA/well and incubated for 2 hr at 22 C. Plates were washed 3 times with LSWB as described above. Then, 100 μl of a 1:25 dilution of each serum in PBS (0.02 M phosphate, 0.15 M NaCl; pH 7.2) were added and incubated O/N at 4 C (this dilution was chosen after a check board titration of 1:10 to 1:200). The next day, plates were washed 5 times for 5 min with high salt wash buffer (HSWB) (0.5 M NaCl, 0.1% Tween 20; pH 7.7), and incubated for 60 min at 22 C with 100 μl/well of the reconstituted antibody IgM MAb-HRP (Oreochromis niloticus) (Aquatic Diagnostics Ltd., Stirling, U.K.). (The MAb is of an IgG1 isotype and recognizes the heavy chain of the IgM molecule [http://www.aquaticdiagnostics.com/products/tiplapia-oreochromis-niloticus-igm-antibody-39.html]). Plates were washed with HSWB as before and 150 μl of the substrate 3‘3’5’5’-tetramethylbenzidine dihydrochloride (TMB) 42 mM in 1:2 acetic acid; distilled water was added to 15 ml of a substrate buffer (sodium acetate/citric acid). Plates were then incubated in dark conditions at 22 C for 45 min. Absorbance values were recorded in an ELISA reader at 450 nm (MRX 20100, Dynex Technologies Ltd., Worthing, U.K.).

All samples were assayed in duplicate wells. The cutoff point was defined as the mean plus 2 standard deviations of the absorbance (OD) values from samples of Group 2.

Absorbance values were tested for normality using Wilk-Shapiro ranking plots. Group values had a normal distribution (Wilk-Shapiro = 0.9853, n = 92).

### Double immunodiffusion (DID) test

To perform the DID test, gels were prepared using a 12% agarosa solution (pH 7.2) in petri dishes of 3.5-cm in diameter (1.5 ml of agarosa/petri dish at 2-mm thickness). Three pairs of 1.5-mm wells were made in each dish. The Cichlidogyrus spp. antigenic extract was placed in the left side and the sera of tilapia diluted in PBS (1:100) was placed in the right side of each pair of wells at increasing volumes of 1, 2, and 3 μl/well. Petri dishes were left for 48 hr in a moist chamber at 37 C, and then stained with 1% Coomassie Blue for 18 hr. They were distilled for 2 hr or until any precipitation band was observed (following the procedures of Craig et al., 1996; Nie and Hoole, 1999).

### Statistical analysis

Departures from normality were observed in the TP and total Igs concentrations by using Wilk-Shapiro ranking plots, and normal log-arithmetic (ln + 1) were used for transformation. Both Group 1 (naturally infected fish) and Group 2 (non-infected fish) had normal distribution values for TP and total Igs (TP: Wilk-Shapiro = 0.8427, n = 71; total IgM: Wilk-Shapiro = 0.8849, n = 71). Differences in the OD values from samples from Group 3 (HS) were compared to the other groups by using a 2-sample t-test.

Comparisons among Groups 1 and 2 for TP and the total Igs were performed using a 1-way ANOVA or unbalanced unifactorial design (UUD). Differences among means were identified by applying the LSD method at the P < 0.05 rejection level. The ANOVA tests were carried out using Statistics (SX) statistical package (version 4.0, Analytical Software, St. Paul, Minnesota).

### RESULTS

Five species of monogeneans were observed on *O. niloticus* in the present study, i.e., *C. sclerosus, Cichlidogyrus dossoi, Cichlidogyrus longicornis longicornis, Cichlidogyrus tilapiae, and Cichlidogyrus haplochromi* (Jiménez-García et al., 2001). We were not able to morphologically classify all monogeneans isolated and we decided to refer them as *Cichlidogyrus* spp. Although there are 5 separate species, all cause cichlidogyrasis (Paperna, 1980).

During the experiment, no mortalities attributed to monogenean infestation were observed, and we could not correlate the parasite burden with ammonia, oxygen, or other physicochemical variables in the experimental system (data not shown). The physicochemical parameters were the average for aquaculture in our facilities (O2 = 5.87 ± 0.46, NH3 = 0.079 ± 0.012).

### ELISA

The cutoff point was 0.09. The OD values in Group 1 (fish infected with monogeneans, n = 45) ranged from 0.031 to 0.118. Only 4 fish were above the cutoff value, i.e., 0.091, 0.093, 0.117, and 0.118, corresponding, respectively, to 3, 336, 106, and 1 monogenean. Some fish from this group had higher parasite burdens but low OD values, i.e., 1 fish had 756 parasites and an OD value of 0.089. Another fish had 4 parasites and an OD value of 0.076.
In Group 2 (fish treated with formalin, n = 26), the OD values ranged from 0.03 to 0.09. In all cases but 1, these were below the cutoff point (Fig. 1). When comparing Groups 1 and 2, significant differences were observed (t = 3.02, P = 0.0047).

The OD values of Group 3 (HS; tilapia inoculated with Cichlidogyrus spp. extract) were above the cutoff point (0.091 to 0.104). These OD values were below the cutoff point before immunization, but increased at different times during the 12-wk postimmunization protocol (Fig. 2). In contrast, Group 4 (FAS; fish inoculated only with adjuvants) had OD values from 0.043 to 0.081. Group 5 (SS; fish inoculated with PBS; pH 7.2) had OD values lower than 0.073 (Fig. 1). In all these cases, there were significant differences in the HS versus the other groups (HS and Group 1, t = 8.60, P < 0.01; HS and Group 2, t = 6.08, P < 0.01; HS and Group 4, t = 4.55, P < 0.05; HS and Group 5, t = 9.74, P < 0.01).

**Double immunodiffusion (DID) test**

The same panel of sera was used with the DID test. A precipitation band was interpreted as a positive reaction in the wells containing 1 µl of the Cichlidogyrus spp. saline extract and 1 µl of serum (1:100 dilution in PBS) of tilapia from the HS group. These precipitation bands were observed consistently from week 2 until the end of the experiment. In contrast, no precipitation bands were observed in the other groups and with the other concentrations of 2 µl and 3 µl (Fig. 3).

**TP and total Igs**

The same panel of sera from Groups 3, 4, and 5 was used to perform the TP and total Igs. They were also divided in 2 categories, the first prior to the inoculation of the Cichlidogyrus spp. extract (weeks −8, −6, −4, −2, and 0), and the second after the inoculation of the antigenic extract (weeks 2, 4, 6, 7, 10, and 12) (Fig. 4). The total protein values of these 3 groups of fish before inoculation ranged from 31 µg/µl to 36 µg/µl for Group 3 (HS), from 34 µg/µl to 46 µg/µl for Group 4 (FAS), and from 39 µg/µl to 55 µg/µl for Group 5 (SS). The values for total protein before inoculation in the HS group were significantly lower than for the FAS and SS groups (n = 15, F2,12 = 6.23, P < 0.05). The values of total Igs for fish before inoculation ranged from 19 µg/µl to 21 µg/µl for Group 3 (HS), from 16 µg/µl to 36 µg/µl for Group 4 (FAS), and from 22

![Figure 1](image1.png)

**Figure 1.** ELISA results from ■, natural infection (NI); †, negative control (NEG); ▲, hyperimmune sera (HS); ×, Freund’s adjuvant sera (FAS), and ○, saline solution (SS).

![Figure 2](image2.png)

**Figure 2.** OD values from the hyperimmune sera (HS) before and after intraperitoneal injection.
FIGURE 3. Double immunodiffusion test (DID) between the Cichlidogyrus spp. saline extract (CSE) and the tilapia Oreochromis niloticus group sera. (1) One μl of CSE and 1 μl of FAS. (2) One μl of CSE and 1 μl of HS. (3) One μl of CSE and 1 μl of the negative infected fish. (4) One μl of CSE and 1 μl of fish from natural infection. The arrow indicates an antigen (Ag)-antibody (Ab) reaction in line 2.

μg/μl to 27 μg/μl for Group 5 (SS). In contrast, there were no significant differences in values for total Igs before inoculation in the HS group as compared to the FAS and SS groups (n = 15, F_{2,12} = 3.34, P > 0.05).

Group 3 (HS) and Group 4 (FAS) showed a primary total protein and total Ig response (first peak) at week 2 (HS: total protein = 111 μg/μl, IgM = 73 μg/μl; FAS: total protein = 77 μg/μl, IgM = 45 μg/μl), followed by a decreasing pattern. At week 10 postinoculation, a smaller peak was observed (HS: total protein = 64 μg/μl, IgM = 37 μg/μl; FAS: total protein = 67 μg/μl, IgM = 42 μg/μl). At the end of the experiment (week 20), the values were as follows: HS: total protein = 45 μg/μl, IgM = 24 μg/μl; FAS: total protein = 60 μg/μl, IgM = 36 μg/μl (Fig. 4). Although the adjuvants alone increased the total protein and the total Igs, these values were always lower than those for the Cichlidogyrus spp. saline extract and adjuvants (Group 3), except during week 10 and 12 (Fig. 4).

DISCUSSION

The results of the ELISA confirmed the presence of IgM-specific antibodies in sera of tilapia injected intraperitoneally with the Cichlidogyrus spp. saline extract (Group 3, HS). The increase of the OD values in sera from the same group of fish after immunization confirms our observations.

In Group 1 (natural infection), the IgM response was variable. For instance, only 4 fish were above the cutoff value, i.e., 0.091, 0.093, 0.117, and 0.118, corresponding, respectively, to 3, 336, 106, and 1 monogenean. One fish had 756 parasites and an OD value of 0.089. Another fish had 4 parasites and an OD value of 0.089.
value of 0.076. There are at least 2 possible explanations for these patterns. First, it could be that the parasite burden was not large enough to elicit an IgM-specific response, or there could have been a fast response that removed the worms which overcame a threshold number, after which the antibody response returned to basal levels. If this is the case, the second explanation is more likely to clarify our findings since it makes sense to start a specific immune response only when the number of worms could become harmful, and then return to basal levels when the parasites are reduced (Williams and Hoole, 1992; Hoole et al., 2003). To assess this hypothesis, in future studies we will need to monitor fish with high and low parasite burdens to determine if variability in the immune response can be related to parasite numbers.

In the same context, it is important to note that fish from Group 1 (parasite burden of ~800 Cichlidogyrus spp.; mean abundance 135 ± 166) and Group 2 (negative control group) were from the same fry and the same pond. Although we were careful to avoid stressing the fish, we cannot be assured that host immunity in this group was not affected by the formalin treatment (Ellis, 1989). However, to the best of our knowledge there are no reports regarding the effect of formalin treatment on the immune response of tilapia. In the present study, the efficacy of the negative control group was corroborated because the OD values were below the cutoff point, with exception of 1 fish (with a value near the cutoff point). To confirm that this group is susceptible to formalin, future studies must include fish with no previous contact with the parasite.

Results in Figure 4 show a characteristic pattern of primary and secondary response in fish injected with antigenic extracts (Van Muiswinkel, 1995). Constant boosts may be required to enhance the immune response, as the second peak at week 10 PI was lower than the first peak during week 2 (Fig. 4). The results from the present study suggest that intraperitoneal injection of the Cichlidogyrus spp. saline extract into tilapia generated the production of specific IgM antibodies. In contrast, inoculation of sterile saline solution in the Group 5 fish (n = 7) produced total protein values that ranged from 39 μg/μl to 52 μg/μl and Igs that ranged from 1 μg/μl to 27 μg/μl, lower than values obtained in the HS group (Fig. 4). A similar pattern of primary and secondary responses was observed in fish inoculated with BSA (Sailendri and Muthukkaruppan, 1975), i.e., Rutillus rutillus infected with metacestodes of Ligula intestinalis (Williams and Hoole, 1992), Cyprinus carpio infected with the cestode Botriocephalus acheniogathi (Nie and Hoole, 1999), and Anguilla anguilla infected with the gill monogenean Pseudodactylogyra bini (Mazzanti et al., 1999).

The results obtained using ELISA were consistent with the DID test. Only the HS sera gave a precipitation band among the fish groups (Nie and Hoole, 1999). In the same way, the total proteins and the total Igs were higher in this group when compared to Groups 4 (FAS) and 5 (SS).

Finally, it has been demonstrated that the immune response is an important regulating factor for parasite numbers in naturally infected fish (Scott, 1985; Thomas and Woo, 1995). However, it remains to be demonstrated whether or not the specific IgM from Group 3 (HS) has a protective role against further infections with Cichlidogyrus spp.

Similarly, even though Cichlidogyrus spp. are not dramatic tilapia killers, it is necessary to seriously consider the parasite’s potential effect upon farmed tilapia in terms of growth, the amount of food supplied to infected and noninfected fish, and the effect of treatment with chemicals on the fish’s immune system.

ACKNOWLEDGMENTS

We acknowledge support from CONACyT for providing a postgraduate studentship to Juan J. Sandoval Gío (Grant 129336). This study is part of the results of the PhD thesis of J. J. Sandoval-Gío. This study was funded by the projects JIRA-CINVESTAV (R. Rodriguez), and “Programa de monitoreo ambiental del sur del Golfo de México” (Campañas Oceanográficas 2-2005 y SGN No. 10-2005), Xcambó-2. (Reference 42881681 & 418815846) (R. Rodriguez and V. Vidal). Special thanks are conveyed to Juan Antonio Pérez-Vega and Gregory Arjona-Torres for their technical support.

LITERATURE CITED


Nie, P., and D. Hoole. 1999. Antibody response of carp, Cyprinus car-
pio to the cestode, *Botriocephalus acheilognathi*. Parasitology **118**: 635–639.


ASSESSMENT OF THE NORTHERN DISTRIBUTION RANGE OF SELECTED PERKINSUS SPECIES IN EASTERN OYSTERS (CRASSOSTREA VIRGINICA) AND HARD CLAMS (MERCENARIA MERCENARIA) WITH THE USE OF PCR-BASED DETECTION ASSAYS

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ABSTRACT: Perkinsus species are protistan parasites of molluscs. In Chesapeake Bay, Perkinsus marinus, Perkinsus chesapeaki, and Perkinsus andrewsi are sympatric, infecting oysters and clams. Although P. marinus is a pathogen for Crassostrea virginica, it remains unknown whether P. andrewsi and P. chesapeaki are equally pathogenic. Perkinsus species have been reported in C. virginica as far north as Maine, sometimes associated with high prevalence, but low mortality. Thus, we hypothesized that, in addition to P. marinus, Perkinsus species with little or no pathogenicity for C. virginica may be present. Accordingly, we investigated the distribution of Perkinsus species in C. virginica and Mercenaria mercenaria, collected from Maine to Virginia, by applying PCR-based assays specific for P. marinus, P. andrewsi, and a Perkinsus sp. isolated from M. mercenaria. DNA samples of M. mercenaria possessed potent PCR inhibitory activity, which was overcome by the addition of 1 mg/ml BSA and 5% (v/v) DMSO to the PCR reaction mixture. All 3 Perkinsus species were found in both host species throughout the study area. Interestingly, the prevalence of P. marinus in M. mercenaria was significantly lower than in C. virginica, suggesting that M. mercenaria is not an optimal host for P. marinus.

Perkinsus species (Perkinsozoa, Alveolata) are the causative agent of perkinsosis in a variety of mollusc species. For some host species, such as the eastern oyster C. virginica, Perkinsus species infections cause widespread mortality in both natural and farmed oyster populations, resulting in severe economic loss for the shellfishery, and detrimental effects on the environment (Andrews, 1988; Ford, 1996; Villalba et al., 2004). Currently, 3 Perkinsus species are recognized along the Atlantic coast of the United States, i.e., P. marinus, isolated from the eastern oyster C. virginica (Mackin et al., 1950), P. chesapeaki from the soft shell clam Mya arenaria (McLaughlin et al., 2000), and P. andrewsi from the Baltic clam Macoma balitica (Coss, Robledo, and Vasta, 2001). In addition, various Perkinsus isolates have been reported, including an isolate from the hard clam M. mercenaria (hereafter referred to as Perkinsus sp. (M. mercenaria)) that appears to be closely related to P. andrewsi (Andrews, 1955; Perkins, 1988; Coss, 2000). It is not yet clear whether P. chesapeaki and P. andrewsi are different species and, although their synonymization has been proposed (Burreson et al., 2005), because of the limited evidence available at present time, we consider P. andrewsi as a distinct Perkinsus species in the present study.

The standard diagnostic method for Perkinsus spp. infections has been the fluid thioglycollate medium (FTM) assay (Ray, 1952, 1966), which is considered to be more sensitive than histological diagnosis (McLaughlin and Faisal, 1999). However, neither method is able to discriminate among Perkinsus species (reviewed in Villalba et al., 2004); diagnostic assays based on anti-Perkinsus sp. antibodies (Choi et al., 1991; Dungan and Robertson, 1993; Ottinger et al., 2001; Montes et al., 2002) have not been rigorously validated, and may exhibit cross-reactivity with dinoflagellates (Dungan et al., 1993; Bushek et al., 2002; Villalba et al., 2004).

The development of culture methods for Perkinsus species

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(Gauthier and Vasta, 1993; Kleinschuster and Swink, 1993; La Peyre et al., 1993) greatly facilitated the development of specific PCR-based diagnostic assays. The first PCR-based assay was developed for P. marinus and was species-specific and more sensitive than the FTM assay (Marsh et al., 1995; Robledo et al., 1998). Subsequently, PCR-based assays specific for Perkinsus olseni (de la Herrán et al., 2000; Robledo et al., 2000), P. andrewsi (Coss, Robledo, Ruiz, and Vasta, 2001), and for other species of Perkinsus (Robledo et al., 2002) were also developed. Quantitative PCR assays for P. marinus (Yarnall et al., 2000; Gauthier et al., 2006), a multiplex PCR assay detecting P. marinus and Haplosporidium species (Penna et al., 2001), and modified PCR-based assays have been developed that can distinguish between P. marinus, P. olseni, Perkinsus mediterraneus, and P. andrewsi/P. chesapeaki or P. marinus and P. olseni, respectively (Elandalloussi et al., 2004; Abollo et al., 2006).

Prior to 2000, all surveys for Perkinsus species were conducted with the use of histology or FTM-based assays, and all Perkinsus infections observed in C. virginica were attributed to P. marinus, the only Perkinsus species described along the Atlantic coast of the Americas at that time. By 2001, 2 new species, P. chesapeaki and P. andrewsi, were described from clams (M. arenaria and M. balthica, respectively) in Chesapeake Bay (Coss, 2000; McLaughlin et al., 2000; Coss, Robledo, Ruiz, and Vasta, 2001). However, in addition to its type host, P. andrewsi was also found in C. virginica (the type host of P. marinus) and in the clams M. mercenaria and M. michelli (Coss, 2000; Coss, Robledo, Ruiz, and Vasta, 2001). Conversely, P. marinus was detected in M. arenaria, M. balthica, and Macoma michelli (Kotob et al., 1999; Coss, 2000; McLaughlin et al., 2000; Coss, Robledo, Ruiz, and Vasta, 2001), suggesting a broad host range for these Perkinsus species.

Perkinsus species infections have been observed in oysters from Tabasco, Mexico, to Maine (reviewed in Burreson and Ragone Calvo, 1996; Ford, 1996; Soniat, 1996). In some areas of the northeastern United States, mortalities in oyster populations were low to moderate, despite high prevalence and infection densities of Perkinsus species (Ford, 1996; Karolus et al., 2000). This observation led us to hypothesize that, in addition
to *P. marinus*, other *Perkinsus* species are present in the northeastern regions that may be less virulent towards *C. virginica*. We therefore surveyed *C. virginica* and *M. mercenaria* obtained from selected sites from Maine to Virginia for the presence of *Perkinsus* species, and specifically for *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) using specific PCR-based assays. This is the first study that assesses the distribution of sympatric *Perkinsus* species in 2 economically important moluscan hosts.

**MATERIALS AND METHODS**

Collection of tissue specimens and DNA extraction

*Crasostrea virginica* (size: 44–142 mm) and *M. mercenaria* (size: 40–73 mm) specimens, collected monthly from June 2002 to September 2002, were obtained from shellfish farmers and academic institutions from 8 sites along the Atlantic coast of the United States as follows: *C. virginica* were obtained from Walpole (Maine), Martha’s Vineyard (Massachusetts), Narragansett Bay (Rhode Island), Oyster Bay (New York), Delaware Bay (New Jersey), and Sandy Point (Maryland); *M. mercenaria* were obtained from Eliot (Maine), Martha’s Vineyard (Massachusetts), and Cheriton (Virginia) (Fig. 1). Upon arrival, the shellfish were stored up to 72 hr at 4°C until further processing.

Eighteen to 60 specimens from each sampling site and collection date were individually dissected. From each individual, gut, gill, and mantle tissues were collected and pooled (50–100-mg wet weight of total tissue/pool), and DNA was extracted with the use of a commercially available kit (DNeasy, 96-well format, QIAGEN, Valencia, California). DNA concentration and purity were estimated by spectrophotometry at wavelengths of 260 and 280 nm. The DNA samples were stored at −20°C until testing.

**PCR assays**

PCR-based assays specific for the genus *Perkinsus*, and the species *P. marinus*, *P. andrewsi*, and *P. olsenii* (syn. *Perkinsus atlanticus*), were used according to Marsh et al. (1995), Coss, Robledo, Ruiz, and Vasta (2001), and Robledo et al. (2000, 2002).

**Development of a PCR-based diagnostic assay specific for the *Perkinsus* species isolated from *Mercenaria mercenaria***

Primers designated M68 (sense, 5'-GGGGGCAATATCTACATCTG AG-3') and M5 (antisense, 5'-AACCATCCCGACTACCATCTGG-3') were designed based on the intergenic spacer of the rRNA gene of *P. marinus* (*M. mercenaria*) with the use of OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). Thermocycler conditions were 94°C for 4 min, 35 cycles of 94°C for 1 min, 65°C for 30 sec with an extension of 1 sec per cycle, 72°C for 1 min, with a final extension at 72°C for 7 min.

Three different PCR reaction mixtures (A–C) were used. PCR reaction mixture A consisted of 1× QIAGEN PCR Master mix (contains *Taq* DNA Polymerase (250 μM/μl), KCl, Tris-Cl, (NH₄)₂SO₄, 1.5 mM MgCl₂, and 200 μM of each dNTP) (QIAGEN), and 40 mM of each primer. To obtain PCR reaction mixture B, heat-treated bovine serum albumin (BSA) (New England Biolabs, Ipswich, Massachusetts) and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri) were added to a final concentration of 1 mg/ml BSA and 5% (v/v) DMSO to PCR reaction mixture A. PCR reaction mixture C consisted of TaKaRa Ex Taq® DNA Polymerase (250 μM/μl) (TaKaRa Bio Inc., Otsu, Shiga, Japan), 1X of the proprietary Ex Taq reaction buffer (contains 2 mM MgCl₂), 200 μM of each dNTP (TaKaRa Bio), 1 mg/ml BSA, 5% (v/v) DMSO, and 40 mM of each primer.

**Assessment of the specificity and sensitivity of the PCR assays**

To assess the specificity of each PCR assay, 50 ng of DNA from *P. marinus* (ATCC 50489), *P. andrewsi* (ATCC 50807), and *Perkinsus* sp. (*M. mercenaria*) were used as templates in the PCR reactions. Sensitivities of the species-specific assays were assessed with the use of decreasing amounts of genomic DNA (100 pg to 1 fg) from the respective *Perkinsus* species. For the genus-specific assay, the sensitivity was assessed with the use of *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) genomic DNA, and for the *Perkinsus* sp. (*M. mercenaria*)–specific assay genomic DNA of *Perkinsus* sp. (*M. mercenaria*) and *P. andrewsi* genomic DNA were used. Assay sensitivities were assessed in the presence or absence of 500 ng of *C. virginica* or *M. mercenaria* genomic DNA. Specificity of the PCR assays in the presence of *C. virginica* genomic DNA was assessed with the use of PCR reaction mixture A. For assessment of the specificity in the presence of other *Perkinsus* species genomic DNA, PCR reaction mixtures A and C were used. Negative controls contained similar PCR reaction mixtures, except that the template was replaced by sterile double-distilled H₂O.

**PCR-based detection of selected *Perkinsus* species in oyster and clam samples**

Five hundred nanograms of DNA extracted from *C. virginica* and *M. mercenaria* were tested for *Perkinsus* species, *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*). For *C. virginica* DNA samples, PCR reagents were used for *M. mercenaria* DNA samples, with the use of PCR reaction mixture A and B. PCR reaction mixtures, except that 1 ng of genomic DNA extracted from cultured *Perkinsus* species was used as a template. In negative PCR controls, the DNA template was substituted by sterile double-distilled H₂O.

To minimize false negatives, the small subunit rRNA gene (SSU) was amplified from all *M. mercenaria* samples with the use of the universal primers UPRA and UPRB from Medlin et al. (1988), which are designed to amplify the SSU of all eukaryotes. For *C. virginica*, 45 of 226 samples that were negative for the presence of *Perkinsus* species were tested for the amplifiability of the SSU. PCR reaction mixtures were identical to those used to detect *Perkinsus* infections in *C. virginica* and *M. mercenaria*. Positive PCR amplification controls consisted of similar PCR reaction mixtures, except that 500 ng of genomic DNA extracted from each *C. virginica* or *M. mercenaria* was used that was known to be amplifiable. Cycling conditions were 94°C for 4 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, with a final extension at 72°C for 7 min.

**Attenuation of inhibitory effects on the PCR amplification**

To establish and optimize PCR conditions that would attenuate the observed inhibition of PCR amplification, experiments were conducted by spiking *M. mercenaria* genomic DNA (500 ng) with 10 pg and 1 pg *P. olsenii* genomic DNA. These mixtures were tested for *P. olsenii* as described elsewhere (Robledo et al., 2000), with the use of the PCR reaction mixtures A, B, and C. PCR amplification controls consisted of PCR reaction mixtures containing only *P. olsenii* genomic DNA. *Perkinsus olsenii* DNA was used because infections with this species have not been reported in the United States. Therefore, it is unlikely that the clams or oysters carried *P. olsenii*, allowing us to control accurately for the amount of target DNA added to the sample.

**Sequencing**

Forty amplicons generated by the *Perkinsus* genus–specific PCR assay of samples that tested negative with all of the *Perkinsus* species–specific assays were cored from agarose gels and reamplified with the use of the genus-specific assay as described above. The amplified products were separated on 1.5% agarose gels, purified from the gels (QIAquick, QIAGEN) and sequenced from both directions with the primers PER1 and PER2 (Robledo et al., 2002). Sequencing services were provided by the Bioanalytical Services Laboratory at the Center of Marine Biotechnology, Baltimore, Maryland. Fragment assembly was performed with the use of the Staden Package v1.6.0 on a Mac OS X (Apple Computer, Inc., Cupertino, California) or Linux Fedora® Core 5 (Red Hat, Inc., Raleigh, North Carolina)–based computer.

**Ribosomal RNA sequences for assay design and sequence comparisons**

Sequences of rRNA genes and intergenic spacers of the rRNA sequences of *P. andrewsi* (Genbank AF102171 and AY305326), *P. marinus* (AF497479), *P. olsenii* (syn. *P. atlanticus*, AF140295), and *Perkinsus* sp. (*M. mercenaria*) (deposited as *Perkinsus* sp. CCA2001, AF252288) were obtained from GenBank®. Sequence alignments were...
FIGURE 1. Sample site locations. *Crassostrea virginica* (●) and *Mercenaria mercenaria* (○) specimens were received from shellfish providers each month from June 2002 to September 2002. 1: Walpole (Maine); 2: Eliot (Maine); 3: Martha's Vineyard (Massachusetts); 4: Narragansett Bay (Rhode Island); 5: Oyster Bay (New York); 6: Delaware Bay (New Jersey); 7: Sandy Point (Maryland); 8: Cheriton (Virginia). The map was generated with the Generic Mapping Tools, v.4.1.4 (Smith and Wessel, 1990; Wessel and Smith, 1998).
**RESULTS**

**Specificity of the diagnostic assays**

To assess the specificity of the 4 PCR diagnostic assays used in this study, 50-ng genomic DNA from clonal cultures of *P. marinus*, *P. andrewsi*, and Perkinsus sp. (*M. mercenaria*) were tested. The genus-specific assay amplified a fragment of approximately 300 bp from each DNA preparation, whereas the assays designed to be specific for *P. marinus* and *P. andrewsi* amplified fragments of expected size (approximately 300 bp) only from genomic DNA preparations of the respective Perkinsus species (Fig. 2). The assay designed for *P. andrewsi* showed intense low molecular bands when *P. marinus* and Perkinsus sp. (*M. mercenaria*) were used as templates. Because this band also appeared with lesser intensity in the negative PCR control, they may represent primer dimers. The PCR assay designed for Perkinsus sp. (*M. mercenaria*) amplified a fragment of expected size (approximately 300 bp) from Perkinsus sp. (*M. mercenaria*). However, it also amplified a 300-bp fragment from *P. andrewsi* (Fig. 2).

**Sensitivity of the diagnostic assays**

The sensitivity of the Perkinsus genus- and species-specific assays was assessed by performing the respective assays on serially diluted genomic DNA (100 pg to 1 fg) with the use of the standard PCR reaction mixture A. The Perkinsus genus-specific assay amplified 100 fg of *P. marinus* and 1 pg of *P. andrewsi* and Perkinsus sp. (*M. mercenaria*) genomic DNA (Fig. 3A). The *P. marinus*— and the *P. andrewsi*—specific assays amplified 1 pg of *P. marinus* and *P. andrewsi* genomic DNA (Fig. 3B, C). The assay designed for the Perkinsus sp. (*M. mercenaria*) amplified 100 fg Perkinsus sp. (*M. mercenaria*) and 1 pg of *P. andrewsi* genomic DNA (Fig. 3D). In the genus-specific assay, the addition of 500 ng *C. virginica* DNA had no effect on the detection limit of *P. andrewsi* and Perkinsus sp. (*M. mercenaria*), but reduced the sensitivity by about 10-fold for *P. marinus*. No effects of 500 ng *C. virginica* DNA were observed on the *P. marinus*— and *P. andrewsi*—specific assays (Fig. 3B, C). In the Perkinsus sp. (*M. mercenaria*)—specific assay, although the 500 ng of *C. virginica* DNA had no effect on the detection limit of *P. andrewsi*, it reduced the sensitivity by about 10-fold for Perkinsus sp. (*M. mercenaria*) (Fig. 3D). The addition of 500 ng of *M. mercenaria* genomic DNA to the PCR reactions reduced the sensitivity by at least 1,000-fold in all 4

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**Table:**

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<th><em>Perkinsus genus</em></th>
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<th><em>P. andrewsi</em> - specific</th>
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**Figure 2:** Specificity of the PCR-based assays. Fifty nanograms of genomic DNA from Perkinsus marinus, Perkinsus andrewsi, and Perkinsus sp. (*M. mercenaria*) were tested with PCR-based assays specific for the Perkinsus genus, *P. marinus*, *P. andrewsi*, and Perkinsus sp. (*M. mercenaria*) with the use of the PCR reaction mixtures A. Perkinsus sp.: Perkinsus sp. (*M. mercenaria*); H₂O: negative control.

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Performed with the use of the Needleman-Wunsch global alignment algorithm within EMBOSS (Rice et al., 2000). Sequence alignments were used to design the Perkinsus sp. (*M. mercenaria*)—specific assay and to identify possible new Perkinsus sp. strains in the study area.

**Statistical analysis**

The main focus of this article was to assess and compare infection frequencies of Perkinsus species collected from 2 hosts at several sampling sites over a relatively short sampling period (4 mo). Therefore, the χ² test and, for pairwise comparison of the sampling site and the 2 host species, the Fisher’s exact test were used. Statistical analyses were performed with the R software suite (R Development Core Team, 2006).

**Climatologic data**

Temperature and precipitation data were obtained from COOP Data/Record of Climatological Observations Forms from selected weather stations made available to the public online by the National Climatic Data Center, U.S. Department of Commerce (http://www7.ncdc.noaa.gov/IPS).

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**RESULTS**

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assays for all Perkinsus species tested (Fig. 3A–D), suggesting that the M. mercenaria genomic DNA preparations possessed potent PCR inhibitory activity.

**Attenuation of the inhibition of the PCR amplification**

To obtain PCR conditions that attenuate the inhibition of the PCR amplification, 500 ng of C. virginica and M. mercenaria DNA were spiked with 10 pg and 1 pg P. olseni genomic DNA. These mixtures were tested for P. olseni with the use of PCR mixtures A, B, and C. PCR reaction mixture A did not amplify P. olseni in the presence of M. mercenaria genomic DNA, confirming PCR-amplification inhibition by DNA extractions from M. mercenaria (Fig. 4). The use of PCR reaction mixture B, which contains BSA (1 mg/ml) and DMSO (5% v/v), alle-
viated PCR inhibitory effects in most clam DNA preparations (Fig. 4). However, DNA extracted from some individual clams was not amplified even in mixture B. With the use of PCR reaction mixture C (containing TaKaRa Ex Taq® 1× of the proprietary Ex Taq reaction buffer, 1 mg/ml BSA, and 5% (v/v) DMSO), no PCR amplification inhibition was observed in any of DNA extractions tested (Fig. 4). Under these conditions, the detection limits of the genus- and the species-specific assays were 1 pg to 10 fg, respectively (Fig. 3).

**False-negative PCR results analysis**

To exclude false-negative PCR results from our analysis, the SSU of DNA samples was amplified with the use of primers that anneal in conserved regions of the SSU (Medlin et al., 1988). Forty-five of 226 *C. virginica* that tested negative with the diagnostic PCR assays were examined. In all samples tested, the SSU was amplified (data not shown). With the use of PCR reaction mixture C, the SSU in 225 out of 244 *M. mercenaria* samples could also be amplified (data not shown). The 19 *M. mercenaria* samples (7.8%) for which no amplification was observed were excluded from further analysis.

**Prevalence of Perkinsus species in Crassostrea virginica**

In total, 625 *C. virginica* collected monthly from June to September 2002 from Walpole (Maine), Martha’s Vineyard (Massachusetts), Narragansett Bay (Rhode Island), Oyster Bay (New York), Delaware Bay (New Jersey), and Sandy Point (Maryland) (Fig. 1) were tested for the presence of *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) (Table I). Overall, by using the genus-specific assay, 449 (66.5%) *C. virginica* tested positive for *Perkinsus* species as far north as Maine. The differences in prevalence between the sampling sites were statistically significant (*P* < 0.001). Generally, prevalences of *Perkinsus* species infections increased from north (Walpole; 10.3%) to south (Sandy Point; 96.5%), with the exceptions of Martha’s Vineyard, which had a significantly higher prevalence compared to Narragansett Bay (Fisher’s exact test, *P* < 0.001), and Oyster Bay, which had lower prevalence compared to Delaware Bay (Fisher’s exact test, *P* < 0.001) and Narragansett Bay (Fisher’s exact test, *P* < 0.001) (Table I). There were no significant differences between prevalences at Sandy Point and Martha’s Vineyard, and Sandy Point and Delaware Bay.

A seasonal trend in *Perkinsus* sp. infection prevalences was observed in Narragansett Bay, Oyster Bay, and Delaware Bay, where infections were lower in early summer (June), as compared to mid- (August; Delaware Bay, Narragansett Bay) or late summer (September; Oyster Bay). In Walpole, prevalences remained low (0–19.4%; Fisher’s exact test, *P* ≥ 0.237) during the sampling period. In Martha’s Vineyard, prevalences were high (86.7–100%; Fisher’s exact test, *P* ≥ 0.173) throughout

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**Figure 4.** Attenuation of PCR amplification inhibition. Five hundred nanograms genomic DNA from 1 individual of *Crassostrea virginica* and 2 *Mercenaria mercenaria* specimens were spiked with 10 pg and 1 pg *Perkinsus olseni* genomic DNA. Samples were tested with a *P. olseni*-specific PCR-based assay with the use of PCR reaction mixtures A, B, and C. In the positive control, host DNA was omitted. H₂O: negative control.
the entire sampling period. Samples from Sandy Point were not available for the months of June and September. Prevalences at this site did not differ between July and August (Fisher’s exact test, \( P = 1 \)) (Fig. 5A).

The 3 Perkinsus species, \( P. \) marinus, \( P. \) andrewsi, and \( P. \) marinus (\( M. \) mercenaria), were detected in \( C. \) virginica from Maryland to Maine. \( P. \) marinus was the dominant species, with 394 (58.4\%) \( C. \) virginica testing positive. In contrast, only 15 (2.2\%) and 3 (1.3\%) \( C. \) virginica tested positive for \( P. \) marinus (\( M. \) mercenaria) and \( P. \) andrewsi, respectively. This trend holds true for all sampling sites with the exception of the sampling site in Walpole, where \( P. \) marinus and \( P. \) andrewsi were both found at low prevalences (Table I). Coinfections with \( P. \) marinus and \( P. \) andrewsi or \( P. \) marinus and \( P. \) andrewsi (\( M. \) mercenaria) species were also observed (Table I). However, none of the oysters that tested positive for \( P. \) andrewsi was positive for \( P. \) marinus (\( M. \) mercenaria).

Fifty-two oysters that tested positive for infection with a Perkinsus species were negative for \( P. \) marinus, \( P. \) andrewsi, or \( P. \) marinus (\( M. \) mercenaria) (Table I). Sequence analysis of amplicons obtained by the genus-specific PCR assay from 22 of the 52 oysters suggests that 13 oysters carried \( P. \) marinus, 3 \( P. \) andrewsi, and 2 \( P. \) marinus (\( M. \) mercenaria). Four samples showed extensive sequence ambiguities, possibly due to infections with more than 1 Perkinsus species.

**Prevalence of Perkinsus species in Mercenaria mercenaria**

To assess the prevalence of Perkinsus species infections in the hard clam \( M. \) mercenaria, 225 specimens were tested with the PCR-based diagnostic assays described above. The specimens tested were collected monthly from June 2002 to August 2002 from Eliot (Maine), Martha’s Vineyard (Massachusetts), and July 2002 to September 2002 from Cheriton (Virginia) (Fig. 1).

Overall, by using the genus-specific PCR-based assay, a total of 72 (32\%) specimens tested positive for a Perkinsus species (Table II). Infection prevalences differed significantly between sites (\( \chi^2 \) test, \( P < 0.001 \)), increasing from north (Eliot) to south (Cheriton) (Table II). A seasonal trend in infection prevalence was only observed in Cheriton, where prevalence was lowest in July and increased over the sampling period. In Martha’s Vineyard, prevalence peaked in July. In Eliot, Perkinsus species infections were not observed in June. Prevalence observed in July and August did not differ considerably (Fisher’s exact test, \( P = 0.765 \)) (Fig. 5B).

Overall, 10 (4.4\%) clam specimens tested positive for \( P. \) marinus, and 3 (1.3\%) and 17 (7.6\%) tested positive for \( P. \) andrewsi and Perkinsus sp. (\( M. \) mercenaria), respectively. Similar to infections in \( C. \) virginica, coinfections in individual host specimens with \( P. \) marinus and Perkinsus sp. (\( M. \) mercenaria) were observed (Table II). None of the clams that tested positive for \( P. \) andrewsi was positive for Perkinsus sp. (\( M. \) mercenaria). Perkinsus marinus infections were observed at all 3 sampling sites. Perkinsus andrewsi infections were observed solely in Martha’s Vineyard, and Perkinsus sp. (\( M. \) mercenaria) were observed in Eliot and Cheriton (Table II).

Forty-four specimens tested positive for a Perkinsus species infection, but negative for any of the Perkinsus species or isolates tested. Eighteen of the 44 amplicons generated by the Perkinsus genus-specific PCR were sequenced. Fourteen of the obtained sequences were highly similar or identical to the sequence of \( P. \) marinus and 3 sequences to \( P. \) andrewsi. One sequence showed extensive ambiguities, suggesting an infection with more than 1 Perkinsus species.

**Comparison of Perkinsus species prevalences in Crassostrea virginica and Mercenaria mercenaria**

Prevalence of Perkinsus species (\( P. \) marinus) in \( M. \) mercenaria was significantly lower compared to prevalence in \( C. \) virginica (Fisher’s exact test, \( P < 0.001 \)). Perkinsus andrewsi prevalence was similar in both host species (Fisher’s exact test, \( P = 1 \)). Prevalence of Perkinsus sp. (\( M. \) mercenaria) was significantly higher in \( M. \) mercenaria (Fisher’s exact test, \( P < 0.001 \)).

Martha’s Vineyard provides a very useful side-by-side comparison of prevalence in both clams and oysters because both hosts were collected from proximal locations. Here, 96.1\% of the \( C. \) virginica were infected with a Perkinsus species, compared to 26.2\% of the \( M. \) mercenaria (Fig. 6; Tables I, II). All infected \( C. \) virginica specimens carried \( P. \) marinus, and \( P. \) andrewsi infected 1.1\% and Perkinsus sp. (\( M. \) mercenaria) 3.9\% of \( C. \) virginica. Each of the oysters infected with \( P. \) andrewsi and Perkinsus sp. (\( M. \) mercenaria) were dually infected with \( P. \) marinus. In \( M. \) mercenaria, \( P. \) marinus and \( P. \) andrewsi were present in low prevalences (3.3\% and 4.9\%, respectively). Per-
Figure 5. Monthly Perkinsus infection prevalence in Crassostrea virginica and Mercenaria mercenaria. Percent prevalences of Perkinsus species (detected by the genus-specific assay) (■), Perkinsus marinus (□), Perkinsus andrewsi (□), Perkinsus sp. (M. mercenaria) (□), dual infections with P. marinus and P. andrewsi (□), as well as P. marinus and Perkinsus sp. (M. mercenaria) (□), and infections with Perkinsus species only (□) are shown for all sampling sites. (A) Percent prevalence in C. virginica. (B) Percent prevalence in M. mercenaria. Jun: June; Jul: July; Aug: August; Sep: September.
Table II. Percent prevalence of Perkinsus species infections in Mercenaria mercenaria collected from June 2002 to September 2002. 2: Eliot, Maine; 3: Martha’s Vineyard, Massachusetts; 8: Cheriton, Virginia; N: number of individuals; n: number of infected individuals; (%): prevalence in percent; P.m.: Perkinsus marinus; P.a.: Perkinsus andrewsi; P.sp.: Perkinsus sp. (M. mercenaria); P.spp.: Perkinsus infections detected with the generic PCR-based assay.

<table>
<thead>
<tr>
<th>Site</th>
<th>N</th>
<th>P.spp.</th>
<th>P.m.</th>
<th>P.a.</th>
<th>P.sp.</th>
<th>P.m. and P.a.</th>
<th>P.m. and P.sp.</th>
<th>P.sp. only</th>
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<td>15</td>
<td>5.1</td>
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<td>85</td>
<td>41</td>
<td>48.2</td>
<td>4</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>23</td>
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<tr>
<td>All</td>
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<td>72</td>
<td>32.0</td>
<td>10</td>
<td>17</td>
<td>0</td>
<td>2</td>
<td>44</td>
</tr>
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</table>

perkinsus sp. (M. mercenaria) was not detected (Fig. 6; Tables I, II). Differences in prevalence between C. virginica and M. mercenaria were statistically significant for Perkinsus species and P. marinus infections (Fisher’s exact test, \( P < 0.001 \)), but not for P. andrewsi (Fisher’s exact test, \( P = 0.105 \)) or Perkinsus sp. (M. mercenaria) (Fisher’s exact test, \( P = 0.208 \)).

**Water temperature and precipitation**

Water temperature and salinity are the main environmental factors affecting Perkinsus species infections (Andrews, 1988; Burreson and Ragone Calvo, 1996). Air temperature is often used as a substitute for water temperature, because it is more frequently recorded and correlates with the temperature of nearby water bodies (Jeffries and Johnson, 1976; Sauriau, 1991). Salinity of coastal water bodies is influenced by freshwater influx from major rivers and precipitation. Thus, to compare prevalences to an earlier study on the distribution of P. marinus, and 2 Haplosporidium species in C. virginica collected in 2000 (Russell et al., 2004), temperature and precipitation data were obtained for May to August 2000 and 2002, from Mineola (New York) (COOP ID 305377), a weather station near Oyster Bay (New York). Mean monthly air temperatures in 2000 were 16.9, 20.4, 21.2, and 21.9 °C in May, June, July, and August, respectively. Monthly precipitation was 108.2, 111.3, 137.7, and 61.2 mm over the same time period, respectively. In 2002, mean monthly temperatures were 15.2, 21.0, 25.9, and 24.8 °C from May to August. Monthly precipitation (May–August) was 93.0, 95.8, 22.9, and 183.9 mm.

**DISCUSSION**

The aim of the present study was to assess the presence of P. marinus, P. andrewsi, and Perkinsus sp. (M. mercenaria) in 2 economically important bivalves, C. virginica and M. mercenaria, with the use of PCR-based assays. Along the Gulf of Mexico and Atlantic coast of the United States, 3 Perkinsus species have been described, i.e., P. marinus (Mackin et al., 1956; type host C. virginica), P. chesapeakei (McLaughlin et al., 2000; type host M. arenaria), and P. andrewsi (Coss, Robledo, and Vasta, 2001; type host M. balitica).

The heterospecificity of P. andrewsi and P. chesapeakei has been controversial and synonymization has been suggested (Burreson et al., 2005). According to the original description, P. chesapeakei is a distinct morphotype (McLaughlin et al., 2000). In contrast, P. andrewsi cannot be distinguished from other Perkinsus species based on morphology, but sequences of the rRNA genes and intergenic regions of P. andrewsi differ from other Perkinsus species (Coss, Robledo, and Vasta, 2001; Coss, Robledo, Ruiz, and Vasta, 2001). The Perkinsus isolate that was analyzed to clarify the relationship of P. andrewsi and P. chesapeakei has been designated as P. chesapeakei because it has been isolated from the appropriate type host. However, this isolate appears to be morphologically identical to P. andrewsi and, thus, may not be the P. chesapeakei originally described. Therefore, until additional evidence is obtained, we retain the P. andrewsi designation for the present study.

The standard diagnostic assay for Perkinsus species that is based on the FTM method does not distinguish between the sympatric Perkinsus species found along the Atlantic coast (Ray, 1952; Coss, 2000; McLaughlin et al., 2000; Coss, Robledo, Ruiz, and Vasta, 2001). However, several Perkinsus species–specific assays are available (Marsh et al., 1995; Yarnall et al., 2000; Coss, Robledo, Ruiz, and Vasta, 2001; Robledo et al., 2002). No PCR-based assay has been developed for P. chesapeakei, mainly due to the lack of a bona fide type culture that would allow design and validation of such an assay.

For the purpose of the present study, we used available PCR-based assays specific for the genus Perkinsus, and the species P. marinus and P. andrewsi (Marsh et al., 1995; Coss, Robledo, Ruiz, and Vasta, 2001; Robledo et al., 2002), and developed a new PCR-based assay for a Perkinsus species isolated from M.
mercenaria [Perkinsus sp. (M. mercenaria)]. The species-specific assays are based on sequence differences within the intergenic spacer (IGS) of the rRNA gene locus of Perkinsus species that links the SSU and LSU genes, whereas the genus-specific assay is based on a conserved region at the 3' end of the IGS (Marsh et al., 1995; Coss, Robledo, Ruiz, and Vasta, 2001; Robledo et al., 2002). As expected, the genus-specific assay detected all Perkinsus species tested in this study, including Perkinsus sp. (M. mercenaria). Perkinsus sp. (M. mercenaria) was not detected by the P. marinus—specific or the P. andrewsi—specific assays, demonstrating the capacity of the genus-specific Perkinsus assay to detect new Perkinsus species or strains.

The assay designed for Perkinsus sp. (M. mercenaria) also amplified P. andrewsi genomic DNA extracted from the P. andrewsi type culture. This is to be expected, for we have previously shown that P. andrewsi has 2 distinct rRNA gene units (types A and B) (Pecher et al., 2004). Sequence analysis of the rRNA gene unit of the Perkinsus sp. (M. mercenaria) revealed only 1 rRNA gene unit that is very similar in sequence to the rRNA-B gene unit of the P. andrewsi type culture (W. T. Pecher and G. R. Vasta, unpubl. obs.). In particular, the IGS of Perkinsus sp. (M. mercenaria) is 98.9% identical to the IGS of the P. andrewsi rRNA-B gene unit (W. T. Pecher and G. R. Vasta, unpubl. obs.), explaining the cross amplification. On the other hand, the P. andrewsi—specific assay does not detect Perkinsus sp. (M. mercenaria), because it has been developed based on the IGS of the rRNA-A gene unit that is only 71.3% identical to the IGS of Perkinsus sp. (M. mercenaria).

The sensitivity of each species-specific assay observed in our study (0.1—1 pg genomic DNA of the respective Perkinsus species) is similar to the sensitivities for the P. marinus and P. andrewsi diagnostic assays reported by Marsh et al. (1995) and Coss, Robledo, Ruiz, and Vasta (2001). Our data suggest that the sensitivity of the genus-specific assay (10 fg to 1 pg genomic Perkinsus species DNA) is equal, or greater, compared to the respective species-specific assays, allowing us to identify low-intensity Perkinsus infections.

Inhibition of PCR amplification is frequently observed in environmental and biological samples (reviewed by Wilson, 1997). Inhibitory substances include organic and phenolic compounds, humic acids, heavy metals, fats, and polysaccharides. In molluscs, PCR inhibition has been attributed to glycogen (Hill et al., 1991; Andersen and Omieckinski, 1992; Atmar et al., 1993). Modified DNA extraction protocols (Atmar et al., 1993), and inclusion of additives in the PCR reaction mixture is commonly used to attenuate the effects of the interfering substances (reviewed in Wilson, 1997a). In addition, commercially available kits have been developed that can be used to extract DNA from plants, animals, and fungi from complex sources such as the soil and other environmental samples.

We did not observe PCR inhibition in C. virginica DNA extracts. However, PCR inhibition was dramatic in DNA from M. mercenaria, and has been observed in scallop DNA (Argopecten irradians) extracted with a commercial tissue kit (W. T. Pecher and G. R. Vasta, unpubl. obs.). We succeeded in attenuating the PCR amplification inhibition in M. mercenaria samples by adding 1 mg/ml BSA in combination with 5% (v/v) DMSO to the PCR reaction mixture. While using regular Taq DNA polymerase with a standard PCR buffer, we were able to PCR amplify the SSU from 70% of all M. mercenaria samples (data not shown). The use of a specialty Taq DNA polymerase with its optimized buffer system designed to amplify large DNA fragments increased the success rate to 92%. However, similar reaction conditions failed to amplify scallop DNA samples (W. T. Pecher and G. R. Vasta, unpubl. obs.). These findings underline the importance of validation of PCR conditions for each sample type used, including template “amplifiability” vis-à-vis inhibition. Once DNA is extracted from samples and PCR conditions are optimized, the PCR-based assays enable detection of any Perkinsus species and different Perkinsus species in the same sample. Thus, the application of the genus- and species-specific assays presents a valuable alternative to the FTM assay.

Studies based on the FTM assay documented the distribution of Perkinsus species in oysters from the Yucatan Peninsula, Mexico, to Maine (Burreson et al., 1994; Ford, 1996; Sotens, 1996). These infections have been attributed to P. marinus. However, the discovery of additional Perkinsus species and the development of specific PCR assays for them have provided tools to test this assumption. In the present study, commercially harvested C. virginica and M. mercenaria populations were tested for the presence of Perkinsus species, P. marinus, P. andrewsi, and a Perkinsus sp. isolated from M. mercenaria with the use of specific PCR-based assays.

In accordance with the studies identified above, with the use of the genus-specific assay in both bivalve host species, Perkinsus species, infections were observed as far north as Maine. The intensity of the amplicons obtained by the PCR-based assays suggested, in the majority of the positive samples, the presence of 10 pg or more of Perkinsus spp. DNA (data not shown), which is roughly equivalent to more than 100 Perkinsus spp. cells (see below). However, it cannot be ruled out that in some specimens that yielded low-intensity amplicons, these actually reveal only the presence of parasite rather than true infections.

With the use of the genus-specific assay, prevalences of Perkinsus species in C. virginica appeared lower in early summer (June) than in mid- (August) or late summer (September) in Delaware Bay, Oyster Bay, and Narragansett Bay. Similar trends were observed in the Chesapeake Bay and other regions. When compared to C. virginica, significantly fewer M. mercenaria specimens tested positive for the genus Perkinsus and P. marinus. Prevalences of Perkinsus species (as assessed by the genus-specific method) differed from site to site in both host species. Generally, prevalences of Perkinsus species decreased from south to north, with the exception of Martha’s Vineyard (Massachusetts) and Oyster Bay (New York) in C. virginica. In Martha’s Vineyard, prevalences were surprisingly high (86.7—100.0% over the 4-mo study period) compared to those in Narragansett Bay and Oyster Bay and are in contrast to observations by Russell et al. (2004). These authors did not observe P. marinus infections in C. virginica specimens that were collected in September 2000 and tested by a multiplex PCR-based assay, suggesting that Martha’s Vineyard may have experienced a localized Perkinsus epizootic in 2002.

Prevalences in Oyster Bay were significantly lower compared to Narragansett Bay. However, the observed prevalences in Oyster Bay in 2002 were higher than those reported by Russell et al. (2004), who observed 0% and 3% P. marinus prevalence in C. virginica collected in June and August 2000, respectively, from a site in Oyster Bay. Similarly, we observed no P. marinus
infections in Oyster Bay in June 2002. In August 2002, however, 17% of C. virginica were infected with P. marinus. These differences may be due to higher temperatures and drier conditions in 2002 compared to 2000, as judged by monthly mean air surface temperatures and precipitation recorded by a nearby weather station (Mineola, New York, COOP ID 305377). Higher temperatures and drier conditions may result in higher water temperatures and higher salinity, both conditions favorable to P. marinus infections (Andrews, 1988; Burreson et al., 1996). Furthermore, differences in the sensitivities of the PCR assays may have contributed to the observed differences. The PCR assay used in the current study detects 1 pg genomic DNA of P. marinus. Based on the following calculation, the PCR-based assay used in the present study is about 2.5-fold more sensitive than the one used by Russell et al. (2004). Based on available sequences, P. marinus has an estimated genome size between 70 and 80 Mb (N. M. El-Sayed, J. A. Fernandez-Robledo, and G. R. Vasta, unpubl. obs.; http://www.tigr.org/tdb/e2kllpmg/), and a single rRNA gene unit is approximately 7 kb long (J. A. Fernandez-Robledo and G. R. Vasta, unpubl. obs.). If a genome size of 80 Mb is assumed, then 1 pg genomic DNA represents 13 genome equivalents. If it is furthermore assumed that, similar to P. olseni (syn. P. atlanticus) (de la Herrán et al., 2000), P. marinus rRNA genes are encoded by 5% of its genome, then about 570 copies of a single rRNA gene unit are present per genome equivalent. Thus, the PCR assay used in the present study would detect 7,500 copies of the rRNA gene unit, or, as each gene unit contains 1 IGS, roughly 7,500 copies of the IGS. The multiplex PCR assay used by Russell et al. (2004) detects 100 fg of plasmid DNA containing the P. marinus IGS (Russell et al. 2004). Because the plasmid with the IGS sequence is approximately 4.5 kb, 100 fg plasmid DNA represents about 20,000 copies of the IGS.

Our data enabled us to extend the observed range of P. andrewsi and Perkinsus sp. (M. mercenaria) from Chesapeake Bay to Maine. It is noteworthy that the prevalence of Perkinsus sp. (M. mercenaria) was highest in the south (Cheriton, Virginia), suggesting that this Perkinsus isolate may prefer warmer waters. However, until investigations on the southern distribution range of this Perkinsus isolate are conducted, this remains speculative. Interestingly, we did not detect P. andrewsi in farmed oysters in Chesapeake Bay. This finding is in contrast to reports of Coss, Robledo, Ruiz, and Vasta (2001) that indicate 65% of 125 C. virginica collected from natural populations throughout Chesapeake Bay were infected with P. andrewsi, 64% with P. marinus, and 34% with both. In our study, 94.7% of oysters were infected with P. marinus and 10.5% with Perkinsus sp. (M. mercenaria). A possible explanation is that the conditions at the particular location were favorable for a P. marinus infection. Alternatively, and not mutually exclusively, it is conceivable that once a P. marinus infection has been established in an oyster population, it may outgrow other Perkinsus infections. To address these questions, further investigations need to be conducted on the infection dynamics of different Perkinsus species.

Perkinsus marinus appears to be the most prevalent Perkinsus species in C. virginica. Therefore, discrepancies between high infection density, prevalence, and low mortality observed in other studies in C. virginica (Ford, 1996; Karolus et al., 2000) cannot be attributed to the presence of a different Perkinsus species with less pathogenicity toward the oyster. Alternative hypotheses that will require further study include the notion of variable P. marinus strains with different pathogenicities, rather than different Perkinsus species, and, perhaps, differences in environmental factors such as cooler summer peak temperatures that could influence the outcome of an infection (Ford, 1996). Of course, the presence of host populations with different genetic backgrounds could be another component leading to lower host mortality. This is exemplified by the observation that in M. mercenaria Perkinsus sp. (M. mercenaria) was the most prevalent Perkinsus species.

Because of sequence similarities of the second rRNA gene unit (rRNA-B) of the P. andrewsi hapantotype to the Perkinsus sp. isolated from M. mercenaria (W. T. Pecher and G. R. Vasta, unpubl. obs.), the P. andrewsi hapantotype is detected by the Perkinsus sp. (M. mercenaria)—specific assay with a 10-fold lower sensitivity. In our study, none of the clams and oysters that tested positive for P. andrewsi also tested positive for Perkinsus sp. (M. mercenaria). Certainly, the lower sensitivity of the Perkinsus sp. (M. mercenaria) toward P. andrewsi may partially explain this observation, but the presence of P. andrewsi isolates that contain only the rRNA-A gene unit and thus are not detected by the Perkinsus sp. (M. mercenaria) assay cannot be ruled out. Because the rRNA unit of Perkinsus sp. (M. mercenaria) and the P. andrewsi rRNA-B unit share high sequence similarities, Perkinsus sp. (M. mercenaria) could be considered a variant of P. andrewsi that possesses only the rRNA-B gene unit. Similar observations have been reported for Trypanosoma cruzi, where isolates have been identified that possess either 2 distinct rRNA gene units (rRNA unit 1 and 2), or one of the 2 rRNA gene units (Souto et al., 1996; Zingales et al., 1999; Stolf et al., 2003).

Application of the genus-specific PCR-based assay to both C. virginica and M. mercenaria specimens resulted in the detection of Perkinsus infections that could not be attributed to P. marinus, P. andrewsi, or Perkinsus sp. (M. mercenaria) by the species-specific PCR assays. However, sequence analysis of selected amplicons generated by the genus-specific PCR failed to reveal novel sequences, which could suggest the presence of yet-undescribed Perkinsus species or strains. Further, it rather suggested that the density of Perkinsus infections in these specimens was below the detection limit of the species-specific assays, but high enough to be detected by the genus-specific assay.

Although Perkinsus species appear to lack strict host specificity, they may have adapted best to the hostile environment of one particular host species. Studies on the effects of plasma of different mollusc species on the in vitro proliferation of P. marinus show that parasite growth is reduced by plasma or sera from bivalve molluscs (Andara ovalis, Geukensia demissa, M. mercenaria, and Mytilus edulis) that are naturally exposed to the parasite as compared to plasma or sera from C. virginica (Anderson, 2001; Gauthier and Vasta, 2002). These observations suggest a preference of P. marinus for its type host C. virginica. Results from our study provide further evidence for a possible host preference of P. marinus. Although P. marinus was detected in M. mercenaria and C. virginica, the prevalence in M. mercenaria was significantly lower. Further studies aimed at elucidating the molecular mechanisms behind this host preference are ongoing.
ACKNOWLEDGMENTS

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LITERATURE CITED


PREVALENCE OF ZOONOTIC TREMATODES IN FISH FROM A VIETNAMESE FISH-FARMING COMMUNITY

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ABSTRACT: The prevalence of fish-borne zoonotic trematode (FZT) metacercariae was investigated in fish farmed by rural households in Nghe An Province, located in northern Vietnam. In total, 716 fish, including tilapia (Oreochromis niloticus) and 6 carp species, i.e., grass carp (Ctenopharyngodon idellus), bighead carp (Aristichthys nobilis), mirgal (Cirrhinus mirgala), common carp (Cyprinus carpio), silver carp (Hypophthalmichthys molitrix), and roha (Labeo rohita), were collected from 53 fish farms were examined. The overall prevalence of FZT metacercariae was 44.6%, ranging from 12.5% to 61.0% in fish species collected from grow-out ponds, which are the production system for growing fish from fingerling size to market size. The overall prevalence was 43.6% in fingerlings cultured in nurseries, ranging from 7.4% to 62.8% for different fish species. The FZT species recovered were heterophyids and echinostomatids and included H. pumilio, H. taichui, H. yokogawai, Centrocestus formosanus, Stellantchasmus falcatus, and Echinococclus japonicus, all of which are intestinal flukes in humans, other mammals, and birds. This is the first report of H. yokogawai and E. japonicus in fish in Vietnam, and the first record for S. falcatus in northern Vietnam. Although a previous cross-sectional survey of the people living in these fish farm households revealed a very low prevalence of FZTs (<1%), our results demonstrate that intestinal flukes are common in farmed fish in this area, suggesting that reservoir hosts such as dogs, cats, and pigs are more important in sustaining the life cycles of these flukes in fish farms than human hosts. This has implications for the effectiveness of control programs focused mainly on treatment of humans.

Fish-borne zoonotic trematodes (FZTs), including liver and intestinal flukes, are significant public health problems worldwide, especially in Asian countries (WHO, 1995, 2004; Chai, Murrell, and Lymbery, 2005; Keiser and Utzinger, 2005; Tesana, 2005; Yoshida, 2005). The number of people currently infected with FZTs was recently estimated by the World Health Organization (WHO) to exceed 18 million; however, the number of people at risk worldwide is more than 500 million (WHO, 2004). Recent figures suggest that about 1.5 million people in Korea, 6 million people in China, and over 5 million in Thailand are infected with liver flukes (Clonorchis sinensis or Opisthorchis viverrini) (Chai, Murrell, and Lymbery, 2005; Tesana, 2005; Yoshida, 2005). However, many of the numerous species of intestinal heterophyids and echinostomatids are also important, but less well recognized, compared to liver flukes, fish-borne zoonoses in China (Yu and Xu, 2005). The dominant trematode parasites are those parasites as also an important national public health problem (De et al., 2003). Clonorchis sinensis is reported from 9 northern provinces of Vietnam, with human prevalence ranging from 0.2% in Thai Binh Province to 26% in Nam Dinh Province. In contrast, O. viverrini has been reported only from 3 southern provinces, with prevalences ranging from 0.3% in Da Nang Province to more than 10% in Phu Yen Province (De et al., 2003). Only recently have FZT metacercariae been reported in Vietnamese fish (Hop et al., 2007; Thien et al., 2007; Thu et al., 2007).

Information on the status of FZTs in Vietnamese fish is highly important because of the importance of aquaculture to the economy and national nutritional needs. Freshwater fish production in Vietnam has increased 9.3-fold, from 41,750 tons in 1962 to 390,000 tons in 2005 (Keiser and Utzinger, 2005). As part of the growing importance of aquaculture in Vietnam, various programs have been implemented to encourage the development of household-scale freshwater aquaculture. In Nghe An Province, for example, freshwater aquaculture is highly developed, and fish production has increased from 7,800 tons in 2000 to 13,382 tons in 2004 (data obtained from 2005 records of the Nghe An Province Fisheries Department). Expansion of aquaculture production may inadvertently increase the risk of FZTs in fish because of increased growth of fish farming, which is very common in household-based culture systems. For economic reasons there is frequent reliance on the use of human and animal manure for pond fertilization, which could increase the risk of FZT egg contamination of the aquatic environment (WHO, 2004). This may also promote the proliferation of snail host populations and facilitate transmission of these trematode parasites to fish. Although surveys for human FZT infection in eastern Nghe An Province have indicated a low prevalence (De et al., 2003; Olsen et al., 2006), the status of FZT infection in farmed fish was of interest to assess the food safety risk of fish produced in this major aquaculture area.

MATERIALS AND METHODS

Study area and sampling design

A cross-sectional survey for zoonotic metacerciae in farmed fish was conducted from May 2005 to August 2005 in Nghe An Province, located about 300 km south of Hanoi. Five districts in the eastern section of the province (Tan Ky, Thanh Chuong, Yen Thanh, Nam Dan, and Hung Nguyen) were selected for fish sampling because of their highly developed aquaculture. Fifty farms were selected from a total of 1,281 fish farms present in these districts. Because the number of farms in the 5 districts was not equal, farms for sampling were selected by a random proportional sampling design. For example, Thanh Chuong district represented 20% of the total number of farms, and, therefore, 10 farms were randomly selected. The number of farms selected in each district was Thanh Chuong (10/263 farms), Nam Dan (15/384 farms), Tan Ky (8/199 farms), Hung Nguyen (8/196 farms), and Yen Thanh (9/239 farms). A previous human prevalence survey of the farm households in these districts identified 9 farms with cases of human FZT infection (Olsen et al., 2006), and these farms were also included in the study bringing the total to 59 study sites; however, only 53 farms were sampled because 6 of the 59 farms did not have fish in their ponds at the

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time of the survey. Two fish production systems, i.e., nursery ponds and grow-out ponds, were included in the study. Nursery ponds were used to grow fish from larvae (1 cm in length) or fry (30 days after hatch) to about 100 g in weight; fish density in these ponds was usually very high. After 2 to 4 mo, the fish were supplied to grow-out pond operators who cultured the fish at low density until harvesting and marketing. Fish were collected from each farm using a seine net or scoop net dragged through the pond up to 3 times.

**Examination of fish for metacercariae**

Fish species cultured in the farms sampled included grass carp (*Ctenopharyngodon idellus*), bighead carp (*Arichthys nobilis*), mirgal (*Cyprinus carpio*), common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), tilapia (*Oreochromis niloticus*), *SRS* species, and 3 unidentified species. Other species of fish not intentionally stocked by the farmer often occurred in ponds and were defined as self-recruiting species (SRS), a term used to describe aquatic animals that can be harvested from farmer-managed systems without intentional stocking. When these species were obtained during sampling, they were retained and examined for the presence of metacercariae.

After collection, the fish were placed on ice and transported to the laboratory of the Research Institute for Aquaculture no. I station in district farms. The randomly collected and examined for FZT metacercariae. The prevalences and densities are expressed as the mean, with 95% confidence intervals. Density of metacercariae is expressed as number of metacercariae/g fish digested. Fish weight and length averages are expressed as mean ±SD. Sigmastat (3.00 SPSS Inc., Chicago, IL) was used to compare differences in prevalence and density using Kruskal Wallis 1-way ANOVA on ranks (*P* ≤ 0.05 was considered significant).

**RESULTS**

**Species of fish and fish sizes collected in each fish culture system**

In total, 716 fish from the 53 fish farms, including 41 grow-out ponds (508 fish) and 12 nursery ponds (208 fish), were collected and examined for FZT metacercariae. The randomly caught fish from each pond type included both cultured and self-recruiting species (Table I). The mean ±SD weight of the sampled fish was 41.1 ± 54.8 g for nursery ponds, and 97.4 ± 153.2 g for grow-out ponds. The mean length ±SD was 13.0 ± 5.7 cm for fishes from nursery ponds, and 16.4 ± 9.7 cm for grow-out ponds.

**Comparisons by districts**

The prevalence of metacercariae was significantly different among the 5 districts (Table II). Prevalence of metacercariae in...
Hung Nguyen, Nam Dan, and Yen Thanh was significantly greater than from Tan Ky (36.3%) and Thanh Chuong (12.3%) districts ($P \leq 0.001$). In contrast, there was a significant difference in infection density of metacercariae in fish from Tan Ky compared to fish from the other 4 districts ($P \leq 0.000001$).

The species of FZT metacercariae, their prevalence, and intensities in cultured fish

The FZT prevalence and intensity data for cultured fish (excluding SRS) from both nurseries and grow-out ponds are presented in Tables II and III. The overall prevalence of metacercariae in all fish collected was 44.7% (CI: 41.1–48.4). All species of metacercariae recovered were potentially zoonotic in intestinal flukes belonging to either the Heterophyidae or the Echinostomatidae. The prevalence of Stellantchasmus falcatus, Echinocotmus japonicus, and Haplorchis yokogawai was very low ($\leq 0.5$%); the 3 most numerous species were H. pumilio, H. taichui, and C. formosanus. Haplorchis pumilio was significantly more common than the other 2 species in both nursery and grow-out ponds ($P \leq 0.05$) (Table IV). The prevalence of C. formosanus was significantly higher in fish from nursery ponds than from grow-out ponds ($P \leq 0.05$). Neither the prevalence nor density of FZT metacercariae was significantly different between nursery ponds and grow-out ponds (Tables II, IV). The density of H. pumilio was significantly greater than that of the other FZT species recovered ($P \leq 0.000001$).

Comparisons of FZT infections between species of cultured fish

The FZT prevalence and density data for all cultured fish species are shown in Table III. The prevalence of metacercariae varied significantly between the species of fish examined ($P < 0.000001$). Grass carp from both nursery and grow-out ponds had the highest FZT prevalence and infection densities. The apparent higher prevalence in bighead carp from nursery ponds was based on only 4 fish.

Prevalence and intensity of FZT in self-recruiting species of fish

Fourteen SRS fish species ($n = 142$ fish) were collected from 53 ponds in the 5 districts in Nghe An Province (Table I). The

Table III. Prevalence and infection density of FZTs in different species of cultured fish, excluding SRS.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>No.</th>
<th>% Prevalence (95% CI)</th>
<th>Mean density (95% CI)</th>
<th>Trematode species recovered*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nursery pond (total)</td>
<td>188</td>
<td>43.6 (36.7–50.8)</td>
<td>1.8 (0.2–3.4)</td>
<td>Ht, Hp, Sf, Cf, Ej</td>
</tr>
<tr>
<td>Bighed carp, <em>Aristichthys nobilis</em></td>
<td>4</td>
<td>75.0 (24.9–98.7)</td>
<td>0.2 (0.1–0.4)</td>
<td>Ht, Hp, Cf</td>
</tr>
<tr>
<td>Common carp, <em>Cyprinus carpio</em></td>
<td>46</td>
<td>54.3 (39.8–68.6)</td>
<td>0.4 (0.6–1.0)</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Grass carp, <em>Ctenopharyngodon idellus</em></td>
<td>43</td>
<td>62.8 (47.7–75.9)</td>
<td>7.1 (5.5–13.7)</td>
<td>Ht, Hp, Sf, Cf</td>
</tr>
<tr>
<td>Mrigal, <em>Cirrhinus mrigala</em></td>
<td>16</td>
<td>56.3 (30.6–79.2)</td>
<td>0.4 (0.1–0.6)</td>
<td>Ht, Hp, Cf</td>
</tr>
<tr>
<td>Rohu, <em>Labeo rohita</em></td>
<td>27</td>
<td>7.4 (1.3–23.7)</td>
<td>0.0 (0.0–0.1)</td>
<td>Ht, Hp, Ej</td>
</tr>
<tr>
<td>Silver carp, <em>Hypophthalmichthys molitrix</em></td>
<td>35</td>
<td>40.0 (25.0–57.2)</td>
<td>0.2 (0.1–0.4)</td>
<td>Ht, Hp, Cf</td>
</tr>
<tr>
<td>Tilapia, <em>Oreochromis niloticus</em></td>
<td>17</td>
<td>11.8 (2.1–35.0)</td>
<td>0.0 (0.0–0.0)</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Grow-out pond (total)</td>
<td>386</td>
<td>44.6 (39.6–49.6)</td>
<td>0.7 (0.4–1.1)</td>
<td>Ht, Hp, Sf, Cf, Hy</td>
</tr>
<tr>
<td>Bighed carp, <em>Aristichthys nobilis</em></td>
<td>11</td>
<td>45.5 (20.0–73.5)</td>
<td>0.1 (0.0–0.3)</td>
<td>Ht, Hp, Cf</td>
</tr>
<tr>
<td>Common carp, <em>Cyprinus carpio</em></td>
<td>89</td>
<td>42.7 (32.5–53.4)</td>
<td>0.1 (0.1–0.2)</td>
<td>Hp, Hp, Cf</td>
</tr>
<tr>
<td>Grass carp, <em>Ctenopharyngodon idellus</em></td>
<td>105</td>
<td>61.0 (51.0–70.1)</td>
<td>1.9 (0.6–3.1)</td>
<td>Ht, Hp, Hy</td>
</tr>
<tr>
<td>Mrigal, <em>Cirrhinus mrigala</em></td>
<td>22</td>
<td>59.1 (38.3–77.8)</td>
<td>0.5 (0.7–0.2)</td>
<td>Ht, Hp, Cf</td>
</tr>
<tr>
<td>Rohu, <em>Labeo rohita</em></td>
<td>23</td>
<td>39.1 (21.3–61.1)</td>
<td>0.1 (0.0–0.2)</td>
<td>Ht, Hp, Cf</td>
</tr>
<tr>
<td>Silver carp, <em>Hypophthalmichthys molitrix</em></td>
<td>72</td>
<td>48.6 (36.9–60.4)</td>
<td>0.8 (0.1–1.4)</td>
<td>Ht, Hp, Cf</td>
</tr>
<tr>
<td>Tilapia, <em>Oreochromis niloticus</em></td>
<td>64</td>
<td>12.5 (5.9–23.2)</td>
<td>0.1 (–0.1–0.3)</td>
<td>Hp</td>
</tr>
</tbody>
</table>

*Hp = Haplorchis pumilio; Ht = H. taichui; Hy = H. yokogawai; Cf = Centrocestus formosanus; Sf = Stellantchasmus falcatus; Ej = Echinocotmus japonicus.

Table IV. Comparisons of FZT species prevalence and mean densities in fish from nursery and grow-out systems including SRS.*

<table>
<thead>
<tr>
<th>Culture system</th>
<th>FZT species</th>
<th>% Prevalence (95% CI)</th>
<th>Mean density (95% CI)</th>
<th>% Prevalence (95% CI)</th>
<th>Mean density (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nursery</td>
<td>H. pumilio</td>
<td>45.2 (38.4–52.2)</td>
<td>2.2 (0.7–3.7)</td>
<td>41.3 (36.9–45.5)</td>
<td>0.7 (0.4–1.0)</td>
</tr>
<tr>
<td>Nursery</td>
<td>H. taichui</td>
<td>1.9 (0.7–4.9)</td>
<td>0.0</td>
<td>1.0 (0.4–2.3)</td>
<td>0.0</td>
</tr>
<tr>
<td>Nursery</td>
<td>H. yokogawai</td>
<td>0.0 (0.0–1.8)</td>
<td>0.0</td>
<td>0.2 (0.0–1.1)</td>
<td>0.0</td>
</tr>
<tr>
<td>Nursery</td>
<td><em>S. falcatus</em></td>
<td>0.5 (0.0–2.8)</td>
<td>0.0</td>
<td>0.2 (0.0–1.1)</td>
<td>0.0</td>
</tr>
<tr>
<td>Nursery</td>
<td>C. formosanus</td>
<td>16.3 (11.7–22.1)</td>
<td>0.1 (0.0–0.1)</td>
<td>8.5 (6.3–11.2)</td>
<td>0.1 (0.0–0.3)</td>
</tr>
<tr>
<td>Nursery</td>
<td>E. japonicus</td>
<td>0.5 (0.0–2.8)</td>
<td>0.0</td>
<td>0.0 (0.0–0.7)</td>
<td>0.0</td>
</tr>
<tr>
<td>Grow-out</td>
<td>H. pumilio</td>
<td>45.2 (38.4–52.2)</td>
<td>2.2 (0.7–3.7)</td>
<td>41.3 (36.9–45.5)</td>
<td>0.7 (0.4–1.0)</td>
</tr>
<tr>
<td>Grow-out</td>
<td>H. taichui</td>
<td>1.9 (0.7–4.9)</td>
<td>0.0</td>
<td>1.0 (0.4–2.3)</td>
<td>0.0</td>
</tr>
<tr>
<td>Grow-out</td>
<td>H. yokogawai</td>
<td>0.0 (0.0–1.8)</td>
<td>0.0</td>
<td>0.2 (0.0–1.1)</td>
<td>0.0</td>
</tr>
<tr>
<td>Grow-out</td>
<td><em>S. falcatus</em></td>
<td>0.5 (0.0–2.8)</td>
<td>0.0</td>
<td>0.2 (0.0–1.1)</td>
<td>0.0</td>
</tr>
<tr>
<td>Grow-out</td>
<td>C. formosanus</td>
<td>16.3 (11.7–22.1)</td>
<td>0.1 (0.0–0.1)</td>
<td>8.5 (6.3–11.2)</td>
<td>0.1 (0.0–0.3)</td>
</tr>
<tr>
<td>Grow-out</td>
<td>E. japonicus</td>
<td>0.5 (0.0–2.8)</td>
<td>0.0</td>
<td>0.0 (0.0–0.7)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Results to 1 decimal place.
FZT prevalence and density data for all SRS are presented in Table V. The prevalence and density of FZT metacercariae in SRS fish were similar to that for the cultured fish species from the same ponds. Statistical comparisons of prevalence between species of SRS fish were not undertaken because of the small sample sizes for most species.

**TABLE V. Prevalence of FZTs from different species of SRS fish.**

<table>
<thead>
<tr>
<th>Fish species</th>
<th>No. infected/ no. examined</th>
<th>FZT species recovered*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nursery pond (total)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crucian carp, <em>Carassius auratus</em></td>
<td>17/20</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Snakehead, <em>Ophioccephalus maculatus</em></td>
<td>3/3</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Sailfish, <em>Istiophorus spp.</em></td>
<td>1/2</td>
<td>Hp</td>
</tr>
<tr>
<td>Featherback, <em>Notopterus notopterus</em></td>
<td>2/2</td>
<td>Hp</td>
</tr>
<tr>
<td>Hemiculter, <em>Hemiculter lencisculus</em></td>
<td>5/5</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Looch, <em>Mastacembelus armatus</em></td>
<td>4/5</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Unidentified species</td>
<td>1/2</td>
<td>Hp</td>
</tr>
<tr>
<td>Grow-out pond</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crucian carp, <em>Carassius auratus</em></td>
<td>47/122</td>
<td>Ht, Hp, Cf</td>
</tr>
<tr>
<td>Snakehead, <em>Ophioccephalus maculatus</em></td>
<td>9/12</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Sailfish, <em>Istiophorus spp.</em></td>
<td>2/5</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Featherback, <em>Notopterus notopterus</em></td>
<td>14/47</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Hemiculter, <em>Hemiculter lencisculus</em></td>
<td>10/27</td>
<td>Ht, Hp, Cf</td>
</tr>
<tr>
<td>Anabas, <em>Anabas testudineus</em></td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>Oomegaika, <em>Leucaspis delineatus</em></td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>Mai, <em>Rashorinus lineatus</em></td>
<td>3/9</td>
<td>Hp, Cf</td>
</tr>
<tr>
<td>Macropodus, <em>Macropodus opercularis</em></td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>Red-eyed carp, <em>Squaliobarbus curriculus</em></td>
<td>1/1</td>
<td>Hp</td>
</tr>
<tr>
<td>Unidentified species</td>
<td>7/17</td>
<td>Hp, Cf</td>
</tr>
</tbody>
</table>

* Hp = *Haplorchis pumilo*; Ht = *Haplorchis taichui*; Cf = *Centrocestus formosanus.*

DISCUSSION

The results from this investigation reveal a high prevalence of intestinal fish-borne zoonotic trematodes (FZTs) in farm-raised fish from a community of fish farmers with a very low human prevalence (0.6%) (Olsen et al., 2006). For the first time, *Haplorchis yokogawai* and *Echinochasmus japonicus* metacercariae were recovered from fish in Vietnam, although *H. pumilio*, *H. taichui*, *C. formosanus*, and *S. falcatus* were only recently reported from Vietnamese fish (Arthur and Te, 2006; Hop et al., 2007; Thu et al., 2007). While the fish-borne liver fluke *Clonorchis sinensis* has been reported from 9 northern provinces of Vietnam (De et al., 2003), the recent discoveries of potential zoonotic intestinal trematode metacercariae in fish may reflect an emerging public health problem for Vietnam (Thu et al., 2007). More than 50 species of fish-borne intestinal flukes belonging to the Heterophyidae and Echinostomatidae are widespread and emergent in Southeast Asia (De et al., 2003), Laos (Chai, Murrell, and Lymbery, 2005), Thailand (Waikagul and Radomyos, 2005), Cambodia (Stauffer et al., 2004), China (Yu and Xu, 2005), and Korea (Chai, 2005). The fish-borne intestinal flukes are much less well characterized clinically than the liver flukes, but are reported to cause significant pathology in the heart, brain, and spinal cord of humans (Africa et al., 1940; WHO, 1995; Chai, Murrell, and Lymbery, 2005). With expanding freshwater and marine/brackish water fisheries in Asia, the economic impact on commercial aquaculture due to FZT food quality and safety issues will become more burdensome (WHO, 2004; Chai, Murrell, and Lymbery, 2005; Murrell and Crompton, 2006; Duarte et al., 2007).

The relatively high prevalence of intestinal flukes in fish from Nghe An Province in the present study and the low prevalence in their human residents suggests reservoir hosts, such as dogs, cats, and pigs, may be important in sustaining the life cycles of these flukes in fish farms. This possibility deserves further investigation because the role of reservoir hosts is not regarded as important in many proposed control strategies (reviewed in Chai, Murrell, and Lymbery, 2005). If animal reservoir hosts are capable of maintaining the transmission of FZTs in the absence of environmental contamination with fluke eggs from infected humans, the long-term impact of control efforts focused on drug treatment of people in rural communities alone is unlikely to remove the threat from FZTs. Therefore, there is a need to improve the management of domestic animals on fish farms to help achieve FZT control.

In the present study, *H. pumilio* and *C. formosanus* were the most common FZTs in fish from Nghe An Province. Although common in other Southeast and East Asian countries, the reasons for the high prevalence of *H. pumilio* in this area, and in the Mekong Delta (Thu et al., 2007; Thiem et al., 2007), may include better adaptation to cultured species of fish, or the availability of preferred definitive hosts (such as cats, dogs, pigs, and fish-eating birds) associated with aquaculture activities. There may also be differences in ecological conditions required by suitable vector snail populations in fish ponds; however, important ecological and epidemiological aspects of these parasites and their hosts are too poorly characterized to draw any conclusions.

The factors responsible for the observed differences in prevalence among different fish species are also difficult to explain, and we can only speculate that certain specific behaviors and innate differences may be important determinants. Grass carp, for example, which had a high prevalence of FZT infection in both nursery and grow-out culture systems, also had a highly diverse parasite species fauna (5 FZT species), including *H. pumilio, H. taichui, H. yokogawai, C. formosanus,* and *S. falcatus.* In this instance, the habitat preferences of grass carp may render it more exposed to infection because it is normally associated with the littoral zone of the pond where vegetation favored by snails is most abundant and possibly exposure to cercariae. In contrast, species with low prevalence such as rohu and tilapia frequent deeper zones of the water column, perhaps reflecting a less suitable habitat for the snail vector. Other host-specific factors also merit consideration, especially innate and acquired host characteristics, which are known to have an influence on susceptibility to cercariae infection. These include skin thickness, scale structure, protective properties of superficial mucus, and the fish’s immune status (Chun, 1964; Rhee et al., 1988; Lan et al., 2005). For example, fish epidermal mucus cells have been shown to produce attractants that influence host-finding and host-specificity in monogenean trematodes (Kearn, 1967).

The comparable prevalence between nursery and grow-out ponds was unexpected and raises an important issue for the aquaculture industry. Obviously the wide distribution of infected fingerlings negatively affects whatever efforts are made by grow-out pond operators to control FZT infection. Several fac-
tors could have contributed toward the prevalence observed in nursery and grow-out ponds. These include susceptibility of the fish, density, or, more likely, surface area of fish in the pond and number of infective organisms. Each one of these variables could be subdivided; for example, susceptibility could be physiological or behavioral, and the number of infective organisms could vary in both time and space. We do not have the data to explain the higher prevalence in nursery ponds, but this is an area for further study.

The high prevalence of SRS fish in these farm ponds suggests that improved pond management could have an impact on the occurrence of FZTs. The presence of SRS fish indicates that the ponds are not well prepared before fish restocking. Management practices such as removing noncultured fish species and snails from ponds before restocking and restricting access of fish and snails during the production cycle could have a significant effect on reducing FZT infections.

In conclusion, the present study has demonstrated the occurrence of FZTs in Vietnamese cultured fish and the potential risk posed to human health. At present they do not appear to represent a significant public health problem in these particular Nghe An districts, presumably because of local eating habits that do not favor consumption of raw fish. Elsewhere in Vietnam, however, the habit of consuming improperly cooked fish is widespread and may be increasing (De et al., 2003; Chai, Murrell, and Lynbbery, 2005); therefore, fish moved to other parts of Vietnam for consumption may pose a greater threat, emphasizing the need to control these parasites in food for human use. A pilot project employing a HACCP (Hazardous Critical Control Points) approach was carried out in Vietnam in 1996–1997 to control liver flukes in Vietnamese aquaculture systems (Lima dos Santos, 2002). This project had initial success, but there has not been any follow-up to assess its long-term effects. The results of the study reported here strongly suggest that any efforts to control transmission will require controlling infections in reservoir hosts and the thorough control of snails in ponds.

ACKNOWLEDGMENTS

We would like to thank the “Fishborne Zoonotic Parasites in Vietnam” (FIBOZOPA) project of the Danish International Development Assistance (DANIDA) for their financial support of this study. The fish farmers, staff of “Support to Freshwater Aquaculture” (SUFA) in Nghe An Province, and the Research Institute for Aquaculture no. 1 station in Cua Lo, especially Nhu Van Can and Nguyen Thi Hien, are thanked for their participation and information provided during field work. We also would like to thank the staffs of the FIBOZOPA project, particularly Jesper Clausen, Jacob Fjalland, Nguyen Thi Hung, and Bui Ngoc Thanh for their assistance provided in the collection and identification of samples. We are grateful to Phan Thi Van and staff of the Centre for Environment and Disease Monitoring in Aquaculture (CEDMA), Research Institute for Aquaculture no. 1 (RIA1), Vietnam, for their constant support and advice throughout the work.

LITERATURE CITED


Thu, N. D., L. T. T. Loan, A. Dalsgaard, and K. D. Murrell. 2007. Survey for zoonotic liver and intestinal trematode metacercariae in...


The composition of the parasite fauna in marine fishes is influenced by host and parasite phylogeny, host ecological factors such as diet and habitat (Poulin, 1995), and by oceanographic characteristics, such as temperature, depth, and specific mass of water (Rohde et al., 1995; Oliva et al., 2004), which, joined or individually, can influence the spatial distributional patterns of the parasites. In the last years, studies using parasites as biological indicators of the biology and movement of fish populations have increased significantly (Mackenzie, 2002), and currently it has been recognized that parasites are a good model to study the biogeography of both the parasites and the hosts (Poulin, 2003; Oliva and González, 2005; González et al., 2006). However, there is a scarcity of studies using parasites of the same fish host as indicators of zoogeographical patterns (Blaylock et al., 1998; Rohde, 2002; González and Moreno, 2005; González et al., 2006). Additionally, fish hosts distributed along extensive geographical areas or different biogeographical areas offer a unique opportunity to examine the distributional patterns of their parasites in order to test the impact of different factors that might be determining the host–parasite relationships (e.g., Oliva and González, 2005).

The bigeye flounder, *H. macrops*, is distributed in an extensive latitudinal range along the southeastern Pacific (Ojeda et al., 2000). This species inhabits in a bathymetric range from shallow waters, in the littoral coast, up to 600 m of depth (Yañez and Barbieri, 1974). It is 1 of the most common pleuronectiform species in the demersal habitat along the northern and central Chilean coast (Villarroel and Acuña, 1999).

In the present study, we examine the parasite fauna of the bigeye flounder, *H. macrops*, from different localities in the southeastern Pacific coast (24°S–33°S) to determine whether their parasites show latitudinal and/or biogeographical patterns, and to evaluate if the observed (if any) distributions are associated with the known zoogeographical patterns of free-living organisms. The bigeye flounder, *H. macrops*, feeds mainly on benthic crustaceans, and it does not show significant latitudinal movements, although bathymetrical migration in older fish has been suggested (Villarroel et al., 2001). Therefore, it is expected that the parasite communities of this fish host changes geographically as a consequence of different physical and biological conditions that could affect the ectoparasites’ dispersal ability and by the zoogeographical breaks that could affect the distribution of intermediate hosts (invertebrate and vertebrate prey), subsequently affecting the transmission of endoparasites.

**MATERIALS AND METHODS**

From March to September 2001, 331 fish were captured by fishing vessels. The sampled localities (Fig. 1), number of fish analyzed, depth of capture, and fish ages are shown in Table I. The fish caught were bagged and frozen at –18 °C until dissection. The total length of each fish was measured (to the nearest centimeter) and the sex was determined by macroscopic observation of the gonads. The parasites were collected with the use of traditional techniques as described in González et al. (2001). Parasitological descriptors (abundance and prevalence) were used according to Bush et al. (1997).

Following Oliva et al. (2004), the age of each fish host was estimated with the use of the von Bertalanffy growth function parameter (males: $L_m = 37.29$ cm, $L_0 = -0.373$, $k = 0.325$; females: $L_m = 41.57$ cm, $L_0 = -0.4157$, $k = 0.281$). The fish age and depths of capture among localities were compared with the use of ANOVAs. Because parasitological data (species richness and abundances) showed a nonnormal distribution, nonparametric tests were also performed (Zar, 1999). The Kruskal–Wallis test was used to compare total parasite abundances and parasite species richness by fish host (separately for ecto- and endoparasites) among localities. Spearman correlations were used to evaluate the association between (1) fish age and depth, (2) fish age and species richness, (3) fish age and total parasite abundances, (4) depth of capture and species richness, and (5) depth of capture and total parasite abundances.

Analyses of the composition of the parasite fauna were carried out at both component and infracommunity levels (Bush et al., 1997). Parasite richness by host and parasite richness by locality were calculated. At the component community level, distributional patterns were evaluated initially with the use of cluster analyses, which were performed with the unweighted pair-group algorithm for the Bray–Curtis similarity index for prevalence data and abundance data following log($a + 1$) transformation. For each cluster analysis, bootstrapping techniques were used to determine the level of taxonomic similarity among localities at which biogeographical regions could be recognized. For this, we re-shuffled each original data set 100 times, obtaining the corresponding pseudovalues of similarity that were then pooled to construct a frequency distribution histogram. We compared our observed similarity values with the generated frequency distribution, and considered those values that exceeded the 95th percentile as corresponding to greater
FIGURE 1. Map of the southeastern Pacific coast showing sampling localities. Codes for localities as in Table I.

similarity in taxonomic composition than expected by chance alone (Manly, 1997). These analyses were performed considering all recovered species, but including only those species with prevalence >10% in at least 1 locality. At the infracommunity level, patterns of similarity in ectoparasite fauna composition between localities were investigated with multivariate discriminant analyses with the use of log(n + 1)-transformed abundance data for each parasite species. At the component community level, a correspondence analysis was used to evaluate the association between all the parasite species and the sampled localities (Digby and Kempton, 1987). The analyses were performed with the software Statistica 6.0.

TABLE I. Localities sampled, sample size (N), mean depth of capture (meters), and mean fish age (years) for each locality. SD = standard deviation.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Latitudinal range</th>
<th>N</th>
<th>Depth (SD)</th>
<th>Age (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taltal (TAL)</td>
<td>24°39'-25°58'S</td>
<td>34</td>
<td>367 (105.5)</td>
<td>2.7 (0.89)</td>
</tr>
<tr>
<td>Caldera (CAL)</td>
<td>26°29'-26°40'S</td>
<td>19</td>
<td>317 (47.7)</td>
<td>3.1 (0.86)</td>
</tr>
<tr>
<td>Huasco (HUA)</td>
<td>28°20'-28°59'S</td>
<td>76</td>
<td>341 (58.5)</td>
<td>3.0 (0.56)</td>
</tr>
<tr>
<td>Coquimbo (COQ)</td>
<td>29°26'-30°12'S</td>
<td>60</td>
<td>323 (86.7)</td>
<td>3.1 (0.79)</td>
</tr>
<tr>
<td>El Teniente (TTE)</td>
<td>30°39' S</td>
<td>40</td>
<td>196 (0.0)</td>
<td>2.9 (1.20)</td>
</tr>
<tr>
<td>Valparaíso (VAL)</td>
<td>32°20'-32°44'S</td>
<td>50</td>
<td>280 (84.7)</td>
<td>2.8 (0.94)</td>
</tr>
</tbody>
</table>

RESULTS

The ages of the 331 fish varied between 1 and 6 yr (X = 2.8; SD = 1.04). The fish age, depth of capture, and number of analyzed specimens by locality are shown in Table I. There were significant differences in the fish age (F(5,325) = 9.69; P < 0.001) and depth of capture (F(5,325) = 55.237; P < 0.001) among the sampled localities. To avoid a potential effect of fish age on parasite burden, only fish with similar ages (1.6-5.5 yr old; n = 279) among localities were compared (F(5,273) = 1.438; P = 0.211). In the new data set, differences in the depth of capture remain (F(5,273) = 33.411; P < 0.001), with samples from Taltal (25°S) being the deepest, and samples from Teniente (31°S) being the shallowest. In the whole sample, there was no significant association between fish age and depths of capture (r_s = -0.06; P = 0.27; n = 279). The ectoparasite (r_s = 0.021; P = 0.718; n = 270) and endoparasite (r_s = 0.026; P = 0.43; n = 279) species richness were also not correlated with depths of capture. Similarly, the ectoparasite (r_s = 0.027; P = 0.65; n = 279) and endoparasite (r_s = -0.009; P = 0.87; n = 279) total abundances were not correlated with depths. Although endoparasite richness (r_s = -0.07; P = 0.89; n = 279) and endoparasite abundances (r_s = -0.114; P = 0.06; n = 279) were not correlated with fish age, ectoparasite richness (r_s = 0.33; P
TABLE II. Abundance and prevalence (in parentheses) of infection for each parasite species recorded in *H. macrops* from the sampled localities. Codes for localities are given in Table I.

<table>
<thead>
<tr>
<th>Species</th>
<th>TAL</th>
<th>CAL</th>
<th>HUA</th>
<th>COQ</th>
<th>TTE</th>
<th>VAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectoparasites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neoheterobothrium chilensis</td>
<td>0.29 (14.7)</td>
<td>2.53 (47.4)</td>
<td>2.70 (69.7)</td>
<td>3.98 (66.7)</td>
<td>1.30 (50.0)</td>
<td>0.44 (22.0)</td>
</tr>
<tr>
<td>Protochondria longicauda</td>
<td>1.03 (41.2)</td>
<td>1.37 (47.4)</td>
<td>1.12 (38.2)</td>
<td>1.22 (51.7)</td>
<td>0.38 (22.5)</td>
<td>0.06 (6.0)</td>
</tr>
<tr>
<td>Holobolomochus chilensis</td>
<td>0.26 (17.6)</td>
<td>0.58 (26.3)</td>
<td>0.34 (19.7)</td>
<td>1.02 (38.3)</td>
<td>0.50 (30.0)</td>
<td>1.32 (52.0)</td>
</tr>
<tr>
<td>Glyptophobdella sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gnathia sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoparasites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floridosentis sp.</td>
<td>6.15 (73.5)</td>
<td>1.42 (52.6)</td>
<td>4.76 (47.4)</td>
<td>0.75 (26.7)</td>
<td>0.18 (15.0)</td>
<td>0.18 (16.0)</td>
</tr>
<tr>
<td>Corynosoma australis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arhythmomorhynchus sp.</td>
<td>0.01 (1.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bolbosoma sp.</td>
<td>0.01 (1.3)</td>
<td></td>
<td>0.01 (1.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nybelinia surmenicola</td>
<td>1.23 (55.9)</td>
<td>0.73 (21.1)</td>
<td>0.14 (6.6)</td>
<td>0.27 (16.7)</td>
<td>0.20 (7.5)</td>
<td>0.14 (10.0)</td>
</tr>
<tr>
<td>Scolex pleurocercis</td>
<td>0.11 (11.8)</td>
<td>0.11 (10.5)</td>
<td>0.02 (2.6)</td>
<td>0.02 (1.7)</td>
<td>0.02 (2.5)</td>
<td>0.06 (4.0)</td>
</tr>
<tr>
<td>Neobothriocephalus aspinosus</td>
<td>0.18 (17.6)</td>
<td>0.73 (15.8)</td>
<td>0.17 (14.5)</td>
<td>0.13 (5.0)</td>
<td>0.28 (15.0)</td>
<td>0.06 (6.0)</td>
</tr>
<tr>
<td>Anisakis sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Philometra sp.</td>
<td>0.05 (5.3)</td>
<td></td>
<td>0.01 (1.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemiuriidae</td>
<td>0.01 (1.3)</td>
<td></td>
<td>0.02 (1.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecithochirium sp. 1</td>
<td>0.04 (3.9)</td>
<td></td>
<td>0.10 (1.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecithochirium sp. 2</td>
<td>0.01 (1.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecithophyllium sp.</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

< 0.001; n = 279) and total ectoparasite abundances (*r* = 0.40; *P* < 0.001; n = 279) were positively correlated with fish age.

In total, 18 parasite species were recovered from the analyzed fish, i.e., 5 ectoparasites (2 Copepoda, 1 Monogenea, 1 Hirudinea, and 1 Isopoda) and 13 endoparasites (4 Digenea, 4 Acanthocephala, 3 Cestoda, and 2 Nematoda). A large number of fish (94.2%) harbored at least 1 parasite species (1–7 parasites species by host). The abundance, prevalence, and parasite species richness by locality are shown in Table II. The most prevalent (>30%) and abundant (>0.7) species were *N. chilensis* (Monogenea), *Protochondria longicauda*, and *Holobolomochus chilensis* (Copepoda), and *Floridosentis* sp. and *Corynosoma australis* (Acanthocephala). The ectoparasite richness (*H*(5,299) = 31.92; *P* < 0.001) and total ectoparasite abundances (*H*(5,299) = 37.122; *P* < 0.001) by fish were significantly different among localities; i.e., fish from Coquimbo (30°S) harbored more ectoparasite species and individuals, and fish from the most northern locality (Taltal, 25°S) harbored fewer species and individual parasites. Endoparasite richness (*H*(5,299) = 9.762; *P* = 0.09) and total endoparasite abundances (*H*(5,257) = 5.445; *P* = 0.360) did not show significant differences among localities.

Most ectoparasite species were present in all sampled geographic areas, but their prevalence and abundance varied among localities (Table II). Among the endoparasites recovered, only 6 species showed extended distribution along the studied geographical area. The abundance and prevalence of *Floridosentis* sp., *C. australis*, and *Nybelinia surmenicola* varied significantly among localities (Table II). The remaining species were present in only some localities, but with low abundance and prevalence.

The cluster analyses for ectoparasite and endoparasite communities of bigeye flounder showed different distributional patterns. The cluster analysis of the ectoparasites, based on prevalence data, showed the following grouping of localities: a first group including Huasco (28°S) and Coquimbo (30°S), a second group was formed by Caldera (26°S) and Teniente (31°S), and the extreme localities, Taltal (25°S) and Valparaíso (32°S) appeared as separate localities (Fig. 2). Based on prevalence data, endoparasites communities showed 2 major divisions: the first includes the most northern localities, Taltal (25°S) and Caldera (26°S), and the second division consisted of 2 groupings, 1 formed by intermediate localities, Huasco (28°S) and Coquimbo (30°S), and the second group by the most southern localities, Teniente (31°S) and Valparaíso (32°S) (Fig. 3).

Clustering based on ectoparasite (Fig. 4) and endoparasite (Fig. 5) abundance data indicated patterns roughly similar to those obtained from prevalence data. The contiguous localities showed greater similarities. For ectoparasites, the most distant localities were arranged separately. For endoparasites, 2 major divisions are recognized. The most northern localities (25°S–28°S) form a group, and the southern localities form a second group (30°–32°S). A similar result was obtained considering all parasites and excluding those with prevalence of less than 10%.

Discriminant analysis with infracomunities of *H. macrops* was roughly concordant with component community patterns. This analysis showed significant differences in the parasite abundances of fish from different latitudes (*F*(60,1230) = 8.315; Wilks’s lambda: 0.203; *P* < 0.001). The discriminant variables were the abundances of *N. chilensis, Floridosentis* sp., *C. australis*, and *N. surmenicola*. Similarly, the correspondence analysis (considering ecto- and endoparasites together) showed significant differences in the parasite prevalence among latitudes. Total inertia reached 83.6% (62.2% and 21.4% for the first and second dimensions, respectively). *Scolex pleurocercis, N. surmenicola*, and *C. australis* were most prevalent in the northern latitudes (25°S–26°S), whereas *Floridosentis* sp. and *Anisakis* sp. were associated with the central and southern latitudes (30°S–32°S) (Fig. 6).
Several studies have used parasites as biological tags for tracing host migrations and for distinguishing host populations (e.g., George-Nascimento, 1996; Oliva and Ballón, 2002; Timi, 2003; Oliva and González, 2004), but very few studies have used parasites of the same host species, whether as indicators of marine biogeographical patterns (Blaylock et al., 1998; Rohde, 2002; González and Moreno, 2005; González et al., 2006) or to evaluate changes in the similarity of their parasites along the geographical distance (Poulin, 2003; Oliva and González, 2005). Along the Chilean coast, 2 faunistic provinces are recognized, i.e., a northern warm temperate region (Peruvian faunistic province) extending from Peru to the northern Chilean coast up to ca. 30°S, and a cold temperate region (Magellanic faunistic province) extending southward of 42°S along the southern Chilean coast (Briggs, 1974). Between both areas is a transitional zone in which species are of both northern and southern origin (Brattström and Johanssen, 1983; Lancellotti and Vásquez, 1999). In this transitional area, between 50 and 400 m of depth, is located the core of a water mass identified as the Eastern South Pacific Intermediate Waters, which shows a unique salinity and temperature (Schneider et al., 2003). Along the northern and central Chilean coast, the parasite composition of H. macrops show latitudinal patterns, but those are different for ecto- and endoparasites. The most ectoparasite species (Table II) were present along the whole latitudinal range analyzed (24°S–33°S). However, among recorded endoparasites, some predominant species were present along the latitudinal range analyzed, whereas other species (mainly digeneans) were present only in some latitudes, where their prevalence and abundance were low (see Table II). Cluster analyses performed with all recorded parasites, and cluster analyses considering...
only species with prevalence higher than 10%, in at least 1 locality, showed similar results. Therefore, the observed distributional patterns for the parasites of *H. macrops* result mainly from quantitative differences among latitudes.

Along the southeastern Pacific coast, there are only 2 previous studies that have analyzed the distributional patterns for parasites of the same fish host (González and Moreno, 2005; González et al., 2006). Despite the latitudinal range analyzed, the parasite assemblages of *H. macrops* are not as extended as those for rockfish, *Sebastes capensis* (20°S–52°S). The latitudinal patterns for ectoparasites of *H. macrops* were, however, concordant with the patterns recorded for ectoparasites of *S. capensis* (González and Moreno, 2005). In both host species, the ectoparasites do not follow a distributional pattern in agreement with the biogeographical areas established on the basis of invertebrates and/or fishes (Lancellotti and Vásquez, 1999; Ojeda et al., 2000; Peñero, 2000).

The higher species richness and prevalence of ectoparasites in *H. macrops* from central latitudes (Table II, Fig. 4) might be explained because in those localities the fish were captured from approximately 300-m depth (Table I), where fish densities are higher. However, in TAL (25°S) and VAL (32°S) the fish were caught from deeper and shallow waters (Table I), respectively, where fish densities are lower (Villarroel et al., 2001). On the contrary, the endoparasites of *H. macrops* show a distributional pattern (see Figs. 3, 5), which is roughly associated with the biogeographic areas known for free-living organisms. This pattern was also described for the endoparasite fauna of *S. capensis* (González et al., 2006).

In the case of *H. macrops*, the division of the northern and southern latitudes is explained by the higher prevalence of *C. australis* and *N. surmenicola* in the most northern localities, and for the absence and low prevalence of the acanthocephalan *Floridosentis* sp. in Taltal (25°S) and Caldera (26°S), respectively.

The parasite species are found only in those areas where conditions are suitable for their transmission. For parasites with direct life cycles, the distribution is determined mainly by en-
environmetal conditions, whereas for endoparasites with indirect life cycles, an additional requirement is that suitable hosts for all developmental stages must be present (MacKenzie and Abaunza, 1998). The low occurrence and/or complete absence of Floridosentis sp. in the most northern latitudes can be explained by the absence of intermediate hosts in that area. Although the life cycle of this acanthocephalan remains unknown, González et al. (2001) suggested that the crustaceans Cer vis munida johni and Pleuroncodes monodon might be intermediate hosts for this parasite species. The abundances of these crustaceans are low in northern latitudes of the Chilean coast, and their bathymetric range varies between 50 and 300 m of depth (Villarroel et al., 2004). In the present study, samples from Tal tal (25°S) were captured in deeper waters, where these crustacea ns are scarce. In the case of the larval C. austral e, this species is present along the whole geographical range analyzed, but their abundance and prevalence decrease with latitude. Similar latitudinal patterns for this larval species have been recorded by George-Nascimento (1996) in the jack mackerel Trachurus symmetricus and by González et al. (2006) in the rockfish S. capensis along the southeastern Pacific. In addition, Timi (2003) recorded a similar pattern in the Argentinean anchovy Engraulis anchoita along the southwest Atlantic coast.

At approximately 30°S, an important upwelling system is present; it has been attributed by different authors as responsible for distributional breaks of free-living organisms (see references in Camus, 2001). The parasite load and species richness of H. macrops captured from intermediate latitudes (28°S–30°S) was higher than in northern latitudes (24°S–26°S). We suggest that larval ectoparasites can be affected by hydrographic discontinuities associated mainly with the minimum oxygen zone, and the presence of more saline waters in this latitude (Acuña et al., 1989; Schneider et al., 2003). Likewise, endoparasite transmission can be affected by distributional breaks of intermediate host (prey) around latitude 30°S (González et al., 2006).

Temperature and depth can be important factors in determining parasite distributions (Campbell, 1980; Oliva et al., 2004). In the present study, the depth of captured fish was not a significant factor associated with the parasite abundance within each locality. However, the prevalence of N. chilensis and Neobothri ocephalus aspinosus showed significant differences with depth in some latitudes. For this monogenean, the higher prevalence in fish living from 250 to 350 m can be explained because at these depths there is a higher density of H. macrops (Villarroel et al., 2001). Thus, host density, added to the gregarious habits of this host, might produce a higher prevalence and abundance for parasites like monogeneans, which are transmitted directly (Oliva et al., 2004). On the other hand, the decrease of the cestode N. aspinosus with increased depth (>350 m) may be explained by the decreased availability of interme-
diate hosts. Along the coasts of the southeastern Chilean Pacific, *N. aspinus* has been recorded parasitizing the 100% of the pelagic fish species *Seriolletia violacea* (Iannacone, 2003), which suggest that the intermediate host of this parasite is a prey of pelagic habits.

The geographic distribution of marine parasites has not been well studied and is largely, although not entirely, limited to some aspects of the zoogeography of parasites of marine fishes (Rohde, 2002). González and Moreno (2005) and González et al. (2006) demonstrated that in a latitudinal gradient along the coast of southeastern Pacific, the spatial distributions shown by ectoparasites and endoparasites of the same littoral fish host species does not follow a common pattern, and the observed dissimilarities are related to different processes that explain the presence of parasites and their differential prevalence and abundance (González and Poulin, 2005). Specifically, patterns for endoparasites are related to the availability of intermediate host prey. A similar picture is evident for bigeye flounder of deeper waters. Consequently, the endoparasite assemblages (but not ectoparasites) of *H. macrops* are useful as indicators of the marine biogeographical areas present in the southeastern Pacific coast, and they support the recorded distributional breaks of free-living organisms around 30°S. More studies following a similar line must be done to define whether the described patterns can be generalized to other fish–parasite systems from this geographical region.

**ACKNOWLEDGMENTS**

We thank Dr. Marcelo Oliva (U. de Antofagasta) for comments on an earlier draft of the manuscript. The fish samples were captured during the development of Fishery Investigation Projects 2006–04 and 2006–11. This study was funded by project FONDECYT Postdoctoral 3060054 granted to M.T.G.

**LITERATURE CITED**


DETERMINANTS OF HOST SPECIFICITY AND COMMENTS ON ATTACHMENT SITE
SPECIFICITY OF TETRAPHYLLIDEAN CESTODES INFECTING RAJID SKATES FROM THE NORTHWEST ATLANTIC

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ABSTRACT: The objectives of this study were to (1) describe the host range for 6 tetraphyllidean species and quantify their host specificity using 5 specificity indices; (2) determine the role of morphological determinants in the host specificity of tetraphyllideans by comparing villar and bothridial measurements of species examined herein; and (3) determine the role of a physiological component in the host specificity of tetraphyllideans by exposing tetraphyllideans to blood sera from different fish species and other solutions. Our results indicate that Echeneibothrium dubium abyssorum (ex Amblyraja radiata), Echeneibothrium canadensis (ex A. radiata), and Zyxibothrium kamianae (ex Malacoraja senta) exhibit the highest degree of specificity, followed by Echeneibothrium vernetiae (ex Leucoraja erinacea and Leucoraja ocellata), Pseudanthobothrium hanseni (ex A. radiata and M. senta), and Pseudanthobothrium purtoni (ex Leucoraja erinacea and L. ocellata). However, these results vary based on the specificity index used. Compatible bothridial and villar measurements indicate that there is no morphological determinant of host specificity but that there is a morphological determinant to attachment site specificity. Our data indicate that attachment site specificity may also be phylogenetically determined. Additionally, the exposure of parasites to blood sera from various hosts confirms that host specificity in this system has a physiological determinant. Therefore, host specificity in this system is determined, at least in part, by physiological factors, whereas attachment site specificity is an extension of host specificity and is phylogenetically determined.

Echeneibothriine cestodes were once thought to exhibit oioxenous specificity (or strict host specificity) (Williams, 1966; as defined by Euzet and Combes, 1980). Randhawa et al. (2007) assessed the host specificity of Echeneibothrium vernetiae Euzet, 1956, Pseudanthobothrium hanseni Baer, 1956, and Pseudanthobothrium purtoni (Randhawa et al., 2008) using anatomical observations and partial sequence data of a variable region (D2) from the large subunit ribosomal DNA (LSU). We established that adult specimens of all 3 species infect ecological pairs (McEachran et al., 1976) of rajid host species. These findings question the strictness of the host-parasite relationship for Pseudanthobothrium spp. and Echeneibothrium spp. (Williams, 1966), suggesting that these genera exhibit a lesser degree of host specificity, i.e., stenoxenous specificity (parasites that infect a few, closely related host species) as defined by Euzet and Combes (1980).

“The literature of parasitology contains no general, heuristic, testable, and nonteleological explanation for why some parasites are discriminatory and others are not” (Janovy et al., 1992). Although it is generally accepted that there are several determinants of host specificity or host range of parasites, there is a lack of experimental work to support the generalization of these determinants. The concept of filters was introduced by Euzet and Combes (1980) to illustrate the determinants of host range, defined as the number of different host species infected by a parasite species at a specific stage of its life cycle. The concept of host range has also been discussed by Holmes (1987), Lymbery (1989), Combes (1995, 2001), and Poulin (2007). The 4 parameters restricting host range (Euzet and Combes, 1980; Combes, 1995, 2001; Poulin, 2007) are: (1) biodiversity (geographical component); (2) behavior (spatial or ethological component); (3) resource (morphological, metabolic, or ecological component); and (4) defense (physiological component). The biodiversity and behavior parameters determine the probability of encounter between potential host and parasite (encounter filter), whereas the resource and defense parameters determine the probability of establishment of the parasite in the potential host (compatibility filter). Additionally, the relatedness between host species is a phylogenetic determinant used to quantify host specificity and provide insights into the evolutionary history of parasites (Poulin and Mouillot, 2003), whereas the relative prevalence, average intensity, and abundance of infection of a parasite (as defined by Margolis et al., 1982; Bush et al., 1997) are parameters used to quantify the relative importance of different host species as a resource for the parasite (Rohde, 1980; Lymbery, 1989; Poulin, 2007; Rohde and Rohde, 2005). Rohde (1980) developed indices of host specificity based on prevalence (S, [frequency]) and average intensity of infection (S, [density]), where prevalence is defined as the proportion of hosts examined that is infected with one or more individuals of a given parasite species (Margolis et al., 1982; Bush et al., 1997), and average intensity of infection is defined as the relative density of a given parasite species in a host population (Rohde, 1980). Rohde’s (1980) definition of average intensity of infection is in fact that of abundance (Margolis et al., 1982; Bush et al., 1997). Poulin (2007) suggested that abundance is a better indicator of an individual parasite’s success at exploiting different host species and would be a more appropriate parameter for quantifying specificity of parasites. Cairns et al. (2003) and Poulin and Mouillot (2003) developed measures of host specificity that quantify the phylogenetic relatedness of the host species used by a particular parasite species based on the standard five taxonomic levels (species, genus, family, order, class) (HS and STD, respectively). However, until Poulin and Mouillot’s (2005) STD* measure, no host specificity index took into account phylogenetic relatedness and ecological parameters.

Although we can assess and measure specificity, there is little yet known about the deterministic features of this fundamental property of parasites. Adaptations of the scolex to the host mucosa have been correlated to host specificity in tetraphyllideans (Williams, 1960, 1966, 1968a, 1968b). Tetraphyllideans belonging to Phyllobothriidae Braun, 1900 include species of
Echeneibothrium, Pseudanthobothrium, and Zyxibothrium (Euzet, 1994) and are characterized by 4 unarmed bothridia (Southwell, 1925; Wardle and McLeod, 1952; Schmidt, 1986; Euzet, 1994). The Echeneibothriinae de Beauchamp, 1905 is characterized by an apical glandomuscular myzorhynchus (Euzet, 1994) and includes species in Echeneibothrium and Pseudanthobothrium. The bothridia and myzorhynchus are recognized as attachment organs (e.g., Williams, 1966; Butler, 1987) and were subdivided into 2 types each by Williams (1966). Williams (1966) described type I bothridia as shallow, open, spoon-shaped, divided into 10 to 20 loculi, and, with the exception of E. dubium, they attach to large areas of gut mucosa. Type II bothridia were described as possessing smaller, cup-shaped openings, with fewer loculi; each bothridium attaches to a single villus; and the number of loculi is inversely proportional to villus length. Williams (1966) described type I myzorhynchi as spherical or hemispherical, and their size is inversely proportional to the number of loculi per bothridium. Type II myzorhynchi are elongate and cylindrical, their length is inversely proportional to the number of loculi per bothridium, and they penetrate the wall of the villus (Williams, 1966; McVicar, 1972).

The factors relating to the specificity of tetraphyllideans are not well understood. The specificity of tetraphyllideans has been attributed to: (1) morphological adaptations of the scolex to the host mucosa (Williams, 1960, 1966, 1968a, 1968b); (2) physiological adaptations of hosts to the parasites, such as serum factors (McVicar and Fletcher, 1970); and (3) ecological factors, such as substrate preference and diet of the hosts (Randhawa et al., 2008). Williams (1960) described the spiral valve mucosa of 3 rajid species and 10 species of elasmobranchs (Williams, 1968a) and concluded that the differences in topography of the gut mucosa contribute to the observed specificity of tetraphyllidean cestodes. McVicar and Fletcher (1970) exposed Acanthobothrium quadrupartitum Williams, 1968, a parasite specific to Leucoraja naevus in the North Sea, to Amblyraja radiata and L. naevus blood sera and concluded that complement and/or a specific antibody response are responsible, at least partially, for the host specificity of this parasite. Using ecological data (McEachran and Musick, 1975; McEachran et al., 1976; Packer et al., 2003a, 2003b, 2003c, 2003d) and gut contents (Randhawa et al., 2008), we have determined that host substrate preference and corresponding prey biota are possible factors relating to the specificity, and lack of strict specificity, of Pseudanthobothrium spp. and Echeneibothrium spp. from the region of the West Isles of the Bay of Fundy, New Brunswick (NB), Canada (Randhawa et al., 2008).

The attachment site preference of tetraphyllideans has been studied in the rajid skates Dipturus chilensis (see Carvajal and Dailey, 1975), L. naevus (see Williams, 1968b; McVicar, 1979), and Raja montagui (see Williams, 1961), and in 6 shark species: Gymnolobomastoma cirratum (see Borucinska and Caira, 1993), Mustelus canis (see Cislo and Caira, 1993), Mustelus mustelus (see Euzet, 1959), Mustelus schmitti (see Alarcos et al., 2006), Priornace glauca (see Curran and Caira, 1995), and Scyliorhinus stellaris (see Rees and Williams, 1965). Attachment site preference has been linked to: (1) adaptations of the bothridia to the spiral valve topography (Williams, 1961, 1968b; Rees and Williams, 1965; Carvajal and Dailey, 1975; McVicar, 1979; Borucinska and Caira, 1993); (2) resource availability, such as nutrients (Williams, 1961; McVicar, 1979; Cislo and Caira, 1993; Curran and Caira, 1995); (3) physicochemical variables, such as gut pH (McVicar, 1979); (4) scolex size (Borucinska and Caira, 1993); and (5) parasite phylogenetics (Cislo and Caira, 1993; Alarcos et al., 2006).

In an extension of the survey by Randhawa, Saunders, and Burt (2007), the level of host specificity for each tetraphyllidean species is established by defining the host range as determined by the host distribution, and it is quantified by employing 5 indices of host specificity: (1, 2) Rohde’s (1980) S, [frequency] and S, [density] indices; (3) Caira et al.’s (2003) HS index; and (4, 5) Poulin and Mouillot’s (2003, 2005) taxonomic relatedness indices (S_H and S_T*, respectively). These results are compared to values obtained for Grillotia sp., a trypanorhynch cestode recovered from all 4 rajid hosts examined. Additionally, the mode of attachment of Zyxibothrium kamienae (Hayden and Campbell, 1981) is described herein. Further, bothridial measurements from 4 of the 6 tetraphyllidean species examined are compared to villar measurements from individual spiral valve chambers in all 4 rajid hosts to determine whether the observed host and attachment site specificity are attributable to scolex adaptations for host mucosa. The attachment sites and distribution of individual species along the length of the spiral valve of each host species are compared using Levin’s niche breadth and Renkonen’s niche overlap indices, in order to determine whether there is a phylogenetic component to attachment site specificity. Finally, McVicar and Fletcher’s (1970) experiment, in which a tetraphyllidean was exposed to sera of different hosts and other solutions, is revisited and expanded to determine whether there is a physiological determinant to the host specificity observed in this study.

**MATERIALS AND METHODS**

**Collection and examination of material**

From May to August of 1997 and from June 2002 to September 2004, 31 Amblyraja radiata (Donovan, 1808), 208 Leucoraja ernacea (Mitchell, 1825), 11 Leucoraja ocellata (Mitchell, 1815), and 33 Macroraja senta (Garman, 1885) samples were collected from Passamaquoddy Bay and waters surrounding the West Isles of the Bay of Fundy, New Brunswick, Canada (samples include collections reported in Randhawa et al., 2007). Skates were collected and processed following the methods outlined in Randhawa et al. (2007). Most spiral valves were examined immediately; however, some were preserved for later examination by injecting either 10% formalin, 10% Lillie’s buffered formalin, or 10% gluteraldehyde in the pyloric end until fully distended and treated as per Randhawa et al. (2008). Only scolices were counted to determine the numbers of parasites present in each individual spiral valve, and the attachment site (whorl number) of those attached was noted. The number of whorls for each spiral valve examined was counted and noted prior to its disposal.

**Host range and host specificity**

Presence in a particular rajid host species was used to determine the host range of tetraphyllidean cestodes. Prevalence, intensity of infection, and abundance data, as defined in Margolis et al. (1982) and Bush et al. (1997), herein, and including those reported in Randhawa et al. (2007, 2008), were quantified for calculations of the 5 host specificity indices. Phylogenetic relatedness was measured based on McEachran and Dunn’s (1998) phylogenetic study of rajid skate genera. Values for both of Rohde’s (1980) indices (S) range between 0 and 1. In both cases, parasites exhibiting the greatest degree of specificity will tend toward 1. Values for Caira et al.’s (2003) HS index range between 0 and 10; parasites exhibiting the greatest specificity will tend toward 0. Values for the S_T* and S_H* indices (Poulin and Mouillot, 2003, 2005, 2008).
TABLE I. Summary of survival of different parasite species following exposure to different blood sera and solutions expressed as percentage survival (number of worms exposed to solution).

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>Tav*</th>
<th>Pe</th>
<th>Po</th>
<th>Pr</th>
<th>Ps</th>
<th>Ed</th>
<th>Ev</th>
<th>Zk</th>
<th>Bs</th>
<th>fp Tav</th>
<th>fp Pe</th>
<th>fp Po</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. acanthias</td>
<td>100(2)</td>
<td>0(26)</td>
<td>0(1)</td>
<td>0(2)</td>
<td>100(4)</td>
<td>100(33)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. erinacea</td>
<td>0(2)</td>
<td>100(8)</td>
<td>50(4)</td>
<td>0(4)</td>
<td>0(1)</td>
<td>100(1)</td>
<td>0(4)</td>
<td>100(3)</td>
<td>0(15)</td>
<td>100(2)</td>
<td>100(2)</td>
<td></td>
</tr>
<tr>
<td>L. ocellata</td>
<td>0(1)</td>
<td>36(11)</td>
<td>100(2)</td>
<td>0(2)</td>
<td>0(1)</td>
<td>100(1)</td>
<td>0(4)</td>
<td>100(2)</td>
<td>0(15)</td>
<td>100(2)</td>
<td>100(2)</td>
<td></td>
</tr>
<tr>
<td>A. radiata</td>
<td>0(1)</td>
<td>0(7)</td>
<td>0(1)</td>
<td>100(4)</td>
<td>0(1)</td>
<td>100(1)</td>
<td>0(2)</td>
<td>0(4)</td>
<td>100(1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. senta</td>
<td>0(1)</td>
<td>0(7)</td>
<td>0(1)</td>
<td>25(4)</td>
<td>100(1)</td>
<td>100(4)</td>
<td>0(15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. americanus</td>
<td>0(2)</td>
<td>0(5)</td>
<td>0(1)</td>
<td>0(2)</td>
<td>100(4)</td>
<td>0(1)</td>
<td>0(2)</td>
<td>100(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elasmo. saline</td>
<td>100(2)</td>
<td>100(15)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(4)</td>
<td>100(3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seawater</td>
<td>100(2)</td>
<td>100(10)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
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<td></td>
</tr>
<tr>
<td>Saline</td>
<td>100(2)</td>
<td>100(5)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>0(2)</td>
<td>0(5)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(2)</td>
<td>100(4)</td>
<td>0(1)</td>
<td>0(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Tav = Trilocularia acanthioeulgaris (n = 17) from Squalus acanthias; Pe = Pseudanthobothrium purtoni from Leucoraja erinacea (n = 99); Po = P. purtoni from L. ocellata (n = 14); Pr = P. hansenii from Amblyraja radiata (n = 24); Ps = P. hansenii from Malacoraja senta (n = 5); Ed = Echeneibothrium dubium abyssorum (n = 5); Ev = E. verneta (n = 4) from L. erinacea; Zk = Zyxibothrium kamienae (n = 20) from M. senta; Bs = Bothriocephalus sp. (n = 19); fp Tav = free proglottids from T. acanthioeulgaris (n = 190); fp Pe = free proglottids from P. purtoni (L. erinacea) (n = 11); fp Po = free proglottids from P. purtoni (L. ocellata) (n = 8); blank = not tested; bold = indicates differential survival.

respectively) range between 1 and 5; parasites exhibiting the greatest degree of specificity will tend toward 1. The variance in taxonomic distinctness (VarS±) was not calculated because not a single tetraphyl­lidan species was recovered from more than 2 host species as adults, a requirement for calculations of VarS± (Poulin and Mouillot, 2003; Krasnov et al., 2006). Molecular data have shown that the distribution of immature Pseudanthobothrium hansenii and P. purtoni specimens mirrors that of adults (mature specimens) (Randhawa et al., 2007); therefore, data for both immature and mature Pseudanthobothrium spec­imens were used to measure the degree of specificity of these parasites using the aforementioned indices. However, since specificity scores postattachment in Echeneibothrium (Randhawa et al., 2007), only adult specimens were used to measure the degree of specificity, prevalence, intensity of infection, and abundance of species belonging to this genus. The influence of this choice on the results will be discussed later.

Examination of bothridia and villi

One hundred and thirty-four worms were used for bothridial measurements (14 E. d. abyssorum, 21 E. vernetae, 43 P. hansenii, 40 P. purtoni, and 16 Z. kamienae). Measurements were taken using light microscopy. Specimens were retrieved from fresh spiral valves, fixed in hot, almost boiling 70% ethanol, and stored in fresh 70% ethanol before being stained in 2% Acetic Acid Alum Carmine (AAC) and processed according to routine histological procedures. All bothridial measurements are represented by the mean ± standard deviation (and range) and are in micrometers (μm) unless otherwise noted. The number of measurements taken is identified by “n” and indicates the number of individual bothridia measured rather than the number of worms examined.

Spiral valves, from which villar measurements were taken, were injected with either 10% Lillie’s buffered formalin or 10% gluteraldehyde. Spiral valves from 5 adult male specimens of L. erinacea, and 2 adult male specimens each of L. ocellata, A. radiata, and M. senta, were excised at their base from the spiral valve walls, from each whorl, using a scalpel under a binocular dissecting microscope. Spiral valve material (individual villi from spiral valves, from which villar measurements were taken, were in­

TABLE II. Summary of the prevalence, average intensity (range), and abundance of infection for: Echeneibothrium canadensis, E. dubium abyssorum, E. vernetae, Grillotia sp., Pseudanthobothrium hansenii, P. purtoni, and Z. kamienae.

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>Host species</th>
<th>Prevalence</th>
<th>Average intensity of infection (range)</th>
<th>Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echeneibothrium canadensis*</td>
<td>Amblyraja radiata</td>
<td>6.5% (2 of 31)</td>
<td>1.5 (1 or 2)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>E. dubium abyssorum*</td>
<td>A. radiata</td>
<td>41.9% (13 of 31)</td>
<td>3.8 (1–20)</td>
<td>1.6</td>
</tr>
<tr>
<td>E. vernetae*</td>
<td>Leucoraja erinacea</td>
<td>63.5% (132 of 208)</td>
<td>11.3 (1–143)</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>L. ocellata</td>
<td>18.2% (2 of 11)</td>
<td>8.5 (8 or 9)</td>
<td>1.6</td>
</tr>
<tr>
<td>Grillotia sp.</td>
<td>A. radiata</td>
<td>16.1% (5 of 31)</td>
<td>5.0 (1–16)</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>L. erinacea</td>
<td>3.4% (7 of 208)</td>
<td>1.3 (1 or 2)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>L. ocellata</td>
<td>27.3% (3 of 11)</td>
<td>6.7 (1–14)</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Malacoraja senta</td>
<td>3.0% (1 of 33)</td>
<td>1.0 (1)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Pseudanthobothrium hansenii</td>
<td>A. radiata</td>
<td>90.3% (28 of 31)</td>
<td>19.6 (1–55)</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>M. senta</td>
<td>48.5% (16 of 33)</td>
<td>10.1 (1–73)</td>
<td>4.9</td>
</tr>
<tr>
<td>P. purtoni</td>
<td>L. erinacea</td>
<td>92.3% (192 of 208)</td>
<td>13.7 (1–96)</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>L. ocellata</td>
<td>81.8% (9 of 11)</td>
<td>14.4 (1–41)</td>
<td>11.8</td>
</tr>
<tr>
<td>Z. kamienae</td>
<td>M. senta</td>
<td>48.5% (16 of 33)</td>
<td>29.5 (1–212)</td>
<td>14.3</td>
</tr>
</tbody>
</table>

* Immature E. canadensis, E. d. abyssorum, and E. vernetae specimens from A. radiata were not included due to the absence of reliable species-diagnostic features.
TABLE III. Summary of the specificity indices values: $S_i$ [frequency] and $S_d$ [density] (Rohde, 1980), HS (Caira et al., 2003), and $S_{TD}$ and $S_{TD}^*$ (Poulin and Mouillot, 2003, 2005, respectively) for *Echeneibothrium canadensis*, *E. d. abyssorum*, *E. vernetae*, Grillotia sp., *Pseudanthobothrium hanseni*, *P. purtoni*, and *Z. kamienae*.

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>$S_i$ (frequency)</th>
<th>$S_d$ (density)</th>
<th>HS</th>
<th>$S_{TD}$</th>
<th>$S_{TD}^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Echeneibothrium canadensis</em></td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td><em>E. d. abyssorum</em></td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td><em>E. vernetae</em></td>
<td>0.91</td>
<td>0.91</td>
<td>0.3</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Grillotia sp.</td>
<td>0.83</td>
<td>0.83</td>
<td>3.3</td>
<td>1.67</td>
<td>1.87</td>
</tr>
<tr>
<td><em>Pseudanthobothrium hanseni</em></td>
<td>0.89</td>
<td>0.89</td>
<td>3</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td><em>P. purtoni</em></td>
<td>0.76</td>
<td>0.76</td>
<td>0.3</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Z. kamienae</em></td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Immature *E. canadensis*, *E. d. abyssorum*, and *E. vernetae* specimens from *A. radiata* were not included due to the absence of reliable species-diagnostic features.

FIGURES 1-4. (1) Scanning electron micrographs of a single bothridium of *Pseudanthobothrium purtoni* recovered from *Leucoraja erinacea*. (2) Scolex of *P. hanseni* recovered from *Amblyraja radiata* bearing 4, stalked bothridia and a myzorhynchus, showing 1 bothridium attached to a villus. (3) Scolex of *Echeneibothrium dubium abyssorum* recovered from *A. radiata* bearing 4 stalked bothridia, each divided into 10 loculi, and a myzorhynchus. (4) Scolex of *Zyxibothrium kamienae* recovered from *Malacoraja dentata* bearing 4 sessile bothridia. B, bothridia; L, loculi; M, myzorhynchus; V, villus. Scale bars: $1 = 50 \mu m$; $2 = 600 \mu m$; $3$ and $4 = 100 \mu m$. 
oxide and mounted on stubs using double-sided adhesive carbon tape. Silver paste was used around the tape to provide additional contact. All villar measurements taken, using 2 to 12 villi per whorl, are represented by the mean ± standard deviation (and range) and are in micrometers (µm) unless otherwise noted.

Within and between species, villar differences were also assessed by t-tests, Mann-Whitney U-tests, and analysis of variance (ANOVA). Furthermore, between species, villar differences were also assessed by ANOVA.

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>n*</th>
<th>Length (range)</th>
<th>Width (range)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. dubium abyssorum</td>
<td>L = 33</td>
<td>452 (320-585)</td>
<td>268 (215-355)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>W = 23</td>
<td>36*</td>
<td>448 (350-520)</td>
<td>303 (200-380)</td>
</tr>
<tr>
<td>E. vernetiae</td>
<td>58?</td>
<td>362 (245-510)</td>
<td>300 (155-440)</td>
<td>This study; Euzet (1956)</td>
</tr>
<tr>
<td>Pseudanthobothrium hanseni</td>
<td>L = 135</td>
<td>242 (140-380)</td>
<td>201 (135-306)</td>
<td>Randhawa et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>W = 61</td>
<td>245 (186-370)</td>
<td>161 (88-250)</td>
<td></td>
</tr>
<tr>
<td>P. purtoni</td>
<td>L = 83</td>
<td>405 (260-695)</td>
<td>300 (225-400)</td>
<td>This study</td>
</tr>
<tr>
<td>Zyxibothrium kamienae</td>
<td>L = 55</td>
<td>440 (204-695)</td>
<td>300 (225-400)</td>
<td></td>
</tr>
</tbody>
</table>

* Measurements from “N” specimens; n = number of individual measurements; L = length; W = width.

<table>
<thead>
<tr>
<th>Amblyraja radiata</th>
<th>Leucoraja erinacea</th>
<th>L. ocellata</th>
<th>Malacoraja senta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of whorls*</td>
<td>9</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>L W1</td>
<td>763 ± 53.4 (690-850)</td>
<td>536 ± 36.8 (465-585)</td>
<td>860 ± 48.3 (795-955)</td>
</tr>
<tr>
<td></td>
<td>208 ± 30.3 (165-250)</td>
<td>151 ± 23.7 (115-175)</td>
<td>227 ± 13.8 (210-250)</td>
</tr>
<tr>
<td></td>
<td>717 ± 79.9 (625-825)</td>
<td>464 ± 34.5 (425-525)</td>
<td>588 ± 59.1 (505-635)</td>
</tr>
<tr>
<td></td>
<td>213 ± 32.6 (165-260)</td>
<td>144 ± 14.1 (130-175)</td>
<td>206 ± 22.5 (175-225)</td>
</tr>
<tr>
<td>W2</td>
<td>685 ± 31.3 (555-640)</td>
<td>368 ± 38.3 (325-445)</td>
<td>532 ± 32.4 (480-575)</td>
</tr>
<tr>
<td></td>
<td>147 ± 27.0 (115-190)</td>
<td>126 ± 12.2 (108-145)</td>
<td>218 ± 14.0 (200-245)</td>
</tr>
<tr>
<td>W3</td>
<td>440 ± 32.8 (395-475)</td>
<td>379 ± 24.6 (335-420)</td>
<td>497 ± 37.1 (415-535)</td>
</tr>
<tr>
<td></td>
<td>153 ± 11.5 (135-165)</td>
<td>116 ± 15.6 (95-143)</td>
<td>175 ± 15.6 (150-195)</td>
</tr>
<tr>
<td>W4</td>
<td>388 ± 29.8 (355-425)</td>
<td>371 ± 50.8 (325-465)</td>
<td>398 ± 23.1 (365-435)</td>
</tr>
<tr>
<td></td>
<td>133 ± 32.8 (90-165)</td>
<td>195 ± 22.8 (170-235)</td>
<td>151 ± 18.6 (115-165)</td>
</tr>
<tr>
<td>W5</td>
<td>348 ± 25.2 (325-375)</td>
<td>232 ± 31.1 (190-305)</td>
<td>380 ± 20.6 (355-420)</td>
</tr>
<tr>
<td></td>
<td>142 ± 15.3 (125-155)</td>
<td>158 ± 24.8 (125-203)</td>
<td>195 ± 18.7 (165-215)</td>
</tr>
<tr>
<td>W6</td>
<td>297 ± 20.2 (275-315)</td>
<td>213 ± 21.0 (188-235)</td>
<td>366 ± 25.5 (340-410)</td>
</tr>
<tr>
<td>W7</td>
<td>250 ± 35.4 (225/275)</td>
<td>208 ± 27.2 (165-235)</td>
<td>316 ± 29.5 (275-365)</td>
</tr>
<tr>
<td></td>
<td>195 ± 36.6 (155/235)</td>
<td>268 ± 49.5 (225-360)</td>
<td>258 ± 21.6 (235-295)</td>
</tr>
<tr>
<td>W8</td>
<td>202 ± 41.9 (175-250)</td>
<td>523 ± 46.0 (490-555)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 ± 27.8 (170-225)</td>
<td>643 ± 53.0 (605-680)</td>
<td></td>
</tr>
<tr>
<td>W9</td>
<td>392 ± 36.8 (350-455)</td>
<td>431 ± 73.5 (340-525)</td>
<td></td>
</tr>
<tr>
<td>W10</td>
<td>392 ± 36.8 (350-455)</td>
<td>431 ± 73.5 (340-525)</td>
<td></td>
</tr>
</tbody>
</table>

* W = Whorl number; n = number of measurements.
Table VI. Comparisons of villar measurements (t-test and Mann-Whitney U-test, respectively), from Table V, between the 4 rajid species examined herein: Amblyraja radiata, Leucoraja erinacea, L. ocellata, and Malacoraja senta. Since the number of whorls is proper to each species, for comparative purposes, the spiral valves are divided into the anterior, middle, and posterior thirds. Grouping of whorls is based on similarities in length and width identified using t-test and Mann-Whitney U-test. The lower triangle compares villar lengths, while the upper triangle compares villar widths. Statistical significance is set to P < 0.05 and is accompanied by an asterisk (*), whereas nonsignificance is indicated by “ns.”

<table>
<thead>
<tr>
<th></th>
<th>Amblyraja radiata</th>
<th>Leucoraja erinacea</th>
<th>Leucoraja ocellata</th>
<th>Malacoraja senta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior third</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amblyraja radiata</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Leucoraja erinacea</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Leucoraja ocellata</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Malacoraja senta</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Middle third</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amblyraja radiata</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Leucoraja erinacea</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Leucoraja ocellata</td>
<td>ns; ns</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Malacoraja senta</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Posterior third</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Amblyraja radiata</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Leucoraja erinacea</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Leucoraja ocellata</td>
<td>ns; ns</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
<tr>
<td>Malacoraja senta</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
<td>—</td>
<td>P &lt; 0.001*; P &lt; 0.001*</td>
</tr>
</tbody>
</table>

Parasite distributions

As mentioned previously, the attachment site (whorl) for each worm was noted whenever possible. These data were used to illustrate the distribution of cestode parasites along the length of the spiral valve of each rajid skate species and to calculate Levin’s niche breadth (LNB) and Renkonen’s niche overlap (RNO) (see Simkova et al., 2000). Values for LNB range between 1 and the size of the niche examined, i.e., number of whorls per host; higher values indicate wider niches. Values for RNO range between 0 and 1, where 0 indicates the absence of niche overlap and 1 represents complete niche overlap. Differences in niche breadth were compared between species using t-tests and Mann-Whitney U-tests. The attachment site of unattached cestodes could not be determined; therefore, these worms were not included in niche breadth calculations.

Exposure of parasites to blood sera and other solutions

For the purpose of this in vitro experiment, blood and parasites were collected from 6 L. erinacea, 3 L. ocellata, 3 A. radiata, and 1 M. senta, as well as S. squamosus (spiny dogfish) (squalid shark) and 3 Hemipterius americanus (sea raven) (teleost fish). All fish were collected on 22 and 25 July 2003 in Passamaquoddy Bay, New Brunswick, Canada. Blood was collected by cardiac puncture in skates and from the caudal vein of S. acanthias and H. americanus using 3-mL and 7-mL heparin-coated Vacutainers (Becton Dickinson Vacutainer Systems, Rutherford, New Jersey). Serum was collected by centrifugation for 10 min at 2,000 rev/min and maintained at ambient seawater temperature. Parasites were collected, placed in elasmobranch saline (Read et al., 1960), and maintained at ambient seawater temperature for a period of 3 hr before being used. Using plates with 24 wells, whole worms or free proglottids from each recovered species were individually placed in 0.75 ml of sera, elasmobranch saline, saline, unfiltered seawater, and distilled water (Table I). For a detailed account of the number of worms from each species exposed to the various solutions, refer to Table I. The elasmobranch saline was used as a control. The blood sera and other solutions were maintained at ambient seawater temperatures by placing the underside of each individual plate in running seawater. Worm survival was monitored every hour, for up to 6 hr, and the time taken to die was noted in each case. Cessation of bothridial movement in whole worms was used as a criterion for death, because lack of movement of the scolex would lead to detachment from the host mucosa and expulsion from the spiral valve. Furthermore, dead worms exposed to blood sera were usually covered with a deposit, with disintegrating strobila, and no movement could be observed in either the scolex or strobilar regions (see McVicar and Fletcher, 1970). Cessation of contraction of free proglottids was used as a criterion for death. Again, dead proglottids exposed to blood sera were usually covered with a deposit similar to that found on whole worms. Exposure time to different solutions was 6 hr. When multiple individuals from a single host species were available, some worms were exposed to blood serum from a different host individual of the same species, depending on the adequacy of the supply of blood serum. Blood serum was never pooled for a different host species. The differences between different treatments (1) exposure to “right” host blood serum; (2) exposure to “wrong” host blood serum; and (3) exposure to elasmobranch saline (control) were assessed using an ANOVA.

RESULTS

The host distribution (for all 6 tetraphyllidean species), prevalence, and intensity data for Pseudanthobothrium purtoni, P. hansenii, Echeneibothrium canadensis, and E. vernetae reported herein include those reported in Randhawa et al. (2007, 2008). Six species of tetraphyllidean cestodes were identified: P. purtoni and E. vernetae Euzet, 1956 were observed in Leucoraja erinacea and L. ocellata; P. hansenii Baer, 1956 was recovered from Amblyraja radiata and Malacoraja senta; E. dubium abys­ sorum Campbell, 1977 and E. canadensis Keeling and Burt, 1996 were found in A. radiata; and Zyxibothrium kamienae Hayden and Campbell, 1981 was also recovered from M. senta.
Another cestode, the trypanorhynch *Grillotia* sp., was seen in all 4 rajid skate species. As mentioned previously, immature *Pseudanthobothrium* specimens were taken from all 4 rajid hosts, and their host distribution mirrors that of adults (see Randhawa et al., 2007). Immature *E. vernetae* specimens were also recovered from *A. radiata* and *L. erinacea* (Randhawa et al., 2007) and, as mentioned already, these were not included in calculations of prevalence, average intensity, and abundance of infection. Prevalence, average intensity (including range), and abundance of infection data are summarized in Table II.

**Host specificity**

Based on the results of the S; [frequency] index, *E. canadensis, E. d. abyssorum*, and *Z. kamienae* exhibited the highest degree of host specificity with scores of 1. They were followed by *E. vernetae* (S; [frequency] = 0.91), *P. hanseni* (S; [frequency] = 0.89), *Grillotia* sp. (S; [frequency] = 0.83), and *P. purtoni* (S; [frequency] = 0.76), respectively (Table III). Results of the S; [density] index were identical to S; [frequency] (Table III). Similarly, HS indicated *E. canadensis, E. d. abyssorum*, and *Z. kamienae* as exhibiting the highest degree of host specificity with scores of 0, followed by *E. vernetae* and *P. purtoni* (HS = 0.3), *P. hanseni* (HS = 3), and *Grillotia* sp. (HS = 3.3), respectively (Table III). In contrast, the *S* and *S* indices indicated that *E. canadensis, E. d. abyssorum, E. vernetae, P. purtoni, and Z. kamienae* exhibited the highest degree of host specificity with scores of 1, whereas *P. hanseni* scored 2 (Table III). The *S* and *S* indices indicated that *Grillotia* sp. (1.67

Table VII. Comparison of villar measurements from Table V (*t*-test and Mann-Whitney U-test, respectively) of *Amblyraja radiata*. The lower triangle compares villar lengths, while the upper triangle compares villar widths. Statistical significance set to *P* < 0.05. Significant differences are indicated by an asterisk (*), and nonsignificant differences are identified by "ns."

<table>
<thead>
<tr>
<th></th>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
<th>W6</th>
<th>W7</th>
<th>W8</th>
<th>W9</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>—</td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>W2</td>
<td><em>P &lt; 0.01</em></td>
<td>—</td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>W3</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>W4</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td>—</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>W5</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>W6</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td>—</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>W7</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>W8</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
</tbody>
</table>

Table VIII. Comparison of villar measurements from Table V (*t*-test and Mann-Whitney U-test, respectively) of *Leucoraja erinacea*. The lower triangle compares villar lengths, while the upper triangle compares villar widths. Statistical significance set to *P* < 0.05. Significant differences are indicated by an asterisk (*), and nonsignificant differences are identified by "ns."

<table>
<thead>
<tr>
<th></th>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
<th>W6</th>
<th>W7</th>
<th>W8</th>
</tr>
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<td><em>P &lt; 0.01</em></td>
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<tr>
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<td><em>P &lt; 0.01</em></td>
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<tr>
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<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
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<td><em>P &lt; 0.01</em></td>
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<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
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<td><em>P &lt; 0.01</em></td>
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<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
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</table>
and 1.87, respectively) exhibited a greater degree of host specificity than *P. hansenii* (Table III).

**Bothridial and villar measurements**

Consistent with their respective generic descriptions, both *Pseudanthobothrium* spp. possessed unloculated, pedicellate, cup-shaped bothridia (Figs. 1, 2) (see Baer, 1956; Schmidt, 1986; Euzet, 1994); all 3 *Echeneibothrium* spp. possessed loculated (number of loculi varied between species), pedicellate bothridia (Fig. 3) (see Euzet, 1956, 1994; Campbell, 1977; Schmidt, 1986; Keeling and Burt, 1996); and *Z. kamienae* possessed sessile and loculated bothridia (Fig. 4) (see Hayden and Campbell, 1981). Additionally, both species of *Pseudanthobothrium* were found to enclose an individual villus with each individual bothridium (Fig. 2), whereas the 3 species of *Echeneibothrium* grabbed hold of the apex of an individual villus with each bothridium. Individual bothridia of *Z. kamienae* grabbed hold of individual villi from the side at their base. Consistent with generic and subfamily descriptions (see Baer, 1956; Euzet, 1994), only *Pseudanthobothrium* spp. and *Echeneibothrium* spp. possessed a retractable myzorhynchus (Figs. 2, 3).

The bothridia measurements for both *Pseudanthobothrium* species were first reported in Randhawa, Saunders, Scott, and Burt (2008) and can be found in Table IV herein, which also includes bothridial measurements from *E. d. abyssorum*, *E. verretae*, and *Z. kamienae* and lists measurements from original descriptions or redescriptions. Bothridial measurements were not taken for *E. canadensis* due to the low numbers recovered (Table II). In light of the different mode of attachment described for *Z. kamienae*, this species was not included as part of the analyses comparing bothridial and villar measurements.

Spiral valves from all 4 host species examined in this study possessed villar projections without crypts and ridges.

<p>| Table IX. Comparison of villar measurements from Table V (t-test and Mann-Whitney U-test, respectively) of <em>Leucoraja ocellata</em>. The lower triangle compares villar lengths, while the upper triangle compares villar widths. Statistical significance set to <em>P</em> &lt; 0.05. Significant differences are indicated by an asterisk (*), and nonsignificant differences are identified by “ns.” |
|---|---|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
<th>W6</th>
<th>W7</th>
<th>W8</th>
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<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.01</em></td>
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</tr>
<tr>
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<td>ns</td>
<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.001</em></td>
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<td><em>P &lt; 0.05</em></td>
</tr>
<tr>
<td>W3</td>
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<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.05</em></td>
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<tr>
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<td>ns</td>
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<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.05</em></td>
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<tr>
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<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.05</em></td>
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<tr>
<td>W6</td>
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<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.01</em></td>
<td>ns</td>
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<tr>
<td>W7</td>
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<td>ns</td>
<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.05</em></td>
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<tr>
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<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>W10</td>
<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.01</em></td>
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<td><em>P &lt; 0.01</em></td>
<td>ns</td>
<td>ns</td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
</tbody>
</table>

<p>| Table X. Comparison of villar measurements from Table V (t-test and Mann-Whitney U-test, respectively) of <em>Malacoraja senta</em>. The lower triangle compares villar lengths, while the upper triangle compares villar widths. Statistical significance set to <em>P</em> &lt; 0.05. Significant differences are indicated by an asterisk (*), and nonsignificant differences are identified by “ns.” |
|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>W1</th>
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<th>W3</th>
<th>W4</th>
<th>W5</th>
</tr>
</thead>
<tbody>
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<td><em>P &lt; 0.05</em></td>
<td>ns</td>
</tr>
<tr>
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<td>ns</td>
<td><em>P &lt; 0.001</em></td>
</tr>
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<td>W3</td>
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<td><em>P &lt; 0.01</em></td>
<td>—</td>
<td>ns</td>
</tr>
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<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.05</em></td>
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</tr>
<tr>
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<td><em>P &lt; 0.001</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
</tbody>
</table>
number of whorls or chambers of the spiral valve of each species was different between each rajid skate species (Table V) \((P < 0.00001)\), was consistent, and did not vary within a species regardless of sex, maturity, or size \((P > 0.05)\). This characteristic was distinguishable to the naked eye and could be used to identify the host species postmortem when identity based on external morphometric data was questionable. Villar measurements were obtained from male individuals only; therefore, within species male versus female villar measurement comparisons were not possible. The size and shape of the intestinal villi varied not only from 1 species to the next (ANOVA, \(P < 0.001\); nested ANOVA, \(P < 0.001\)), but also within individual spiral valves (ANOVA, \(P < 0.001\)). Results of individual \(t\)-tests and \(U\)-tests for between-species comparisons are summarized in Table VI, whereas those for within-species comparisons are summarized in Tables VII–X. In all 4 species, the length of the villi generally decreased posteriorly (Table V). In \(A.\ radiata\), \(L.\ ernacea\), and \(L.\ ocellata\), the anterior half of the spiral valve was composed of cylindrical villi (whorls 1 to 5; 1 to 4; and 1 to 5, respectively), whereas the posterior half was composed of flattened (in cross section) villi (Figs. 5–31). In \(M.\ senta\), the villi were cylindrical only in the first whorl, whereas the other 4 whorls were composed of flattened pyramidal- or rectangular-shaped villi (Figs. 32–36). Villar lengths and widths from individual whorls for each rajid skate species examined are summarized in Table V.

The 3 \(Echeneibothrium\) species and 2 \(Pseudanthobothrium\) species possessed type II bothridia (Williams, 1966), and each bothridium was found to attach to a single villus. Based on observations herein and original descriptions of \(E.\ canadensis\) (Keeling and Burt, 1996) and \(E.\ vernetae\) (Euzet, 1956), both possessed type I myzorhynchi (Williams, 1966), which are characterized by a spherical or hemispherical myzorhynchus, whereas \(E.\ d.\ abyssorum\) (Campbell, 1977), \(P.\ hanseni\) (Baer, 1956), and \(P.\ purtoni\) possessed type II myzorhynchi (Williams, 1966), which are characterized by an elongate cylindrical myzorhynchus. Furthermore, by comparing bothridial (Table IV) to villar (Table V) measurements, the latter would not prevent
FIGURES 14–21. Scanning electron micrographs of villi from the spiral valve of *Leucoraja erinacea*, whorls 1 through 8, respectively. Scale bars: 14–17 and 19–21 = 300 μm; 18 = 400 μm.
Villi from all whorls in *A. radiata* were compatible with bothridial measurements for *P. hanseni* (see Table XII). However, villi in the posterior third of the spiral valve tended to be larger than the bothridial opening (*t*-test and Mann–Whitney *U*-test; *P* < 0.001; Table XI). In spite of this, the low number of villi examined from *A. radiata* limits the inferences that can be drawn from the data. Additionally, of 60 *E. d. abyssorum* specimens, only 2 (3.3%) were recovered from the posterior third of the spiral valve; villi in the posterior third of the spiral valve were too short to provide a suitable site of attachment for *E. d. abyssorum* (*t*-test and Mann–Whitney *U*-test; *P* < 0.05; Tables IV [fourth row], V [first column], XI [first row × first column]). Data presented in Tables IV (first and fifth rows), V (first column), XI (first column × second and third rows), and XII (second and third rows) indicate that there were adequate attachment sites for *E. vernetae* and *P. purtoni* along the spiral valve of *A. radiata*. Figure 37 summarizes the distribution of *E. canadensis*, *E. d. abyssorum*, *Grillotia* sp., and *P. hanseni* along the length of the spiral valve of *A. radiata*.

Villi from the last whorl in *L. erinacea* were too wide for *P. purtoni* to attach (*t*-test and Mann–Whitney *U*-test; *P* < 0.05; Tables IV [first row], V [second column], XI [third row × second column], and XIII [third row × eighth column]). Of the 2,622 *P. purtoni* specimens recovered from *L. erinacea*, only 17 (<0.7%) were recovered from the last whorl. Furthermore, excepting the last whorl, villi were smaller or not significantly different than the *P. purtoni* bothridial opening length or width (Tables IV [first row], V [second column], and XIII [third row]), thereby potentially providing adequate attachment sites. Additionally, of 1,478 *E. vernetae* specimens, only 23 (<1.6%) were recovered from the posterior third (whorls 6, 7, and 8). Although the width of the villi in all 8 whorls seemed to provide a potentially suitable attachment site for *E. vernetae*, villi in whorls 6, 7, and 8 were too short for *E. vernetae* to attach (Tables IV [fifth row], V [second column], and XIII [second row]). Data presented in Tables IV (second and fourth rows), V (second column), XI (second and third rows × second column), and XIII (second and fourth rows) indicate that there were adequate attachment sites for *E. d. abyssorum* and *P. hanseni* along the spiral valve of *L. erinacea*. Figure 38 summarizes the distribution of *E. vernetae*, *Grillotia* sp., and *P. purtoni* along the length of the spiral valve of *L. erinacea*.

Villi from whorls 9 and 10 in *L. ocellata* were wider than the bothridia of *P. purtoni* (*t*-test and Mann–Whitney *U*-test; *P* = 0.05; Tables IV [first row], V [third column], XIV [third row × ninth and tenth columns]). Of the 92 *P. purtoni* recovered from *L. ocellata*, none was recovered from the ninth and tenth whorls. Additionally, no *E. vernetae* specimen was recovered from the posterior half of the spiral valve of *L. ocellata*, even though whorls 8 (villi too short; *t*-test and Mann–Whitney *U*-test; *P* < 0.01; Tables IV [fifth row], V [third column], XIV [third row × eighth columns]) and 10 (villi too wide; *t*-test and Mann–Whitney *U*-test; *P* < 0.01; Tables IV [fifth row], V [third column], XIV [third row × tenth column]) were the only ones not to provide suitable attachment sites for this species. Data presented in Tables IV (second and fourth rows), V (third column), XI (first and fourth rows × third column), and XIV (first and fourth rows) indicate that there were adequate attachment sites for *E. d. abyssorum* and *P. hanseni* along the spiral valve of *L. ocellata*. The distribution of *E. vernetae*, *Grillotia* sp., and

*E. d. abyssorum*, *E. vernetae*, *P. purtoni*, and *P. hanseni* from attaching to any of the 4 host’s spiral valve mucosa (Table XI). Additionally, no significant correlation was observed between the number of loculi and villus length (*P* > 0.05; *r*² = 0.039), or between number of loculi and myzorhynchus length (*P* > 0.05; *r*² = 0.027).

Dimensions of the villi can restrict the attachment site for some tetraphyllidean species. However, as shown here, absence of worms was also noted in whorls (or host species) where villi seemed to provide suitable attachment sites.
FIGURES 32–36. Scanning electron micrographs of villi from the spiral valve of Malacoraja senta, whorls 1 through 5, respectively. Scale bars: 32–36 = 500 μm.
TABLE XI. Comparison of bothridial measurements (t-test and Mann-Whitney U-test, respectively) of *Echeneis* *vernetae*, *Pseudanthobothrium hanseni*, and *P. purtoni*, with villar measurements from spiral valves of all 4 rajid hosts studied herein. The spiral valve has been divided into thirds to increase the number of measurements included in each comparison. Statistical significance is set to $P < 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Amblyraja radiata</th>
<th>Leucoraja erinacea</th>
<th>Leucoraja ocellata</th>
<th>Malacoraja senta</th>
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<tbody>
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<td>Mid.</td>
<td>Post. 1/3</td>
<td>Ant. 1/3</td>
</tr>
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<td>***</td>
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<tr>
<td></td>
<td>W × W</td>
<td>***</td>
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<td>***</td>
</tr>
<tr>
<td><em>E. vernetae</em></td>
<td>L × L</td>
<td>***</td>
<td>*</td>
<td>***</td>
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<td></td>
<td>W × W</td>
<td>***</td>
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<td>***</td>
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<tr>
<td><em>P. hanseni</em></td>
<td>L × L</td>
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<td></td>
<td>W × W</td>
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<td>***</td>
</tr>
<tr>
<td><em>P. purtoni</em></td>
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<td>***</td>
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<tr>
<td></td>
<td>W × W</td>
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</table>

† Comp. indicates the type of bothridial and villar comparison, reflecting different mode of attachment of the different parasites. L × L indicates bothridial length compared with villar length; W × W indicates bothridial width compared with villar width; L × W indicates bothridial length compared with villar width; ns indicates that differences are nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

*P. purtoni* along the length of the spiral valve of *L. ocellata* is illustrated in Figure 39.

Although villi from *M. senta* do not provide a suitable attachment site for *P. hanseni* (Tables XI [fourth row × fourth column] and XV [fourth column]), only villi from the first whorl in *M. senta* overlapped with bothridial measurements (see ranges in Tables IV [second row] and V [fourth column]). Of the 161 *P. hanseni* specimens recovered from *M. senta*, only 5 (3.1%) were recovered from the second whorl, and none was recovered posterior to the second whorl. The bothridial opening is not significantly different between *P. hanseni* and *P. purtoni* (t-test and Mann–Whitney U-test; $P > 0.05$). Figure 40 illustrates the distribution of *Grillotia* sp., *P. hanseni*, and *Z. kamienae* along the length of the spiral valve of *M. senta*.

All specimens of *Z. kamienae* were found in the anteriormost whorl. Whether examined live or fixed in situ, only the strobila was visible and found to extend beyond the distal tip of the villi. Contrary to the other 5 tetraphyllidean species examined herein, the scolex of *Z. kamienae* was found at the proximal base of the villi with each sessile loculated bothridium (Fig. 4) grasping the proximal base of an individual villus.

**Parasite distributions**

The distribution of tetraphyllideans along the spiral valve of the 4 rajid hosts differed from one species to the next. The frequency distribution of all cestode species examined in *A. radiata* is presented in Figure 37, while those for *L. erinacea*, *L. ocellata*, and *M. senta* are presented in Figures 38, 39, and 40, respectively.

*Echeneis* *vernetae* occupies similar attachment sites (Figs. 38, 39) and niche sizes (t-test and Mann–Whitney U-test; $P > 0.05$ for Levin’s niche breadth [LNB]) in the 2 Leucoraja species. In contrast, the distribution of *P. purtoni* in *L. ocellata* is more posterior than in *L. erinacea* (Figs. 38, 39); however, it occupies similar niche sizes (t-test and Mann–Whitney U-test; $P > 0.05$ for LNB). *Pseudanthobothrium hanseni* occupies very different attachment sites in both its host species. It attaches primarily in the most anterior whorl in *M. senta*, whereas it has a broader distribution in *A. radiata* (Figs. 37, 40). This is reflected in the significant difference in niche breadth in the 2 hosts (t-test and Mann–Whitney U-test; $P < 0.001$). All LNB values are included in Table XVI, and frequency distribution of LNB values are illustrated in Figures 41, 42, 43, and 44. Niche breadth was greatest for *P. purtoni* and differed significantly (t-test and Mann–Whitney U-test) from that for *E. canadensis* ($P < 0.01$), *E. d. abyssorum* ($P < 0.001$), *E. vernetae* ($P < 0.001$), *P. hanseni* ($P < 0.001$), and *Z. kamienae* ($P < 0.001$). Niche breadth for *E. d. abyssorum*, *E. vernetae*, *P. purtoni*, and *P. hanseni* was found to be significantly dependent ($P < 0.01$) on intensity of infection ($r = 0.33, 0.75, 0.60, 0.49$, respectively), and the breadth generally extended posteriorly with greater intensities. Niche breadth for *Z. kamienae* was independent of intensity of infection, since all worms were recovered from the most anterior whorl.

Further, niche breadth comparisons between *P. purtoni* and *P. hanseni* (from *A. radiata*) showed that they were similar (t-test and Mann–Whitney U-test; $P > 0.05$). This comparison did not include specimens from *M. senta*, since attachment site of *P. hanseni* in this host species is clearly limited by incompatibility of villi and bothridia size posterior to the first whorl. Comparisons between *E. d. abyssorum* and *E. vernetae* revealed similarities in niche breadths for these 2 species ($P > 0.05$). Comparisons between *P. purtoni* and *E. vernetae*, *P. hanseni* (in *A. radiata*) and *E. vernetae*, *P. purtoni* and *E. d. abyssorum*,
and P. hansenii (in A. radiata) and E. d. abyssorum revealed significant differences in niche breadths (t-test and Mann–Whitney U-test; P < 0.001, P < 0.001, P < 0.001, P < 0.001, respectively).

A total of 9 different parasite pairs was recovered from various infracommunities: E. canadensis–P. hansenii (n = 1), E. d. abyssorum–P. hansenii (n = 14), E. d. abyssorum–Grillotia sp. (n = 3), E. vernetae–P. purtonii (n = 131), E. vernetae–Grillotia sp. (n = 5), P. purtoni–Grillotia sp. (n = 8), P. hansenii–Grillotia sp. (n = 4), P. hansenii–Z. kamieniae (n = 4), and Z. kamieniae–Grillotia sp. (n = 1). Renkonen’s niche overlap (RNO) values were low for most species pairs examined (<0.5) (Table XVII).

### Exposure of parasites to different solutions

Parasites used in this experiment included: 3 E. d. abyssorum from A. radiata; 4 E. vernetae from L. erinacea; 5 P. hansenii from M. senta and 24 from A. radiata; 99 P. purtoni from L. erinacea (plus 11 free proglottids); 14 from L. ocellata (plus 8 free proglottids); 20 Z. kamieniae from M. senta; 17 Trilocularia acanthias from Squalus acanthias (plus 190 free proglottids); and 19 Bothriocephalus sp. from Hemitripterus americanus (Table I). All worms survived the initial 3-hr exposure to elasmobranch saline and were then exposed to blood sera or other solutions. Disintegrating strobilae in blood sera were usually covered with a deposit, and no movement could be observed in either the scolex or strobilar regions. Dead worms in distilled water were not covered with such a deposit. Table I summarizes results of the in vitro experiments and provides the number of replicates. Exposure to “blood serum from the ‘wrong’ host” treatment caused significantly higher mortality than exposure to either “blood serum from the ‘right’ host” treatment or the control (ANOVA, P < 0.001). Furthermore, no significant differences in survival following exposure to the control solution, saline, and seawater (ANOVA, P > 0.05) were observed. However, the differences in survival were significant when comparing the latter 3 treatments to exposure with distilled water (ANOVA, P < 0.001). There were some cases of differential survival.

Bothriocephalus sp., a pseudophyllidean parasite commonly found in H. americus, was exposed to sera of H. americus (n = 2), L. erinacea (n = 3), L. ocellata (n = 2), and S. acanthias (n = 4) and to elasmobranch saline (n = 2), seawater (n = 2), saline (n = 2), and distilled water (n = 2). Survival was observed in all solutions at 6 hr postexposure (PE), except in distilled water, where death was observed at 4 hr PE. Some eggs were shed from all worms exposed to elasmobranch saline, seawater, and saline within 1 hr PE, whereas eggs retained within the worms turned black within 2 hr PE.

Echeneibothrium d. abyssorum, a tetraphyllidean parasite restricted to A. radiata, was exposed only to A. radiata (n = 1) and M. senta (n = 1) blood sera and elasmobranch saline (n = 1). Survival was observed in its natural host serum, in M. senta serum, and in elasmobranch saline at 6 hr PE. Survival in M. senta serum could be attributed to low amounts of serum available (0.25 ml vs. 0.75 ml).

Echeneibothrium vernetae, a parasite common to both L. erinacea and L. ocellata, was exposed to 3 solutions: elasmobranch saline (n = 1), L. erinacea (n = 1) blood serum, and A.
radiata (n = 2) blood serum. Survival was observed 6 hr PE to both elasmobranch saline and L. erinacea serum. Death occurred within 1 hr PE to A. radiata serum.

Survival of P. hansenii recovered from A. radiata was observed only in its own natural host blood serum (n = 4), elasmobranch saline (n = 1), seawater (n = 1), and saline (n = 1) (6 hr PE). Death occurred within 1 hr PE in all other solutions except for 1 of 4 worms exposed to M. senta blood serum, which died 8 hr PE.

Survival of P. hansenii recovered from M. senta was observed only in its natural host serum (n = 1), and elasmobranch saline (n = 1) (6 hr PE). Death occurred within 1 hr PE to blood serum from the 3 other rajid species. Due to the lack of available material, P. hansenii specimens from M. senta were not exposed to any of the other solutions.

Pseudanthobothrium purtoni, recovered from L. erinacea, died within 1 hr of being exposed to sera of S. acanthias (n = 26), H. americanus (n = 5), A. radiata (n = 7), and M. senta (n = 7), but survived 6 hr PE to L. erinacea (n = 8) serum. Differential survival was observed in L. ocellata serum (n = 11). In the first set of exposures, 4 of the 6 worms survived 6 hr PE, whereas the other 2 worms died within 1 hr PE. In the second set of exposures, all 5 worms died 6 hr PE. Survival for a period of 6 hr occurred when worms were exposed to elasmobranch saline (n = 15), seawater (n = 10), and saline (n = 5). However, worms stopped moving and were fully distended within 1 hr PE in distilled water (n = 5). Survival of all free proglottids was observed in L. erinacea (n = 2) and L. ocellata (n = 2) blood sera, as well as in elasmobranch saline (n = 2), seawater (n = 1), and saline (n = 2). However, all gravid proglottids in these solutions shed their oncospheres within 1 hr PE. None of the free proglottids survived in distilled water (n = 2). Due to unavailability of material, P. purtoni free proglottids were not exposed to the other solutions.

Exposure of P. purtoni, recovered from L. ocellata, to different solutions yielded similar results to P. purtoni recovered from L. erinacea, except that specimens from L. ocellata survived 6 hr PE in L. ocellata (its natural host) blood serum (n = 2), and differential survival was observed in L. erinacea blood serum (n = 4). In the first set of exposures, 1 of the 3 worms survived 6 hr of exposure to L. erinacea serum, whereas the other 2 died within 1 hr PE. In the second trial, 1 individual worm survived 6 hr of exposure to L. erinacea serum. Free proglottids of this species were exposed to the same solutions as free proglottids from the ones recovered from L. erinacea and yielded identical results to those described previously.

Trilocularia acanthiaevulgaris, a tetraphyllidean parasite recovered only from S. acanthias, survived only in its own host blood serum (n = 2). All worms died within 1 hr PE in sera from H. americanus (n = 2) and from the 4 rajid species. Survival for a period of 6 hr was also observed for worms exposed to elasmobranch saline (n = 2), seawater (n = 2), and saline...
However, worms stopped moving and were fully dis­tended within 1 hr after being exposed to distilled water (n = 2). The same results were observed when free proglottids of T. acanthias vulgaris were exposed to the same solutions. The only difference was that lysis occurred 4 hr PE in distilled water (n = 15).

Zyxibothrium kamienae from M. senta was exposed to sera from the 4 Rajidae species and to elasmobranch saline. The worms survived 6 hr PE in its own natural host blood serum (n = 4) and elasmobranch saline (n = 4). Death was observed within 1 hr PE in the other 3 blood sera.

**DISCUSSION**

Prior to work conducted in our laboratory, Echeneibothrium vernetae and Pseudanthobothrium hansenii were each reported from only 1 rajid host species (Randhawa et al., 2007, 2008). The expanded host range for E. vernetae, P. hansenii, and P. purtoni challenges the narrow specificity of tetraphyllideans from those 2 genera. Furthermore, the low abundance of E. canadensis (Table II; Keeling and Burt, 1996) may lead to an underestimation of the host range for this species, since over-estimating host specificity (restricted host range) due to low sampling effort or sample size (Poulin, 1992) would be more common in species with low prevalence and/or abundance of infection. Immature Echeneibothrium spp. cannot be identified unequivocally to species using morphometric data; therefore, they were not included in these analyses. Molecular identification of additional immature Echeneibothrium specimens using the D2 domain of the large subunit ribosomal DNA (LSU) (Randhawa et al., 2007) may increase the prevalence and/or abundance of infection of either one, or all 3, of E. canadensis, E. d. abyssorum, or E. vernetae.

Parasite species with a host range restricted to a single host species would score a value of “1” for 4 of the 5 quantitative host specificity indices used herein, i.e., Sf [frequency], S[ density], SrDS [density], and Sk*, and “0” for HS. Only 3 of the 6 tetraphyllidean species examined obtained those scores for the Sr [frequency] and Sk [density]: E. canadensis, E. d. abyssorum, and Z. kamienae. Scores for E. canadensis should not be accepted unequivocally since it exhibited low prevalence (6.5%) and abundance of infection (<0.1 per Amblyraja radiata examined). Host specificity tends to be overestimated in species with small sample sizes (Poulin, 1992); therefore, the specificity of E. canadensis, E. d. abyssorum, and Z. kamienae could be overestimated due to the small sample size of A. radiata, Leu­coraja ocellata, and Malacoraja senta (n = 31, 11, and 33, respectively). This is unlikely for P. purtoni since prevalence and abundance data in L. ocellata are similar to those observed in L. erinacea (t-test and Mann-Whitney U-test; P > 0.05). However, both these parameters are significantly lower (P < 0.01) for E. vernetae infecting L. ocellata compared to those infecting L. erinacea. Zyxibothrium is a monotypic genus that has only been recovered from M. senta, and its relatively high prevalence (48.5%), average intensity (6.7 per infected M. sen­ta), and abundance (2.4 per examined M. senta) of infection indicate that infections are not acquired accidentally and are not rare in M. senta. Average intensity and abundance of infection parameters reported herein cannot be compared statistically to those reported in Hayden and Campbell (1981) (7 per infected...
M. senta, and 4.8 per examined M. senta, respectively). However, the prevalence for this parasite (16 of 33 examined M. senta) is not significantly different (t-test; $P > 0.05$) from that reported in Hayden and Campbell (1981) (24 of 35 examined M. senta). These data indicate that plerocercoids are fairly common in this environment and are likely encountered by other sympatric rajid species, e.g., A. radiata (Packer et al., 2003a, 2003b). The $S_f$ [frequency] and $S_d$ [density] scores for E. vernetae overestimate specificity if immature specimens recovered from a third host species are considered, i.e., A. radiata (Randhawa et al., 2007), which represents a “true” final host for this parasite. However, as E. vernetae attaches in A. radiata (Randhawa et al., 2007) initially, but does not establish as mature worms, the $S_f$ [frequency] and $S_d$ [density] values are considered to reflect the adult specificity of this parasite. However, had immature E. vernetae been easily identifiable in L. erinacea and L. ocellata, both $S_f$ values would likely have scored higher. Values for both $S_f$ [frequency] and $S_d$ [density], calculated for Grillotia sp., are 0.83. Considering that this parasite infects 4 different host species in the study area examined in this study, this result highlights 2 weaknesses of these indices: (1) similar scores for parasites using different numbers of host do not necessarily reflect, accurately, patterns of host utilization (Poulin, 1998); and (2) they do not consider taxonomic distances between host species. Rohde and Rohde (2005) proposed a modification that would increase sensitivity of the indices to the number of host species infected. However, this modification assumes that values for prevalence and abundance are approximately the same (Rohde and Rohde, 2005). In the present study, these data are significantly different ($P > 0.05$); therefore, the proposed modification would not fulfill its proposed role. Caira et al.’s (2003) HS index and Poulin and Mouillot’s (2005) STD index were proposed to consider the taxonomic distance between the hosts utilized by the parasite. However, neither is sensitive to unevenness in the distribution of hosts at higher taxonomic levels (Rohde and Rohde, 2005). Poulin and Mouillot (2003) proposed to measure the variance in host distribution ($\text{Var}_{STD}$), which can only be calculated when a parasite species is recovered from more than 2 host species as adults (Poulin and Mouillot, 2003; Krasnov et al., 2006), a criterion fulfilled only by Grillotia sp. Rohde and Rohde (2005) stated that even with $\text{Var}_{STD}$, the shortcoming of the STD index is the lack of consideration given to host utilization. The STD* index was proposed by Poulin and Mouillot (2005), and it considers host utilization and taxonomic relatedness of the hosts in measuring specificity. Unfortunately, the only available rajid phylogeny (McEachran and Dunn, 1998) was done at the generic level; therefore, our STD and STD* results may have been different if we had access to a rajid skate phylogeny at the species level. However, even by dividing taxonomic ranks into subtaxonomic ranks, e.g., tribe, subfamily, etc., we obtain a $S_{TD}$* value of 2.07 (vs. 1.87) for Grillotia sp., while that of P. hansenii remains.
Table XIV. Comparison of bothridial measurements (mean and Mann-Whitney U-test, respectively) of *Echinobothrium dabinii* abdonlyi, *E. vernene*, *Pseudanthobothrium hanseni*, and *P. parvum*. NS, non-significant differences are indicated by *ns*. **Shading indicates suitable attachment sites.**

<table>
<thead>
<tr>
<th>Species</th>
<th>L × L (w × w)</th>
<th>W × W</th>
<th>ns</th>
<th>P</th>
<th>ns</th>
<th>P</th>
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<tr>
<td><em>E. d. abyssorum</em></td>
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<tr>
<td><em>E. vernene</em></td>
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<tr>
<td><em>Pseudanthobothrium hanseni</em></td>
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<td></td>
</tr>
<tr>
<td><em>P. parvum</em></td>
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</table>

**A comparison of bothridial to villar measurements does not provide evidence that host specificity for the 6 tetraphyllidean species examined is due to morphological factors. The spiral valves of all 4 rajid skate species examined are characterized by villar projections and the absence of crypts and ridges. Williams (1960) compared the mucosal topography of the spiral valves of *R. montagui* (characterized by the absence of villi and the presence of a complicated network of folded ridges), *R. clavata* (characterized by the absence of villi and the presence of a fine network of ridges with tubular crypts), and *L. naevus* (characterized by the presence of villi). Williams (1966) described the attachment organs of *Echeneibotheirium* and *Pseudanthobothrium* spp. and classified them on the basis of their modes of attachment, i.e., adapted to attach to villi or between ridges. Williams (1968a) described the mode of attachment of species of *Phylobothrium* in relation to the topography of the gut mucosa of elasmobranch species they infect and concluded that there is a correlation between the mucosal folds (and villi) of the spiral valve and the parasites they harbor. Williams (1968b) described a new species of *Phylobothrium* and its mode of attachment in *L. naevus* and concluded that this species possesses bothridia, and each bothridium is capable of attaching to up to 12 villi simultaneously. It is, therefore, possible that the 6 tetraphyllidean species examined herein possess bothridia adapted to attach to villi and would be unable to attach in species that have gut mucosa to which they harbor. Williams (1968b) described the modes of attachment, i.e., adapted to attach to villi or between ridges. Furthermore, as stated already, villi from all 4 host species are compatible with all echeneibothriines studied herein, and they thereby provide suitable attachment sites. Consequently, factors other than scolex adaptations to host mucosa are involved in the specificity of echeneibothrine tetraphyllideans. Host specificity may have a morphological determinant, but not for the hosts examined here.

Contrary to Williams (1966), we found no evidence of an inverse relationship between depth of villi and number of loculi per bothridium. According to Williams (1966), we would expect the species with more loculi to be found more posterior location than those with fewer loculi, since villar length decreases posteriorly in the spiral valves of all 4 rajid species examined (Table V). All tetraphyllidean species reported in this
FIGURE 39. Distribution of cestode parasites infecting the spiral intestine of *Leucoraja ocellata*.

TABLE XV. Comparison of bothridial measurements (t-test and Mann-Whitney U-test, respectively) of *Echeneibothrium dubium abyssorum*, *E. vernetae*, *Pseudanthobothrium hanseni*, and *P. purtoni*, with villar measurements from all whorls from *Malacoraja senta*. Statistical significance is set to $P < 0.05$. Significant differences are indicated by an asterisk (*), and nonsignificant differences are identified by "ns." Shading indicates suitable attachment sites.

<table>
<thead>
<tr>
<th>Comp.†</th>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
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<tr>
<td><em>Echeneibothrium dubium abyssorum</em></td>
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<tr>
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<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.05^*$</td>
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</tr>
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<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.01^*$</td>
<td>$P &lt; 0.001^*$</td>
<td>ns</td>
</tr>
<tr>
<td><em>E. vernetae</em></td>
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</tr>
<tr>
<td>$L \times L$</td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001^*$</td>
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<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.01^*$</td>
<td>$P &lt; 0.001^*$</td>
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<tr>
<td><em>Pseudanthobothrium hanseni</em></td>
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<tr>
<td>$L \times W$</td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001^*$</td>
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<tr>
<td>$W \times W$</td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001^*$</td>
<td>ns</td>
<td>$P &lt; 0.01^*$</td>
<td>$P &lt; 0.001^*$</td>
</tr>
<tr>
<td><em>P. purtoni</em></td>
<td></td>
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<tr>
<td>$L \times W$</td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001^*$</td>
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<td>$P &lt; 0.001^*$</td>
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<tr>
<td>$W \times W$</td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001^*$</td>
</tr>
</tbody>
</table>

† Comp. indicates the type of bothridial and villar comparison, reflecting different mode of attachment of the different parasites. $L \times L$ indicates bothridial length compared with villar length. $W \times W$ indicates bothridial width compared with villar width. $L \times W$ indicates bothridial length compared with villar width.
study showed a preference for the anterior portion of the spiral valve, regardless of number of loculi (see Figs. 37–40).

Additionally, *E. canadensis* and *E. vernetae* possess a type I myzorhynchus, and we found no evidence of an inverse relationship between size of the myzorhynchus (measurements for *E. canadensis* taken from only 2 specimens) and number of loculi (12 vs. 8, respectively), as suggested in Williams (1966). However, too few measurements were included from *E. canadensis* to support or disprove Williams’ (1966) hypothesis of an inverse relationship between myzorhynchus size and number of loculi per bothridia.

The myzorhynchus is a contractile organ (Linton, 1889; Southwell, 1925; Baer, 1956; Williams, 1966; Euzet, 1994); therefore, length and width cannot be used to assess robustness of this organ. Williams (1966) described the strength of the myzorhynchus as increasing with size. However, if the volume of the myzorhynchus is used, calculated as the volume of a cylinder, i.e., \( \pi r^2 h \), and using either the minima or the maxima for length and width of the myzorhynchus, there is still no evidence of the myzorhynchus of *E. vernetae* being more robust than that of *E. canadensis*. Campbell (1977) described the myzorhynchus of *E. d. abyssorum* as subspherical (160–270 μm

<table>
<thead>
<tr>
<th>Tetraphyllidean</th>
<th>Rajid host</th>
<th>LNB</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Echeneibothisium canadensis</em></td>
<td><em>Amblyraja radiata</em></td>
<td>1.00 ± 0.00 (1)</td>
</tr>
<tr>
<td><em>E. dubium abyssorum</em></td>
<td><em>A. radiata</em></td>
<td>1.57 ± 0.88 (1–3.60)</td>
</tr>
<tr>
<td><em>E. vernetae</em></td>
<td><em>Leucoraja erinacea</em></td>
<td>1.70 ± 0.83 (1–6.03)</td>
</tr>
<tr>
<td><em>L. ocellata</em></td>
<td><em>A. radiata</em></td>
<td>2.04 ± 1.07 (1.28 or 2.79)</td>
</tr>
<tr>
<td><em>Pseudanthobothrium hansenii</em></td>
<td><em>Malacoraja senta</em></td>
<td>2.96 ± 1.43 (1–5.44)</td>
</tr>
<tr>
<td><em>P. purtoni</em></td>
<td><em>L. erinacea</em></td>
<td>2.06 ± 0.17 (1–1.55)</td>
</tr>
<tr>
<td><em>Z. kamienae</em></td>
<td><em>L. ocellata</em></td>
<td>3.41 ± 1.34 (1–6.37)</td>
</tr>
<tr>
<td></td>
<td><em>M. senta</em></td>
<td>3.61 ± 1.55 (1–6.25)</td>
</tr>
</tbody>
</table>

Table XVI. Levin’s niche breadth (LNB) values (mean ± standard deviation, and range) shown for each tetraphyllidean from individual host species.
by 170–260 µm), which corresponds to a type I myzorhynchus. However, measurements taken from specimens collected during this study (n = 14) show the myzorhynchus to be elongate and cylindrical (200 to 465 µm long by 95 to 190 µm wide), which corresponds to a type II myzorhynchus. *Echeneibothisrium d. abyssorum* is described as a subspecies of *E. dubium* (Campbell, 1977). Furthermore, according to Williams (1966), *E. dubium* possesses a type II myzorhynchus. We, therefore, consider *E. d. abyssorum* to possess a type II myzorhynchus. With respect to *E. d. abyssorum* (10 loculi), *P. hansenii* (unloculated), and *P. purtonii* (unloculated), which all exhibit type II myzorhynchi, the mean length of the myzorhynchus of *E. d. abyssorum* (Campbell, 1977) is not significantly different than that for either *P. hansenii* (H. Randhawa, pers. obs.) or *P. purtonii* (H. Randhawa, pers. obs.) (P > 0.05). Therefore, Williams’ (1966) hypothesis—for species possessing type II myzorhynchi, the length of the myzorhynchus is inversely proportional to number of loculi—cannot be confirmed or refuted based on the data presented herein.

Attachment site data for the 6 tetraphyllideans examined are consistent with those reported previously for tetraphyllideans (Williams, 1961, 1968b; Rees and Williams, 1965; Carvajal and Dailey, 1975; McVicar, 1979; Cislo and Caira, 1993; Curran and Caira, 1995) and are further evidence in support of a morphological component to attachment site specificity of tetraphyllideans (Williams, 1961, 1968b; Rees and Williams, 1965; Carvajal and Dailey, 1975; McVicar, 1979). Furthermore, consistent with other studies on the attachment site specificity of tetraphyllideans, the posterior region of the spiral valve was almost always devoid of worms in all 4 rajid species (Williams, 1961; Carvajal and Dailey, 1975; McVicar, 1979; Cislo and Caira, 1993; Curran and Caira, 1995; Alarcos et al., 2006), even if the topography of the mucosa was suitable for attachment. The absence of tetraphyllideans from the posterior region of the spiral valve has been attributed to nutrient availability being greater in the anterior portion of the spiral valve (Williams, 1961; McVicar, 1979; Cislo and Caira, 1993; Curran and Caira, 1995). *Echeneibothisrium* spp. exhibit some sort of countertransport mechanism for maltose (McVicar, 1979), a disaccharide absorbed further down the length of the spiral valve (Read, 1957; Laurie, 1961; Carlisky and Huang, 1962); this observed pattern of parasite distribution should not be surprising. *Echeneibothisrium* spp. exhibit some sort of countertransport mechanism for maltose (McVicar, 1979), a disaccharide absorbed further down the length of the spiral valve (Read, 1957; Laurie, 1961; Carlisky and Huang, 1962); this observed pattern of parasite distribution should not be surprising. *Echeneibothisrium* spp. exhibit some sort of countertransport mechanism for maltose (McVicar, 1979), a disaccharide absorbed further down the length of the spiral valve (Read, 1957; Laurie, 1961; Carlisky and Huang, 1962); this observed pattern of parasite distribution should not be surprising.
explain the presence of *P. hanseni* and *P. purtoni* in the anterior whorls of the spiral valve. The presence of *Z. kamienae* in the anterior portion of the spiral valve of *M. senta* suggests that this tetraphyllidean has an affinity for glucose. However, the nutrient absorption in *M. senta* and absorption of sugars in *Z. kamienae* have not been studied; therefore, this hypothesis should not be accepted without question. *Phyllobothrium piriei*, a cestode known to infect *L. naevus* from the northeastern Atlantic (Williams, 1968a; McVicar, 1972, 1977, 1979), has a high affinity for maltose (McVicar, 1979); thus, it is not surprising that its preferred habitat is the posterior half of the spiral valve (Williams, 1968a). The nutrient requirements of tetraphyllideans have probably been the driving force behind the morphological adaptations that have led to attachment site specificity.

Closely related parasite species are expected to exploit similar habitats and resources in closely related host species (Cislo and Caira, 1993; Alarcos et al., 2006). Our data support parasite phylogenetics as a determinant of attachment site specificity: (1) niche breadth for *P. purtoni* and *P. hanseni* is similar; (2) niche breadth for *E. abyssorum* and *E. vernetae* is similar; and (3) all comparisons of niche breadth between any *Pseudanthobothrium* species and *Echeneibothrium* species reveal significant differences. However, the exact phylogenetic relationships within individual genera of the Phyllobothriidae have not been studied, and it has been suggested that *Pseudanthobothrium* is paraphyletic (Caira et al., 2001). An investigation into the interspecific relationships within *Echeneibothrium* and *Pseudanthobothrium* may provide insights into the exact nature of the relationship between phylogenetics and attachment site specificity.

Selective site segregation, where evolutionary competitive pressures lead to narrow niche selection, has also been suggested as an explanation for the differing attachment sites of tetraphyllideans (Friggens and Brown, 2005). These results were based on an analysis of 2 skates belonging to the same order but different families (FishBase web database: www.fishbase.org). Even though they are consistent with Friggens and Brown (2005) and Alarcos et al. (2006), our data reveal little niche overlap between tetraphyllideans infecting the same host species, and so selective site segregation should not be unequivocally accepted as a determinant of attachment site specificity until further studies are conducted on congeners, or at least species belonging to the same tribe of rajid skates. Furthermore, the high Renkonen scores obtained for the *E. canadensis–P. hanseni*, *Z. kamienae–Grillotia* sp., and *Z. kamienae–P. hanseni* species pairs (Table XVII) should not be accepted without question. Low prevalence of these species pairs could overestimate niche overlap. Additional sampling of *A. radiata* and *M. senta* is necessary to verify this trend. However, it is likely that the *Z. kamienae–P. hanseni* species pair overlaps in their niche distributions since *Z. kamienae* was only recovered

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![Graph](image-url)

**Figure 42.** Frequency distribution of Levin’s niche breadth values for tetraphyllideans infecting the spiral intestine of *Leucoraja erinacea.*
from the first whorl and *P. hanseni* could only physically attach to villi in the anterior-most whorl in *M. senta*.

The exposure of 7 species of cestodes to different blood sera and solutions was an extension of the experiment conducted by McVicar and Fletcher (1970). They concluded that the tetraphyllidean *Acanthobothrium quadripartitum* survived when exposed to blood serum from *L. naevus*, its natural host (Williams, 1968c), but it did not survive in blood serum from *A. radiata*, a host from which the parasite has not been recovered (Williams, 1969). Death of the parasite was attributable to serum factors described as components of complement and natural antibodies (McVicar and Fletcher, 1970). Results described herein are generally consistent with those of McVicar and Fletcher (1970). However, the survival of *E. d. abyssorum* in *M. senta* blood serum, an atypical host for this parasite, may be explained by exposure of the parasite to a lesser quantity of blood serum (0.25 ml vs. 0.75 ml). Dilution can affect the activity of blood components found in the serum (McVicar and Fletcher, 1970). Although blood serum was not diluted herein, exposure to one-third volume of blood serum would represent an exposure to one-third of the blood components found in the other serum exposures, which may not be enough to kill the parasite. Differential survival of *P. hanseni*, a parasite reported in *A. radiata* (Baer, 1956; Williams, 1966) and in *M. senta* (Randhawa et al., 2007, 2008), in serum from *A. radiata* and *M. senta* may be explained by phenotypical differences or plasticity, where a given phenotype may be better adapted to blood serum components of one host rather than the other, assuming there is a difference in blood components between both host species. The same mechanisms may explain the differential survival of *P. purtoni*, a parasite reported from *L. erinacea* and *L. ocellata*, in *L. erinacea* and *L. ocellata* blood serum. Length of exposure is not a factor considered in relation to differential survival (*P* < 0.001). Lengthy exposure to blood serum without providing nutrients and where metabolic wastes accumulate could lead to death explained by lack of resources or exposure to its own waste. However, all worms exposed to elasmobranch saline, under the same conditions, survived exposures for the same length of time as those exposed to blood sera that eventually died following exposures of 6 hr. Survival of *Bothriocephalus* sp. in all solutions, regardless of length of exposure, can be explained by its size in relation to volume of solution to which it was exposed. *Bothriocephalus* sp. is a large cestode, 10 to 100 times longer and wider than the tetraphyllideans used herein (H. Randhawa, pers. obs.). The effect of exposing a larger worm to the same volume of solution would have a similar effect to diluting these solutions, therefore increasing survival of the parasite (see McVicar and Fletcher, 1970).

Although tetraphyllideans live in the spiral valve, they are exposed to blood and its components. Tetraphyllideans with type II myzorhynchi possess a myzorhynchus that penetrates the wall of the villus (Williams, 1966; McVicar, 1972), whereas

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**Figure 43.** Frequency distribution of Levin's niche breadth values for tetraphyllideans infecting the spiral intestine of *Leucoraja ocellata*.  

<table>
<thead>
<tr>
<th>Index value</th>
<th>Proportion of hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1.99</td>
<td>0.1</td>
</tr>
<tr>
<td>2-2.99</td>
<td>0.2</td>
</tr>
<tr>
<td>3-3.99</td>
<td>0.3</td>
</tr>
<tr>
<td>4-4.99</td>
<td>0.4</td>
</tr>
<tr>
<td>5-5.99</td>
<td>0.5</td>
</tr>
<tr>
<td>6-6.99</td>
<td>0.6</td>
</tr>
</tbody>
</table>

- *Pseudanthobothrium purtoni*  
- *Echeneibothrium vemetae*
those with type I myzorhynchi erode tissue between the crypts and ridges of the spiral valve mucosa using their myzorhynchus (Williams, 1966), which causes considerable host-tissue reactions at the site of attachment of the parasite (Williams, 1966). Damage to the spiral valve mucosa has been observed at the attachment site of E. vernetae, a species with a type I myzorhynchus, in L. erinacea (H. Randhawa, pers. obs.) but has not been characterized.

By applying Euzet and Combes’ (1980) concept of filters to define the host range of tetraphyllideans from the present study, we can determine the different parameters or determinants involved in the specificity of these parasites. All 4 rajid skate species are sympatric (McEachran and Musick, 1975; McEachran et al., 1976) and, therefore, are likely to encounter the larvae of all 6 tetraphyllidean species discussed herein (biodiversity parameter). However, L. erinacea and L. ocellata prefer sandy and gravely bottoms (Packer et al., 2003c, 2003d), whereas M. senta shows a strong preference for soft muddy substrate (Packer et al., 2003a). Although A. radiata shows little preference for habitat (Packer et al., 2003b), it is positively associated with M. senta (McEachran and Musick, 1975; McEachran et al., 1976). Each substrate type harbors its own corresponding biota, and this may explain, at least in part, some of the dietary differences between the rajid species (McEachran et al., 1976; Packer et al., 2003a, 2003b, 2003c, 2003d). The different substrate preferences and corresponding prey biota represent the behavior parameter. Additionally, from results presented herein, all 4 rajid host species have spiral valve mucosal topography characterized by the presence of well-developed villi. All 6 tetraphyllidean species examined herein are adapted to attach to villi. Bothridial measurements (Table IV) correspond to villar measurements (Table V); therefore, all 4 rajid skate species provide suitable attachment sites for the parasites (Tables XI–XV). Furthermore, based on the presence of glucose

### Table XVII. Niche overlap (Renkonen’s index) between pairs of cestode species recovered from all 4 rajid hosts examined in this study. Values represent means ± standard deviation (maximum).

<table>
<thead>
<tr>
<th>Cestode Species</th>
<th>Grillotia sp.</th>
<th>E. dubium abyssorum</th>
<th>E. vernetae</th>
<th>Z. kamienae</th>
<th>E. canadensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grillotia sp.</td>
<td>0.19 ± 0.15 (0.34)</td>
<td>0.24 ± 0.21 (0.75)</td>
<td>0.56 ± 0.51 (1.00)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>P. purtoni</td>
<td>0.24 ± 0.38 (1.00)</td>
<td>0.31 ± 0.23 (1.00)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudanthobothrium hansenii</td>
<td>Grillotia sp.</td>
<td>0.10 ± 0.17 (0.30)</td>
<td>0.51 ± 0.38 (1.00)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>P. purtoni</td>
<td>0.24 ± 0.38 (1.00)</td>
<td>0.31 ± 0.23 (1.00)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
and maltose in the elasmobranch spiral valve (Laurie, 1961) and the affinity of *Echeneiboethrium* spp. to glucose (McVicar, 1979), elasmobranchs provide the adequate nutritional requirements for the tetraphyllideans examined. The attachment and nutritional requirements represent the resource parameter. Finally, the results from the in vitro experiments in which live tetraphyllideans were exposed to different blood sera and solutions are consistent with an immune response to the parasite, which therefore represents the defense parameter. There is no evidence of a biodiversity or resource parameter restricting the host range of these parasites in this system.

In summary, the 6 tetraphyllidean species examined exhibit host specificity of varying degrees. A specificity index incorporating both parasite ecology data, e.g., abundance of infection and taxonomic relatedness (including branch length data) of the host species infected with a given parasite, would provide a more accurate measure of host specificity. The adaptation of bothridia to host spiral valve mucosa topography does not seem to play a role in the host specificity of parasites examined in this study. However, while attachment site specificity of tetraphyllideans is, at least in part, phylogenetically determined, bothridial adaptations to specific niches may be driven by nutrient requirements, where tetraphyllideans requiring glucose attach in the anterior portion of the spiral valve and worms with disaccharide nutritional requirements attach more posteriorly, and each has attachment organs adapted to the site at which they attach, i.e., attachment site specificity. The results of exposure of live tetraphyllideans to different blood sera and solutions support the hypothesis that a complement and/or antibody-mediated mechanism is involved in the host specificity of tetraphyllideans. However, the exact identity of the component(s) involved has not been elucidated.

**ACKNOWLEDGMENTS**

The authors are grateful to staff of the Huntsman Marine Science Centre (HMSC): F Purton for his professionalism and availability; D. Parker for his technical assistance and availability; Peter Rose and Jeff Markey for assistance with collection of specimens; and E. Carter who went beyond the call of duty as captain of the R/V *W. B. Scott*. The help of D. Loveless and W. Minor, mate/engineer and captain of the CCGS *Pandalus III*, respectively, in collections conducted by Department of Fisheries and Oceans Canada (DFO) personnel is also gratefully acknowledged. We thank S. Belfry of the Electron Microscopy Unit at the University of New Brunswick, who was instrumental in teaching the senior author the essentials of scanning electron microscopy and who assisted in the preparation of the material. The senior author is grateful for the support, encouragement, and advice of his co-supervisor; Dr. G. W. Saunders. The diligence and helpful comments of Dr. S. B. Heard contributed to vastly improve this manuscript. The HMSC and the Department of Biology at the University of New Brunswick (UNB) provided laboratory space and other research facilities.

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**LITERATURE CITED**


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ANALYSIS OF TRANSCRIPTS FROM INTRACELLULAR STAGES OF EIMERIA ACERVULINA USING EXPRESSED SEQUENCE TAGS

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ABSTRACT: Coccidiosis in chickens is caused by 7 species of Eimeria. Even though coccidiosis is a complex disease that can be caused by any combination of these species, most of the molecular research concerning chicken coccidiosis has been limited to Eimeria tenella. The present study describes the first large-scale analysis of expressed sequence tags (ESTs) generated primarily from second-stage merozoites (and schizonts) of E. acervulina. In total, 1,847 ESTs were sequenced; these represent 1,026 unique sequences. Approximately half of the ESTs encode proteins of unknown function, or hypothetical proteins. Twenty-nine percent of the E. acervulina ESTs share significant sequence identity with sequences in the E. tenella genome. Additionally, EST hits seem to be much different compared with those of E. tenella. One of the differences is the very low number of ESTs that encode putative microneme proteins. This study underlines the potential differences in the molecular aspects of 2 Eimeria species that in the past were thought to be highly similar in nature.

Coccidiosis in chickens is caused by 7 species of Eimeria. This complex disease is of major economic importance to poultry producers in the United States and worldwide (Allen and Fetterer, 2002; Williams, 2002). Currently, coccidiosis is treated predominantly with vaccines containing live (attenuated or virulent) oocysts derived from several Eimeria species (Vermeulen et al., 2001; Chapman et al., 2002). The use of chemotherapeutic agents represents the other predominant method of treating coccidiosis; however, drug resistance, cost of new drug development, and changes in laws regulating drug use (especially in Europe) have led to the increasing popularity of using vaccines to counter coccidiosis (Jenkins, 2001; Blake et al., 2006; Williams, 2006a). Because of the relatively high cost of producing live oocyst vaccines, and the need for alternative control measures, there has been some effort to produce a recombinant subunit vaccine. These efforts have not been successful (Jenkins, 2001). The difficulty in producing a subunit vaccine lies in identification of cross-reactive antigens that are also immunoprotective (Jenkins, 2001; Blake et al., 2006). The discovery of these antigens has been partially hampered due to the fact that the bulk of research concerning gene and antigen discovery using expressed sequence tag (EST) analysis, genomics, and proteomics has been limited to a single Eimeria species, i.e., Eimeria tenella (Li et al., 2003; Refeaga et al., 2003; Miska et al., 2004; de Venevelles et al., 2006). The wealth of information concerning the molecular characteristics of E. tenella is not surprising as recovery of this parasite is relatively easy because it infects the ceca. However, it has also been reported that E. tenella seems not to be as immunogenic as E. maxima or E. acervulina (Rose and Long, 1962), and it may not be as predominant as either of those species in the field (Jenkins et al., 2006).

To add a comparative component into the gene discovery data, we carried out a study investigating a relatively large number of ESTs generated from second-stage merozoites and some schizonts of E. acervulina. This species is highly abundant, and it has been found in every field sample analyzed in Europe, and in the United States (Jenkins et al., 2006; Peek and Landman, 2006; Williams, 2006b). Recent reports have also found that drug resistance is common for E. acervulina (Peek and Landman, 2006; Williams, 2006b). The pathogenic effects of E. acervulina involve lesion formation in the duodenum and jejunum that result in weight loss and low feed conversion ratios (Long, 1973). Even though infections with E. acervulina typically do not result in mortality (Long, 1973), the parasite has a high reproductive capacity (Williams, 2001); therefore, high oocyst outputs are associated with E. acervulina infections. Because E. acervulina infects a different portion of the intestine than E. tenella, the recovery of the intracellular stages is difficult. This has led to a far larger body of molecular data being generated for E. tenella. For example, >34,000 ESTs from E. tenella have been deposited in the GenBank database, compared with 59 ESTs generated from E. acervulina. Analyzing ESTs from multiple species of Eimeria that are infectious to chickens is important because it will help identify genes and proteins whose sequence and expression are conserved. This analysis will help identify potential vaccine targets that are not only immunogenic but also cross-protective among Eimeria species.

MATERIALS AND METHODS

Host infection and parasite recovery

Chickens (Moyer’s Hatcheries Inc., Quakertown, Pennsylvania) were infected with 15 × 10⁵ E. acervulina oocysts, using gavage (Fetterer and Barfield, 2003). Infected birds were killed by cervical dislocation 89 hr postinfection (PI), and the duodenum and jejunum were removed. Isolation of E. acervulina merozoites was carried out as described previously (Xie et al., 1990; Miska et al., 2005). By microscopic examination, the preparation contained primarily merozoites with some schizonts. After isolation, the merozoites were pelleted and snap frozen at −70 C.

Recovery of RNA

Pelleted merozoites were resuspended in 10 ml of TRizol reagent (Invitrogen, Carlsbad, California), and then they were vortexed for 1 min, followed by incubation on ice for 1 min (4 times). The remainder of the total RNA isolation procedure was carried out using the manufacturer’s recommended protocol. RNA pellets were resuspended in DNase/RNase-free water (Invitrogen), and they were placed at −70 C until 5 mg of total RNA was obtained. Ethanol and 3.5 M NaAc were added to the RNA, and the samples were shipped on dry ice to the Ampliton Express facility (Pullman, Washington) for cDNA library construction.

cDNA library construction and screening

Briefly, mRNA was isolated from the total RNA, which was then reverse transcribed into cDNA using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, California). The E. acervulina merozoite-derived

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cDNA was cloned into the Uni-ZAP XR Vector (Stratagene) incorporating the EcoRI and XhoI cloning sites at the 5' and 3' end of the insert, respectively. A mass in vivo excision was carried out producing a pBluescript SK (−) plasmid library (Stratagene). The effective library titer was approximately 8 × 10⁸ colony-forming units/ml. The excised library was propagated in the SOLR E. coli strain, and stocks were frozen in Luria-Bertani (LB) medium containing 15% glycerol. The library was plated onto LB/Amp plates, and colonies were picked at random and grown overnight in LB/Amp medium. Plasmid DNA was purified using the QIAprep Spin Miniprep kit (QIAGEN, Valencia, California). By carrying out a restriction digest of 24 clones, it was estimated that the average insert size of this library was approximately 1.6 kilobases (kb). Clones were sequenced using universal M13 Forward and Reverse primers. All sequencing reactions were performed using the Big Dye sequencing kit version 3.1 (Applied Biosystems, Foster City, California) with nonisotopic dye terminators, and they were analyzed on an automated sequencer (3730xl DNA sequencer; Applied Biosystems).

Sequence analysis

All sequences generated were edited using the Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, Michigan). Vector sequences were located and trimmed from the 5' and 3' ends of each sequence. Additionally, any remaining vector sequences were identified by screening each sequence against the UniVec database (http://www.ncbi.nlm.nih.gov/VedicScreen/VedicScreen.html). Reliability of the sequences was ascertained manually, and any low-quality sequence was removed. To identify clones overlapping in sequence, each was compared against all other sequences using the "assemble contigs" function of Sequencher 4.7. All unique sequences were compared with the nonredundant protein sequence database (nr), and Swiss-Prot database using BLASTX algorithm (Altschul et al., 1990). BLASTX algorithm (Altschul et al., 1990) was used to search the dbEST database (Boguski et al., 1993). Finally, sequences were compared against the E. tenella genome assembly available at the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast?e.tenella/omni) using BLASTN. A match between sequences was designated as significant if the E value was lower than 10⁻³. All ESTs were submitted to the dbEST database under accession numbers EH385064–EH386434. All unique sequences were also deposited in a database located at http://129.24.144.92/et2/parsecity/zea.display.php, where results of each similarity search can be accessed directly.

RESULTS

Screening of cDNAs expressed by E. acervulina merozoites and schizonts

In total, 1,847 clones were sequenced from the E. acervulina cDNA library. From these, 1,426 high-quality sequences were obtained, representing 1,026 unique contiguous sequences (contigs); 205 contigs were composed of single pass sequences from multiple clones containing identical or partially overlapping sequences. A pdf file containing the overview of the similarity search analysis for each unique sequence generated in this study is available for download at http://129.24.144.92/et2/parsecity/zea.display.php, where results of each similarity search can be accessed directly.

Table I. Breakdown by functional category of E. acervulina contigs that share significant sequence identity with entries in the nr and/or Swiss-Prot databases (n = 408 contigs).

<table>
<thead>
<tr>
<th>Functional category</th>
<th>% of contigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothetical proteins of unknown function</td>
<td>22</td>
</tr>
<tr>
<td>Ribosomal proteins</td>
<td>15.7</td>
</tr>
<tr>
<td>Metabolism</td>
<td>15</td>
</tr>
<tr>
<td>DNA replication/transcription</td>
<td>10.5</td>
</tr>
<tr>
<td>Heat shock proteins/chaperonins</td>
<td>6.4</td>
</tr>
<tr>
<td>Transport</td>
<td>6.4</td>
</tr>
<tr>
<td>Translation</td>
<td>5.9</td>
</tr>
<tr>
<td>Structural</td>
<td>4.2</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>3.9</td>
</tr>
<tr>
<td>Various enzymes</td>
<td>3.5</td>
</tr>
<tr>
<td>Cell cycle control</td>
<td>2</td>
</tr>
<tr>
<td>Signaling</td>
<td>1.5</td>
</tr>
<tr>
<td>Redox</td>
<td>1.5</td>
</tr>
<tr>
<td>Surface antigens</td>
<td>0.7</td>
</tr>
<tr>
<td>Microneme proteins</td>
<td>0.5</td>
</tr>
</tbody>
</table>

To determine the type of cellular processes that E. acervulina merozoites may undergo, all contiguous sequences that shared significant sequence similarity with sequences in the nr and Swiss-Prot databases were converted into 15 functional categories that are shown in Table I. In total, 408 contigs were included in this analysis. Sequences matching hypothetical proteins of unknown functions that have been identified from other protists represent the most common classification. Sequences encoding ribosomal proteins represent the second most common classification. ESTs encoding proteins involved in metabolism, DNA replication, transcription, and transcript processing were also widely represented. Proteins that share homology with known chaperonins and other heat shock proteins represented 6.4% of the contigs in this study. Surprisingly, only 0.7 and 0.5% of E. acervulina contigs shared sequence homology with surface antigens and microneme proteins, respectively, found in other apicomplexans.

Most abundant ESTs

To determine which of the E. acervulina ESTs were most abundant, contigs containing the greatest number of overlapping sequences were examined (Table II). The most abundant contig (9) is made up of 16 ESTs and contains the full-length homolog of a protein designated SO7 described from E. tenella.
TABLE II. Eighteen contigs containing the highest number of overlapping ESTs.

<table>
<thead>
<tr>
<th>Contig no.</th>
<th>Putative ID</th>
<th>No. of ESTs</th>
<th>Length (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Unknown protein RB-1a (SO7)</td>
<td>16</td>
<td>1,732</td>
</tr>
<tr>
<td>15</td>
<td>Unknown</td>
<td>11</td>
<td>1,024</td>
</tr>
<tr>
<td>31</td>
<td>Heat shock protein 70 (Hsp70)</td>
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<td>77</td>
<td>α-Tubulin</td>
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<tr>
<td>6</td>
<td>Polypyrinquin</td>
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<td>18</td>
<td>Ribosomal protein L7a</td>
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<td>High mobility group protein</td>
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<td>127</td>
<td>Ribosomal protein S16</td>
<td>6</td>
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(Debe and Augustine, 1989; Liberator et al., 1989). Interestingly, another contig (9a) composed of 3 ESTs, spanning 1,045 nucleotides (nt), seems to also contain sequence that is very similar to that of contig 9; however, it is clear that these contigs were generated from 2 different transcripts. Four of the contigs shown in Table II encode proteins of unknown function, which does not share similarity to any sequences present in the 4 databases searched. Four of the highly abundant contigs encode ribosomal proteins; the other contigs had similarity to proteins associated with metabolism, translation, stress response, and structural elements of cells. These results are congruent with the overall classification of ESTs described above.

Results of querying dbEST

Of the E. acervulina ESTs described here, 287 shared significant identity with sequences in dbEST. The majority (64.5%) of these were most similar to ESTs generated from E. tenella. Three ESTs were identical in sequence to E. acervulina Open Reading Frame ESTs. An additional 10.5% of the ESTs were most similar in sequence to ESTs generated from other apicomplexans, whereas 16.7% were most similar to ESTs generated from other organisms, most commonly bacteria, plants, and vertebrates. Interestingly, 21 ESTs of E. acervulina identically matched ESTs from chickens. Two of these ESTs probably represent contamination of merozoite material with chicken tissue. However, 19 of these E. acervulina sequences were identical or highly similar to ESTs generated from a library made from intestinal intraepithelial lymphocytes, which were generated from chickens infected with E. acervulina and E. maxima (Min et al., 2005).

DISCUSSION

Because of the high proportion of unique genes present in apicomplexan parasites, screening of ESTs has proven to be a useful tool in gene discovery, expression profiling, comparative genomics, and protein function prediction (Wan et al., 1999; Li et al., 2003; Klotz et al., 2005; Boyle et al., 2006). The 1,426 sequences from E. acervulina presented here represent the first EST analysis from this species. The cDNA library from which these sequences are derived seems to be of high quality, with inserts measuring an average of 1.6 kb, almost half of which encode full-length ORFs. Additionally, the library is not highly redundant, with almost 80% of the clones representing unique sequences. By comparing the 1,026 unique sequences to 4 databases, it seems that most of the sequences encode novel proteins of unknown function. This in itself is not unexpected because similar results have been observed in other apicomplexans (Boyle et al., 2006). One of the surprising findings of this study has been the low percentage of sequences that are significantly similar to E. tenella genome and ESTs sequences. Approximately 34,000 ESTs from various stages of the E. tenella life cycle have been deposited in dbEST (Wan et al., 1999; Ng et al., 2002; Li et al., 2003). Only 29% of the unique E. acervulina sequences shared significant similarity with entries in dbEST, and 64.5% of these were most similar to E. tenella ESTs. This translates to only 185 E. acervulina sequences from the total of 1,026 unique contigs (or 18% of total sequences) matching an E. tenella sequence. The comparison of E. acervulina ESTs to the E. tenella genome resulted in less than half of the ESTs finding a match in the genome. This represents the first comparative molecular analysis of these 2 species, and it suggests that they may be divergent. However, it is also likely that many more homologs will be identified when the E. tenella genome assembly is completed and annotated. Additionally, the presence of introns made this comparison more difficult, because intervening sequences will lower the E values of BLAST search results. Therefore, it is likely that the result of this comparison is skewed in a negative manner.

In E. tenella merozoites, some of the most abundantly expressed transcripts (10–20% of all ESTs) encode microneme proteins, which play an important role in parasite invasion (Tomley and Soldati, 2001; Ng et al., 2002; Li et al., 2003), and they are limited to apicomplexans. In contrast, only 2 E. acervulina ESTs were found (which represents 0.002% of the total unique sequences reported) that encode homologs of E. tenella microneme 5 (Brown et al., 2000), and micronemal protein 4 from Toxoplasma gondii (Brech et al., 2001). This is interesting because these 2 proteins are the only Apple domain-containing microneme proteins that have been so far identified in these 2 taxa (Tomley and Soldati, 2001). It will be very interesting to further investigate the microneme proteins in E. acervulina to determine whether the repertoire of these proteins is limited, and whether the expression of these genes is significantly lower than observed in E. tenella, or whether these results were produced due to a bias in clone composition of the cDNA library.

Although ESTs encoding microneme proteins seem to be underrepresented in E. acervulina merozoites, the most abundant sequence found in this study encodes a homolog of SO7, an antigen that has been investigated as a possible cross-species protective vaccine candidate (Danforth and Augustine, 1989). This protein seems to also be associated with refractile bodies (RBs), which are organelles specific to the Eimeriidae whose function is unknown (de Venevelles et al., 2006). Interestingly, RBs are not likely to be present in second-stage merozoites.
(Hammond et al., 1970). However, the library from which these clones were isolated was collected mainly from second-generation merozoites; therefore, it will be interesting to investigate whether this protein has several functions and whether its expression profile is different in *E. acervulina* and *E. tenella*. Additionally, 2 unique sequences were identified that encode the *E. acervulina* SO7 homolog. These most likely represent a splice variant; however, it should also be investigated whether *E. acervulina* contains multiple copies of SO7 in its genome.

Altogether, we found that screening *E. acervulina* merozoite library yielded many novel genes for which homologous sequences have not yet been described in other apicomplexans. Also, EST encoding genes that are abundantly expressed in *E. tenella* merozoites, such as those encoding microneme proteins, are underrepresented in the *E. acervulina* library. The results of the present study indicate that potential molecular differences exist between these 2 seemingly similar species. Even though *E. tenella* is the most studied member of the species that cause poultry coccidiosis, it is imperative that multiple *Eimeria* species be investigated both for basic comparative analysis and for development of effective coccidiosis control. It is highly likely that at the molecular level, poultry *Eimeria* species are quite diverse and should not be treated as a single organism.

**ACKNOWLEDGMENTS**

We acknowledge the expert technical assistance of J. Herrmann, R. Barfield, and G. Wilkins. This work was supported by USDA–ARS CRIS project 1265-31320-070-00D. Support was also provided by the University of New Mexico’s Molecular Biology Facility, which is supported in part by NIH grant R22 RR18754 from the Institute Development Award (IDeA) Program of the National Center for Research Resources.

**LITERATURE CITED**


Williams, R. B. 2001. Quantification of the crowding effect during infections with the seven *Eimeria* species of the domesticated fowl:


GYMNOPHALLOIDES SEOI EGGS FROM THE STOOL OF A 17TH CENTURY FEMALE MUMMY FOUND IN HADONG, REPUBLIC OF KOREA

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Department of Parasitology, College of Medicine, Dankook University, Chonan 330-714, South Korea, e-mail: cyj@snu.ac.kr

ABSTRACT: It was previously reported that paleoparasitological clues for parasites infecting humans could be found in the feces of mummies of the Joseon Dynasty (1392–1910) in the Republic of Korea. Here, we report the presence of trematode eggs, including Clonorchis sinensis, Metagonimus yokogawai, and Gymnophalloides seoi (a human parasite known in Korea since 1993) in the feces of a recently excavated female mummy in Hadong, Republic of Korea. This is the first report of the discovery of a G. seoi infection in a human mummy. Since Hadong is currently not an endemic area for G. seoi, we speculate that the parasite might have occurred frequently along coastal areas of the Korean peninsula several hundred years ago and that the endemic areas contracted to, more or less, restricted regions since that time.

The database for paleoparasitological findings has been expanding for several decades. Researchers are starting to provide invaluable information on the past patterns of parasite infections (Aufderheide, 2003). As to paleoparasitological samples acquired in the Republic of Korea, we have previously shown that stools collected from mummies of the Joseon Dynasty (1392–1910) may become a good resource for studying parasites that infected medieval Korean people. For example, eggs of Trichuris trichiura, Ascaris lumbricoides, and Clonorchis sinensis were identified in a stool collected from a child mummy of Yangju, Korea (Seo et al., 2007). Since we now have access to a number of Korean mummies found in tombs with a lime-soil mixture barrier (LSMB), we can report additional cases that should be helpful in understanding past patterns of parasite infections in the medieval Korean society.

On 6 April 2006, a new female mummy was found within a medieval tomb possessing a LSMB. The tomb was located in Hadong, a coastal county where the Seomjin River flows into the South Sea of Korea (Fig. 1A, B). Archeologists have concluded that she lived at some point between the second half of the 16th century and the first half of the 17th century. Both the river and the sea are located within a 10-km radius from the region where she was buried (Fig. 1B); therefore, freshwater or marine fish, and other sorts of seafood, would have been available for consumption at that time.

Considering that this is the first mummy ever found in the coastal region of Korea, we expected that a paleoparasitological study might show patterns different from those of previously reported cases for inland counties (Table I). In addition, we also expected that the current mummy might be used for confirming a change in the prevalence of parasites during the past several hundred years, based on comparisons with recent national parasitological surveys (Korea Association of Health Promotion, 2004; Chai et al., 2006).

MATERIALS AND METHODS

The mummy

The mummy was found at a construction site for a thermoelectric power plant. As seen in previously reported mummy cases, the coffin was encapsulated by a LSMB (Fig. 2A). After the LSMB was broken, well-preserved double-layered coffins were exposed, in which the mummified female was found (Fig. 2B). The mummy was transferred to the Seoul National University for further scientific investigation. In the Department of Forensic Medicine, Seoul National University College of Medicine, we performed an autopsy (Fig. 2C); it was during the autopsy that feces were collected from the luminal surface of the rectum (Fig. 2D).

Parasitological investigations

The fecal samples recovered were rehydrated in 0.5% trisodium phosphate solution (van Cleave and Ross, 1947; Pike, 1968; Han et al., 2003; Reinhard and Urban, 2003). To ensure complete rehydration, the feces were totally immersed and shaken vigorously in the solution on a daily basis for 1 wk. The rehydrated samples were filtered through several layers of gauze, using the trisodium phosphate solution. After disintegration, the samples were let stand for 2 d for spontaneous sedimentation, and the upper turbid layer was discarded. The precipitate was dissolved in 10% neutral buffered formalin and then pipetted onto microscope slides. The slides were examined for the presence of helminth eggs using light microscopy (BH-2, Olympus, Tokyo, Japan). Eggs found were compared morphologically with those from contemporary samples. The contemporary egg samples were obtained from the distal part of the uteri of C. sinensis, M. yokogawai, and G. seoi adult worms, which had been harvested from human infections after anthelminthic treatment and purging. The number of eggs per g of feces (EPG) was calculated by multiplying 100 times the number of eggs counted in 10-μl fecal samples.

Examination of oysters

Naturally produced oysters, Crassostrea gigas, the second intermediate host for G. seoi (Lee et al., 1996), were generously provided by K. S. Kang (Fig. 1B) in a locality about 1 km from the site where the mummy was found. To confirm that the oysters were free from infection...
with G. seoi metacercariae, we examined them as previously described (Lee et al., 1996). Briefly, 132 oysters were collected and transported to the laboratory, and weighed after their shells were removed. The oysters were digested with artificial gastric juice (12 g pepsin and 16 g HCl in 2 L of distilled water) for 3–4 min. After washing 3 times with physiological saline, the presence or absence of G. seoi metacercariae in the sediment was determined using stereomicroscopy (SZ 3060, Olympus, Tokyo, Japan).

RESULTS

Using a low-magnification field, we were able to identify widely scattered eggs of C. sinensis, M. yokogawai, and G. seoi in fecal samples taken from the mummy. The egg sizes and EPGs are summarized in Table II. Gymnophalloides seoi eggs were the most common. No nematode or cestode eggs were detected.

Under higher magnification, typical morphological characteristics of each of the 3 trematode species could be identified. Thus, for example, eggs of C. sinensis were brown in color, ovoid, with musk melon pattern on the egg shell, and with a remarkable operculum and shoulder rim (Fig. 3). For M. yokogawai, the eggs were dark brown and elliptical; their operculum was less prominent than that of C. sinensis, and musk melon patterns were not observed on the eggshell (Fig. 3).

We also identified G. seoi eggs that exhibited typical char-

<table>
<thead>
<tr>
<th>Name of sample</th>
<th>Parasite eggs found</th>
<th>Investigating institution</th>
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<tbody>
<tr>
<td>Yangju child mummy</td>
<td>* Trichuris trichiura</td>
<td>Dankook University (2006)</td>
</tr>
<tr>
<td></td>
<td>Ascaris lumbricoides</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clonorchis sinensis</td>
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<tr>
<td>Hakbong mummy*</td>
<td>T. trichiura</td>
<td>Korea University (2004)</td>
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<tr>
<td></td>
<td>C. sinensis</td>
<td></td>
</tr>
<tr>
<td>Papyeong Youn mummy†</td>
<td>Nematode§</td>
<td>Korea University (2003)</td>
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<tr>
<td></td>
<td>A. lumbricoides</td>
<td></td>
</tr>
<tr>
<td>Chilgok-gun and Uljin-gun‡</td>
<td>T. trichiura</td>
<td>Seoul National University (2003)</td>
</tr>
<tr>
<td></td>
<td>A. lumbricoides</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. sinensis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown trematodes</td>
<td></td>
</tr>
</tbody>
</table>

* Official report has not been published. The finding was unofficially reported in YTN news, Korea, July 27, 2004.
† Official report was published in Korean, Papyeong Youn Ssi Mo Ja Mi Ra Jong Hap Youn Gu Non Mun Jip, Korea University Museum, 2003.
‡ Detection of parasite eggs in soil samples from archeological excavation sites (Han r, 2003).
§ The finding was observed by a scanning electron microscope.
characteristics, i.e., small, elliptical, transparent, and with a thin eggshell (Fig. 3). Though the larvae within the eggs were not well preserved compared to those from contemporary samples, the general morphology was well preserved.

Finally, no G. seoi metacercariae were found in the tissue digests of naturally produced oysters found close by the location of the mummy.

DISCUSSION

Although it is believed that the tradition of eating raw fish was not common in medieval Korea, we found that raw fish cuisine was enjoyed by the people of the Joseon Dynasty when we searched historical documents. According to “Sallim Gyongje” (Hong, 1718), a reference book for farm management, a recipe for making raw fish cuisine is described as follows: “a dish of raw fish could be prepared by removing tail, intestine, scales of the fish; drying upon the white paper; slicing like a thread; mixing with minced radish, mustard seed, red pepper, and vinegar.” In historical documents such as “Joseon Wangji Sillok” (Bureau of State Records 1392–1910) and “Seungjeongwon Ilgi” (Royal Secretariat 1623–1910) there are many descriptions referring to the people of the Joseon Dynasty as enjoying raw fish dishes. Considering these records, trematode infections observed in the present study should not be surprising.

In addition, as far as historical records are concerned, the species of trematode parasites identified in the present study could reflect the socio-cultural conditions in which the mumified woman was easily infected by these parasites. “Sinjeung Dongguk Yeoji Seungnam” (translated as “Newly Verified Survey of the Geography of Korea”) was published in Lee (1530) as a national survey resource book on human geography of the Joseon Dynasty, and included all of the staple products in a given locality. The specialties of Hadong County were described as follows, “Codfish, ear shell, octopus, sweetfish, shellfish, gudgeon, thornback, common octopus, a kind of herring, oyster, crab, . . . croaker, gray mullet, sea bass.” Interest-

<table>
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<th>Parasite species</th>
<th>Egg size (μm)</th>
<th>EPG*</th>
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<tr>
<td>Clonorchis sinensis</td>
<td>28.8 ± 1.3 × 15.9 ± 0.7 (n = 20)</td>
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<tr>
<td>Metagonimus yokogawai</td>
<td>30.8 ± 1.6 × 16.9 ± 0.9 (n = 20)</td>
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</tr>
<tr>
<td>Gymnophalloides seoi</td>
<td>20.0 ± 1.7 × 12.0 ± 0.7 (n = 20)</td>
<td>21,417</td>
</tr>
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</table>

* Number of eggs per g of feces. The values represent an average of 6 time measurements on each sample. In each measurement, the number of eggs present in 10 μl feces was multiplied by 100.
FIGURE 3. Eggs of *Clonorchis sinensis*, *Metagonimus yokogawai*, and *Gymnophalloides seoi* recovered in the mummy feces samples. The morphologies of eggs of each species are compared with those acquired from contemporary samples. In the case of *C. sinensis*, the operculum, shoulder rims, and muskmelon patterns on the eggshell could be clearly observed in both the mummy and contemporary samples. *Metagonimus yokogawai* eggs from the mummy and contemporary samples showed typical characteristics, including the presence of a less prominent operculum (arrows) without shoulder rims, and clean eggshell surface. *Gymnophalloides seoi* eggs from the mummy and contemporary samples showed typical characteristics, including the elliptical shape, and transparent and thin eggshell. Scale bars, 5 μm.

Interestingly, among these specialties in Hadong County, we could find all the intermediate hosts for the parasites that were observed in the feces of the mummy, i.e., *C. sinensis*, *M. yokogawai*, and *G. seoi* (Table III). This means that infection by trematode parasites identified in the present study could have been easily produced by gudgeon, sweetfish, and oysters, the intermediate hosts for *C. sinensis*, *M. yokogawai*, and *G. seoi*, respectively, in Hadong County during the Joseon Dynasty.

Since Hadong County is still noted to be an area of infection with *C. sinensis* and *M. yokogawai* (Fig. 4A, B), it would appear that the infection patterns of these 2 trematode parasites have not changed significantly in the last several hundred years in the Republic of Korea. However, the case with *G. seoi* is thought to be unique. Originally, *G. seoi* was reported from a Korean woman suffering from acute pancreatitis and gastrointestinal troubles who resided on a coastal island (Lee et al.,
1993). In a series of studies on this fluke species (Chai et al., 2003), the second immediate host, i.e., the oyster *C. gigas*, and the final hosts, i.e., humans and the oystercatcher *Haematopus ostralegus*, were identified. A nationwide survey revealed that human *G. seoi* infection was mainly distributed in 2 western coastal areas (Fig. 4C) of the Korean peninsula, excluding Hadong County, where the mummified woman was found (Chai et al., 2001). This suggests that the geographical distribution of *G. seoi* might have contracted from wider coastal areas of the Korean peninsula to a restricted region as shown in recent national surveys.

In paleoparasitological studies performed in the Republic of Korea, *G. seoi* infection was not found in samples from inland counties (Table I), but was identified for the first time in a coastal county. This suggests that the geographical distribution of *G. seoi* was restricted to the region in which fresh seafood could be easily supplied during the Joseon Dynasty. Historical documents described difficulties with transporting fresh fish to inland counties. From this, it was suggested that the nobility in the countryside were not wealthy enough to afford beef or pork, whereas people in the countryside did not. From this, it was suggested that the nobility in France ate undercooked or raw beef or pork, whereas people in the countryside did not. From this, it was suggested that the nobility in France ate undercooked or raw beef or pork, whereas people in the countryside did not.

*Clonorchis sinensis* exhibits a different geographical distribution pattern from *G. seoi*. As seen in Tables I and II, eggs of *C. sinensis* were identified in nearly all the samples from the counties where freshwater fish could be obtained from rivers. Since the intermediate hosts of this parasite are known to be freshwater fishes, people could be infected by *C. sinensis* even if they were living in inland counties. The possibility of these eggs being those of *Opisthorchis viverrini*, a closely related species existing in Thailand and Laos with similar egg morphology, is negligible, because *O. viverrini* has never been reported from the Republic of Korea.

In the case of soil-transmitted parasites such as *A. lumbricooides* and *T. trichiura*, previous cases have shown the presence of eggs from various regions of the Korean peninsula (Table I). Considering that these helminths were among the most frequently observed parasites in other countries (Bouchet et al., 2003), it is not unusual that the Korean people were commonly infected with these nematodes during the Joseon Dynasty. However, in the current study, we could not find nematode eggs in the samples. Since we could not be sure if the absence of nematode eggs really reflects the unique infection pattern of parasites among the people living in seashore counties such as Hadong, this question needs further attention.

Differences in paleoparasitological patterns in different population groups of the same age were reported by Matsui et al. (2003) and Bouchet et al. (2003). Matsui et al. (2003) observed that *Diphyllobothrium* spp. eggs were identified only in the samples from Yanagi-no-gosho Site in Japan, but not from nearby Akiita Castle. As raw cherry salmon is a part of Yanagi-no-gosho’s regionally famous local cuisine and easily caught from the nearby Kitakami River, consumption of this fish is likely to be the main cause of *Diphyllobothrium* spp. infection. The difference in the eating habit of this fish might explain the difference in the parasite’s presence or absence in the 2 adjacent locations. Similar differences in parasite species between population groups living in the same time period were also observed by Bouchet et al. (2003). According to them, fecal samples from wealthy homes of the French nobility typically exhibited *Taenia* sp. eggs while those from the surrounding countryside did not. From this, it was suggested that the nobility in France ate undercooked or raw beef or pork, whereas people in the countryside were not wealthy enough to afford beef or pork.
and would not, accordingly, be exposed to Trichuris solium or Trichuris saginata cysticerci (Bouchet et al., 2003). Taken together, all these studies, including the present one, could successfully show that paleoparasitological investigation can be performed not only for identifying the presence of specific types of parasites in very old samples, but can be used for reconstructing the historic interactions within ancient populations, as well as socio-cultural conditions that may have impacted their diets.

ACKNOWLEDGMENTS

M. Seo and D. H. Shin equally contributed as the first authors of this paper. All queries about the Korean mummies (including the current Hadong mummy case), except for those regarding parasitological examination, can be sent to D. H. Shin (drdoogi@snu.ac.kr). This study was conducted in compliance with The Vermillion Accord on Human Remains, World Archaeological Congress, Vermillion, South Dakota, 1989. We especially appreciate the generous decision of the descendants of the mummified person for permitting the scientific investigation on oysters in Hadong to us, so that our study could be successfully concluded. This study was supported by the grant No. 04-2006-081 from SNUH Research Fund.

LITERATURE CITED


ASEXUAL BLOOD STAGES OF *PLASMODIUM FALCIPARUM* EXHIBIT SIGNS OF SECONDARY NECROSIS, BUT NOT CLASSICAL APOPTOSIS AFTER EXPOSURE TO FEBRILE TEMPERATURE (40°C)

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**ABSTRACT:** It has been shown by others that after cultures of *Plasmodium falciparum* were exposed to a febrile temperature of 40°C, parasitemia was reduced in the subsequent generation, suggesting a temperature-induced inhibition of trophozoites and schizonts. In the current study, influences unique to cultivation were ruled out, demonstrating that 40°C impacted the parasites directly. Metabolic profiling of DNA synthesis, protein synthesis, and glucose utilization clearly indicated that febrile temperatures had a direct effect on parasite development, beginning 20–24 hr after erythrocyte invasion. The mechanism of parasite death was investigated for evidence of temperature-induced apoptosis. Lack of typical physiological hallmarks, namely, caspase activation, characteristic mitochondrial membrane potential changes, and DNA degradation as indicated by DNA laddering, eliminated ‘classical’ apoptosis as a mechanism of parasite death. Parasites dying under the influence of heat, staurosporine, and chloroquine initially appeared pyknotic by light and electron microscopy (as in apoptosis), but eventual swelling and lysis of the food vacuole membrane led to secondary necrosis. Chloroquine did induce DNA laddering, but it was later attributed to occult white blood cell contaminants. While not apoptosis, the results do not rule out other forms of temperature-induced programmed cell death.

In vitro experiments have demonstrated that when erythrocytic stages of *Plasmodium falciparum* were exposed to febrile temperatures of 40°C, the parasites did not show the expected increase in parasitemia in the subsequent generation. Furthermore, parasites appeared to be more affected by 40°C during the second half of their erythrocytic cycle (Kwiatkowski, 1989). These observations have been confirmed and expanded by Long et al. (2001) to show that parasites became susceptible to 40°C beginning 24 hr after parasite–erythrocyte invasion (24 hr postinvasion [PI]). Exposure to febrile temperatures apparently exerted a growth-limiting effect on the parasite, slowing an otherwise mounting parasitemia. Several mathematical models have suggested that fevers play an important function in limiting the rate of parasite reproduction during the erythrocytic cycle in humans, promoting host survival and maintaining a persistent parasitemia supporting mature gametocyte transmission (Gravenor and Kwiatkowski, 1998; Molineaux et al., 2001; Rouzine and McKenzie, 2003).

A number of protozoan parasites have been shown to exhibit apoptosis-like death as a means of self-limitation, or following exposure to drugs (Ameisen et al., 1995; Zangger et al., 2002). Evidence of an apoptosis-like process has also been reported in *Plasmodium berghei* oocokites during the mosquito stages of that parasite (Al-Olayan et al., 2002) and in the *P. falciparum* erythrocytic cycle after exposure to chloroquine (Picot et al., 1997). Furthermore, a recent report using microarray analysis in cultured *P. falciparum* has shown that mRNA profiles are significantly altered in parasites after exposure to 41°C, suggesting initiation of a temperature-induced programmed cell death (PCD), possibly apoptotic in nature (Oakley et al., 2007).

The purpose of the current investigation was to characterize the death process in *P. falciparum* during exposure to 40°C. We examined the possibility that parasite death initiated by 40°C in vitro might be due to the deleterious effect of heat on parameters unique to the culture system of *P. falciparum* such as the nutritional status of the culture medium, or the suitability of the cultured erythrocytes to facilitate merozoite invasion and support parasite development. Because parasite death, after exposure to 40°C, appeared to be stage-specific and self-limiting in nature, we examined the possibility that febrile temperatures might induce parasite death through apoptosis. We attempted to find evidence of apoptosis in *P. falciparum* during exposure to 40°C by characterizing visual changes as seen in Giemsa-stained thin films and electron microscopy, changes in mitochondrial membrane potential (Δψm), caspase activity, metabolic profiles, and determining status of parasite DNA by measurement of oligonucleosomal DNA fragmentation (DNA laddering). We also looked for evidence of apoptosis in parasites exposed to lethal doses of chloroquine and the protein kinase inhibitor staurosporine, a classical inducer of apoptosis in many different cell types.

**MATERIALS AND METHODS**

**Parasite culture**

*Plasmodium falciparum* strain CSC-1 (Honduras) was cultured using the Trager and Jensen (1976) method, with modification (Jensen, 2002). Erythrocytes were stored for 1–12 days at 4°C in the blood preservative CPDA-1. Culture media consisted of RPMI 1640 (16.2 g/L) supplemented with NaHCO3 (0.2% w/v), penicillin-streptomycin (18 units/ml; 180 µg/ml), and serum substitute (Albumax I [0.27% w/v]; hypoxanthine [70 µM]) (Invitrogen, Carlsbad, California). Parasites were cultured in modular incubation chambers (Billups-Rothenberg, Del Mar, California) having an atmosphere of 94% N2, 5% CO2, and 1% O2 at 2% hematocrit. Generally, parasites were incubated at 37°C except when subjected to febrile temperatures (40°C). In chloroquine experiments, stock solutions of chloroquine phosphate (Sigma, St. Louis, Missouri) were diluted into culture media to a final concentration of 100 µM. Comparisons were made between cultures fed with chloroquine-supplemented media or control. In staurosporine investigations, comparisons were made between identical cultures treated with staurosporine (Alexis Biochemicals, San Diego, California). (5 µM) or control media, each containing equal concentrations of the solvent dimethylsulfoxide (DMSO). In all growth curve experiments, parasitemia was estimated by [3H]-phenylalanine incorporation into parasite protein.

**Synchronization of cultures**

Parasites were synchronized to a 4 hr window using a combination of the gelatin separation (Jensen, 1978) and sorbitol (Vernes et al., 1984) techniques. Briefly, parasitized erythrocytes were suspended in a gelatin solution for 30 min at 37°C. Once the uninfected and ring-infected erythrocytes had settled, the mature-stage parasites were isolated from...
the supernatant by gentle centrifugation and allowed to invade fresh erythrocytes for 4 hr after which all trophozoite and schizont infected cells were lysed by sorbitol treatment, leaving highly synchronous ring-stage cultures (Jensen, 2002).

**Protein and DNA synthesis assays**

Microcultures of synchronized parasites were established in 96-well plates with a 4% hematocrit and <5% parasitemia. Beginning at 0 hr Pl and throughout the erythrocytic cycle, protein synthesis was determined in 6 hr increments during exposure to 40 C, staurosorpin, chloroquine, or control by incorporation of [1H]-phenylalanine (4 μCi/well; L-Phenylalanine-[Ring-2,6-3H(N)]; Sigma). Results were analyzed using scintillation spectrometry (Cell Harvester, Inotech Biosystems, Rockville, Maryland). DNA synthesis was measured using a similar protocol with the exceptions that [1H]-phenylalanine (4 μCi/well) (hypoxanthine-[2,8-3H]; Sigma) was used for incorporations and parasites were fed during the experiments with culture media containing minimal cold hypoxanthine (17 μM).

**Glucose assay**

Glucose consumption throughout synchronized parasite development was determined during exposure to 40 C, chloroquine, staurosorpin, or control. Culture medium was removed after treatment period and was assayed using the Glucose (HK) Assay Kit (Sigma) following the manufacturer’s instructions.

**Caspase activation assay**

Pan-caspase activation was assayed using the CaspaTag Flourescein Caspase (VAD) Activity Kit. Activity of specific caspases 8, 9, and 10 were determined using CaspaTag Caspase-8 (LETD) Activity Kit, CaspaTag Caspase-9 (LEHD) Activity Kit, or CaspaTag Caspase 10 (AEVD) Activity kit, respectively (Millipore, Billerica, Massachusetts). All assays were conducted according to manufacturer’s instructions. Stained cells were analyzed using flow cytometry, 488 nm argon laser, FL1-525 nm emission, 10,000 cell counts per sample (Coulter Epics XL flow cytometer, Beckman-Coulter, Fullerton, California) and confirmed visually by fluorescent microscopy.

**Mitochondrial membrane potential**

Mitochondrial membrane potential (∆Ψm) was assayed using the method described by Srivastava et al., (1997), with modification. Parasites were synchronized and exposed to 40 C, staurosorpin, chloroquine, or control. Parasitized red blood cells were isolated from uninfected erythrocytes by micro-centrifugation on Percoll gradient (35/45%) and then incubated with the protonophore carbonylcyanide m-chlorophenylhydrazone (CCCP) (Molecular Probes, Eugene, Oregon) at 200 μM or ethanol control for 1 hr at 37 C or 40 C. The cationic fluorescent probe DiOC6(3) (Molecular Probes) was added to the mixture (10 nM final concentration) for 30–40 min. Cells were then washed twice in media and immediately analyzed by flow cytometry, 488 nm argon laser, FL1-525 nm emission, 10,000 cell count per sample (Coulter Epics XL, Beckman-Coulter).

**Oligonucleosomal DNA fragmentation (DNA laddering)**

Synchronized ring-stage parasites were allowed to progress to the early trophozoite stage before being exposed to 40 C, chloroquine, staurosorpin, or control for 10–16 hr. DNA extraction was performed using the protocol described by Cary et al. (1994). After extraction some DNA samples were subjected to micrococcal nuclease digestion (50 units/ml; Molecular Probes) or DNase I digestion (1% w/v; Fisher Scientific, Waltham, Massachusetts). Laddered controls consisted of DNA extracted from the established cell line, HL-60, which had been exposed to staurosorpin (5 μM) to induce apoptosis. All lanes contained 5–10 μg DNA unless otherwise indicated. DNA was visualized by 1.5% agarose gel electrophoresis (100 volts; model, manufacturer, city, state) and post-stained with SYBR Green I dye (Molecular Probes).

**White blood cell depletion**

Erythrocytes used in some chloroquine experiments were exhaustively depleted of white blood cells (WBCs) using a Dynabeads CD45 kit (Invitrogen) following manufacturer’s instructions. Erythrocytes were further incubated with 1% (w/v) DNase I at 37 C for 30 min to remove extracellular DNA. Concentrated parasite cultures were then allowed to expand into the purified erythrocytes for 1–2 cycles before DNA ladder experiments were initiated.

**Transmission electron microscopy**

Synchronized parasites were partitioned into 6 identical cultures and placed in experimental or control conditions at 24 hr Pl. Every 3–5 hr, control and experimental parasites were collected, concentrated by gel separation (Jensen, 1978), and fixed for transmission electron microscopy using a modification of Yaron et al. (1984). Briefly, parasites were washed twice in phosphate-buffered saline (pH 7.4) then suspended in fixative (1.6% gluteraldehyde, 0.1 M sucrose, 0.2 mM CaCl, and 0.1 M cacodylate) for 0.5 hr. Samples were washed 3 times in buffer (0.12 M sucrose, 0.1 M cacodylate) then stained with buffered osmium (1% OsO4, 0.12 M sucrose, 0.1 M cacodylate) for 0.5 hr. After 2 washes, the pellets were stored in distilled H2O at 4 C until all samples had been prepared to this point. Parasites were then stained overnight in 0.5 M uranyl acetate followed by 3 H2O washes. Samples were dehydrated by sequential washes in ethanol (10–100% at 10% increments) and finally in 100% acetone. The cells were embedded in Spurr’s resin by gradual increase in resin concentration (1 part resin:2 parts aceton; 2 parts resin:1 part acetone; 100% resin) and polymerized overnight at 70 C. Thin sections were cut by diamond knife, post-stained with Reynolds’ lead citrate (2.6% Pb(NO3)2, 3.5% sodium citrate, 0.15 N, NaOH, pH 12) and visualized on a Tecnai T-12 transmission electron microscope (FEI Company, Hillsboro, Oregon).

**Statistical analysis and confirmation**

Data from experimental replicates were analyzed using unpaired t-tests with significance determined by P value <0.05. Error bars represent 95% confidence intervals. All experiments were confirmed by 2–3 independent trials and by visual inspection of Giemsa-stained thin films.

**RESULTS**

To rule out changes in culture parameters as a cause of parasite death at 40 C, we pre-exposed complete culture medium or uninfected erythrocytes to 40 C for 24–48 hr before use in parasite cultures. We compared parasite invasion and growth rates in cultures using treated and nontreated media or erythrocytes. Results indicated that 40 C had no effect on the ability of culture media to support growth of the parasite, nor did the higher temperatures have a deleterious impact on the erythrocyte’s capacity to serve as host to invading merozoites, or subsequent parasite development. Using highly synchronized parasite cultures, we noted that ring-stage parasites up to 18–24 hr Pl were unaffected by 40 C, advancing typically through the life cycle when returned to 37 C. However, young trophozoites (24 hr Pl) were irreversibly damaged if exposed to 40 C for 6 hr or more. Allowing parasites to recover at 37 C for up to 36 hr did not improve survival. Moreover, late trophozoites and schizonts became increasingly sensitive to 40 C, requiring ≤4 hr of exposure to induce irreversible parasite death. Long-term exposure (48 hr) to 40 C led to a >95% parasite death rate in cultures. There were no living parasites detected after 2 continuous cycles at 40 C.

In an attempt to identify metabolic processes directly affected by febrile temperatures, we compared alterations in parasite metabolic profiles of protein synthesis, DNA synthesis, and glucose consumption during exposure to 40 C or control (Fig. 1). Clearly, 40 C had a significant impact on parasite metabolism, especially on trophozoite and schizont stages. Protein synthesis, DNA synthesis, and glucose consumption profiles were also...
determined for parasites exposed to staurosporine and chloroquine. Results were similar, with the exception that staurosporine-treated parasites showed significant decrease in protein synthesis, DNA synthesis, and glucose consumption several hours earlier than parasites exposed to 40°C or chloroquine (data not shown).

To determine whether parasite death as seen under the influence of 40°C, chloroquine, or staurosporine might be due to apoptosis, we examined the cultured parasites for markers often associated with this form of PCD. Synchronized parasite cultures assessed for pan-caspase activity and for activation of specific caspases 8, 9, and 10 showed unexpected caspase activity in control cultures. Paradoxically, dying and dead parasites showed decreased activity of caspases in general, as well as for the specific caspases 8, 9, and 10 (results not shown).

In 40°C treated cultures, parasites exhibited permanent loss of mitochondrial membrane potential coincident with initiation of sensitivity to heat at 24 hr PI (Fig. 2). Similarly, trophozoites exposed to chloroquine and staurosporine exhibited permanent loss of \( \Delta \Psi \text{m} \) after less than 8 hr of treatment (data not shown).

There were no observable DNA ladders in parasites exposed to 40°C or staurosporine (Figs. 3A, B). In early experiments, DNA extracted from parasites exposed to chloroquine often exhibited DNA laddering (Fig. 3C, lanes 5, 6). However, DNA laddering was observed in preserved blood devoid of parasites after exposure to chloroquine as well (Fig. 3D, lane 1). To determine if the observed ladders in cultures were from occult WBC contamination, we purged WBCs from the erythrocytes before culturing with parasites. Once cultures were depleted of WBCs, DNA ladders could no longer be demonstrated in parasites treated with chloroquine, even though the cultures contained dead and dying parasites (Fig. 3E, lane 6).

Ring-stage parasites appeared normal in Giemsa-stained thin films during exposure to 40°C. However, after 24 hr PI, para-
FIGURE 3. No detectible oligonucleosomal fragmentation (DNA laddering) during death of *Plasmodium falciparum* initiated by exposure to 40 C, chloroquine, or staurosporine. Arrows indicate DNA laddering. (A) Parasites incubated at 37 C or 40 C for 16 hr. Lane one is 100 bp size ladder; lane 2 is 37 C; lane 3 is 40 C; lane 4 is 37 C DNA digested with DNase I; lane 5 is HL-60 cells; lane 6 is HL-60 cells treated with staurosporine (5 fM) for 12 hr to induce apoptosis with typical DNA laddering. (B) Parasites treated with staurosporine or DMSO control for 10 hr. Lane 1 is size marker; lane 2 is staurosporine; lane 3 is DMSO. (C) Parasites exposed to chloroquine or control for 8 hr. Lane 1 is size marker; lane 2 is control; lanes 3 and 4 are control digested with micrococcal nuclease (1 min, 3 min); lanes 5 and 6 are chloroquine. (D) Preserved blood kept at 4 C for 3 days. Lane 1 is stored blood cells treated with chloroquine 8 hr; lane 2 is stored blood cells digested with micrococcal nuclease; lane 3 is size ladder. (E) Parasites grown in WBC-depleted erythrocytes exposed to chloroquine or control for 8 hr. Lane 1 is size marker; lane 2 is WBC-depleted erythrocytes; lane 3 is WBC-depleted erythrocytes digested with micrococcal nuclease; lane 4 is control parasites; lane 5 is control parasites digested with micrococcal nuclease; lane 6 is chloroquine-treated parasites; lane 7 is chloroquine-treated parasites digested with micrococcal nuclease. Lanes 2 and 3 contained <1 fLg DNA.

sites exposed to 40 C began showing abnormalities such as obviously retarded development, most appearing pyknotic with a lesser proportion showing vacuolation. Chloroquine-treated parasites appeared normal until the trophozoite stage, after which they appeared markedly vacuolated, with a few pyknotic forms. Every stage of the erythrocytic cycle appeared affected by staurosporine treatment, with obvious retardation of development, revealing a mixture of pyknotic and vacuolated cells (Fig. 4).

Ultrastructural analysis of trophozoites exposed to 40 C initially showed cytoplasmic condensation (6 hr), followed by food vacuole swelling and organelle lysis, becoming obviously necrotic after 9 hr of exposure. Staurosporine-treated cells were similar in appearance to parasites cultured at 40 C, although they exhibited food vacuole swelling and lysis earlier than those exposed to 40 C (5 hr). Parasites treated with chloroquine displayed notable cytoplasmic vacuolation, followed by food vacuole swelling and lysis beginning as early as 8 hr after exposure (Fig. 5).
DISCUSSION

Many cells have the ability to initiate an organized pathway of PCD in response to outside stimuli or intrinsic messages originating within the cell. The most studied and recognizable form of PCD is apoptosis. This form of PCD consists of specific morphological and physiological changes that are usually precipitated by activation of a family of cysteine-aspartate proteases called caspases (reviewed in Kerr, 2002; Guimaraes and Linden, 2004). The phenomenon of PCD is in contrast to another type of cell death known as necrosis, an uncontrolled degradation of the cell occurring after extreme physical or chemical insult (Cohen, 1993). Determination of the types of cell death employed in malarial parasites is of particular interest because of their potential role as targets for antimalarial therapy.

Initial studies by Kwiatkowski (1989) and Long et al. (2001) determined that temperatures of 40 C caused a marked reduction in parasite number in cultured *P. falciparum*. Their conclusions were based on notable decreases of parasites in the subsequent culture generation, with the logical assumption that febrile temperatures were responsible for reduction of parasitemia. We have confirmed and extended these earlier observations by determining that the reduction in the parasite population was indeed a parasite phenomenon and not an artifact of the in vitro system. Furthermore, we have confirmed that parasites became sensitive to febrile temperature 20–24 hr PI and determined that ring stages exposed to 40 C developed normally when returned to 37 C, unlike trophozoite and schizont stages, which, once committed, were unable to recover. Additional experimentation yielded direct biochemical evidence of parasite death by showing significant decreases in DNA synthesis, protein synthesis, and glucose utilization during exposure of the parasites to 40 C. Later stage parasites are extremely sensitive
to exposure to 40 C, while the ‘ring’ stages are refractive to heat treatment. This may be due to the fact that later stage parasites are exceptionally metabolically active, exhibiting a marked increase in protein and DNA synthesis, possibly increasing the parasites susceptibility to higher temperatures.

It has long been known that malarial parasites are able to modulate apoptotic pathways in their human and arthropod hosts (Hurd and Carter, 2004; James and Green, 2004). Moreover, recent reports of apoptoticlike pathways during self-limitation of P. berghei ookinetes and of temperature-induced alterations of mRNA expression during heat treatment (Oakley et al., 2007) have raised the question of possible apoptosis in P. falciparum when exposed to 40 C. There exists reasonably consistent hallmarks of apoptosis, including temporary loss of mitochondrial transmembrane potential, upregulation of caspases, and specific DNA degradation into multimers of 180 bp that appear on agarose gels as DNA ladders (reviewed in Cohen, 1993). Because parasite death at a febrile temperature was reasonably stage specific, exhibiting a pattern of increasing irreversibility, and since 40 C is probably insufficient in itself to induce necrosis, we initially postulated that febrile temperature might be inducing an apoptoticlike pathway in the parasites. The evolutionary rationale for such a pathway was that of self-limitation, sparing the host long enough to allow sufficient time for the development and transmission of gametocytes to the mosquito host (reviewed in Ameisen, 2004; Welburn, 1997).

Often in the process of apoptosis, ΔΨm is lost early as molecules used to initiate the apoptotic pathway are released from mitochondria. However, apoptosis is an active process, and the early initial decrease in ΔΨm is often partially recovered to allow for energy generation throughout the rest of the pathway (Kroemer et al., 1998; Skulachev, 2006). Loss of ΔΨm during heat-induced death of P. falciparum was a relatively early and permanent event and was, therefore, not consistent with apoptosis.

Caspases are proteases that function as signal transduction molecules and molecular promoters of apoptotic pathways in higher eukaryotes (Degterev et al., 2003). Caspase-like activity has been detected in apoptotic pathways in protozoans such as Leishmania spp. (Arnoldt et al., 2002) and the malarial parasite P. berghei (Al-Olayan et al., 2002) despite the fact that no caspase-homologous genes have been discovered in unicellular eukaryotes (Greenberg, 1996). We determined that there was no increase of caspase activation during P. falciparum death. In fact, caspase activity was notable in healthy parasites and unexpectedly decreased as parasite death progressed. These data are in direct contrast to Al-Olayan’s (2002) work in P. berghei. There may be an explanation as to why our results differ. The activity that we observed could have been caspase activity in the erythrocyte host and not the parasite itself (Zetmati et al., 2001). The P. berghei study was conducted on the extracellular ookinete stage. It is unlikely that the caspase-like activity we observed was due to parasite caspase activation because the P. falciparum genome contains no caspase sequence (Wu et al., 2003). It is possible that the caspase probe used could be cross-reacting with other parasite proteases. Research has shown that DEVD-FMK type probes, such as the one we used, are able cross-react with other cysteine proteases (Rozman-Pungercar et al., 2003); in viable parasites, these probes may have been reacting to cysteine proteases in the parasite food vacuole. Nevertheless, apparent caspase activity, which usually increases in cells undergoing apoptosis, notably decreased in the parasites with the onset of death.

DNA degradation, specifically oligonucleosomal fragmentation leading to multimers of ~180 bp, resulting in DNA ladders on agarose gels, is another common marker of apoptosis (Cohen, 1993). Earlier work reported by Picot et al. (1997) showed DNA laddering in P. falciparum after exposure to chloroquine. Initially, we visualized DNA ladders in parasites exposed to chloroquine as well, but upon further experimentation determined that the DNA ladders were due to occult WBC contamination in the parasite cultures. It has previously been demonstrated that chloroquine is able to induce apoptosis in peripheral blood lymphocytes (Meng et al., 1997), which may account for the ladders seen when parasite cultures were exposed to chloroquine. After measures were taken to deplete the erythrocytes of all WBCs, chloroquine-induced DNA ladders disappeared. It is probable that the DNA ladders reported by Picot et al. (1997) were due to chloroquine-induced apoptosis in WBCs in parasite cultures. This experience should serve as a warning and reminder that parasite cultures and preserved blood may contain foreign DNA contributed by WBC contamination. In other attempts to demonstrate parasite DNA degradation during temperature-induced parasite death, we used the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay to determine overall DNA damage. Although we tried several modifications of this assay we were unable to produce consistent results. Caution must be taken when using this assay as it gives unreliable results in rapidly dividing cells such as the mature stages of P. falciparum. Furthermore, DNA replication and karyokinesis in malaria parasites is not a completely defined process. Extensive research will have to be accomplished to determine if the TUNEL assay can be used as a viable marker of DNA damage in P. falciparum.

In early studies of apoptosis and necrosis, these 2 forms of cell death were characterized by morphological changes during cell demise (Kerr et al., 1972). Apoptotic cells were described as appearing shrunk or pyknotic, having condensed cytoplasm and chromatin, intact organelles, and blebbing from the cell plasmalemma. Necrotic cells, on the other hand, exhibited cytoplasmic and organelle swelling, followed by rupture of the cell membrane. Previous studies examining death of P. falciparum, initiated by exposure to immune serum, showed developmentally retarded and pyknotic parasites, known as crisis forms, during death of the parasite (Jensen and Boland, 1982). These changes appeared morphologically similar to cells undergoing apoptosis. In the current study, Giemsa-stained thin films of parasites undergoing temperature-induced death showed many pyknotic cells reminiscent of apoptosis, while other parasites appeared swollen and heavily vacuolated, similar to changes seen in necrosis. Parasites killed by staurosporine and chloroquine appeared grossly similar. Those parasites that were pyknotic by light microscopy (Fig. 4) exhibited condensation of the cytoplasm, with some food vacuole swelling when examined ultrastructurally (Fig. 5). We postulate that the parasites that appeared vacuolated under light microscopy may represent parasites with necrotic cytoplasm seen by electron microscopy. Thus, early visual changes were often reminiscent of cells undergoing apoptosis, but subsequent food vacuole swelling and lysis may have induced secondary necrosis. The malarial parasite food vacuole is an acidic digestive organelle con-
taining cysteine and aspartic proteases and lipases, among other enzymes, used to digest hemoglobin. The homologue to the parasite food vacuole in higher eukaryotic cells is the secondary lysosome. Research on the role of these lysosomes during PCD has shown that breakdown of the lysosomal membrane leads to necrosis (Bursch, 2001; Artal-Sanz et al., 2006). Because the current study has focused on changes seen in parasite death during the later stages of the erythrocytic cycle, it would be of interest to characterize parasite death when no food vacuole was present or attempt to ameliorate action of food vacuole enzymes.

Recently, microarray analysis determined that exposure to 41°C for 2 hr produced alterations in several mRNA expression profiles (Oakley et al., 2007). These observations indicate that some organized pathway is initially involved in death during exposure to febrile temperature. Our results do not rule out the possibility that P. falciparum may exhibit some form of PCD, though we were unable to find biochemical or physiological evidence of apoptosis per se. Other studies examining drug-induced death of the erythrocytic cycle of P. falciparum were also unable to detect classic physiological hallmarks of apoptosis (Pankova-Kholmyanskaya et al., 2003; Nyakeriga et al., 2006). In many cells undergoing apoptosis or another form of PCD, necrosis is often the eventual end-game of cell death (Unal-Cevik et al., 2004). Due to limitations in achieving and maintaining tight parasite developmental synchrony in vitro, the possibility remains that temperatures of 40°C may induce some form of PCD that eventually results in the loss of membrane integrity of the parasite’s food vacuole. Lysis of the food vacuole membrane would then lead to an uncontrolled digestion of the parasite, resulting in secondary necrosis as appears to be the case in our study. Further research into other forms of PCD should increase our understanding of heat-induced parasite death.

**LITERATURE CITED**


MORPHOLOGICAL PHYLOGENETIC ANALYSIS OF THE AFRICANA GENUS
(NEMATODA: HETERAKIDAE)

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ABSTRACT: A morphological phylogenetic hypothesis of the relationships among the species of Africana is provided. The phylogenetic analysis suggests that Squamata or Amphibia were ancestral hosts for species of Africana, with dispersal events to Testudinidae; a second transfer to amphibians is also suggested. On the basis of biological data for these parasites and the paleogeographic reconstructions, a South American origin for Africana spp. is indicated, with subsequent dispersal events to Africa. It is further proposed that Chamaeleonidae acquired species of Africana after their ‘out-of-Madagascar’ dispersal events to Africa.

Species of Heterakidae Railliet and Henry, 1912 (Ascaridida) are gut parasites of Tetrodota (Baker, 1987). Travassos (1920) erected the Spinicaudinae (Heterakidae) for species of Spinicauda and Africana, nematode parasites of amphibians and reptiles, and transferred Heterakis africana Gendre, 1909, parasites of Kenixys belliana Gray, 1831, in Guinea to a new genus, Africana, along with 2 other species, Heterakis acuticeps Gedoelst, 1916 and H. brodeni (Gedoelst, 1916), parasites of Chamaeleonidae (Chamaeleon gracilis Hallouay, 1842 and C. dilepis Leach, 1819) in the Democratic Republic of Congo. Six species have since been described (see Bouamer and Morand, 2007; Table I), with 4 species from the Afrotropical region (with 2 of these in amphibians and 2 from reptiles) and 2 parasite species of reptiles from the Neotropical region. Presently, the heterakid species parasitizing herptiles are present only on the actual Gondwanian landmasses, with species of Africana restricted to Ethiopian and Neotropical regions.

In the present study, we propose a morphological phylogenetic analysis of all species in the genus. The results of the analysis, together with information on the life history traits of the parasite and the behavior and ecology of their hosts, permit us to propose scenarios of biogeographical origin and diversification of the genus.

MATERIALS AND METHODS
Phylogenetic reconstruction

Because of the old and incomplete description of Africana astylosterni, we performed 2 analyses, one including the taxon, the other not including the taxon. In total, 26 characters were considered, all referring to morphological and anatomical features (see Appendix). States of characters were obtained from the present study and from original descriptions (Fig. 1). Unfortunately, the cephalic end is often not described. All of these characters, traditionally used in the taxonomy of this group, were checked for their diagnostic value. Additional characters were recognized as informative and thus were considered in the analysis. Characters representing autapomorphies were excluded.

The list of species, hosts, and localities are given in Table I. A data matrix was compiled using characters derived from the comparative morphology of species based on 26 morphological characters (Table II). Outgroup

Characters states were coded using an outgroup comparison (Watrous and Wheeler, 1981; Maddison et al., 1984). The outgroup species was chosen on the basis of the morphological systematic of Travassos (1920) and according to recent description, Spinicauda voltaensis Baker and Bain (1981) was selected as the outgroup species.

Phylogenetic analysis

Characters were treated as unordered or unweighted and were coded as either binary or multistate (Maddison, 1993). Character states found to be unclear (or missing) were scored as ambiguous. All analyses were run using PAUP* 4.0b10 (Swofford, 2002). Heuristic searches in PAUP* 4.0b10 (Swofford, 2002) were performed using the following options, i.e., branch swapping = tree bisection and reconnection (TBR), with MULPARS operational. Associated statistics included the Consistency Index (CI), Rescaled Consistency (RC), Retention Index (RI), and Homoplasy Index (HI).

RESULTS
First analysis, with A. astylosterni Sandground, 1933 included

The analysis using all characters, equally weighted, resulted in 2 parsimonious trees (Figs. 2, 3) and a strict consensus tree (Fig. 4) with the length = 60 steps, CI = 0.77, HI = 0.23, CI excluding uninformative characters = 0.74, HI excluding uninformative characters = 0.25, RI = 0.64, and RC = 0.49.

The 2 parsimonious trees had a similar topology, except for the basal nodes. In the first parsimonious tree (Fig. 2), A. telfordi was a sister group of the group formed by A. astylosterni and A. chabaudi. However, in the second parsimonious tree (Fig. 3), these 3 species formed a paraphyletic group, with A. astylosterni and A. chabaudi forming a monophyletic group at the base of the tree.

The 2 cladograms (Figs. 2, 3) support the monophyly of Africana, with 4 synapomorphic characters (21, 22, 23, and 24). The Ethiopian species (A. africana, A. acuticeps, A. brodeni, A. taylori, A. kinixysae, and A. congensis) form a monophyletic group supported by 3 synapomorphies (characters 10, 25, 26). The parasites of the Ethiopian Squamata, A. acuticeps and A. brodeni, also form a monophyletic group, with this group being the basal members of the Ethiopian clade. The 2 species, A. kinixysae and A. congensis, are sister taxa, supported by the synapomorphic character 20; each is a sister group of A. africana and A. taylori.

Second analysis, with A. astylosterni Sandground, 1933 excluded

The analysis using all characters, equally weighted, resulted in 1 parsimonious tree (Fig. 5) with length L = 55, CI = 0.80, HI = 0.20, the CI excluding uninformative characters = 0.75, HI, and excluding uninformative characters = 0.24, RI = 0.64.
Table I. List of *Africana* species, and the outgroup, with their hosts and localities.

<table>
<thead>
<tr>
<th>Nematode</th>
<th>Host</th>
<th>Localities and biogeographical domain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Africana africana</em></td>
<td><em>Kenixys bella</em> <em>Testudo hermanni</em></td>
<td>Guinea, Ethiopian, Afrotropical</td>
<td>Gendre (1909)</td>
</tr>
<tr>
<td><em>A. astylosterni</em> Sandground, 1933</td>
<td><em>Astylosternus robustus</em></td>
<td>Democratic Republic of Congo, Ethiopian, Afrotropical</td>
<td>Sandground (1933)</td>
</tr>
<tr>
<td><em>A. taylori</em> Fitzsimmons, 1961</td>
<td><em>Bufo regularis</em></td>
<td>Nigeria, Ethiopian, Afrotropical</td>
<td>Taylor (1924)</td>
</tr>
<tr>
<td><em>A. telfordi</em> Bursey and Goldberg, 2002</td>
<td><em>Enyalioides heterolepis</em></td>
<td>Panama, Northern of Neotropical</td>
<td>Bursey and Goldberg (2002)</td>
</tr>
</tbody>
</table>

and RC = 0.51. The parsimonious tree was fully resolved, and all species of the ingroup formed a monophyletic group supported by 4 synapomorphies (characters 21, 22, 23, and 24; Table III). The Neotropical parasites of Squamata, *A. telfordi* and *A. chabaudi*, do not form a monophyletic group, with *A. chabaudi* sp. being the basal member of the ingroup. The Ethiopian parasites form a monophyletic group ([*A. kinixysae*, *A. congoensis*] [A. *africana*, A. *taylori*) supported with 3 synapomorphic characters (10, 25, and 26). The parasites of the Ethiopian Squamata, *A. acuticeps* and *A. brodeni*, also form a monophyletic group that is supported by the synapomorphy character, 7. This group is basal for the clade of parasite species of Testudinidae and Anura (*A. kinixysae*, *A. congoensis*) and (*A. *africana*, A. *taylori*), respectively. The 2 new species form a monophyletic group supported by the synapomorphic character, 20.

**DISCUSSION**

Nematode parasites of amphibian reptiles appear to be only weakly host specific, and differences in the behavior and ecol-
FIGURE 4. Strict consensus of 2 equally cladograms, L = 60, CI = 77, RI = 0.68.

FIGURE 5. One parsimonious tree generated by the cladistic analysis, L = 0.55, CI = 0.80, RI = 0.64. Black bars indicate synapomorphies.

<table>
<thead>
<tr>
<th>Characters</th>
<th>CI Tree I Analysis I</th>
<th>CI Tree II Analysis I</th>
<th>CI Tree Analysis II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Car1 Number of pre-sucker papillae</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Car2 Number of post-median sucker papillae</td>
<td>0.667</td>
<td>0.667</td>
<td>0.500</td>
</tr>
<tr>
<td>Car3 Number of ventral papillae surrounding the sucker</td>
<td>0.750</td>
<td>0.750</td>
<td>0.750</td>
</tr>
<tr>
<td>Car4 Number of lateral complex papillae</td>
<td>1.000</td>
<td>0.800</td>
<td>1.000</td>
</tr>
<tr>
<td>Car5 Lateral complex papillae, pre- and ad-sucker</td>
<td>0.500</td>
<td>0.500</td>
<td>0.667</td>
</tr>
<tr>
<td>Car6 Number of pre-anal median papillae</td>
<td>0.500</td>
<td>0.500</td>
<td>0.500</td>
</tr>
<tr>
<td>Car7 Presence or absence of ad-anal papillae</td>
<td>0.500</td>
<td>0.500</td>
<td>1.000</td>
</tr>
<tr>
<td>Car8 Number of ventral pair papillae surrounding the cloaca</td>
<td>0.667</td>
<td>0.667</td>
<td>0.667</td>
</tr>
<tr>
<td>Car9 Absence or presence of sub-ventral and post-anal papillae</td>
<td>0.500</td>
<td>0.500</td>
<td>0.500</td>
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<tr>
<td>Car10 Number of lateral and post-anal pair papillae</td>
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<tr>
<td>Car11 Number of dorsal caudal pair papillae</td>
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</tr>
<tr>
<td>Car12 Number of dorsal caudal pair papillae</td>
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<tr>
<td>Car13 Caudal alae ending at</td>
<td>0.800</td>
<td>0.800</td>
<td>0.750</td>
</tr>
<tr>
<td>Car14 Equal spicules or unequal spicules</td>
<td>0.500</td>
<td>0.500</td>
<td>0.500</td>
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<tr>
<td>Car15 Shape of the tail</td>
<td>0.667</td>
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</tr>
<tr>
<td>Car16 Shape the anterior end</td>
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</tr>
<tr>
<td>Car17 Shape of the vulva</td>
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<td>0.500</td>
</tr>
<tr>
<td>Car18 Position of the vulva</td>
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<td>1.000</td>
</tr>
<tr>
<td>Car19 Presence or absence of lateral alae</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Car20 Position of sub-ventral and post-anal papillae</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Car21 Presence of the sub-lateral caudal end pair of papillae</td>
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<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Car22 Position of dorsal caudal pair papillae</td>
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</tr>
<tr>
<td>Car23 Presence or absence of caudal alae</td>
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</tr>
<tr>
<td>Car24 Presence or absence of median sucker papillae</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Car25 Position of lateral complex papillae</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Car26 Presence or absence of ventral and post-anal papillae</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>
ogy of hosts seem to be largely responsible for observed host specificities (Goater et al., 1987; McAlpine, 1997). As a consequence, many common nematode species are found in a large number of hosts. The present phylogenetic analysis supports the hypothesis that the presence of species of *Africana* in these different hosts may then be explained by a “phenomenon capture or lateral transfer” (Combes, 1995; Bouamer and Morand, 2007) via ingestion of parateneic invertebrate hosts.

Most paleogeographic reconstructions depict the Cretaceous period as the most active period of Gondwanian fragmentation, with Indo-Madagascar separating from Antarctica by 130–125 mya and South America separating from Africa approximately 100 mya (Scotese et al., 1988; Scotese, 1991; Pitman et al., 1993). The connections between these 2 continents, however, could have persisted via volcanic islands on mid-ocean bridges until 95 mya (Raven and Axelrod, 1972). Moreover, magnetic anomaly data and Mesozoic time scales reveal that Madagascar first broke away from Africa 165 mya ago, with movement ending by 121 mya ago (Rabinowitz et al., 1983).

On one hand, no parasitological study concerning Squamata and Amphibia of Africa and South America has mentioned the presence of *Africana* species (Chabaud and Brygoo 1960; Moraive et al., 1987; Sanchez et al., 1994; Al-Deen et al., 1995; Saber et al., 1995; Goldberg and Bursey, 1996, 2002; Boquimpani-Freitas et al., 2001; Ramallo et al., 2002; Vrcibradic et al., 2002; Iannacone, 2003; Rocha and Vrcibradic, 2003). On the other hand, there are no indications of *Africana* species in Chamaeleonidae or other Squamata from Madagascar (Chabaud and Brygoo, 1960; Caballero, 1968; Petter, 1968), India (Deshmukh and Choudhari, 1980; Nagpur, 1981; Sood, 1999), or Australia and New Guinea (Owen and Moorhouse, 1980; Moravec, 1990; Cameron and Cogger, 1992; Bursey and Goldberg, 1999, 2001).

The phylogenetic analyses suggest a South American origin for *Africana*, with dispersal events to Africa, which should have occurred before the separation between these 2 continents, i.e., between 110 and 100 mya (Scotese et al., 1988) and 95 mya (Raven and Axelrod, 1972). Moreover, Chamaeleonidae were parasitized by species of *Africana* after their “out-of-Madagas­car” dispersal events to Africa, according to the Madagascar origin of this family (Raxworthy et al., 2002).

Our phylogenetic analyses also suggest that *Africana* spp. originated as parasites of Squamata or Amphibia, with dispersal events to Testudinidae, and with a second transfer from Squama­ta to Amphibia in the Afrotropical region. These hypotheses concerning the biogeographic origin and radiation are supported by the life cycles of these parasites, the paleogeographic reconstructions, the absence of representatives of this genus in other Gondwanan land masses (even if many species of Squamata, such as chamaeleons, Testudinidae, and Amphibia, are present), and the absence of this genus outside of the northern of Neotropical or Afrotropical regions.

**LITERATURE CITED**


APPENDIX

1. Number of pre-sucker papillae: 0 = 0, 2 = 1, 3 = 2, 4 = 3, 6 = 4, 6 = 5

2. Number of post-median sucker papillae: 0 = 0, 1 = 1, 2 = 2

3. Number of ventral papillae surrounding the sucker: 0 = 0, 1 = 1, 2 = 2, 3 = 3, 6 = 4

4. Number of lateral complex papillae: 0 = 0, 2 = 1, 3 = 2, 4 = 3, 5 = 4

5. Lateral complex papillae: pre and ad-sucker = 0, and post-sucker = 1, post-sucker = 2

6. Number of pre-anal median papillae: 0 = 0, 1 = 1, 2 = 2

7. Presence or absence of ad-anal papillae: absence = 0, presence = 1

8. Number of ventral pair papillae surrounding the cloaca: 0 = 0, 1 = 1, 2 = 2

9. Absence or presence of sub-ventral and post-anal papillae: absence = 0, presence = 1

10. Number of lateral and post-anal pair papillae: 0 = 0, 1 = 1, 2 = 2, 3 = 4

11. Presence or absence of pair papillae supporting the caudal alae: absence = 0, presence = 1

12. Caudal alae ending at: pre-anal = 0, ad-anal = 1, post-anal = 2, middle half of caudal = 3, posterior end of tail = 4

13. Equal spicules = 0, unequal spicules = 1

14. Shape of the tail: acutely pointed = 0, conical = 1, truncated with hyaline spine = 2

15. Shape of the anterior end with a cephalic region larger than the other part of the anterior end: absence = 0, presence = 1

16. Shape of the vulva: not prominent = 0, prominent = 1

17. Presence of the vulva: near oesophageal bulb = 0, at the level of the mid-body = 1, near the posterior end = 2

18. Lateral alae: absence = 0, presence = 1

19. Position of sub-ventral and post-anal papillae: closed = 0 (ventral position), separated = 1

20. Presence or absence of the sub-lateral caudal end pair of papillae (sub-lateral or lateral): absence = 0, presence = 1

21. Position of dorsal caudal pair papillae: pre-anal = 0, post-anal = 1

22. Caudal alae: absence = 0, presence = 1

23. Presence or absence of median sucker papillae: absence = 0, presence = 1

24. Presence or absence of ventral and post-anal papillae: absence = 0, presence = 1

25. Position of lateral complex papillae: ventral = 0, lateral = 1

26. Presence or absence of ventral and post-anal papillae: absence = 0, presence = 1
NEW GENUS AND SPECIES OF APOROCOTYLIDAЕ (DIGENEA) FROM A BASAL ACTINOPTERYGIAN, THE AMERICAN PADDLEFISH, POLYODON SPATHULA, (ACIPENSERIFORMES: POLYODONTIDAE) FROM THE MISSISSIPPI DELTA

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ABSTRACT: Acipensericola petersoni n. gen., n. sp. (Digenea: Aporocotylidae) infects the heart of the American paddlefish Polyodon spathula (Walbaum, 1792) in the Mississippi Delta. It has robust, spike-like body spines arranged in ventrolateral transverse rows; a bowl-shaped anterior sucker centered on the mouth and having minute spines on the inner anteroventral surface only; a pharynx; an inverse U-shaped ceca extending to near the posterior body end; intercel testes comprising a pre-ovarian testicular column plus a single testis posteriorly; an extensively lobed ovary located medially and immediately posterior to the testicular column; a spherical ootype that is intercecal and post-ovarian; a Laurer’s canal; and a common genital pore. The new species is the first-named aporocotylid collected from a basal actinopterygian. It resembles the chondrichthyan aporocotylids Chimaerohemecus trondheimensis, Orchispirium heterovitellatum, and Hyperandrotrema cetorhini in having an inverse U-shaped ceca, but it is morphologically most similar to the anguilliform aporocotylid Paracardicoloides yamaguti in having that feature plus a comparable anterior sucker, a single testis posteriorly, an intertesticular ovary, and a common genital pore. Sequence data for the complete small subunit ribosomal DNA (18S) do not refute its membership within Aporocotylidae nor its affinity to 1 of those aforementioned aporocotylids. A. petersoni was basal to the few teleost aporocotylids analyzed, and C. trondheimensis was the only taxon basal to A. petersoni. We regard the specimens of Spirochis sp. previously reported from the shortnose sturgeon Acipenser brevirostrum Lesueur, 1818 as congeneric with the new species.

Adult blood flukes (Digenea: Schistosomatoidea) infect jawed vertebrates (Gnathostomata) and historically have been grouped into 3 families correlating to the broad phylogenetic affiliations of their definitive host groups: Aporocotylidae, Odhner, 1912 for those blood flukes that infect non-tetrapod gnathostomes, i.e., fishes (Smith, 1997a, 1997b), Spirorchidae, Stunkard, 1921 for those of turtles (Platt, 2002); and Schistosomatidae, Stiles and Hassall, 1898 for those of birds and mammals (Khalil, 2002). We concur with Stunkard (1921) who stated that, “In my opinion, the Aporocotylidae of fishes, the Spirorchidae of turtles, and the Schistosomatidae of birds and mammals constitute a well-defined group with inherent natural relationships.” At present, Aporocotylidae includes only 5 nominal species that infect cartilaginous fishes (Chondrichthy- es) (Bullard et al., 2006), plus >100 species that infect bony fishes (Actinopterygii: Teleostei) (see Smith, 1997a, 1997b). As such, aporocotylids infect definitive hosts allocated to widely separated gnathostome lineages, whereas all adult spirorchids and schistosomatids are reportedly restricted to members of Tetrapoda (Gnathostomata: Sarcopterygii). Although Aporocotylidae is the most diverse blood fluke family, with respect to the number of named species and accepted genera, at least tens of aporocotylid species remain unnamed (S. Bullard, unpubl.), and many potential host lineages seem vastly under-explored for the presence of aporocotylid infections. Fewer than 200 (Smith, 1997b) of the nearly 28,000 valid fish species (Nelson, 2006) are reported as hosts, and most fish orders (46 of 63) reportedly lack infections. This void of information is a barrier to understanding the relationship between host ancestry and the evolution of fish blood flukes. Most notable among those under-explored host lineages are the lower (basal) actinopterygians (sensu Grande and Bemis, 1996), which form a non-monophyletic group of convenience that includes extant members allocated to 2 distinct lineages, i.e., Acpensieriformes (including paddlefishes [Polyodontidae] and sturgeons [Acipenseridae]) and Polypterygiformes (including bichirs [Polypteridae] only) (e.g., Grande and Bemis, 1996; Nelson, 2006). Herein, we provide the first name and description for an aporocotylid that infects a basal actinopterygian, the American paddlefish Polyodon spathula (Walbaum, 1792) (Acipenseriformes: Polyodontidae) and propose a new genus to accommodate this new species.

MATERIALS AND METHODS

American paddlefish from the Mississippi Delta were captured with gill nets in April of 2004 and 2006. All fish were killed by spinal severance, and immediately afterwards the heart was extracted, placed in a sample bag, bisected to expose its lumen, immersed in an anticoagulant solution of ~5.0 gm NaCl and ~2.0 gm NaCl-citrate/L of distilled water, and kept in a cooler with a small amount of ice for several hours. Upon returning to the laboratory, the contents of the bag were examined with the aid of a dissecting microscope. Observations of living flukes were made with the aid of dissecting and compound microscopes. Flukes intended as whole mounts were killed with heat from an ethanol-burner flame, under little or no coveship pressure, and transferred to a vial of 5% neutral buffered formalin (n.b.f.). Whole mounts were stained in Van Cleave’s hematoxylin with several additional drops of Ehrlich’s hematoxylin, made basic at 70% ethanol with lithium carbonate and butyl-amine, dehydrated, cleared in clove oil, and mounted in Canada balsam. Two specimens were embedded in paraffin, serially-sectioned at 4 μm, and stained with Gill’s hematoxylin and eosin. Three specimens for scanning electron microscopy (SEM) were dehydrated, immersed in hexamethyldisilazane for 30 min, air-dried for 45 min, and sputter-coated with gold-palladium. Drawings were made with the aid of a drawing tube and facilitated by differential interference contrast (DIC) optical components. Measurements are reported in μm and given as ranges with the sample size in parentheses. The specimen of “Spirochis sp.” of Appy and Dadswell (1978) (USNPC No. 73138) was lost and presumably destroyed during Hurricane Katrina on 29 August 2005.

Fish classification and higher level taxon names used herein follow primarily Grande and Bemis (1996) and Nelson (2006). Because “fishes” comprise a paraphyletic assemblage if one excludes Tetrapoda, we herein use that term not as a taxonomic rank but rather, as stated by Nelson (2006), “as a matter of convenience, essentially to describe
those vertebrates studied by ichthyologists and covered in ichthyologi­
cal courses.** The testes specimens intended for molecular analyses were fixed directly
in 95% EtOH, and genomic DNA was extracted using a DNeasy tissue
kit (Qiagen, Valencia, California) according to the manufacturer’s in­
structions. Polymerase chain reaction (PCR) was used to amplify SSU
rDNA with the forward primer 18S (5'-CCG AAT TCG TCG ACA
ACC TGG TGT ATC CTG CCA GT) and the reverse primer WORMB
(5'-CCT GTT AGG ACT TTT ACT TCC) (Littlewood and Olson,
2001). Reactions were performed in a total volume of 25 µl and con­
sisted of approximately 20 ng of gDNA, 0.2 µM of each primer, and
12.5 µl FideliTaq PCR Master Mix (USB Corporation, Cleveland,
Ohio). Reaction volume was brought to 25 µl with sterile deionized water. Amplification was performed on a Perkin Elmer GeneAmp 2400
thermocycler (Perkin Elmer, Waltham, Massachusetts) under the follow­ing
conditions: 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 sec,
50–56 °C for 30 sec, and 72 °C for 2 min, followed by 1 cycle of 72 °C for 5 min. Unincorporated PCR primers and nucleotides were removed from PCR products using exonuclease I and shrimp alkaline phosphatase from a PCR Product Pre-Sequencing Kit (USB Corpora­tion). Sequences were determined directly from PCR templates using Big Dye terminator chemistry and an ABI Prism 3100 (Applied Bio­
systems, Forest City, California). Primers used in sequencing SSU
rDNA included the PCR primers and the internal forward primers 388F
(5'-AGG GCT CGA TCG AGG AG) and 1100F (5'-CAG AGT TTC
GAA GAC GAT C) and the reverse primers CEST1R (5'-TTT TTC
GTC ACT ACC TCC CC) and 1270R (5'-CCG TCA ATT CCT TTA
AGT) (Littlewood and Olson, 2001). Sequence data from the new spe­
cies were aligned with sequences taken from GenBank. The ingroup included the new species (GenBank DQ534192, Aporocotyle spinosica­
alis (AY222177), Neoparacardicola nasonis (AY22097), Phe­
thorhachis acanthus (AY22098), and Cladopelmatoides longicollis
(AY22089). Sequences were assembled using Contig Express (v. 8.0, InforMax, Invitrogen, Carlsbad, Califor­
nia) and provisionally aligned using Clustal W (Thompson et
al., 1994), followed by alignment by eye in MacClade v. 4.06 (Sinaur and
Positions for which alignment was ambiguous were removed before analysis. Maximum parsimony analysis of these data was performed using the “branch and bound search,” “random sequence addition,”
and “TBR branch-swapping” options of PAUP* (v. 4.0b10, Sinaur and
Al, 2001). Gaps were treated as missing data, and characters were unordered with equal weight. Nodal support was as­
sesed using bootstrap resampling (Felsenstein, 1985) (1,000 bootstrap replicates, 100 heuristic searches/replicate).

** DESCRIPTION **

**Acipeciserolina n. gen.**

(FIGS. 1–28)

**Diagnosis:** Body flat, ventrally concave, elongate, <4 times longer than wide, with anterior and posterior gads tapering approximately equally, spined; tegumental body spines robust, spike-like, lacking recurved tip, in ventrolateral transverse rows. Rosethorn-shaped spines absent. Lateral nerve cord extending nearly entire body length, appear­ing subterminal in anterior body end, with commissure anteriorly. Dor­solateral nerve cords indistinct. Sensory papillae abundant, occupying ventrolateral body surface between lateral nerve cord and body margin. Anterior sucker bowl-shaped, centered on mouth, demarcated from the anterior body end by peduncle, having minute spines on inner antero­ventral surface only. Pharynx between anterior sucker and nerve com­
missure, highly muscular, intensely basophilic. Esophagus medial, straight, ventral to anterior sucker commissure, extending ap­proximately 1/6 of body length; posterior esophageal swelling present immediately anterior to cecal bifurcation. Intestine inverse U-shaped, with long posterior ceca only and no anterior cecum, smooth, lacking diverticula or secondary rami, extending posteriorly to near body end. Testes intercellic, non-contiguous, an anterior testicular column plus 1
tests posteriorly, with extensively lobed margins. Cirrus sac clearly delineated from surrounding tissue, enveloping seminal vesicle, pro­
static gland cells and cirrus; cirrus evertting dorsally near dextral body
margin; prostatic gland cells spheroid, encircling ejaculatory duct at base of cirrus and proximal to common genital pore. Ovary single, medial, intercel, separating anterior column of testes from single pos­
terior testis, immediately posterior to testicular column, deeply lobed, located within posterior 1/4 of body. Oviduct sinistral, functioning as oviducal seminal receptacle. Vellarium an extensive network of narrow, interconnecting branching bands, situated both dorsal and ventral to gonads and ceca, not extending laterally beyond ventrolateral nerve
cords. Laruer’s canal present. Ootype spherical, intercel, inter-telic­
ular, post-ovarian, medial to cirrus sac. Uterus near ovarian level, inter­
cel, typically containing several large eggs; uterine eggs oblong, >1/3 the width of uterus and appearing markedly collapsed or com­
pressed in whole mounts. Metraterm indistinct. Male and female repro­
ductive tracts sharing common opening, lacking posteralteral protu­
berance of body associated with pore. Excretory vesicle large, Y-shaped, cradling posterior testis, thin-walled. In blood vascular sys­
tem of members of Acipeciseriformes.

**Differential diagnosis:** Body elongate, <4 times longer than wide; tegumental body spines robust, spike-like, lacking recurved tip, in ven­
tralateral transverse rows. Rosethorn-shaped spines absent. Anterior sucker bowl-shaped, centered on mouth, demarcated from the anterior body end by peduncle, having minute spines on inner antero­ventral surface only. Pharynx highly muscular, intensely basophilic. Posterior esophageal swelling present. Intestine inverse U-shaped and lacking an­
terior cec. Testes intercel, an anterior testicular column plus 1 post­
teriorly. Cirrus sac enveloping seminal vesicle, prostatic gland cells, and cirrus; cirrus evertting dorsally near dextral body margin; postero­

**Type species:** Acipeciserolina petersoni n. sp. Other species: Acipeciserolina sp. (as Spirorhachis sp. of Appy and Dadswell [1978]).

**Remarks**

drotrema* Maillard and Ktari, 1978, and *Paracardicoloiides* Martin,
1974, in having inverse U-shaped ceca. It also has affinities to both *Chimaerohemecus* and *Hypan­
drotrema* by having a Laerer’s canal, not present, or at least not reported, in any other aporocotyliid genera. *Acipeciserolina*, however, is most similar to *Paracardicoloiides* because it has a comparable anterior sucker (oral disc of Martin [1974]), a sep­
ate testis posteriorly, an intercel omphalosome, and a common genital pore. It is noteworthy that the ventrolateral nerve cords of both *A. pe­
tersoni* and *Paracardicoloiides yamagutii* Martin, 1974 appear subter­
mlinal, i.e., they are blind-ending in the anterior end of the body pos­
terior to the level of the mouth. *Acipeciserolina* is easily differentiated from these and all other aporocotyliid genera in having a large, bowl­
shaped anterior sucker with minute spines on inner antero­ventral
surface only (Figs. 1, 7, 12, 19) and an obvious, highly muscular phar­
ynx (Figs. 1, 7, 12). In addition to that combination of features, the shape and orientation of the ventrolateral body spines differentiates the new genus. It has ventrolateral body spines that are spike-like and ar­
ranged in distinct transverse rows (Fig. 2). *Chimaerohemecus* and Hy­
pan­drotrema* each have 1 or 2 ventrolateral columns of C-shaped body spines (Van der Land, 1967; Maillard and Ktari, 1978; Bullard et
al., 2006), *Paracardicoloiides* has straight body spines that are distrib­
uted in a narrow ventrolateral field (Martin, 1974; Nolan and Cribb,
2004), and *Orchispirium* lacks spines altogether (Madhavi and Rao,
1970). None of the remaining accepted genera of Aporocotyliidae, all of which comprise species that infect bony fishes (Teleostei) only, have a bowl-shaped anterior sucker, strongly muscular pharynx, inverse U-shaped ceca, or Laerer’s canal.

**Acipeciserolina petersoni n. sp.**

(FIGS. 1–28)

**Diagnosis of adult (measurements and illustrations based on 10 whole mounted specimens, 2 serially-sectioned specimens, and 3 SEM...**
FIGURES 1–4. *Acipensericola petersoni* from the heart of *Polyodon spathula*, ventral view. (1) Body of holotype showing anterior sucker (as), pharynx (ph), nerve commissure (nc), esophagus (es), cecal bifurcation (cb), testes 1–6 (t1–t6), ovary (o), and excretory pore (ep). Bar = 500 μm. (2) Transverse rows of ventrolateral body spines, paratype. Bar = 50 μm. (3) Juvenile, body showing anterior sucker (as), pharynx (ph), esophagus (es), and intestinal anlagen (ia). Bar = 200 μm. (4) Genitalia, composite, ventral view, showing posterior-most testis of testicular column (t5), uterus (u), uterine eggs (ue), anterior trunk of vasa efferentia (ave), vas deferens (vd), ovary (o), everted cirrus (ec), seminal vesicle (sv), primary vitelline collecting duct (vt), Laurer’s canal (lc), ootype (oo), posterior trunk of vasa efferentia (pve), oviduct (ov) with sperm and serving as oviducal seminal receptacle, and posterior-most testis (t6). Bar = 200 μm.

*prepared specimens*: Body 2,405–4,026 (10) long, 810–1,325 (10) wide, 2.9–3.2 times longer than wide (Fig. 1); dorsum with honeycomb-like surface features (Figs. 21, 22, 28); ventral surface relatively smooth medially (Fig. 18). Body spines 7–12 (8) long (Figs. 2, 11), nearly indistinct in some whole mounted specimens; proximal end broadly rounded (Fig. 2); distal end with sharp tip protruding only slightly from tegument (Fig. 24). Spine rows 10–12 (7) long, numbering 110–135 (3) per side, indistinct in posterior region of some specimens, not contiguous posteriorly. Ventrolateral nerve cord becoming confluent with paired cord 75–233 (5) or 4–6% of body length from posterior body.
end (Figs. 1, 5); ventrolateral nerve commissure 139–273 (9) or 5–8% of body length from anterior body end, 64–140 (9) across width of worm, 20–35 (7) in diameter, perpendicular to midline of body (Fig. 1). Ventral sensory papillae 5–12 (10) in diameter, appearing volcano-shaped with light microscopy (Fig. 11) and nipple-like with SEM (Figs. 18, 20, 23), arranged in ventrolateral bands, delimited by approximate track of ventrolateral nerve cord and lateral body margin (Figs. 18, 23), indistinct or absent along midline of ventral and dorsal body surface. Anterior sucker 75–159 (9) in diameter or 9–13% of body width (Figs. 1, 7, 12, 18, 19, 21, 22, 25); anterior sucker spines about 3–5 long, conical, directed posteriorly, clustered, not occurring in clearly delin­eated rows (Figs. 24, 26, 27). Pharynx 139–199 (10) long or 38–53% of esophagus length, 109–144 (10) wide or 4–11 times esophagus width, with muscular wall 30–55 (10) thick, nearly as thick as anterior body end (Figs. 1, 7, 12). Esophagus 273–497 (10) long or 10–14% of body length, 10–25 (10) wide, with wall 5–10 (10) thick at level immediately posterior to pharynx; posterior swelling 40–100 (10) wide or 2–7 times width of anterior esophagus at level immediately posterior to pharynx, 104–224 (10) long or 31–67% of esophagus length, with wall 15–35 (10) thick (Figs. 1, 13). Esophageal gland indistinct surrounding swelling. Ceca bifurcating immediately posterior to esophageal swelling, 388–647 (10) or 14–19% of body length from anterior body end, extending posteriad in parallel 1,792–2,995 (10) or 72–79% of body length, ending 259–408 (10) or 9–13% of body length from posterior body end, 40–135 (10) wide, not extending laterally beyond ventrolateral nerve cord (Figs. 1, 3), with wall having cuboid basophilic mononucleate cells (Figs. 5, 14), containing granular material within lumen in some individuals; granular material blackish, probably comprising hematin and other compounds formed in decomposition of hemoglobin and erythrocytes, nearly filling cecal lumen in some specimens (Figs. 8, 10).

Testes spheroid, 6 in number, each approximately equal in diameter, comprising 5 testes (1–45) oriented in a single testicular column plus 1 separate testis (6) posteriorly; testicular column intercecal, 933–1,743 (10) long or 35–50% of body length, with anterior-most testis in column 221–565 (10) or 9–21% of body length from cecal bifurcation, 173–393 (10) wide or 20–32% of body width at widest level; posterior-most testis intercecal, between distal ends of ceca, 259–512 (10) long or 9–13% of body length, 114–299 (10) wide or 11–24% of body width, 313–597 (10) or 11–16% of body length from posterior margin of an-
Ovary 189–448 (10) long or 8–10% of body length, 213–472 (10) wide or 26–41% of body width, 0.9–1.5 times wider than long, dorsal to vas deferens and seminal vesicle; post-ovarian space 465–870 (10) long or 17–24% of body length (Fig. 4). Oviduct sinus, ultimately extending posteriorly while curving medially, recuring dorsally at anterior margin of t6, extending anteriorly while curving toward midline, containing sperm for entire length, 25–99 (7) in maximum width (Fig. 4). Laurer’s canal extending 74–136 (7) anteromedially from distal region of oviduct, immediately before union of vitelline duct and oviduct, opening on dorsal surface, 7–10 (7) wide, highly glandular. Primary vitelline collecting duct sinistral, ventral, extending posteriorly and following a track between or ventral to testicular column and intestinal cecum, extending 174–448 (9) posterior from margin of t5, uniting with oviduct immediately before joining ootype and short distance distal to where Laurer’s canal joined (Figs. 1, 4, 6). Ootype 45–69 (9) in diameter, medial, at level of cirrus sac, anterior to major posterior loop of oviduct, dorsal to ova efferentia (Figs. 1, 5, 17); Mehlis’ gland diffuse, surrounding ootype, with many hair-like ducts (Fig. 17). Uterus primarily dextral for most of length, running anteriad a short distance along midline from ootype, ventral to ovary, dorsal to vas deferens, becoming somewhat convoluted lateral to ovary, looping anteriad before running posteriorly, 20–37 (10) in maximum width (Figs. 1, 4). Uterine eggs 35–50 (7) long, large relative to many other aporocotylids, curved, crenulated, or collapsed completely in many specimens, lacking apparent operculum, with thin membranous shell when in distal portion of uterus (Fig. 4); ejected eggs seemingly more regular in shape (Fig. 15); mature egg in gill of definitive host not observed.

Excretory vesicle oblong, 71–146 (9) long, 10–32 (9) wide; excretory pore subterminal, dorsal, 42–128 (9) or 2–3% of body length from posterior body end (Figs. 1, 5, 9, 16); system difficult to observe in fixed specimens.

**Diagnosis of juvenile** (based on 1 whole mounted specimen from heart): Body 663 long, 98 wide; ventrolateral body spines indistinct. Nervous system indistinct. Sensory papillae not evident. Anterior sucker 35 in diameter. Pharynx 60 long, 40 wide. Esophagus 102 long or 15% of body length, 8 wide immediately posterior to pharynx, extending directly posteriorly; posterior esophageal swelling 28 long, 12 wide.
**Acipensercola petersoni** from the heart of *Polyodon spathula*, adult specimens, scanning electron micrographs. (18) Body, ventral view. Bar = 100 μm. (19) Ventral aspect of anterior sucker, lateral view. Bar = 10 μm. (20) Sensory papillae (circled) on ventral surface of body. Bar = 5 μm. (21) Body, dorsal view. Anterior sucker (as). Bar = 100 μm. (22) Anterior sucker (as), dorsal view. Bar = 100 μm. (23) Ventrolateral surface of body showing dispersion of sensory papillae (sp). Bar = 10 μm. (24) Spine row showing protruding tips of ventrolateral body spines (s). Bar = 0.5 μm. (25) Anterior sucker (as) and mouth (m), ventral view. Bar = 10 μm. (26) Higher magnification of Figure 25 showing anterior sucker spines (s). Bar = 4 μm. (27) Higher magnification of Figure 26 showing cluster of spines on the inner anteroventral surface of the anterior sucker. Bar = 2 μm. (28) Tegegument of body, dorsal view. Bar = 10 μm.

**Prevalence of infection**: Nine of 11 (82%) from Six Mile Lake and 6 of 6 (100%) from Lower Lake.

**Etymology**: The specific name ‘petersoni’ honors Jody Lee Peterson (Parasitology Section, Gulf Coast Research Laboratory) for his intuition and skill as a fisherman and for his invaluable field assistance to SAB during 1997 through 2007.

**Remarks**

Live specimens of *Acipensercola petersoni* used their flat, ventrally-concave surface and their ventrolateral body spines for attachment and locomotion. Like some other crawling aporocotylids that have been observed (Bullard and Overstreet, 2002, 2003, 2004), these flukes adhere to the walls of the heart, as well as to glass and plastic surfaces, by using the lateral body margin, presumably as a gasket that creates and maintains an internal negative pressure between the fluke’s body and the attachment surface. Specimens crawled by repeated, wave-like un-
dulations of the lateral body margins. As with adults of some species of Cardiola, as well as with Elaphobates euzeti Bullard and Overstreet, 2003, the transverse rows of ventrolateral body spines of A. petersoni probably enhance grip and traction for attaching to, and crawling over, uneven fleshy surfaces. However, these spines apparently are not required for initial attachment and crawling since adults adhered to, and crawled over, impervious surfaces, e.g., glass and plastic, and the juvenile specimen lacked spines altogether (Fig. 3). Regarding the function of the anterior sucker, a few live specimens applied their anterior sucker to the surface of the plastic sample bag and remained anchored there, even after the bag was shaken vigorously. These specimens, however, could be dislodged by inserting the bristles of an artist’s brush beneath the rim of the anterior sucker.

Parsimony analysis of SSU data (Fig. 29) derived from a single specimen of A. petersoni, as well as from several other aporocotylids, produced a single most-parsimonious tree with nodal support ranging from 74–100 and a tree length of 1,311 (224 of the 1,815 total base pairs sequenced were informative). The tree topology showed that C. trondheimensis, a chondrichthyan blood fluke, was the most basal ingroup taxon in the tree, with A. petersoni basal to Sanguinicola cf. inermis, both of which were basal to 3 euteleost (Euteleostei) aporocotylids included in the analysis, i.e., Aporocotyle spinosicanalis, Plethorchis acanthus, and Neoparacardicola nasonis.

**FIGURE 29.** Phylogram based on maximum parsimony analysis of small subunit ribosomal DNA. Nodal support based on bootstrap resampling is to the left of each ingroup node. Note that the aporocotylids infecting Chondrichthyes (Chimaerohemecus trondheimensis), Aipenseriformes (Acipensericola petersoni), and Ostariophysi (Sanguinicola cf. inermis) are basal to those aporocotylids that infect Euteleostei (Aporocotyle spinosicanalis, Plethorchis acanthus, and Neoparacardicola nasonis).

### DISCUSSION

At least 1 species of the new genus infects a sturgeon in North America. This reported (Appy and Dadswell, 1978), previously collected, but unnamed aporocotylid infects the mesenteric vessels of shortnose sturgeon (Acipenser brevirostrum Lesueur, 1818 [Acipenseridae]) in the Saint John River Estuary, New Brunswick, Canada. Appy and Dadswell (1978) detailed 9 immature specimens and identified the specimens as Spirorchis sp.; however, we regard the unnamed species as congeneric with A. petersoni because it has: (1) a prominent, bowl-shaped anterior sucker that is centered on the mouth; (2) a clearly delineated pharynx that is located immediately posterior to the anterior sucker; (3) an inverse U-shaped ceca that extends to near the posterior body end; (4) a testicular column; and (5) ventrolateral body spines. Although ventrolateral body spines were neither described nor illustrated by Appy and Dadswell (1978), we examined a voucher specimen (USNPC No. 73138) and confirmed the presence of ventrolateral spines. However, since we could not discern ventrolateral body spines in the juvenile specimen of A. petersoni in our collection (Fig. 3), perhaps some of the younger specimens collected by Appy and Dadswell also lacked them. In addition, ventrolateral body spines can be overlooked quite easily because of their small size and, in poorly fixed specimens, these spines may be lost because the thin margins of the body seem to be especially vulnerable to autolysis, which can result in the subsequent detachment of the spines. The specimens of Appy and Dadswell (1978) from the sturgeon remain unidentified, and not yet ready to be described, because no adult aporocotylid material from a shortnose sturgeon has been reported or examined by us. We suspect that it represents a species distinct from A. petersoni. As previously stated, the morphological features of the species certainly indicate that it belongs in Acipensericola, but its host’s phylogenetic affiliation (Acipenseridae rather than Polyodontidae) and geographic distribution (New Brunswick River draining to northwestern Atlantic Ocean rather than the Mississippi River drainage) suggest that it represents a new species. Addressing this taxonomic problem should require detailed necropsies of freshly killed, wild-caught shortnose sturgeons. Unfortunately, such an opportunity is rare and has proven logistically difficult for us to arrange because the shortnose sturgeon, like nearly all sturgeon species, is presently protected throughout its range.

Collectively, aporocotylids exhibit several different morphological types of suckers associated with the mouth (Bullard and Overstreet, 2003), and we think that the fine features associated with these various types of suckers help elucidate evolutionary relationships within the group. Hereinafter, we refer to them as...
“anterior suckers.” The shape of the sucker, the spines of the sucker, and the location of the mouth are diagnostic for at least some genera, e.g., *Elaphrophates* Bullard and Overstreet, 2003. Homology of these various anterior suckers presently is uncertain for some taxa, which represents a barrier to understanding the phylogenetic interrelationships among aporocotylid genera. Demonstrating homology of these various suckers is beyond the scope of the present paper because it requires a complete phylogenetic analysis of the family; however, we regard the anterior suckers of *A. petersoni* and *P. yamagutii* as 2 slightly different variations of the same homologous sucker type, and we herein delineate that type from those of other aporocotylids by its general shape and the relative position of the mouth. In *Acipensercola* and *Paracardicoloides*, unlike all other accepted aporocotylid genera, the anterior sucker is bowl-like, centered on the mouth, and demarcated from the anterior body end by a short trunk or peduncle that supports the sucker and can direct it anteroventrally (Figs. 1, 25; Fig. 3 of Martin [1974]). Despite these general similarities, the pharynx and spination associated with the anterior sucker differs among the species of *Acipensercola* and *Paracardicoloides*. The anterior sucker of *A. petersoni* has spines on its inner anterodorsal surface only and is accompanied by a muscular pharynx, whereas that of *P. yamagutii* reportedly lacks exposed spines and an associated pharynx. The bowl-shaped anterior sucker of *Acipensercola* and *Paracardicoloides* is superficially like that of a spirorchiid because it is relatively large and centered on the mouth, but these aporocotylid genera have a peduncle associated with the sucker. In contrast, the spirorchiids studied by 1 of us (S.A.B.) have a sucker that is more strongly muscular and wholly invested in the forebody; there is no obvious peduncle or demarcation between the sucker and body-proper that can be discerned from fixed, stained whole mounts. Based on this feature, we suspect that the anterior sucker (‘oral sucker’) of spirorchiids (Platt, 2002) is not readily comparable to that of *Acipensercola* and *Paracardicoloides*.

Our understanding of the phylogenetic interrelationships of aporocotylid remains unclear because no clade-based phylogenetic hypothesis involving morphological or molecular sequence data for the majority of aporocotylid genera has been published. Two obvious obstacles to completing such a task are that (1) several of the most specious aporocotylid genera need revision, e.g., *Aporocotyle* Odhner, 1900, *Cardiola* Short, 1953, and *Sanguinicola* Plehn, 1905, and (2) type material for many of the named species in those genera are in poor condition or not available to borrow. Although a taxonomic revision and phylogenetic analysis of Aporocotylidae are in preparation by 1 of us (S.A.B.), additional descriptions of new aporocotylid species that infect fishes belonging to previously undocumented host lineages promise to further advance our knowledge of how these flukes evolved among various lineages of “fish.” The potential of the present study offer some preliminary insight into the potential interrelationships among particular aporocotylid genera and their host groups and provide a framework from which to test future hypotheses about aporocotylid-fish co-phylogeny. For example, the morphological similarities we observed between *A. petersoni* and those aporocotylids that infect non-eutelost fishes indicate a strong phylogenetic affiliation among these genera. Further, the available SSU data indicate that non-eutelost aporocotylids are basal to those that infect eutelosts (Fig. 29). This preliminary result contradicts the notion that fish blood flukes lack a detectable level of phylogenetic host specificity to their definitive hosts.

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A NEW SPECIES OF SARCOCYSTIS (APICOMPLEXA: SARCOCYSTIDAE) FROM THE BLACK BEAR (URSUS AMERICANUS)

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ABSTRACT: Infection with Sarcocystis species is common in herbivores but is rare in bears. Histological sections of 374 black bears (Ursus americanus) from Pennsylvania were examined for sarcocysts. In total, 3 sarcocysts were found in 3 bears, with 1 sarcocyst per section. Sarcocysts from 2 bears were considered a new species, Sarcocystis ursusi. Sarcocysts of S. ursusi n. sp. were microscopic and contained only bradyzoites. By light microscopy, the sarcocyst wall was thin (0.5 μm thick) and had minute serrations. Ultrastructurally, the serrations on the sarcocyst wall consisted of villar protrusions (Vp) that were mostly 0.5 μm long. The Vp had bundles of electron-dense microtubules that were as wide as long; these microtubules extended deep into the ground substance layer, a feature that distinguished this species from unnamed sarcocysts from black bear. Bradyzoites were 4.8–6.0 μm long. The sarcocyst from the third bear was structurally different from S. ursusi; its sarcocyst wall was approximately 2 μm thick and had finger-like villi on the cyst wall giving the sarcocyst wall a striated appearance.

Parasites belonging to species of Sarcocystis (Apicomplexa) have a 2-host life cycle (Dubey et al., 1989). The definitive host becomes infected by ingesting the asexual stage (sarcocyst) encysted in the intermediate host’s tissues, whereupon the sexual cycle may commence in the lamina propria of the small intestine of the carnivore. Typically, species of Sarcocystis exclusively parasitize a single intermediate host species. Little is known of the species of Sarcocystis in bears. Previously sarcocysts of an unnamed species were found in tissue sections of 6 of 53 black bears (Ursus americanus) from the southeastern and northwestern United States (Crum et al., 1978), i.e., 1 of 92 in North Carolina (Dubey et al., 1998), 1 of 132 from Florida (Cheadle et al., 2002), and 2 of 46 in Oregon (Foreyt et al., 1999). However, sarcocysts from the black bear have not been named. In the present paper sarcocysts from black bears from Pennsylvania are described.

MATERIALS AND METHODS

Samples of skeletal muscle from 374 black bears, legally shot in November 1997, were fixed in 10% buffered formalin 1–4 days after death. Attempts were made to collect muscle away from gunshot wounds. Routine histologic examination was performed on paraffin-embedded sections (5 μm) stained with hematoxylin and eosin (H and E).

For transmission electron microscopy, a paraffin-embedded section from 1 bear was postfixed in 1% osmium tetroxide in Millonig’s phosphate buffer, rinsed in the same buffer, dehydrated in ethanol, and embedded in epoxy resin. Semithin sections were stained with Toluidine blue in 1% sodium tetraborate. The ultrathin sections were contrasted with uranyl acetate and lead citrate before examination in a transmission electron microscope. Sections of 1 sarcocyst from bear no. 1 were examined ultrastructurally.

RESULTS

Sarcocysts were found in 3 bears. All 3 infected bears were adult females (51, 80, 90 kg). Bear no. 1 (ear tag 97-1035) was from Houston Township, Clearfield County; bear no. 2 (ear tag 97-1626) was from Foster Township, McKean County; and bear no. 3 (ear tag BV 13) was from Venango Township, Clinton County. Only 1 sarcocyst was found in each bear. Sarcocysts were microscopic. In bear no. 1, the sarcocyst was cut longitudinally in 3 parts that probably belonged to a single sarcocyst (the myocyte was cut unevenly, and thus the sarcocyst appeared to be 3 separate sarcocysts). The total length of the sarcocyst was 700 × 50 μm. The sarcocyst from bear no. 2 measured 250 × 75 μm in size and its sarcocyst wall (Fig. 1B) was identical to the sarcocyst in bear no. 1. We were unable to locate this sarcocyst in sections made for transmission electron microscopy (TEM). A new name is proposed for the sarcocysts in bears nos. 1 and 2.

DESCRIPTION

Sarcocystis ursusi n. sp. (Figs. 1–3)

Diagnosis: In 5 μm section stained with H and E; sarcocyst wall thin (<1 μm) with minute serrations (Fig. 1A, B). Sarcocyst interior packed with slender bradyzoites; difficult to measure because boundaries indistinct. Ultrastructurally, outer layer of sarcocyst (parasitophorous vacuolar membrane [PVM], wavy in outline, with minute undulations that did not invaginate toward sarcocyst interior (Fig. 2). Undulations occurred at irregular intervals. Interior of undulations electron-dense, thinned out at irregular distances. Villar protrusions (Vps) on PVM located at irregular distances. Vps up to 3.8 μm long depending on plane of section, most 0.5 μm long (Fig. 2). Vps with tufts of prominent electron-dense microtubules continuing into ground substance (GS) layer. GS 0.3–1.0 μm thick; thickest at origin of septa (Fig. 2). GS continued into interior of sarcocyst as septa (Fig. 2). Both sarcocysts mature, containing fully formed bradyzoites; metrocytes absent. Groups of bradyzoites separated by septa. Bradyzoites butted against GS. Longitudinally cut bradyzoites measured 4.8–6.0 × 1.4–1.8 μm (n = 8). Bradyzoites with conoid, micronemes, 1–2 rhoptries per section, and posteriorly located nucleus (Fig. 3). Rhoptries with long neck, often looped so that blunt end directed toward conoid (Fig. 3). Micronemes numerous, located mostly at conoidal end of bradyzoite (Fig. 3). Nucleus located in posterior half of parasite. Amylopectin granules present throughout bradyzoites.

Taxonomic summary

Type host: Black bear (Ursus americanus).
Other hosts: Unknown.
Type locality: Clearfield County, McKean County, Clinton County, Pennsylvania.
Specimens deposited: Two histological sections stained with H and E were deposited as syntypes for bears nos. 1 and 2 in the United States National Parasite Collection (USNPC nos. 10101 and 10102), United States Department of Agriculture, Beltsville, Maryland. One H and E
Figure 1. (A, B) Sarcocysts of *Sarcocystis ursusi* n. sp. in skeletal muscles of the naturally infected black bears. Note thin sarcocyst wall with minute serrations on sarcocyst wall (arrowheads) and septa (s). (C) Sarcocyst of *Sarcocystis* sp. Note thick sarcocyst wall with finger-like villi.

A stained section from bear no. 3 was deposited as voucher specimen from bear no. 3 (USNPC no. 10103).

*Sarcocystis* sp.

*Diagnosis:* Sarcocyst in bear no. 3 cut in cross section; measured 75 × 50 μm. Sarcocyst wall (Fig. 1C) distinct from those in bears nos. 1 and 2. Its sarcocyst wall approximately 2 μm thick with finger-like villi on sarcocyst wall giving it a striated appearance. Unable to locate sarcocyst in sections made for TEM.

Remarks

Complete life cycles of *Sarcocystis* spp. are known for only a few species of animals, mostly those in livestock (Dubey et al., 1989). Most *Sarcocystis* species have been named based on their intermediate host occurrence and their sarcocyst structure. Dubey et al. (1989) and Dubey and Odening (2001) recognized 35 types of sarcocysts based on their structure.

Morphologically, the sarcocysts from black bears nos. 1 and 2 were distinct from the unnamed species previously reported from black bear. Prior to the present report, only 1 sarcocyst from a black bear was
FIGURE 2. TEM of the sarcocyst of *Sarcocystis ursusi* n. sp. with details of the sarcocyst wall cut at different angles (A, B, C). Note villar protrusions (V) on the sarcocyst wall. Villar protrusions have prominent microtubules (T) that extend (arrow) into the ground substance layer (GS). The parasitophorous vacuolar membrane has undulations (arrowheads). Bradyzoites (B) are located just beneath the sarcocyst wall. Also note prominent septum (S).
studied ultrastructurally (Dubey et al., 1998). In that sarcocyst the sarcocyst wall had 2-μm-long villar protrusions that lacked microtubules. The sarcocyst from bear no. 3 resembled the sarcocyst from black bear from North Carolina (Dubey et al., 1998), but in the absence of electron microscopic examination it was difficult to compare them.

Recently a new species, Sarcocystis arctosi, was described from brown bear (Ursus arctos) from Alaska (Dubey et al., 2007). Sarcocysts of S. arctosi are structurally distinct from S. ursusi; sarcocysts of S. arctosi have minute undulations on the sarcocyst wall instead of long villar protrusions on S. ursusi.

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LITERATURE CITED


A NEW TRIAENOPHORID TAPEWORM FROM BLACKFISH CENTROLOPHUS NIGER

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ABSTRACT: Milanella familiaris n. gen. and n. sp. (Bothriocephalidea: Triaenophoridae) is proposed to accommodate a new cestode from blackfish Centrolophus niger (Gmelin) (Perciformes: Centrolophidae). Milanella is characterized as follows: trapeziform, i.e., markedly craspedate proglottids with a velum-like posterior margin and horn like lateral projections; pyriform uterine sac in the first gravid proglottids; arrow-shaped scolex with well-developed apical disc and prominent posterior margins; strobila with intensively stained corpuscles, most numerous in the anterior part; deeply lobated ovary; absence of a neck; a large, pyriform, thin-walled cirrus-sac with the proximal part bent anteromedially; vagina posterior to the cirrus-sac; and cortical vitelline follicles. Milanella most closely resembles Bathycystus Kuchta and Scholz, 2004, Pistana Campbell and Gartner, 1982, and Probothriocephalus Campbell, 1979, differing mainly in the shape of proglottids and uterine sac.

Bothriocephalidean tapeworms are common parasites, mostly of marine fish. Three species are now known from the blackfish, Centrolophus niger (Gmelin, 1789) (Perciformes: Centrolophidae), a bathypelagic fish with cosmopolitan distribution (Kuchta and Scholz, 2007). One species belongs to the Triaenophoridae, whereas the remaining 2 taxa are in the Echinophallidae (Bray et al., 1994; Kuchta and Scholz, 2007). In the present paper, another triaenophorid is reported from the blackfish and a new genus is proposed to accommodate this new taxon.

MATERIALS AND METHODS

Tapeworms were found in freshly killed C. niger (a total of 4 specimens examined from August to September 2004) from the North Atlantic Ocean to the west of the Outer Hebrides (56°20'–57°00'N, 9°00’–9°12’W; depth 550–1,000 m). The fish were caught during an expedition of the research vessel Scotia of the Fisheries Research Services, Scotland, U.K., in autumn 2004.

Most tapeworms found were fixed with hot (almost boiled) 4% formaldehyde solution for morphological studies, including scanning electron microscopy (SEM) and histology. Remaining tapeworms were fixed with 96% alcohol for molecular study or with 3.5% glutaraldehyde for ultrastructural (transmission electron microscopy) studies. The specimens for a morphological study were stained with Mayer’s hydrochloric carmine solution and mounted as permanent slides in Canada balsam. The strobila were also cross-sectioned, using standard histological procedure (Scholz and Hanzelova, 1998). One scolex and several proglottids were prepared for SEM observations using the methodology outlined by Scholz et al. (1998). Illustrations were made using a drawing attachment of an Olympus BX51 microscope with Nomarski interference contrast. Measurements in descriptions are given in micrometers (μm) unless otherwise stated.

To confirm that the new tapeworms do not represent a morphologically distinct form (morphotype) of Amphicoelos heteropleura (Diesing, 1850), which possesses the scolex of a fairly similar shape and occurs simultaneously in blackfish, partial sequences of 3 genes (cox1, ITS-2, and 28S rRNA) of 1 isolate of the new cestode (sample TS 04/155) and 2 isolates of A. heteropleura from the North Atlantic Ocean off Outer Hebrides (TS 07/101) and the Pacific Ocean off New Zealand, collected by Claire Healy (TS 05/45), were compared.

Genomic DNA was extracted from strobila of adult worms using the JETQUICK Tissue DNA Spin Kit (GENOMED, St. Louis, Missouri). To amplify the parts of the above-mentioned genes, primers from FLO1 and ITSS (Logan et al., 2004), CO1A2 and CO1B2 (Štefka, unpubl. obs.), and LSU5 and 1500R (Littlewood et al., 2000; Olson et al., 2003) were used. Standard 25–μl polymerase chain reaction tests were performed with 0.2 units Taq DNA polymerase (Top-Bio, Prague, Czech Republic), and the cycling conditions were as follows: initial denaturation 96°C/15 min followed by 30 cycles of 96°C/1 min, 50°C/1 min, and 72°C/2 min.

Amplified products and directly cycle-sequenced on ABI PRISM 3100 automated sequencer. When sequencing the 28S rRNA gene, internal sequencing primers 300F, 400R, and 900F (Littlewood et al., 2000; Olson et al., 2003) were used. Contiguous sequences were assembled with Seqman (DNASTAR, Madison, Wisconsin); sequences of individual genes were aligned in program MAFFT (Katoh et al., 2005) by the L-INS-i method. Pairwise distances among the 3 taxa were determined using PAUP* ver. 4.0b10 (Swofford, 2002).

DESCRIPTION

Milanella n. gen.


Taxonomic summary

Type and only species: M. familiaris n. sp.

Etymology: The genus is named for Milan Říha, Institute of Hydrobiology, BC AS CR, who helped the senior author with sampling cestodes of marine fish in Indonesia.

Remarks

The new genus is placed in Triaenophoridae Lönning, 1889, because it possesses a marginal genital pore, follicular vitellaria, and a ventral uterine pore. This family currently includes 20 genera (Kuchta and Scholz, 2007; Kuchta et al., 2008).

Milanella is typified by a combination of the following characteristics: (1) trapeziform, markedly craspedate proglottids with velum-like posterior margins and horn-like posterolateral projections; (2) pyriform uterine sac in the first gravid proglottids, becoming widely oval to elongate in the posterior part of the strobila; (3) arrow-shaped scolex with prominent posterior margins, well-developed apical disc and elongate, simple bothria; (4) intensively stained corpuscles, most numerous in the anterior part of the strobila; (5) large pyriform, thin-walled cirrus-sac with the proximal part bent anteromedially; (6) deeply lobated ovary; (7) absence of a neck.

Milanella most closely resembles Bathycystus Kuchta and Scholz, 2004, Pistana Campbell and Gartner, 1982, and Probothriocephalus Campbell, 1979. However, it can be distinguished from these genera by...
possessing the above characteristics, especially in having a different shape of proglottids and a pyriform uterine sac. Morphological similarity of *Milanella* with *Bathycestus* and *Probothriocephalus* is also reflected in their phylogenetic relatedness, because these taxa formed a sister group in comparative analyses of partial sequences of the 18S and 28S rRNA genes (Brabec et al., 2006; Fig. 3; Kuchta, 2007—Fig. 10; in both cases, *Milanella* was misidentified as *Amphicoelius*. *Milanella* is most closely related to *Probothriocephalus*, whereas *Amphicoelius* forms a sister clade with *Bathycestus* (J. Brabec, unpubl. obs.).

**Milanella familiaris** n. sp. (Figs. 1–16)

Description (based on holotype [field number DS 263a]—complete worm on 4 whole mounts and 4 slides with cross-sections, 1 paratype [DS 269]—1 complete and 3 slices of cross-sections, and 2 vouchers [DS 137 and 146]—2 incomplete specimens): Strobila up to 40 cm long in holotype; maximum width 1 mm. External and internal segmentation present; secondary segmentation present. Proglottids trapeziform, from slightly wider than long to longer than wide in last gravid proglottids, markedly crespedate, with velum-like posterior margin and horn-like posterolateral projections (Figs. 1–4, 15).

Surface covered with filiform microtriches similar in shape and size; posterior margin of proglottids with narrow band of spiniform microtriches (Figs. 1, 15, 16). Margins of bothria with tumuli-like globular structures (Figs. 13, 14). Two pairs of elongate, median, pyriform in first gravid proglottids; dorsoventrally (diameter 11–20); ventral canals wide (diameter 23–88), connected by transverse anastomoses. Longitudinal musculature well developed, formed by large bundles of muscle fibers (Fig. 11). Numerous intensively stained corpuscles (diameter 11–25; n = 25) present throughout strobila, reaching up to scolex, less numerous and transparent in posterior part of strobila (Figs. 1–5, 11).

Scolex arrow-shaped in lateral view (Fig. 1), 693–1,016 long by 510–597 wide (n = 4), with prominent posterior margins freely projecting over anterior half of first proglottid. Apical disc well developed, 200 wide dorsally, 185–280 wide laterally, 56–107 high, with deep incisions on dorsal and ventral sides (Fig. 12). Bothria elongated, deep posteriorly, becoming more shallow posteriorly, 584–829 long (n = 7) (Fig. 12). Neck absent, first proglottids appearing immediately posterior to scolex (Fig. 1).

Immature proglottids 292–1,029 long by 294–1,018 wide (n = 18) (Fig. 2). Mature proglottids, i.e., with spermatozoa in vas deferens, wider than long, 565–734 long by 627–1,021 wide (n = 18). Gravid proglottids wider than long or longer than wide, 587–1,273 long by 857–1,133 wide (n = 18) (Figs. 3, 4). Testes medullary, oval, 74–109 in number (n = 10), 47–61 in diameter (n = 14), forming 2 narrow longitudinal bands, continuous between proglottids, with testes lacking medially and near lateral margins (Fig. 3). Cirrus-sac large, thin-walled, pyriform, with proximal part anteromedially (Figs. 3–5), 269–417 long by 72–188 wide (n = 21) (length: width ratio 0.6–1.4:1); representing 34–48% of width of mature proglottid (n = 21). External sperm ducts forming numerous loops posterior to cirrus-sac; internal sperm ducts strongly coiled; cirrus unarmed, opening to deep genital atrium (Fig. 5). Intensively stained cells surround proximal part of cirrus-sac externally (Fig. 5).

Genital pore marginal, alternating irregularly (Fig. 3), equatorial to slightly post-equatorial (at 48–63% of proglottid length). Ovary asymmetrical, deeply lobed, especially apically, slightly submedian, poral, 127–239 long by 318–420 wide (n = 10) (Figs. 3, 4). Vagina thick-walled, 14–18 in diameter, with proximal part straight, almost horizontal, opening posterior to cirrus-sac into genital atrium; vaginal sphincter absent (Fig. 5). Vitelline follicles large, 31–43 in diameter (n = 15), cortical, forming 2 wide longitudinal bands confluent between proglottids, separated mediately, rarely connected by several follicles at postovarian region (Figs. 4, 5, 11).

Uterine duct winding, forming numerous tightly coiled loops, filled with eggs and enlarging in gravid proglottids (Figs. 3, 4). Uterine sac thick-walled, median, pyriform in first gravid proglottids, then becoming widely oval to elongate, occupying less than one-half of proglottid length and width (Figs. 3, 4). Uterine pore thick-walled, in posterior part of uterine sac (Figs. 3, 4). Eggs oval, thin-walled, operculate (not confirmed by SEM), unembryonated, with small, knob-like thickening on aboperculate pole, 56–62 long by 35–41 wide (n = 22) (Figs. 6–10).

**Taxonomic summary**

Type host: Blackfish *C. niger* (Gmelin) (Perciformes: Centrolophidae).

*Site of infection:* Anterior part of the intestine, with the scolex attached to the pyloric caeca.

*Type locality:* North Atlantic west off the Outer Hebrides (57°20'N, 9°33'W; depth 1,000 m; collection date, 3 September 2004).


*Synonyms:* *A. heteropleura* (Diesing, 1850) of Bray et al. (1994) in part; *A. heteropleura* of Brabec et al. (2006).

*Etymology:* The specific name *familiaris* (= belonging to a household, friendly, intimate, current, common) refers to the common occurrence in the fish host (all 4 examined blackfish were infected).

*Prevalence and intensity:* *Milanella familiaris* was found in all 4 blackfish from the North Atlantic, but not in the same host from New Zealand and Italy (number of fish examined not available). The intensity of infection was 1–2 specimens per host.

**Remarks**

Light microscope observation of the eggs of *M. familiaris* liberated from the uterus and kept in ethanol revealed the presence of a very feebly developed operculum, which is difficult to see (Figs. 7, 8). Since no eggs suitable for SEM observation were available, the presence of the operculum should be confirmed in new material, because the presence/absence of an operculum in bothriocephalidean cestodes can often be confirmed with certainty only when eggs are studied using SEM (Bray et al., 1994; Kuchta, 2007). Bray et al. (1994; their Fig. 10.66) illustrated a mature proglottid of a worm identified as *A. heteropleura*. However, this tapeworm markedly differs from the actual *A. heteropleura* described by Diesing (1850) and later redescribed by Schumacher (1914), especially in the shape of proglottids that are very short and wide in *A. heteropleura*, with prominent lateral projections containing vitelline follicles. The tapeworm illustrated by Bray et al. (1994) is undoubtedly conspecific with *M. familiaris*. Similarly, *M. familiaris* found by the senior author in *C. niger* off the Outer Hebrides and then sequenced by Brabec et al. (2006) was also misidentified as *A. heteropleura*.

A comparative analysis of sequences of 3 genes (*cox1, ITS-2*, and 28S rRNA) of *M. familiaris* from the North Atlantic and 2 isolates of *A. heteropleura* from the North Atlantic Ocean and Pacific Ocean has shown marked species-specific differences. A total of 519 bp-long *ITS-2* sequences of both samples of *A. heteropleura* was identical, whereas the sequence of *M. familiaris* differed in 5.78% (sequence similarity 0.942). Partial sequences of the *cox1* gene (426 bp long) of *A. heteropleura* samples differed in 6.57% of the nucleotides (similarity 0.934),
but differed from that of *M. familiaris* in 14.79–15.26% of nucleotides (similarity 0.847–0.852). Sequences of the 28S rRNA gene of *A. heteropleura* samples (length 1467 bp) differed in 0.07% of nucleotides, while that of *M. familiaris* showed a much greater sequence difference (2.38–2.45% of nucleotides) from *A. heteropleura* samples (similarity 0.975–0.976).

**DISCUSSION**

Bothriocephalidean tapeworms are 1 of the most typical parasites of deep-sea teleosts, with several taxa specific to centrolophid fish (Campbell, 1983; Klampel et al., 2001; Kuchta and Scholz, 2007). Blackfish *C. niger* harbors species of as many as 4 different genera of these cestodes. Besides *M. familiaris*, the following taxa have been reported: *A. heteropleura* (Diesing, 1850) (syn. *Amphicotyle typica* Diesing, 1863) (Triacanthophoridae), *Bothriocotyle solinosomum* Ariola, 1900, and *Echinophallus wageneri* (Monticelli, 1890) (syn. *Echinophallus setii* (Ariola, 1895)) (Echinophallidae). Both echinophallids differ from *M. familiaris* in having a submarginal genital pore and spined cirrus (Bray et al., 1994; Kuchta, 2007; Kuchta and Scholz, 2007).

In scolex morphology, *M. familiaris* somewhat resembles *A. heteropleura*, also found in blackfish from the North Atlantic. However, the bothria of the former taxon possess a hole-like depression near their posterior margin (Kuchta, 2007; Fig. 7B). In addition, *A. heteropleura* can be distinguished easily from *M. familiaris* by the shape of the proglottids, which are very short and wide, with prominent lateral projections containing vitelline follicles, and strobilar morphology (Schumacher, 1914). The taxa are also markedly distinct in their sequences of all 3 genes (*cox1*, *ITS-2*, and 28S rRNA) used in the present study.
Milanella is mainly characterized by the shape of the proglottids that are trapeziform, markedly craspedote, with a velum-like posterior margin and horn-like posteralateral projections. Among triaenophorids, trapeziform proglottids are present only in Eubothrioides lamellatus Yamaguti, 1952, from Zenopus nebulosus (Zeiformes), but they are much wider than long and devoid of the velum-like posterior margins and posteralateral projections present in M. familiaris (see Yamaguti, 1952).

Another specific characteristic of Milanella is a thin-walled, pyriform uterine sac in the first gravid proglottids. A slightly similar uterine sac is present also in Amphicotyle ceratias Tkachev, 1979, a parasite of Ceratias holboelli Kröyer (Loxophiiformes), and Pistana eurypharyngis Campbell and Gartner, 1982, from Eurypharynx pelecanaoides Vaillant (Saccopharyngiformes) (Tkachev, 1979; Campbell and Gartner, 1982). However, the uterine sac of P. eurypharyngis is much more elongate (Campbell and Gartner, 1982); in addition, the species differs markedly from M. familiaris in possessing a dendritic ovary and rectangular, elongated proglottids lacking posteralateral horn-like projections. Amphicotyle ceratias has a uterine sac situated almost horizontally in the aporal half of proglottids, which are much wider than long (Tkachev, 1979; Kuchta, 2007).

The strobila of M. familiaris contains numerous intensively stained cupules, most numerous in immature proglottids. Similar cupules are present in all other tapeworms occurring in blackfish, namely A. heteropleura, B. solinosomum, and E. wagneri, which belong to 2 different families. However, intensively stained cupules of similar shape and size were also observed in species of Bathysteus Kuchta and Scholz, 2004, and Fistulicola Lühe, 1899 (R. Kuchta, unpubl. obs.), parasites of shortfin spine eel (Notacanthus bonaparte Risso) and swordfish (Xiphias gladius L.) from the Atlantic Ocean.

Milanella familiaris was found exclusively in the North Atlantic, together with all other 3 tapeworms known from C. niger, but not in the Mediterranean Sea and Pacific Ocean off New Zealand. In blackfish caught in more than 1,000 m of depth, M. familiaris was the only tapeworm found. On the other hand, blackfish caught at around 500 m of depth harbored all 4 bothriocephalidean species, but M. familiaris was represented by immature tapeworms only. This indicates that M. familiaris may be a typical parasite of blackfish living in deeper sea.

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LITERATURE CITED


REDESCRIPTION OF SYNTHESIS PONTOPORIAE N. COMB. WITH NOTES ON S. TURSIONIS AND S. SEYMOURI N. COMB. (DIGENEA: BRACHYCLADIIDAE ODHNER, 1905)

Juliana Marigo*,†, Ana Carolina Paulo Vicente*, Ana Luisa Schifino Valente‡, Lena Measures§, and Cláudia Portes Santos||

ABSTRACT: Synthesis pontoporiae n. comb. is redescribed, together with Synthesium tursionis and Synthesium seymouri n. comb.; the parasites were obtained from stranded and accidentally caught cetaceans. The sucker ratio (ratio between widths of the oral and ventral suckers) in S. pontoporiae was 1:1.8–3.0 (mean 1:2.2); in S. tursionis was 1:0.5–0.7; and in S. seymouri was 1:0.5–0.7. Synthesium pontoporiae differed from its congeners by additional diagnostic characters, including: oval to lobed testes; small cirrus with pyriform proximal region and flexible, tubular distal region formed by evagination of ejaculatory duct; and vitellarium in small follicles extending from the level of the seminal vesicle to the posterior extremity of the body and not forming dendritic radial bunches. Data on the morphology of adult S. pontoporiae and S. tursionis were inferred from confocal laser microscopical observations.

The brachycladiids (see Gibson, 2005), formerly known as campulids, parasitize the hepatic and pancreatic ducts, intestine, lungs, and head sinuses of marine mammals. As indicated by Gibson (2005), good specimens are difficult to obtain from decomposed or frozen hosts, resulting in poorly preserved specimens that make interpretation of the morphology difficult. Their taxonomy is problematic and has a convoluted history (Fernández et al., 1994; Gibson, 2005).

The Campulidae Odhner, 1926 was established for genera previously contained in the Brachycladiinae, based on Brachycladium Loss, 1899, which was believed to be a synonym of Campula Cobbold, 1858. Faust (1929) raised the Brachycladiinae to a family status as the Brachyclidiidae. However, Stunkard and Alvey (1930) preferred to use Campulinae rather than Brachyclidiinae within the Fasciolidae, and included Campula (syn. Brachycladium), Lecithodesmus Braun, 1902, Orthosplanchnus Odhner, 1905, Zalophotrema Stunkard and Alvey, 1930, and Synthesium Stunkard and Alvey, 1930, the latter as a new genus based on Orthosplanchnus tursionis (Marchi, 1873) Odhner, 1926. Price (1932) accepted this latter arrangement, adding Odneriella Skrjabin, 1905 and Hadwennius Price, 1932; Hadwennius seymouri Price, 1932 was considered as the type species of the latter genus, from the type host Delphinapterus leucas (Pallas, 1776) from Canada. Hadwennius seymouri was described as having an oral sucker slightly larger than the ventral sucker, oval testes, and a vitellarium composed of rosette masses of radiating cords of follicles extending from the vicinity of the anterior testis to the posterior extremity of the body. Synthesium (=Orthosplanchnus) tursionis (Marchi, 1873) Stunkard and Alvey, 1930, from the type host Tursiops truncatus (Montagu, 1821) in European waters, was the most closely related species to H. seymouri.


Pontoporia blainvillei Gervais and d’Orbigny, 1844 is a small cestacean, commonly called franciscana and endemic to South Atlantic waters, that occurs from Espírito Santo State, Brazil (18°25’S) to Peninsula Valdez, Argentina (42°35’S) (Siciliano et al., 2002). The species is in danger of extinction, and it is a priority of the Brazilian environmental agency (IBAMA 1997, 2001) to identify and characterize possible stocks or sub-populations.

The intestinal trematode Synthesium pontoporiae (Raga et al., 1994) n. comb. is exclusive to P. blainvillei and was proposed as a biological indicator for stock identification of the franciscana’(Aznar et al., 1995; Andrade et al., 1997; Secchi et al., 2002). To investigate the value of this trematode as a biological indicator, samples were collected since 1997 from franciscanas in different areas off the Brazilian coast, enabling an analysis of S. pontoporiae along the southeastern and southern Brazilian coastlines.

However, during our initial work, we realized that the description of Synthesium was incomplete and that S. pontoporiae was not well differentiated from related species of the genus, as indicated by the original description; i.e., as Hadwennius pontoporiae. Furthermore, Raga et al. (1994) did not differentiate S. pontoporiae from S. tursionis, the most closely related species, since they were, at that time, in separate genera. In the present paper, we redescribe S. pontoporiae based on the para-
type and new specimens. Synanthesium tursiosis from Tursiops truncatus in Brazilian waters and Synanthesium seymouri n. comb. from Delphinapterus leucas in Canadian northwest Atlantic waters were also studied.

MATERIALS AND METHODS

All cetaceans studied, whether accidentally caught or stranded, were in different stages of decomposition. Their collection and transport for research was authorized by the Brazilian environmental agency (IBAMA). Specimens of P. blainvillei were collected on the southeastern and southern coast of Brazil in 6 different areas; i.e., São Paulo North, São Paulo Central, São Paulo South, Paraná, Santa Catarina, and Rio Grande do Sul. Tursiops truncatus specimens were collected from Santa Catarina. Delphinapterus leucas specimens were collected from Saint-André de Kamouraska, St. Lawrence Estuary, Québec, Canada.

During necropsies, intestines were collected and frozen to be examined later in the laboratory. The intestines of D. leucas were examined when relatively fresh, i.e., not frozen first. The intestines were measured and opened; their contents were washed into a 150-μm mesh sieve and examined with a dissecting microscope. Specimens of S. pontoporiae and S. tursiosis were fixed in 70% alcohol and those of S. seymouri were fixed in AFA. Parasites were stained in Gomoris trichrome, delafield hematoxylin, or alcoholic chloridic carmine, cleared in beechnwood creosote, and mounted in Canada balsam. Drawings were made with the aid of a microscope tube. Measurements are presented as a range in micrometers, with the mean in parentheses.

The terminology used to describe testes shapes are: oval, wavy (undulating), or lobed. The sucker ratio refers to the ratio between the widths of the oral and ventral suckers.

Confocal laser scanning electron microscopy (CLSM) studies were performed using an LSM510 META Zeiss, Hambourg, Germany; the specimens analyzed were previously stained with alcoholic chloride fixative. Parasites were stained in Gomoris trichrome, orophiriform proximal region apparently permanent (Fig. 5, 6); small cirrus with pyriform proximal region varying from puntiform (readily lost in dead or fixed material). Genital pore at anterior margin of ventral sucker, 637–1,417 (971) from anterior end. Ovary round to oval, 87–262 × 67–171 (174 × 127), pretesticular. Mehlis’ gland pre-ovarian. Vitellarium arranged in small follicles in lateral fields of body, from level of testis anterior to ventral sucker to posterior testis (readily lost in dead or fixed material). Genital pore at 755 from anterior margin of ventral sucker, 7,080 (939) long, extends dorsally to ventral sucker to posterior testis 190–523 × 97–322 (335 × 222). Cirrus sac elongate-claviform, 708–1,309 (939) long, extends dorsally to ventral sucker to open via genital atrium (Fig. 3). Seminal vesicle long, coiled, occupies almost entire posterior half of cirrus sac, opens into par prostatica via sphincter; pars prostatica tubular, continues as ejaculatory duct within cirrus and opens into genital atrium (Figs. 5, 6); small cirrus with pyriform proximal region apparently permanent (Fig. 7) and flexible, tubular distal region, formed by evagination of ejaculatory duct which may reflex or extend through genital pore (Fig. 3); small spines seen on proximal part of cirrus (readily lost in dead or fixed material). Genital pore at anterior margin of ventral sucker, 637–1,417 (971) from anterior end. Ovary round to oval, 87–262 × 67–171 (174 × 127), pretesticular. Mehlis’ gland pre-ovarian. Vitellarium arranged in small follicles in lateral fields of body, from level of seminal vesicle extending to posterior extremity, not forming dendritic radial bunches. Vitelline reservoir at level of ovary. Uterus coils intercelly, from ovary to genital pore. Metraterm unarmed. Eggs oval, 46–60 × 23–28 (53 × 26), slightly flattened at opercular pole. Excretory vesicle long, tubular, reaches level of gonads. Excretory pore subterminal.

Taxonomic summary

Host: Pontoporia blainvillei.
Locality: Off São Paulo, Brazil (24°00’S, 46°24’W).
Site: Small intestine.
Synonyms: Hadwenius pontoporiae Raga, Aznar, Balbuena and Dailey, 1994
Paratype (USNPC 82916) (Fig. 8)

Adult: Measurements based on 1 specimen. Body elongate, 5,082 × 662 at level of ovary. Spines of tegument not observed. Oral sucker subterminal 201 × 169, ventral sucker 327 × 340. Sucker ratio 1:2.01. Preparhyphyn not observed. Esophagus variable in length. Pharynx pyriform, 239 × 120 at largest width. Intestine H-shaped; anterior ceca reach level of oral sucker; posterior ceca end blindly near posterior extremity. Testes irregular in shape, lobed, in middle third of body. Anterior testis 285 × 230; posterior testis 322 × 207. Cirrus sac elongate-claviform, 1,078 long, opens via genital atrium. Seminal vesicle long, coiled, in posterior half of cirrus sac, opens into par prostatica; pars prostatica tubular, continues as ejaculatory duct within cirrus and opens into genital atrium (Figs. 5, 6). Cirrus sac elongate-claviform, 708–1,309 (939) long, extends dorsally to ventral sucker to open via genital atrium (Fig. 3). Seminal vesicle long, coiled, occupies almost entire posterior half of cirrus sac, opens into par prostatica via sphincter; pars prostatica tubular, continues as ejaculatory duct within cirrus and opens into genital atrium (Figs. 5, 6); small cirrus with pyriform proximal region apparently permanent (Fig. 7) and flexible, tubular distal region, formed by evagination of ejaculatory duct which may reflex or extend through genital pore (Fig. 3); small spines seen on proximal part of cirrus (readily lost in dead or fixed material). Genital pore at anterior margin of ventral sucker, 637–1,417 (971) from anterior end. Ovary round to oval, 87–262 × 67–171 (174 × 127), pretesticular. Mehlis’ gland pre-ovarian. Vitellarium arranged in small follicles in lateral fields of body, from level of seminal vesicle extending to posterior extremity, not forming dendritic radial bunches. Vitelline reservoir at level of ovary. Uterus coils intercelly, from ovary to genital pore. Metraterm unarmed. Eggs oval, 46–60 × 23–28 (53 × 26), slightly flattened at opercular pole. Excretory vesicle long, tubular, reaches level of gonads. Excretory pore subterminal.

Taxonomic summary

Type host: Pontoporia blainvillei.
Type locality: Off Necochea, Argentina (38°37’S, 58°50’W).
Site: Small intestine.
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<tr>
<td>Ratio oral/ventral sucker</td>
<td>1:1,88–2,80 (1:2,34)</td>
<td>1:1,82–2,99 (1:2,21)</td>
<td>1:1,88–2,80 (1:2,43)</td>
<td>1:1,51–2,49 (1:1,99)</td>
<td>1:1,6–2,83 (1:2,16)</td>
<td>1:1,99–2,92 (1:2,52)</td>
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<tr>
<td>Genital pore/anterior end</td>
<td>616–1,817 (1,002)</td>
<td>637–1,417 (971)</td>
<td>662–1,355 (940)</td>
<td>532–1,540 (916)</td>
<td>616–931 (792)</td>
<td>108–1,463 (988)</td>
</tr>
<tr>
<td>Cirrus-sac</td>
<td>665–1,170 (964)</td>
<td>708–1,309 (939)</td>
<td>493–1,309 (820)</td>
<td>504–1,032 (776)</td>
<td>722–1,047 (835)</td>
<td>693–1,540 (930)</td>
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FIGURES 1–3. *Synthesium pontoporiae* n. comb. from *Pontoporia blainvillei*. (1) Total body, ventral view. (2) Testes anterior (top row) and posterior (bottom row)—(a) oval/wavy, (b) oval/lobed and (c) wavy and lobed. (3) Cirrus sac (cs), cirrus (c), ejaculatory duct (ed), genital atrium (ga), pp (pars prostatica), sphincter (s), seminal vesicle (sv) and eggs in uterus (egg). Scale bars: Figs. 1, 3 = 600 μm; Fig. 2 = 400 μm.
Synthesium pontoporiae n. comb. from Pontoporia blainvillei, confocal microscopy. (4) Anterior region showing oral and ventral suckers and pharynx. (5) Detail of genital pore and ventral sucker. (6) Cirrus sac (cs), cirrus (c), ejaculatory duct (ed), genital atrium (ga) and eggs in uterus (egg). (7) Detail of pyriform cirrus. Scale bars: Fig. 4 = 100 μm; Figs. 5, 6 = 50 μm; Fig. 7 = 10 μm.

Synthesium tursionis (Marchi, 1873) Stunkard and Alvey, 1930 (Figs. 9-11)

Adult: Measurements based on 15 specimens. Body elongate, 8,855–21,313 × 477–847 (14,357 × 618), with maximum width at level of ovary (Fig. 9). Tegument spinose. Oral sucker subterminal, 431–722 × 462–675 (600 × 554). Ventral sucker pre-equatorial, 400–760 × 428–684 (560 × 552). Sucker ratio 1:0.8–1.21 (1:1.01). Prepharynx extensible, 200–789 (527); pharynx pyriform, strongly muscular, 409–678 × 185–333 (526 × 258). Esophagus variable in length. Intestine H-shaped; anterior ceca reach posterior level of oral sucker and posterior ceca reach posterior extremity. Testes irregular in shape, varying from oval to lobed, in middle third of body. Anterior testis 409–1,093 × 276–618 (661 × 345); posterior testis 475–1,112 × 266–589 (686 × 369). Cirrus sac elongate-claviform, 1,432–2,911 (2,193) long, extends dorsally to ventral sucker to open into genital atrium. Genital pore at 2,079–3,970 (2,940) from anterior end, close to anterior margin of ventral sucker. Seminal vesicle long, occupies posterior third of cirrus sac; pars prostatica long, occupies middle third of cirrus sac. Cirrus tubular, eversible with small, readily lost spines (Figs. 10, 11). Ovary round to oval, 143–339 × 124–247 (238 × 169), pretesticular. Vitelline reservoir and Mehlis' gland pre-ovarian. Vitellarium arranged in small follicles in lateral fields of body from posterior to cirrus sac to posterior extremity, not

<table>
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<th>Area</th>
<th>Position</th>
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<th>Wavy (%)</th>
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Table II. Variation in the testes of Synthesium pontoporiae n. comb.; shape and percentage in different geographical areas off the coast of Brazil. np = number of parasites.
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8


Taxonomic summary

Host: Tursiops truncatus.
Locality: Off Santa Catarina, Brazil (26°37’S, 48°41’W).
Site: Small intestine.

Synthesium seymouri (Price, 1932) n. comb. (Figs. 12–14)

Adult: Measurements based on 10 specimens. Body elongate 27,258–38,115 × 616–1001 (34,154 × 793), with maximum width at level of ovary (Fig. 12). Tegument spined. Oral sucker subterminal, cup-shaped, 1,786–2,618 × 1,278–2,079 (2,025 × 1,645). Ventral sucker pre-equatorial, 1,078–1,463 × 724–1,309 (1,029 × 839). Sucker ratio 1:0.48–0.70 (1:0.60). Prepharynx extensible, 447–1,232 (792). Pharynx oval, strongly muscular, 939–1,232 × 724–1,309 (1,029 × 839). Esophagus variable in length. Intestine H-shaped; anterior ceca extends close to posterior level of oral sucker; posterior ceca reach posterior extremity of body. Testes regular in shape, oval, in middle third of body. Anterior testis 1,278–1,848 × 477–970 (1,569 × 707); posterior testis 1,263–1,925 × 508–847 (1,588 × 676). Cirrus sac elongate-claviform, 2,464–4,158 (3,253) long, extends dorsally to ventral sucker to open into genital atrium (Fig. 13). Genital pore at 3,696–5,929 (4,777) from anterior end, at anterior margin of ventral sucker. Seminal vesicle occupies proximal third, pars prostatica occupies middle third, and tubular cirrus occupies anterior third, of cirrus sac. Cirrus eversible. Spines of cirrus not observed. Ovary oval, pretesticular, 231–431 × 154–323 (337 × 231). Mehlis’ gland pre-ovarian. Vitelline reservoir close to ovary. Vitellarian arranged in radial dendritic bunches of follicles in lateral fields of body, from level of testes to posterior extremity of body (Fig. 14). Uterus coils intercircally, from level of ovary to genital pore. Metraterm unarmed. Eggs oval, 83–97 × 51–55 (91 × 55), slightly flattened at opercular pole. Excretory vesicle long, tubular. Excretory pore dorsal, subterminal.

Taxonomic summary

Host: Delphinapterus leucas.
Locality: Near Saint-André de Kamouraska, St. Lawrence Estuary, Québec, Canada (47°40’N, 69°43’W).
Site: Intestine.

Synonym: Hadwenius seymouri Price, 1932

DISCUSSION

The redescription of S. pontoporiae, based on a paratype from P. blainvilliei taken off Argentina, 20 specimens from P. blainvilliei from the São Paulo Central region, and an additional 75 measurements from P. blainvilliei from 6 areas off Brazil, indicated a wide range in body and prepharynx length (Table I). This variation, and the observed loss of tegumental and cirrus spines, is almost certainly due to the hosts being dead and frozen, an observation already reported by other authors (Adams and Rausch, 1989; Gibson, 2005). However, the presence of basal disks on the tegument or on the cirrus was observed, indicating that these organs had been spinose.

All specimens studied herein were adults with eggs, and the measurements were similar to those specimens studied by Raga et al. (1994). A valuable diagnostic feature of S. pontoporiae is the sucker ratio which, after accounting for variation in total body length, presented a pattern of 1:1.5–3.0 in all 95 speci-
Figures 9–11. *Synthesium tursionis* from *Tursiops truncatus*. (9) Total body, ventral view. (10) Terminal part of cirrus, confocal microscopy. (11) Detail of cirrus sac. Scale bars: Figs. 9, 11 = 1 mm; Fig. 10 = 100 μm.
Figures 12–14. *Synthesium seymouri* n. comb. from *Delphinapterus leucas*. (12) Total body, ventral view. (13) Detail of cirrus sac. (14) Detail of vitellarium arranged in radial dendritic bunches of follicles. Scale bars: Fig. 12 = 2 mm, Fig. 13 = 1 mm, Fig. 14 = 100 μm.
mens from the 6 sampled areas. The minimum sucker ratio was found in smaller specimens from the Paraná area. The sucker ratio of *S. tursionis* was 1:0.8–1:2 and that of *S. seymouri* was 1:0.5–0.7, which differed from *S. pontoporaria* in which the ventral sucker can be up to 3 times wider than the oral sucker.

The oral suckers of *S. pontoporaria*, *S. tursionis*, and *S. seymouri* are subterminal. However, the round shape of the pharynx of *S. seymouri* differed greatly from that of *S. pontoporaria* and *S. tursionis*, which are pyriform; in *S. tursionis*, the slender anterior part of the pharynx was equal in length to the broad posterior part, while in *S. pontoporaria* the anterior part was shorter than the posterior part. Gibson (2005) stated that the ‘prepharynx may envelop anterior pharynx’ in *Synthesium*; however, in a few specimens of *S. pontoporaria*, we observed the prepharynx enveloping the anterior part of the pharynx, but we interpreted this as being due to body contraction.

The original description of the cirrus sac of *S. pontoporaria* was not given in detail. The cirrus sac was elongate-claviform in the 3 presently studied species and extended dorsally to the ventral sucker, to surface via the genital atrium. Similarly, in these 3 species the seminal vesicle occupies the posterior part of the cirrus sac; the pars prostatica was also tubular and continued as the ejaculatory duct within the cirrus, to open into the genital atrium. The cirrus sacs of *S. tursionis* and *S. seymouri* are tubular, unlike that of *S. pontoporaria*, which has a pyriform, apparently permanent, proximal region and a flexible, tubular distal region, formed by evagination of the ejaculatory duct that extends through the genital pore.

The testes of *S. pontoporaria* were described as oval but, in fact, their shape varied from oval to lobed (generally up to 5 lobes). The variation in testicular shape is described in Table II; based on testis position (anterior/posterior) and shape (oval, lobed) or lobed testes were the most common. The transition of testicular shape from oval to lobed was observed in *S. tursionis* and *S. seymouri*; unlike that of *S. pontoporaria*, which has a pyriform, apparently permanent, proximal region and a flexible, tubular distal region, formed by evagination of the ejaculatory duct that extends through the genital pore.

The testes of *S. pontoporaria* were described as oval but, in fact, their shape varied from oval to lobed (generally up to 5 lobes). The variation in testicular shape is described in Table II; based on testis position (anterior/posterior) and shape (oval, lobed), the combination of oval/lobed was most common, except in specimens from São Paulo South, where wavy/lobed or lobed testes were the most common. The transition of testicular shape (oval – wavy – lobed) may be related to maturation and, if so, the posterior testis probably matures first, since the oval/lobed and lobed/lobed combinations were rare. However, this hypothesis is not entirely supported by our material, since some of the larger individuals still had anterior oval and posterior lobed testes. Further studies are necessary to clarify this pattern.

The testes were nearly situated in the middle third of body in the 3 presently studied species. The variation in testicular shape from oval to lobed was observed in *S. pontoporaria* and *S. tursionis*, as reported by Fernández et al. (1994), but in *S. seymouri*, both testes were oval.

The vitellarium of *S. pontoporaria* formed small follicles in lateral fields of the body from the level of the seminal vesicle to the posterior extremity of the body and did not form dendritic radial bunches. In *S. tursionis*, the arrangement was similar, but follicles began posterior to the cirrus sac and extended to the posterior extremity of the body. *Synthesium seymouri* differed in that the follicular arrangement was in the form of radial dendritic bunches in lateral fields of the body from the level of the testes to the posterior end of the body.

Eggs of *S. pontoporaria* were similar in size to eggs of *S. tursionis* (46–60 × 23–28 vs. 51–55 × 28–32, respectively). This was one of the most conserved characters observed by Fernández et al. (1995) when they studied the biometric variability of *S. tursionis*. Eggs of *S. seymouri* are much larger (83–97 × 51–55).

*Synthesium pontoporaria* is, therefore, characterized by a sucker ratio of 1:1.5–3.0, testicular shape ranging from oval to lobed, a cirrus with a pyriform proximal region and flexible, tubular distal region formed by the evagination of the ejaculatory duct that extends through the genital pore, and a vitellarium in the form of small follicles situated between the level of the seminal vesicle and the posterior extremity of the body, and not forming dendritic radial bunches. This species is known to exclusively parasitize *P. blainvillei* in south Atlantic waters.

The generic diagnosis of *Synthesium* of Gibson (2005) needs to be amended to include characteristics of the suckers of *S. pontoporaria* as follows: suckers well developed; oral sucker smaller, similar in size or larger than ventral sucker; ventral sucker in first quarter of body. The other species of the genus require review so that a full taxonomic key can be accomplished.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


—. 2005. Family Brachycladiidae Odhner, 1905. In *Keys to the


A NEW ANGIOSTRONGYLID (NEMATODA) SPECIES FROM THE PULMONARY ARTERIES OF AKODON AZARAE (RODENTIA: CRICETIDAE) IN ARGENTINA

Maria del Rosario Robles, Graciela T. Navone, and John M. Kinsella
Centro de Estudios Parasitológicos y de Vectores (CEPAVE) (CONICET-UNLP), 1900 La Plata, Argentina. e-mail: rosario@cepave.edu.ar

ABSTRACT: Angiostrongylus morerai n. sp. (Nematoda: Angiostrongylidae) is described from the pulmonary arteries of Azara’s grass mouse, Akodon azarae (Rodentia: Cricetidae) in Argentina. It is distinguished from its congeners principally by the morphology of the dorsal ray, which is as long, or longer, than the externodorsals and has 2 long branches: the spicules lengths are also greater (400–465 μm).

The metastrongylid genus Angiostrongylus Kamensky, 1905, includes species that are parasites of the blood vessels of rodents, felids, canids, and, occasionally, primates, both non-human and human. Some authors (Drozdz, 1970; Anderson, 1978) have divided the genus into 2 subgenera, i.e., Angiostrongylus and Parastrongylus, based on the morphology of the lateral rays of the bursa, while others (Chaubad, 1972; Ubelaker, 1986) have regarded these subgenera as full genera. The latter view has not been widely accepted in the literature, especially with regard to the important human parasites, Angiostrongylus cantonensis (Chen, 1935) and Angiostrongylus costaricensis Moreira and Céspedes, 1971.

Following the definition of Anderson (1978), 2 species can be assigned to the subgenus Angiostrongylus, both parasites of carnivores, i.e., Angiostrongylus vasorum (Baillet, 1886) and Angiostrongylus chabaudi Biocca, 1957. Angiostrongylus raillieti (Travassos, 1927), from the crab-eating fox, Cerdocyon thous (Linnaeus, 1766) in Brazil was considered a synonym of A. vasorum by Costa et al (2003). Twelve species can be assigned to the subgenus Parastrongylus, all parasites of rodents: Angiostrongylus ta­tonensis (Baylis, 1928); Angiostrongylus cantonensis; Angiostrongylus sciu­ri Merdevenci, 1964; Angiostrongylus mackerrasae Bhaibulaya, 1968; Angiostrongylus sandarsae Alicant, 1968; Angiostrongylus petrowi (Tariyanova and Tschertkova, 1969); Angiostrongylus darijardini Drozd and Doby, 1970; Angiostrongylus schmidtii Kinsella, 1971; Angiostrongylus costaricen­sis; Angiostrongylus malaysiensis Bhaibulaya and Cross, 1971; Angiostrongylus ryjikovi (Jushkov, 1971); and Angiostrongylus siamensis Obhayashi, Kamiyi, and Bhaibulaya, 1979. With the exception of A. costaricensis and A. siamensis, which are found in the mesenteric arteries of their hosts, all other species are found in the pulmonary arteries and lungs.

Azara’s grass, or field, mouse, Akodon azarae (Fischer, 1829) (Rodentia: Cricetidae), is a small mammal, averaging 19 g in weight; it prefers high herbaceous cover, often along forest borders and roadsides (Matthews and Myers, 2004). During a study of this mouse in Argentina, a new species of nematode was found in the pulmonary arteries.

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MATERIALS AND METHODS

During a study on the biology and systematics of rodents, 30 specimens of A. azarae were collected in September 1998 (n = 28) and April 1999 (n = 2) in Cerro de la Gloria (Partido de Castelli, 36°06’S, 57°46’W), Buenos Aires Province, Argentina.

Viscera were fixed in 10% formalin and examined in the laboratory. Nematodes were collected from the pulmonary arteries, preserved in 70% ethanol, cleared as temporary mounts in lactophenol, and studied using light microscopy. Drawings were made with the aid of a drawing tube. Some specimens were dried using the critical point method, examined under a scanning electron microscope (Jeol 6360 LV, Tokyo, Japan), and photographed. Measurements are given in micrometers (μm) except when indicated, with the range followed by the mean in parentheses. Voucher specimens of A. vasorum were borrowed from the United States National Parasite Collection (USNPC), Beltsville, Maryland. Type specimens were deposited in the Helmthological Collection of the Museo de La Plata (CHMLP) in Buenos Aires, Argentina.

DESCRIPTION

Angiostrongylus morerai n. sp.
(Figs. 1–9)

General: Body of both sexes filiform and tapering at anterior end. Head smoothly rounded. Mouth without lips, surrounded by symmetrical ring of 6 papillae and 2 large lateral amphids (Fig. 1). Esophagus club-shaped. Excretory pore slightly posterior to end of esophagus. Nerve ring in anterior third of esophagus (Fig. 5).

Male (holotype, 3 complete paratypes, and 13 tail fragments): Body length 17.1–19.2 mm (18.0), width at base of esophagus 121–138 (129), maximum width 265–285 (273), esophagus length 220–285 (249). Anterior end of testis variable in position, sometimes extending to end of esophagus. Spicules slender, with a conspicuously striated flange, equal, similar, 400–465 (427) long. Proximal ends of spicules pointed, with irregular edges, distal ends sharply pointed (Fig. 8). Gubernaculum inconspicuous, curved, 42–54 (45) long. Bursa symmetrical and well developed with a small dorsal incision (Figs. 2, 3, 6, 7). Dorsal ray thick, with 2 long branches, as long as, and sometimes longer than, externodorsals. Externodorsals long, digitiform, separate at base. Lateral rays widely separated from lateral trunk.

Female (allotype and 1 paratype, 4 tail fragments): Body length 29.0–30.5 mm (29.8), width at base of esophagus 170–175 (173), maximum width 285–335 (310), esophagus length 388–395 (392). Anterior ends of ova­ries variable in position, sometimes reaching to middle of esophagus. Uterine tubules spirally wound around intestine, producing “barber-pole” appearance typical of genus. Vulva with slightly elevated lips, 250–335 (292) from tip of tail (Figs. 4, 9). Anus 71–108 (86) from tip of tail. Tail bluntly rounded, with no spike. Eggs not fully developed.
Figures 1–4. Angiostrongylus morerai n. sp. (1) Oral opening with ring of 6 papillae and 2 large lateral amphids (a); (2) ventral view of cloaca and bursa; (3) dorsal view of bursa; (4) female tail showing (b) vulvar opening and (c) anus.

Taxonomic summary

Type host: Azara's grass mouse, Akodon azarae (Fischer, 1829) (Cricetidae: Sigmodontinae: Akodontini), voucher specimen deposited in the Colección de Mammalia del Museo de la Plata, Buenos Aires, Argentina, MLP 26.XII.02.5. Other hosts housed MLP 16.VI.01.9, MLP 3.XII.02.6.

Site of infection: Pulmonary arteries.

Prevalence: Nineteen of 30 total hosts (63%); only hosts captured in September 1998 were infected.

Mean intensity: 2.47.

Type locality: Cerro de la Gloria (Partido de Castelli, 36°06'S, 57°46'W), Buenos Aires Province, Argentina.

Type specimens: Holotype: CHMLP No. 5715; Allotype: CHMLP No. 5716; Paratypes: CHMLP No. 5717.

Etymology: This species is named after Dr. Pedro Morera in recognition of his contributions to our knowledge of the systematics and ecology of A. costaricensis.

Remarks

According to Anderson (1978), species in the subgenus Angiostrongylus have the externolateral ray completely separate from the other lateral rays, while species in the subgenus Parastrongylus have the externolateral ray joined to a common trunk with the other lateral rays. Recently, Costa et al. (2003) redescribed A. vasorum from experimental infections in dogs in Brazil and found that, although deeply clefted, the externolateral rays shared a common trunk with the other lateral rays. Examination of voucher specimens of A. vasorum collected from a red fox, Vulpes vulpes in Italy (USNPC 78165), clearly showed that the lateral rays all share a common trunk (Fig. 10). Therefore, no attempt is made here to assign the new species to a subgenus.

While the externolateral ray of A. morerai n. sp. is deeply clefted, it also shares a common trunk with the mediolateral and posterolateral rays (Figs. 2, 3, 6). It is principally distinguished from other members of the genus by the fact that the mediolateral and posterolateral rays are fused for most of their lengths, the dorsal ray is as long as, and sometimes longer than, the externodorsal rays, and the branches of the dorsal ray are very long (Fig. 2). In all other species, the posterolateral and mediolateral are only fused for one-quarter to one-half their lengths. In all species, except for A. dujardini, the dorsal ray is shorter than the externodorsals. And, in all species, including A. dujardini, the branches of the dorsal ray consist of 2 or more very short digitations.

In addition, the spicules of A. morerai n. sp. (400-465 µm) are shorter than A. cantonensis (1,000–1,420), A. malaysiensis (800–1,200), A. chabaudi (540), and A. tateronae (500–620), but longer than A. sandarsae (330–360), A. petrowi (362–371), A. ryjikovi (320), A. siamensis (339), and A. schmidti (215–279). The remaining 5 species (A. costaricensis, A. dujardini, A. mackerrasae, A. sciuri, and A. vasorum) have spicule lengths in the same general range as A. morerai n. sp.

To date, the only species of Angiostrongylus reported from South America are A. vasorum and A. costaricensis, a parasite of the mesenteric arteries of many species of cricetid rodents, as well as raccoons, opossums, monkeys, and humans (Morera and Cespedes, 1971; Tesh et al., 1973; Miller et al., 2006). Morera (1973) gave the spicule lengths of A. costaricensis from Sigmodon hispidus in Costa Rica as 318–333 µm. Santos (1985) redescribed A. costaricensis from Proechimys sp. in Venezuela and reported spicule lengths of 270–330 µm. Graeff-Teixeira et al. (1989) infected white mice with larvae of A. costaricensis from mollusk intermediate hosts in Brazil, and reported spicule lengths of 390–420 µm. Although there is a slight overlap in the range of spicule lengths between A. costaricensis and A. morerai n. sp., these species differ in the morphology of the dorsal ray (short with 2–3 small digitations in A. costaricensis), the presence of a small spike on the tail of
the female in *A. costaricensis* (absent in *A. morerai* n. sp.), and the site of infection.

**DISCUSSION**

Although the attempts by Drozdz (1970), Anderson (1978), and others to divide the genus *Angiostrongylus* into subgenera that conveniently separated species in carnivores from species from rodents, it appears that this separation may be artificial rather than phylogenetic. The lateral rays of *A. vasorum* have been found to have a common trunk both in the present study and by Costa et al. (2003). The fact that the externolateral ray is more deeply clefted in *A. vasorum* and *A. chabaudi* than in rodent species is a matter of degree, and, in any case, this character is also shared by *A. morerai* n. sp. Phylogenetic studies using molecular analyses are particularly needed for this genus to determine if separation into subgenera is valid, and also to determine how closely related the species dwelling in the pulmonary arteries are to those occurring in the mesenteric arteries.

The finding of *A. morerai* n. sp. is the first report of a metastrogyloid in a sigmodontine rodent in Argentina. To date, the only species of *Angiostrongylus* recorded from Central and South America are *A. costaricensis*, *A. vasorum*, and *A. morerai* n. sp., although there are dozens of rodent species in those areas yet to be studied. In North America, *A. schmidti* has been recorded from the rice rat *Oryzomys palustris* (Harlan, 1937) in Florida by Kinsella (1971, 1988), *A. costaricensis* in the cotton rat *Sigmodon hispidus* (Say and Ord, 1825) in Texas by Ubelaker and Hall (1979), and in cotton rats, raccoons, opossums, and non-human primates in Florida by Miller et al. (2006). *A. vasorum* has been recorded in red foxes, coyotes, and dogs in Canada (Conboy, 2004; Jeffery et al., 2004; Bourque et al., 2005). In addition, *A. cantonensis* has recently successfully invaded Louisiana and several islands of the Caribbean, apparently accompanying *Rattus* spp. on ships, and is now endemic in these areas (Campbell and Little, 1988; Kim et al., 2002; Raccurt et al., 2003).

Kinsella (1987) observed that even light experimental infections (<10 worms) of *A. schmidti* were fatal to smaller hosts like white mice, gerbils, and juvenile hamsters, while larger hosts such as white rats, cotton rats, and rice rats survived to have patent infections. It is possible that *A. morerai* n. sp. may be a source of mortality in a rodent as small as *A. azarae*, and this may explain its absence in other small rodents collected in the same areas.
FIGURE 10. Lateral rays of bursa of Angiostrongylus vasorum from Vulpes vulpes in Italy (NPC 78165).

ACKNOWLEDGMENTS

We offer thanks to Mariano Merino for collection and identification of hosts, and to Carlos Galliari for corroborating their identification; to María Cristina Estivariz for the drawings; and to Patricia Sarmiento for the SEM photographs. This study was funded by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

LITERATURE CITED


HEPATOZOON SPECIES OF THE TIMBER RATTLESNAKE IN NORTHERN FLORIDA: DESCRIPTION OF A NEW SPECIES, EVIDENCE OF SALIVARY GLAND OOCYSTS, AND A NATURAL CROSS-FAMILIAL TRANSMISSION OF AN HEPATOZOOON SPECIES

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ABSTRACT: Two species of Hepatozoon, i.e., H. sauritus and H. horridus n. sp., were present in 1 of 8 timber rattlesnakes, Crotalus horridus. The narrow gamonts of H. sauritus are 15.0–19.0 × 3.5–5.0 μm, with LW 58–86 μm and L/W 3.2–4.7, with a narrow, rounded anterior end. The spherical to slightly ovoid oocysts produce ooid to elongate sporocysts, 21–43 × 12–24 μm, L/W 1.20–2.7, containing on average 22.1 (10–34) sporozoites. This is the first report of a natural cross-familial transfer of a Hepatozoon species. Gamonts of H. horridus n. sp. are 13.0–17.0 × 4.0–6.0 μm, with LW 63–102 μm and L/W 2.6–4.0, and have broadly rounded ends. The gamont cytoplasm is vacuolated. The spherical to ovoid oocysts form spherical to elongate sporocysts 14–45 × 11–25 μm, L/W 1.0–2.3, producing an average of 13.0 (8–21) sporozoites. The salivary gland in 1 of 5 mosquitoes dissected contained 1 mature oocyst.

The timber rattlesnake, Crotalus horridus, is distributed in montane regions of the eastern United States and southward in the Piedmont and Coastal Plain to northern Florida. Lowland populations differ consistently in pattern, coloration, and other characters, and were previously considered a subspecies, i.e., C. h. atricaudatus, the canebrake rattlesnake. There are no records of Hepatozoon species from this host.

An adult C. horridus collected in Baker County, Florida, in April 2006 was infected by morphologically different gamonts of 2 Hepatozoon species, 1 of which resembled Hepatozoon sauritus of colubrid snakes throughout most of Florida (Telford et al., 2004). Sporogony of 2 species obtained in mosquitoes confirmed the presence of H. sauritus and an undescribed species. The presence of a sporulated oocyst within the salivary gland of 1 mosquito is reported here, along with the description of the undescribed Hepatozoon species, and a comparison of H. sauritus from the viperid host with infections from the usual colubrid hosts.

MATERIALS AND METHODS
A total of 8 C. horridus were examined between 2002–2006, 4 from southern Georgia and 4 from north-central Florida. Procedures relating to blood sample collection and sporogony and taxonomic characters used were those described by Telford et al. (2001). Nucleus position was estimated from the slightly narrower end of the gamont. All measurements, obtained by a calibrated ocular micrometer, are in μm, with means and SD, followed by ranges in parentheses, or in the case of LW (length × maximum width), values are in μm². Comparisons were done by 1-way ANOVA on transformed data (log[1 + x]) with significance P ≤ 0.05. Differences noted under “Description” and “Remarks” below (smaller, larger, greater, less, etc.) were statistically significant; the term “similar to” indicates that significant differences were not present. Hapantotype slides were deposited in the U.S. National Parasite Collection (USNPC), Beltsville, Maryland, and other hapantotype slides were reserved for deposition with the Telford collection.

REDESCRIPTION
Hepatozoon sauritus
(Figs. 1–4)
Gamonts (Figs. 1, 2): Gamonts not recurved, slender with a slightly narrower, rounded, and slightly curved anterior and rounded posterior end, 16.6 ± 1.0 × 4.1 ± 0.4 (15.0–19.0 × 3.5–5.0, n = 25), with LW 67.9 ± 8.0 (58–86) and L/W 4.1 ± 0.39 (3.2–4.7). Nuclei 5.3 ± 0.8 × 3.2 ± 0.3 (4.0–7.0 × 2.5–3.5), with LW 16.6 ± 2.9 (12.0–21.0), always present in second quarter of gamont, commonly extending into first quarter (56%) but not into third quarter. Erythrocyte cytoplasm often thin, appearing partially dehemoglobinized (72%), with infected cells commonly distorted (48%). Infected erythrocytes similar in length and width to uninfected cells; erythrocyte nuclei larger but narrower, with similar LW.

Sporogony: Sporogony of H. sauritus in experimental vector Aedes aegypti occurred within head, hemococel of thorax, and abdomen. Oocysts spherical to slightly ovoid, 92.0 ± 26.4 × 89.7 ± 26.7 (55–140 × 50–137, n = 15), and L/W 1.03 ± 0.04 (1.0–1.1). Sporocysts (Figs. 3, 4) ovoid to elongate, 32.8 ± 7.0 × 18.3 ± 3.6 (21.43–12.24, n = 19), LW 61.0 ± 197.3 (252–1,008), and L/W 1.83 ± 0.42 (1.20–2.7), containing 22.1 ± 7.4 (10–34, n = 19) sporozoites.

DESCRIPTION
Hepatozoon horridus n. sp.
(Figs. 5–10)
Gamonts (Figs. 5, 6): Gamonts of medium width with broadly rounded ends, not recurved, 15.7 ± 0.9 × 5.1 ± 0.6 (13.0–17.0 × 4.0–6.0, n = 25), with LW 80.2 ± 11.9 (63–102) and L/W 3.10 ± 0.33 (2.6–4.0). Nuclei 5.0 ± 0.6 × 3.7 ± 0.6 (4.0–6.0 × 3.0–5.0), with LW 18.2 ± 3.7 (13.5–25.0), always present in second quarter of gamont, seldom extending into first quarter (8%) or third quarter (12%). Erythrocyte cytoplasm vacuolated, rarely appearing dehemoglobinized (4%), with infected cells often distorted (48%). Infected erythrocytes and their nuclei similar in dimensions to uninfected cells.

Sporogony: Sporogony in experimental vector Aedes aegypti within head, but less commonly than in hemococel of thorax, and abdomen. One oocyst present within salivary gland in 1 of 5 mosquitoes dissected. Oocysts spherical usually to ovoid (Figs. 7–11), 106.0 ± 40.5 × 97.9 ± 36.8 (40–212 × 32–192, n = 52), and L/W 1.09 ± 0.10 (1.0–1.5), containing 20.4 ± 13.7 (4–61, n = 31) sporocysts. Sporocysts (Figs. 12–16) spherical to elongate, 24.0 ± 4.2 × 20.4 ± 2.4 (14–45 × 11–25, n = 66), LW 491.9 ± 116.4 (196–900), and L/W 1.18 ± 0.22 (1.0–2.3), containing 13.0 ± 3.8 (8–21, n = 36) sporozoites.

Taxonomic summary
Type host: Crotalus horridus Linnaeus (Serpentes: Viperidae).
Other hosts: None known.
Type locality: Florida, Baker County, State Road 250, 3.6 km west of Taylor (30°29.532′N, 82°18.232′W).
Prevalence: One of 8, overall.
Site of infection: Erythrocytes.
Material deposited: Hapantotype blood films U.S. National Parasite Collection (USNPC) Beltsville, Maryland, No. 99676. Hapantotype stained dissection containing oocysts and sporocysts USNPC No. 99678. Other hapantotypes retained for deposition with the Telford collection. Symbiotype host not deposited.

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FIGURES 1-4. *Hepatozoon sauinus* from *Crotalus horridus*. (1, 2) Gamonts. (3, 4) Fresh sporocysts in hemocoel of *Aedes aegypti*. Scale bars = 10 μm.

FIGURES 5-16. *Hepatozoon horridus* n. sp. from *Crotalus horridus*. (5, 6) Gamonts. (7-9) Fresh oocysts in hemocoel of *Aedes aegypti*. (10, 11) Slightly flattened oocysts. (12, 13) Fresh sporocysts. (14-16) Stained sporocysts. Scale bars = 10 μm, except Figs. 7-10, Scale bars = 45 μm.
**Etymology:** The specific name refers to the specific name of the host.

**Remarks**

In 1 of 5 infected *Aedes aegypti*, dissected 21 days after feeding on the infected *C. hordius*, sporulated oocysts of *H. horridus* were present in head (1), thorax (10), and abdomen (69). A sporulated oocyst, 56 × 52 μm, was found within the salivary gland (Figs. 17–19) and contained 7, possibly 8, sporocysts with an ovoid shape consistent with that of *H. horridus*. No parasites were seen in the salivary glands of the other 4 infected mosquitoes examined, and proboscides were not examined. One presumably infected mosquito on day 23 post-feeding was fed to each of 3 small snakes, *Seminatrix pygaea*, *Diadophis punctatus*, and *Storeria dekayi*, none of which survived more than 19 days, too soon to expect patent infections of *Hepatozoon* gamonts.

**DISCUSSION**

All morphological characters of gamonts and sporogonic stages of *H. sauritus* from *Crotalus horridus* are consistent with the redescription of the species from 5 colubrid snake species (Telford et al., 2004). A comparison of DNA would be complicated by the presence of *H. horridus* n. sp. in mixed infection and has not been done. Although other species of *Hepatozoon*, notably *H. rarefaciens* (Ball et al., 1967) and *H. fusifex* (Oda et al., 1971), have experimentally infected host species in other families, and indeed, from snake host to lizard host (Booden et al., 1970), this is the first demonstration of cross-familial transfer of a *Hepatozoon* species among natural populations of snakes.

Among the 15 *Hepatozoon* species thus far described from snakes in Florida (on the basis of their gamont and sporogonic characters), 4 species have sporocysts with average length <30 μm (Table I). One of these, *H. punctatus*, is known only from south Florida and needs no further comparison here except to note that the number of sporozoites within sporocysts averages greater, 20.1 ± 4.7 (15–33), in *H. punctatus* than in *H. horridus*, 13.1 ± 3.8 (8–21). *Hepatozoon sirtalis* has the smallest sporocysts on average (14.7 × 13.2 μm) and produces fewer than one-half the number of sporozoites per sporocyst, on average 5.7 ± 1.5, than do the other 2 north Florida species, *H. priapus* 12.6 ± 3.4, and *H. horridus* n.sp. 13.1 ± 3.8. These are similar in sporozoite numbers and in dimension of sporocysts, 26.3 ± 6.1 × 23.3 ± 5.5 μm and 22.3 ± 4.5 × 20.6 ± 1.8 μm, respectively. However, the gamonts of *H. priapus* can be distinguished from those of *H. horridus* n. sp. by the absence of multiple, rounded vacuoles in the cytoplasm of the gamont. Vacuolated cytoplasm of the gamont is present only in *H. horridus* among the 15 described *Hepatozoon* species of snakes in Florida. Although similar in width and L/W ratio, gamonts of *H. horridus* are shorter, with a greater LW value than those of *H. sauritus*.

The presence of a sporulated oocyst in the salivary gland of one of the dissected mosquitoes is another example of sporozoite proximity to the taking of a blood meal by the mosquito. Although not routinely examined for (during dissections of mosquitoes fed on the *Hepatozoon* species of Florida snakes since 1992), either small oocysts or free sporocysts were found in proboscides that had been removed from infected mosquitoes prior to their dissection. Five of 13 proboscides, examined from mosquitoes fed upon infections of *H. pictiventris* in *Nerodia fasciata*, contained 1, 1, 4, 16, and 21 sporocysts (Telford et al., 2001). In 2 species that host *H. sauritus*, 1 of 5 proboscides, from mosquitoes fed upon infections of *H. sauritus* in *Thamnophis sirtalis*, contained 8 sporocysts (Telford et al., 2004) and in 1 of 13 fed upon *Elaphe guttata*, an unrecorded number of...

**Table I. Dimensions and sporozoite numbers of sporocysts with average length <30 μm in *Hepatozoon* species of snakes in Florida.**

<table>
<thead>
<tr>
<th><em>Hepatozoon</em> species</th>
<th>Length μm</th>
<th>Width μm</th>
<th>L/W</th>
<th>Sporozoite numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sirtalis</em></td>
<td>14.7 ± 2.0</td>
<td>13.2 ± 1.4</td>
<td>1.12</td>
<td>5.7 ± 1.5</td>
</tr>
<tr>
<td><em>priapus</em></td>
<td>12–21</td>
<td>10–18</td>
<td>1.0–1.5</td>
<td>4.8</td>
</tr>
<tr>
<td><em>horridus</em></td>
<td>26.3 ± 6.6</td>
<td>23.3 ± 5.5</td>
<td>1.14</td>
<td>12.6 ± 3.4</td>
</tr>
<tr>
<td><em>punctatus</em></td>
<td>20–45</td>
<td>16–23</td>
<td>1.0–2.3</td>
<td>8–21</td>
</tr>
<tr>
<td><em>sirtalis</em></td>
<td>22.3 ± 4.5</td>
<td>20.6 ± 1.8</td>
<td>1.09</td>
<td>13.1 ± 3.8</td>
</tr>
<tr>
<td><em>punctatus</em></td>
<td>25.1 ± 3.0</td>
<td>22.7 ± 3.0</td>
<td>1.11</td>
<td>20.1 ± 4.7</td>
</tr>
<tr>
<td><em>priapus</em></td>
<td>20–32</td>
<td>16–31</td>
<td>1.0–1.5</td>
<td>15–33</td>
</tr>
</tbody>
</table>

**Figures 17–19.** Oocyst containing sporocysts of *Hepatozoon horridus* n. sp. in salivary gland of *Aedes aegypti*. Scale bars = 10 μm.
sporocysts were present (data not shown). Only 5 proboscides of mosquitoes fed upon Coluber constrictor infected by H. polytopis were examined, and 3 contained 5, 5, and 4 sporocysts (Telford et al., 2005). This indicated that mosquito proboscides often possess Hepatozoon sp. sporocysts in laboratory feedings, and there is no reason to suppose that this does not occur commonly in nature. Taken together with the salivary gland infection by H. horridus n. sp. reported here, there is sufficient reason to conclude that direct salivary transmission may be possible for those Hepatozoon species that do not require a first vertebrate intermediate host in the life cycle, as do Hepatozoon sipedon (Smith et al., 1994) and Hepatozoon sirtalis (Telford et al., 2001). There would not have to be a high prevalence of infected proboscides in the mosquito vector population, given the high density of these mosquitoes in subtropical areas like Florida. It has long been known that human malaria may be maintained in endemic areas by a very low prevalence of infected mosquitoes that have good access to humans. As a recent example, Plasmodium falciparum sporozoite prevalences of 0.8% and 1.61% in Anopheles arabiensis effectively maintained entomological inoculation at levels of 12.8 and 39.3, respectively, in a single village in the western lowland wet zone of Eritrea and in 3 villages of the western escarpments, where rainfall exceeded 200 mm per yr (Shililu et al., 2003).

**LITERATURE CITED**


A NEW SPECIES OF HENNEGUYA (MYXOZOA) IN THE BIG-EYED SCAD (SELAR CRUMENOPHTHALMUS) FROM HAWAI'I

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ABSTRACT: We describe a new myxozoan, Henneguya akule n. sp., infecting the carangid fish Selar crumenophthalmus in Hawaii. Spores were found only in the aortic bulb, characterized by elliptical capsule with 2 tails, and pyriform polar capsules that angled toward the anterior end of the spore. Polar filaments had 3–4 coils. Parasites were present in apparently healthy fishes and caused no evident gross pathology. On microscopy, parasites evinced a mild inflammatory response in the host characterized by accumulations of eosinophilic fibrillar material around spores and a mononuclear infiltrate in the adventitia of the bulbus arteriosus. Overall prevalence was 20%, and prevalence between 2001 and 2006 ranged from 12 to 27%, but did not differ significantly between years. In contrast, prevalence of infection was highest in south-central Oahu. There was no relationship between infection status and body condition or gender of fish, and infection was absent in the smallest and largest fishes. Phylogenetically, H. akule n. sp. is most closely related to other Henneguya species infecting the heart of marine fishes based on ribosomal DNA analysis. This is the first documentation of a myxozoan parasite in marine fishes from Hawaii.

The big-eyed scad (Selar crumenophthalmus), or akule, is an important fisheries resource for the state of Hawaii, with annual landings by commercial and artisanal fisheries ranging from 100 to 600 tons. In Hawaii, akule spawn from April through October and mature at ca. 25 cm (Clarke and Privitera, 1995). For such a commercially important fish, surprisingly little biological information exists. Even less data are available on factors such as disease that could affect populations. Developing some basic understanding of microorganisms that infect this species would seem critical, particularly when it is being considered as a potential candidate for aquaculture (Iwai et al., 1996). As part of a larger study monitoring big-eyed scad at sewer outfalls on Oahu for internal tumors, we discovered a myxozoan in the bulbus arteriosus. Our objective was to describe this parasite, its pathology, and prevalence.

MATERIALS AND METHODS

From 2001 to 2006, fish were collected using line and hook at depths ranging from 15 to 70 m throughout 5 sites on southern Oahu. Fish were killed with an overdose of MS-222 in seawater. Necropsies consisted of measuring total and fork length (0.5 cm) with a caliper, weighing (0.1 g) with an electric scale, and a complete external and internal examination. Sections of spleen, liver, cranial and caudal kidneys, swim bladder, brain, heart, skeletal muscle, gill, and gonad were excised and measured as described (Lom and Arthur, 1989). For electron microscopy, tissues were fixed in Trump’s fixative (McDowell and Trump, 1976), rinsed in 0.1 M Sorenson’s phosphate buffer, and postfixed in 2% osmium tetroxide. Epoxy-embedded tissues were cut into 1-µm-thick toluidine blue stained sections. Ultra-thin sections were stained with uranyl acetate, poststained with lead citrate, and examined with a Zeiss EM 109 electron microscope.

The DNeasy Tissue Kit (Qiagen Inc., Valencia, California) was used to extract DNA from infected heart tissue. Overlapping regions of the small subunit (SSU) ribosomal DNA (rDNA) were amplified with PCR primers of Diamant et al. (2004), using Invitrogen Taq DNA polymerase (Invitrogen Co., Carlsbad, California). PCR products were gel-extracted using the QIAEX II Gel Extraction Kit (Qiagen). Direct sequencing was carried out with the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1, using the ABI PRISM® 3730 DNA Analyzer (Applied Biosystems, Foster City, California). Fragments were assembled with Contig Express (Invitrogen) and edited by visual inspection. The resulting 2058 nucleotide SSU sequence for H. akule n. sp. was deposited in GenBank (accession no. EU016076).

For phylogenetic analysis, the SSU rDNA sequence of H. akule n.
FIGURE 2. Spores of *Henneguya akule* n. sp. (A) Wet mount dorso-ventral view, bar = 7 µm. (B) Wet mount side view, bar = 7 µm. (C) Giemsa-stained impression smear, bar = 6 µm. (D) Electron micrograph *en face* view, note suture of bivalvular spore (arrow), bar = 1 µm. (E) Electron micrograph side view; note polar filaments within the polar capsule (black arrow), glycogen granules (white arrow), and sporoplasm (arrowhead), bar = 1 µm. (F) Aggregate of spores in aortic bulb (Giemsa), bar = 50 µm. (G) Closeup of section F (hematoxylin and eosin), bar = 10 µm. (H) Nidus of eosinophilic debris surrounding individual spores (arrow) in aortic bulb (H&E), bar = 25 µm.
evaluate difference in body condition index between parasitized and parasite-free fish (Daniel, 1987). Alpha for all comparisons was 0.05.

**DESCRIPTION**

*Henneguya akule* n. sp.

Spores ellipsoid with long bifurcated tails (Figs. 1, 2A–E). Morphometrics (μm) based on measurements of 50 spores from aortic bulb of 2 akule. Spore length (mean ± SD, range), 12.1 ± 0.8, 10–14; spore width, 7.4 ± 0.7, 5–9; spore total length, 40.8 ± 5.2, 29–52; spore thickness, 5.3 ± 0.6, 3–7; polar capsule width, 1.4 ± 0.5, 1–2; polar capsule length, 3.4 ± 1, 2–6. Polar filament had 3–4 coils. Plasmodia pleiomorphic ranging from 0.01 to 0.7 mm long (Figs. 2F–H).

**Taxonomic summary**

*Type host:* *Selar crenonophthalmus* (Bloch 1793) (Carangidae), big-eyed scad or akule.

*Site in host:* Bulbus arteriosus.

*Type locality:* Southern Oahu: Barbers Point (21.15°N, 158.00°W).

*Other localities:* Southern Oahu: Mauna Lua Bay (21.15°N, 157.45°W), Waianae coast (21.30°N, 158.15°W), Sand Island (21.18°N, 157.54°W).

*Specimen deposited:* Hapantotypes (giemsa-stained slide, hematoxylin, and eosin sections) deposited at the U.S. Department of Agriculture National Parasite Collection in Beltsville, Maryland (USNPC no. 099994.00 and USNPC no. 099995.00). Small subunit ribosomal DNA sequence deposited in GenBank (EU016076).

*Prevalence and relative intensity:* A total of 347 fish was examined historically for the presence of *Henneguya akule* n. sp.; overall prevalence of infection was 20%. Prevalence in males (15%, n = 140) versus females (22%, n = 205) (2 fish were of unknown sex) did not differ significantly. Annual prevalence ranged from 12 to 27% between 2001 and 2006 and monthly prevalence during January–June ranged from 5 to 20% and 18 to 32% from July to December. Prevalence between year and month did not differ significantly. In contrast, prevalence between sites differed significantly ($\chi^2 = 9.671, P < 0.05$), with the highest prevalence concentrated in south-central Oahu (Fig. 3A). Prevalence was lowest in the smallest and largest size classes (Fig. 3B). Body condition of infected fishes did not differ significantly from non-infected fishes.

*Etymology:* Akule refers to the Hawaiian language name for big-eyed scad.

**Remarks**

The parasite seen here is consistent with species of *Henneguya* based on ellipsoid spores with a suture separating biconvex valves having long caudal projections (Lom and Dyková, 1992). *Henneguya* species infect mainly freshwater fishes and the morphological stages of this parasite are mostly found in the gills, skin, kidneys, musculoskeletal system, or gastrointestinal tract (Eiras, 2002); however, *Henneguya* sp. in the bulbus arteriosus are relatively less common (Yokoyama et al., 2005).

Despite the advent of molecular tools to examine the phylogeny of the Myxosporea, considerable uncertainty continues to exist on exactly how to classify species. Although predominantly freshwater species, marine representatives of these genera clustered together in early analyses (Kent et al., 2001), suggestive of a single marine invasion from freshwater. Increased taxonomic sampling in later studies (Fiala, 2006) placed freshwater and marine species within the same clade, indicating multiple reversals to and from the marine environment. Based on analyses of SSU rDNA (Kent et al., 2001; Fiala, 2006), *Henneguya* and *Myxobolus* form a polyphyletic clade comprised of both marine and freshwater species. Kent et al. (2001) further concluded that taxa seemed to cluster more by development and tissue location than by spore morphology. The importance of tissue specificity in myxozoan evolution has been reinforced by subsequent phylogenetic studies (Blaylock et al., 2004; Eszteterbauer, 2004; Whipp et al., 2004; Fiala, 2006; Burger et al., 2007). Consistent with these findings, *H. akule* n. sp. was phylogenetically most similar to *H. lateolabracis* and *H. pagri*, both parasites of the bulbus arteriosus of marine fishes (Fig. 4). As in previous analyses (Easy et al., 2005; Yokoyama et al., 2005; Fiala, 2006), much of the remaining tree topology was unstable as indicated by numerous polytomies and low bootstrap support (Fig. 4). Bayesian anal-

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**Figure 3.** (A) Map of Oahu (Hawaii) with sites sampled for prevalence of *Henneguya akule* n. sp. Numbers indicate prevalence, and numbers in parentheses are sample size for each area. (B) Prevalence (dots, right axis) of *H. akule* versus frequency histogram of fork length (bars, left axis).

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sp. was aligned to SSU sequences from other species in the relevant subclade based on the phylogenies of Easy et al. (2005), Yokoyama et al. (2005), Fiala et al. (2006), and our preliminary analyses of which-incorporated sequences recently deposited in GenBank. The marine myxozoans *Unicapsula* sp. and *Kudoa alliaria* were used to root the tree. Nucleic acid sequence alignments were conducted in ClustalX (Thompson et al., 1997) using default settings and edited by eye. Phylogenetic analyses using parsimony and maximum likelihood algorithms were conducted in PAUP*4.01 (Swofford, 1998). Bayesian analyses (BA) was conducted using MrBayes v. 3.0 (Ronquist and Huelsenbeck, 2003). Parsimony analyses (MP) were conducted using a heuristic search algorithm with 10 random additions of sequences and tree bi-section-reconnection (TBR) branch swapping. Bootstrap values were calculated with 100 replicates using a heuristic search algorithm with simple sequence addition and TBR branch swapping. An optimal evolutionary model (GTR+G+I) for the alignment was determined using Modeltest 3.06 (Posada and Crandall, 1998). Maximum likelihood (ML) analysis employed a heuristic search algorithm with random sequence addition and TBR branch swapping. Bootstrap confidence values were calculated with 100 repetitions. Tree construction using BA was run with 4 simultaneous Monte Carlo chains for 104 generations, sampling every 100 generations, with a burn-in of 100 trees.

Body condition indices (weight/fork length2) were calculated (Petrusheski and Kogteva, 1954). Data were tested for normality and equal variance. A chi square analysis was performed to evaluate relationship between parasitism and sex, parasitism and year of collection, and parasitism and location of collection. Student’s t-test was used to
Figure 4. Phylogenetic analysis of small subunit ribosomal DNA of Henneguya akule n. sp. Strict consensus of the 3 most parsimonious trees generated by maximum parsimony. In-group taxa were selected to represent an entire myxobolid subclade supported by previous analyses (Easy et al., 2005; Fiala, 2006) including relevant BLAST matches and sequences derived from unclassified actinospore stages. Unicapsula sp. and Kudoa alliaria were used to root the tree. GenBank accession numbers are shown adjacent to names. Numbers on branches indicate bootstrap support resulting from maximum parsimony, bootstrap support from maximum likelihood, and clade confidence values from Bayesian analysis. Values below 50 are not shown.
a|mined). Henneguya akule n. sp. has caudal filaments similar to those seen in H. pagri (Yokoyama et al., 2005) (Figs. 1, 2A–B), but these are considerably shorter and adopt a tortuous appearance. H. akule also has a longer spine. Other Henneguya species that have been documented from the heart of fish (reviewed in Yokoyama et al., 2005) can be differentiated from H. akule n. sp. in having much larger spores, or, in the case of Henneguya ootolithi, much longer caudal appendages. Given its location in the host (bulbus arteriosus only), its morphology, and the geographic isolation of the parasite’s host (Hawaii), we believe the naming of H. akule n. sp. as a new species is justified. Furthermore, DNA sequence data support distinction of H. akule n. sp. from its morphologically similar relatives. Despite morphological similarities, the genetic similarity of the SSU rDNA of H. akule n. sp. to H. lateolabracios and H. pagri was 88.7 and 87.2 percent, respectively, which is well in excess of the 1–2% distance that might be considered intraspecific variation for myxozoans (Whipp and Diggles, 2006).

Host response to the parasite was minimal, and gross lesions were not present. On microscopy, host response was mild and characterized by accumulations of eosinophilic fibrillar material around spores (Fig. 2H) and occasional mononuclear infiltrates in the adventitia (seen in 17% of infected fish). Unlike Meyers et al. (1977), we did not observe rodlet cells as a component of the inflammatory response to H. akule n. sp. It appears that pathological changes due to infection with Henneguya sp. are more severe in cultured fish (Yokoyama et al., 2003, 2005) where, presumably, opportunities for infection are greater than for wild fish. Like Ganapati (1941) and Meyers et al. (1977), parasites were absent in very young and very old fishes, but prevalence was relatively constant for intervening sizes. Mild pathology and constant prevalence with age suggests this parasite may have little measurable detrimental effect on the host.

Although there appeared to be an increase in prevalence from June–December, this was not significant and contrasted with seasonality observed in infections by H. pagri, which had a peak prevalence in August, and H. ootolithi, which had a peak prevalence in March. Overall prevalence of infection of akule with H. akule n. sp. was generally lower than that reported in other studies (Ganapati, 1941; Meyer et al., 1977) and similar to that reported for H. lateolabracios (Yokoyama et al., 2003) and H. sebasta in selected species of rock fish (Moser and Love, 1975). Few studies have examined geographic variation of infection with marine Henneguya species; the high prevalence of infection in fishes from south-central Oahu was intriguing. This region is adjacent to the highest urban concentration (Honolulu) and is close to Pearl Harbor, suggesting that certain physiographic aspects of these areas may be contributing to high prevalence there. Explaining this higher prevalence in south-central Oahu will require more detailed epizootiologic studies and further knowledge about the life cycle of H. akule n. sp.

Acknowledgments

We thank Christine Densmore, Frank Morado, and anonymous reviewers who provided constructive critiques of the manuscript. Thanks to A. Muranaka, G. Voitovich, and the staff of the Oceanographic Team, City and County of Honolulu, for collecting the fish for this study. This study was funded, in part, by the Water Resources Division of the University of Hawaii. C.M.W. is grateful for the services of Nevada Genomics Center, which is supported in part by a grant from the Nevada IDEA Network of Biomedical Research Excellence (2 P20 RR16463).

Literature Cited


**BOOK REVIEW . . .**


Samuel Taylor Darling, MD, achieved eminence in parasitology, pathology, and public health and was abundantly endowed with the personal qualities that elevate an expert into positions of leadership. Today, more than 80 years after he rose to prominence in our field of scholarship, he is little remembered. In this new biography, Chaves-Carballo provides a wide vista of Darling’s work and a glimpse of his nonworking life. Perhaps there was not much room, in man or book, for anything but work.

Darling was a man of vast medical learning and much practical experience. Before his untimely death in a car accident, he spent about a decade in Panama, working for the Isthmian Canal Commission, and about a decade working for the Rockefeller Foundation in what is now known as Malaysia and Indonesia, and in Brazil, and the southern United States. He made medical forays into other countries and was acclaimed worldwide as an authority on tropical medicine. Darling’s reputation in parasitology rested chiefly on his work on hookworm disease and malaria, but he also published observations on anemia, trypanosomiasis, leishmaniasis, filariasis, schistosomiasis, piroplasmosis, and strongyloidiasis, as well as notes on mites, ticks, bedbugs, and fleas. He described an early clinical case of what was probably toxoplasmosis. He described the first cases of histoplasmosis and discovered its etiological agent. He considered the agent a protozoan parasite and named it *Histoplasma capsulatum* (later declared to be a yeast-like fungus).

With the appearance of Chaves-Carballo’s book we have a biography written by one who for many years has immersed himself, as a labor of love, in the history of Darling and his tropical experience. That undoubtedly accounts for its strength—and, alas, its weakness. The volume is a scholarly compendium of facts and figures and is referenced and annotated to a fare-thee-well. Narrative is overwhelmed by the onslaught of information. Fortunately some sense of the man’s style as a pioneer investigator in exotic places does percolate through the layers of fact.

Darling was a doer as well as a thinker. He was endlessly curious and unafraid to explore uncharted intellectual territory. He was a clinical pathologist, yet he made himself an expert on the fine points of mosquito morphology and taxonomy and on anything else he needed to know. He was industrious and accustomed since his college days to excelling at whatever he did. His originality is exemplified by experiments in which he weighed mosquitoes before and after feeding, in order to calculate the volume of a blood meal. He then fed mosquitoes on blood with a known concentration of macrosomocytes. Subsequent dissection of the insects enabled him to conclude that the great majority of macrosomocytes do not become fertilized, or at least do not form viable zygotes. His common sense is reflected in his insistence that hookworm control should be focused on the community rather than the individual. In the teaching and promotion of public health, Darling was indefatigable and effective. For all these things he should be remembered—but for a variety of reasons Darling and his like are apt to be forgotten.

A good biography will place its subject in the appropriate historical, geographical, and cultural setting. This the author tries to do, but the text is bloated by excessive digression and devotion to minutiae. It is fine to speculate that medical student Darling was influenced by his pathology professor—but is it helpful to know that the professor had closely cropped hair and married late in life? The problem is one of proportion. Darling worked in Panama at the beginning of the 20th century, and the contemporary Panamanian scene, with canal building as a dominant theme, is relevant here. Bringing in Balboa’s early 16th-century exploits in the region is a bit of a stretch. We are even given, for no apparent purpose, the name of Balboa’s dog—in English and Spanish. It almost gives the reader the feeling of having slipped into the wrong book! In a work of this sort, surplus or marginal information can often be relegated to an endnote or appendix; but in the present case there is so much that is extraneous, and the “back matter” is already so replete with supplement, that this would hardly be a practical solution.

In 3 different chapters we are told that the French handed over their canal-building equipment to the Americans in 1904 (not just in that year but on May 4 of that year). And in 3 different chapters we are told that Darling traveled to Panama about 2 days after getting married. These repetitions would be justified if the information had been made pertinent to each context, but this is not the case. Referring to a famous tragedy that befell a family during the French attempt to build the Panama Canal, the author specifies the family members who died of disease. In the next chapter, he refers to the incident again, and does use it to make a different point, but repeats the same details without reference to the previous chapter. Throughout the book one gets the impression that many details were added because they had been found, not because they were relevant, and that the chapters were drafted in isolation from each other and not subsequently harmonized. Good editing could have done much to rid the text of its repetitions, its excessively detailed tangential accretions, and its occasional syntactic quirks. The publisher must bear much of the responsibility for not seeing that this was done.

Dr. Chaves-Carballo deserves our gratitude for amassing so much information about Samuel Taylor Darling, his milieu, and his work. He has also told us a little about the nonprofessional side of Darling’s life. Perhaps further personal observations and insightful anecdotes are simply not to be had. Still, one longs for a more rounded picture of Darling as a human being. Here, after all, was a man who could exclaim (in a letter), “Oh it is good to be alive and see all the wonders in the tropical world.”

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DETECTION OF ENTAMOEBA HISTOLYTICA ANTIGEN IN STOOL SAMPLES IN MERSIN, TURKEY

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ABSTRACT: Entamoeba histolytica, 1 of the 2 Entamoeba species with similar morphology that infect humans, causes invasive intestinal and extraintestinal diseases, whereas Entamoeba dispar is found commensally and is noninvasive. Because of their morphologic similarity, E. histolytica and E. dispar cannot be differentiated microscopically. The antigens of E. histolytica and E. dispar, however, may be detected by the ELISA method. Previous studies have found that the detection of antigens in the stool is as sensitive and specific as cultures and isoenzyme analyses. Stool samples from 272 patients with diarrhea in the province of Mersin, Turkey, were examined for the presence of Entamoeba species microscopically and for Entamoeba (E. histolytica/E. dispar) antigens using the ELISA method. An E. histolytica–specific ELISA test was used to examine 29 E. histolytica/E. dispar–positive samples. Twenty-four (8.82%) of the samples tested positive for E. histolytica/E. dispar by trichrome staining, and 29 (10.6%) of the samples tested positive for E. histolytica/E. dispar by the Entamoeba screening test. Entamoeba histolytica was positive in 21 (7.72%) and E. dispar positive in 8 (2.94%) samples. The detection of true E. histolytica infection is possible with the use of E. histolytica–specific antigen ELISA tests. Thus, real cases of amoebiasis can be detected and treated, and overtreatment of the patients with E. dispar, which is the nonpathogenic species, will be prevented.

Entamoeba histolytica is an intestinal protozoan, and the agent of amoebiasis, which is responsible for more than 100,000 deaths per year and is the second leading cause of death due to protozoans, following malaria (World Health Organization [WHO], 1997). Entamoeba histolytica, 1 of the 2 Entamoeba species with similar morphology that infect humans, causes invasive intestinal and extraintestinal diseases, whereas E. dispar is a noninvasive commensal (WHO, 1997; Haque et al., 1998; Pillai et al., 1999). It has been reported that 10% of the world population is colonized by E. histolytica or E. dispar; 90% have the nonpathogenic E. dispar, whereas the remaining 10% are infected with E. histolytica (Braga et al., 1998). The WHO (1997) recommends specific identification and treatment of E. histolytica, but not E. dispar.

Entamoeba histolytica infections are commonly observed in tropical and subtropical regions of the world. Humans are the primary hosts and the infection is transmitted via ingestion of water or food contaminated by feces containing E. histolytica cysts (Leber and Novak, 1999; Stanley, 2003).

The diagnosis of E. histolytica infection is traditionally made by microscopic examination of fresh, or fixed, stool samples. However, E. histolytica and E. dispar cannot be differentiated by microscopic examination because of their morphological similarity (Pillai et al., 1999). The current reference methods for differentiating between E. histolytica and E. dispar are in vitro culture and isoenzyme analysis. However, these methods are not widely used and they are not practical for routine diagnostic laboratories (Haque et al., 1995).

New approaches to the detection of E. histolytica and E. dispar are based on antigen detection in stool or detection of E. histolytica–specific DNA by polymerase chain reaction (PCR) amplification (Haque et al., 1998; Ackers, 2002). Detection of antigens in the stool is as sensitive and specific as culture and isoenzyme analyses (Haque et al., 1995, 1998). The antigens of E. histolytica and E. dispar are detected easily and quickly by the enzyme-linked immunosorbent assay (ELISA) method (Haque et al., 1995). PCR is, however, more sensitive and specific than ELISA (Gonin and Trudel, 2003), but this technique has some disadvantages; it is technically complex, time consuming, and expensive (Haque et al., 1998).

Stool samples from diarrheic patients that were sent to our laboratory and stool samples retrieved from diarrheic patients presenting at other health institutions (such as Social Insurance Institution Hospital, the State Hospital, and local health institutions) in our province were collected in order to discriminate between the pathogenic and nonpathogenic species of Entamoeba, and to determine an accurate prevalence of E. histolytica, which is the true pathogen. These samples were treated with trichrome stain for microscopic examination, and the ELISA method was used for the detection of E. histolytica/E. dispar antigen. The aim was to compare microscopic findings and results from E. histolytica/E. dispar antigen tests, in addition to detecting the actual prevalence of E. histolytica by examining the E. histolytica/E. dispar antigen–positive cases with an E. histolytica–specific ELISA test.

MATERIALS AND METHODS

Stool samples from diarrheic patients that were sent to the Microbiology Department of Mersin University, Faculty of Medicine Research and Practice Hospital, Mersin Social Insurance Hospital, and Local Central Health Institution Number 1, in Mersin, Turkey, were collected between July 2004 and October 2005. The samples were obtained from 272 patients, whose ages ranged between 1 mo and 66 yr; 137 of the samples were from children. Stool samples were examined microscopically with the use of wet mount (0.9% saline and lugol) and trichrome-stained slides. Subsamples of stools were kept at −20°C to evaluate the presence of antigens by the ELISA method. When a sufficient number of samples were obtained, the ELISA screening test was used to search for E. histolytica/E. dispar antigens; samples positive for E. histolytica/E. dispar antigen were screened with the use of the E. histolytica–specific ELISA test. In total, 272 cases were examined with the ELISA test (Ridascreen Entamoeba; R-Biopharm, Darmstadt, Germany) to detect the E. histolytica/E. dispar antigen. Twenty-nine samples that tested positive from the screening tests were examined with the ELISA test, which detects the specific E. histolytica adhesins (Entamoeba CELISA Path, Cellabs, Brookvale, Australia).

RESULTS

Twenty-four (8.82%) of the samples were positive for E. histolytica/E. dispar with the trichrome staining, and 29 (10.6%) were positive for E. histolytica/E. dispar antigens with the non-specific ELISA screening test (Table I). When microscopy was
TABLE I. Results of microscopy and the ELISA test.

<table>
<thead>
<tr>
<th></th>
<th>Positive n (%)</th>
<th>Negative n (%)</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy (Trichrome staining)</td>
<td>24 (8.82)</td>
<td>248 (91.1)</td>
<td>272</td>
</tr>
<tr>
<td>ELISA (Entamoeba screening test)</td>
<td>29 (10.6)</td>
<td>243 (89.3)</td>
<td>272</td>
</tr>
<tr>
<td>Ridascreen Entamoeba</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA (Entamoeba histolytica–specific antigen)</td>
<td>21 (7.72) (E. histolytica)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entamoeba CELISA path</td>
<td>8 (2.94) (Entamoeba dispar)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

compared with ELISA, its sensitivity was 60% and its specificity was 94% (Table II). *Entamoeba histolytica* was positive in 21 (7.7%) and *E. dispar* was positive in 8 (2.9%) samples. The *E. histolytica/E. dispar* antigen was present in 8.8% of children, and *E. histolytica* was positive in 5.0% of the children, whereas the prevalences were 12.6% and 10.3% in adults, respectively. The age distributions of *E. histolytica*–positive and –negative samples are shown in Table III. From the patients whose *E. histolytica* antigens were positive, 1 (4.7%) of the stools was bloody, 9 (42.8%) had blood and mucous, 5 (23.8%) had mucous, and 6 (28.5%) had watery stools. From the *E. histolytica*–negative patients, 4 (1.6%) had bloody stools, 116 (46.2%) had mucous, 10 (3.9%) stools were bloody and had mucous, and 121 (48.2%) had watery stools. Erythrocytes were present in direct microscopic examination of stools in 47.6% (10) of patients whose *E. histolytica* antigens were positive and 13.5% (34) of patients whose *E. histolytica* antigens were negative.

**DISCUSSION**

*Entamoeba histolytica* causes intestinal infections, as well as extraintestinal lesions. In contrast, *E. dispar* is nonpathogenic (Haque et al., 1998). The diagnosis of intestinal amoebiasis is usually made by the history of the patient, clinical findings, and detection of *E. histolytica* as a cyst or trophozoite in the stool. The microscopic examination is made on preparations of fresh stools diluted by physiological saline, and treated with a trichrome or iron-hematoxylin stain (Ravdin, 2000). However, *E. histolytica* and *E. dispar* cannot be differentiated by this method (Stanley, 2003). WHO (1997) stated that because *E. histolytica* and *E. dispar* were morphologically similar and could not be differentiated from each other, reports should use the term "*E. histolytica/E. dispar.*" Moreover, because light microscopy cannot discriminate between the 2 species, asymptomatic cases of *E. histolytica/E. dispar* should not be treated when specific diagnosis cannot be made and treatment should be saved for cases with the specific diagnosis of *E. histolytica* (WHO, 1997). The specific diagnosis of *E. histolytica* in stool specimens depends on isoenzyme analysis, antigen detection, hybridization with species-specific DNA probes, and sensitive and specific tests based on PCR (Haque et al., 1998; Ackers, 2002). The detection of *E. histolytica* antigens in stools has opened a new era in diagnosis due to its practical application, high sensitivity, and specificity (Ravdin, 2000).

Haque et al. (1995) found that the *E. histolytica* ELISA was more sensitive and specific than microscopy in a study in which they compared microscopy, culture, and antigen detection tests for 202 patients with diarrhea. They reported that the *E. histolytica*–specific ELISA method is faster and more reliable than zymodeme analysis in discriminating between *E. histolytica* and *E. dispar*, i.e., 95% sensitive and 93% specific when compared to isoyzme analysis. Subsequently, Haque et al. (1998) reported that PCR was 87% sensitive, whereas antigen detection was 85% sensitive in a study in which they compared PCR, isoenzyme analysis, and the *E. histolytica*–specific antigen detection test. They concluded that the antigen detection test is rapid, easy, and that it could be used in the diagnosis of *E. histolytica* infection because it did not require special equipment.

In a study searching for the frequency of *E. histolytica* and *E. dispar* in stool samples in Mexico by PCR, Ramos et al. (2005) detected 14% *E. histolytica*, 7.2% *E. dispar*, and 24% double infections of *Entamoeba* species. Braga et al. (2001) found the presence of *E. histolytica/E. dispar* complex in 25.4% of the stools examined in Brazil, and *E. histolytica* infection alone in 14.9% of the stools with the use of the antigen detection method. The prevalence of *E. histolytica* infection was found to be 4.7% in a study conducted on 680 asymptomatic children between the ages of 2 and 5 in Bangladesh (Haque et al., 1999). Tanyüksel et al. (2005) detected 24% *E. histolytica/E. dispar* positivity by trichrome staining and 13% *E. histolytica* positivity by the ELISA method in 380 stool samples from diarrheic patients in Van and Sariyer, Turkey. In a previous study we

**TABLE II. Comparison of microscopy and *Entamoeba* species screening test.**

<table>
<thead>
<tr>
<th>Sample size (yr)</th>
<th><em>E. histolytica</em> positive n (%)</th>
<th><em>E. histolytica</em> negative n (%)</th>
<th>Sample size total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1-4</td>
<td>3 (5)</td>
<td>55 (94.8)</td>
<td>58</td>
</tr>
<tr>
<td>5-14</td>
<td>4 (5)</td>
<td>75 (94.9)</td>
<td>79</td>
</tr>
<tr>
<td>15-24</td>
<td>2 (8)</td>
<td>23 (92)</td>
<td>25</td>
</tr>
<tr>
<td>25-34</td>
<td>6 (19.3)</td>
<td>25 (80.6)</td>
<td>31</td>
</tr>
<tr>
<td>35-44</td>
<td>4 (16.6)</td>
<td>20 (83.3)</td>
<td>24</td>
</tr>
<tr>
<td>&gt;45</td>
<td>2 (3.6)</td>
<td>55 (96.3)</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>21 (7.72)</td>
<td>251 (92.2)</td>
<td>272</td>
</tr>
</tbody>
</table>

**TABLE III. Age distribution of *Entamoeba histolytica*–positive and –negative samples.**
conducted in our province in Turkey, we detected *Entamoeba histolytica* and *E. dispar* positivity in 20.4% of the cases by trichrome staining and 29.5% by ELISA. In that study, specific *E. histolytica* antigen could not be examined because of fixation problems. When compared to ELISA, we found that microscopy had higher sensitivity, but lower specificity, with a low negative predictive value and high positive predictive value (Delialioglu et al., 2004).

In conclusion, laboratory studies have a supportive and important role in the clinical diagnosis of intestinal amoebiasis. Although direct microscopic examination is cheaper than antigen-detection tests in stools, its subjective characteristics, requirement for experienced observers, and difficulty in discriminating between leukocytes and other parasites hinder its reliability. In addition, microscopy was found to be less sensitive than ELISA. Amoebic culture and isoenzyme analyses require considerable time and, because of the requirement for special equipment, neither diagnostic technique is recommended in clinical diagnosis. True *E. histolytica* infection can be detected by ELISA tests in which *E. histolytica*-specific monoclonal antibodies are used. The routine application of specific ELISA tests will facilitate the diagnosis of true amoebiasis and prevent the unnecessary treatment of patients with *E. dispar*, the non-pathogenic species.

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LITERATURE CITED


VALIDATION OF THE WORLD HEALTH ORGANIZATION’S RAPID ASSESSMENT METHOD FOR URINARY SCHISTOSOMIASIS IN SOUTHEASTERN NIGERIA

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ABSTRACT: Urinary schistosomiasis is a helminth disease that causes high morbidity in endemic areas of tropical and subtropical regions. Efforts are being made to evolve a cost-effective method for diagnosing the infection in large populations. A study supported by the World Health Organization (WHO) has established the Rapid Assessment (Questionnaire) method in which diagnosis is based on the respondent’s ability to answer yes to the presence of hematuria. This method has been validated in some African countries and elsewhere. The aim of the present study was to validate the Rapid Assessment method in a community in southeastern Nigeria where the disease is endemic. A survey was carried out using both the parasitological diagnosis of the presence of the characteristic egg of Schistosoma haematobium in urine samples and the WHO Rapid Assessment method. Positive results in the 2 methods were calculated as percentages, and a correlation analysis of the percentages was done using product moment statistics. This gave a significant value of \( r = 2.9435 \) (\( P < 0.05 \)). Sex-related prevalence was observed at significant correlation values of \( r = 1.0011 \) and \( r = 1.574 \) (\( P < 0.05 \)). The diagnostic performance of the Rapid Assessment method was calculated using Baker’s procedure method. A high sensitivity of 93.4%, specificity of 99%, positive predictive value of 96.6%, and negative predictive value of 99.4% were calculated. The consistent high correction performance values confirm that the Rapid Assessment method may be a useful alternative to the parasitological tests for use in schools and community surveys in identifying high-risk individuals for urinary schistosomiasis in southeastern Nigeria.

Urinary schistosomiasis is caused by Schistosoma haematobium in 44 African countries and affects the lower abdomen and genito-urinary tract (Ukoli, 1991; World Health Organization [WHO], 1991). Individuals infected with urinary schistosomiasis suffer severe pathological defects, including carcinoma of the bladder (Ukoli, 1991), perportal thickening and portal enlargement, glomerulonephritis, and pulmonary hypertension (Lambertucci et al., 2000).

Intervention efforts on transmission control have been mainly directed at environmental and other vector-control measures. In recent years, however, attention has shifted to the control of morbidity in infected individuals. In response to this effort, various techniques of screening infected individuals have evolved, especially those that can combine cost effectiveness and safety with rapid results. Diagnostic methods include sedimentation technique (McCullough, 1957; Garcia and Ash, 1979), enzyme-linked immunosorbent assay (ELISA) (von Lieshout et al., 2000), reagent strips (Pugh et al., 1978; Savioli and Mott, 1989), ultrasound technique (Vennervald et al., 2000), and the Rapid Assessment method (Lengeler, 1992, 2002). The Rapid Assessment method involves the use of simple questionnaires to determine the frequency of urinary schistosomiasis in a community or school (WHO, 1995). The school is considered the most appropriate target population because children of school age are likely to have the highest infection prevalence.

Extensive validation studies have been carried out on Rapid Assessment method in some African countries by the Red Urine Group (WHO, 1995) and Lengeler et al. (2002). These studies revealed that the technique of using questionnaires in school and community-wide diagnosis of urinary schistosomiasis is both feasible and cost effective. The present study, therefore, sought to evaluate the diagnostic performance of the questionnaire (blood in urine) method in several selected schools of southeastern Nigeria and to rank schools according to prevalence of the disease as a prerequisite for a possible intervention program.

MATERIALS AND METHODS

Study locality

The present study was conducted in Umuchieze autonomous community, Umunneochi Local Government Area of Abia State, Nigeria, which is located along the “north-south line” between Ihube in Imo State and Awgu in Enugu State; it lies between longitude 7°10’E and 7°34’E and latitude 5°4’N and 6°3’N. The area experiences tropical climatic conditions. The vegetation is entirely tropical rainforest to the south, which changes to the woodland-savanna region of Nigeria. The source of permanent water for domestic use is the Aku River. Other sources of water are seasonal and cannot be used for domestic purposes during the dry seasons. Mining activities in the area have led to the presence of abandoned quarry pits retaining permanent water for domestic use.

There are 7 primary schools and 3 secondary schools included in the study. The area is owned by the government and located at varied distances from the quarry pits. Stratified random sampling was used in selecting 40% of the pupils, class by class, in the schools, taking cognizance of the male-female ratios in each class. Being a rural agricultural community, the primary schools include children of varying ages (from 6 to 18 yr). Only pupils in junior classes 1–3 in the 3 secondary schools (of similar ages with those in the primary schools) were involved in the study. These schools (located within a 1–10 km radius from the quarry pits) were identified as follows: (A) Lokpaukwu Community School, (B) Amaubiri Primary School, (C) Lekwesi Community School, (D) Comprehensive Secondary School Umuchieze, (E) Lokpanta’Secondary School, (F) Leru Secondary School, (G) Leru Primary School, (H) Lekwesi Central School, (I) Urub Primary School, and (J) State Primary School Lokpanta.

Urine collection, sedimentation, and examination

A preliminary visit was made to the schools with a copy of an introductory letter to the school heads. On the appointed days of the visit, clean, labeled specimen bottles were used to collect fresh urine specimens from pupils aged between 8 and 18 yr of age; the sex of each pupil was also recorded. Collections were made from between 1,000 and 1,400 daily. The urine specimens were taken to the laboratory immediately. About 10 ml of each sample was centrifuged using the method of Garcia and Ash (1979). The supernatant of each centrifuged sample was poured away; the sediment was placed on a slide and examined for the characteristic eggs of S. haematobium using a light microscope equipped with a 10× objective and 10× eye piece.
**Questionnaire preparation, distribution, and collection**

The questionnaires were adopted from WHO (1995) and included 2 primary diagnostic focuses, i.e., "schistosomiasis" and "blood in urine." The questionnaire was pretested in 3 schools. It was observed that the term "schistosomiasis" was unknown to the children of this community, leading to its exclusion. The term "blood in urine," requiring yes or no responses, was then evaluated for diagnostic performance.

The questionnaires were distributed to teachers on the day a urine collection was made. The teachers were educated on the method of completing the questionnaires; they were given 2 wk to interview the pupils who had provided their urine samples. Completed questionnaires were collected from the teachers after a specified period.

**Statistical analysis**

The positive questionnaire rates were calculated using percentages and correlated using product-movement statistics. Responses on the questionnaire include true positive (TP), true negative (TN), false positive (FP), and false negatives (FN). The results obtained were subjected to statistical evaluation using the methods of Baker (1976), which uses the parasitological test results as an index of active infection by urinary schistosomiasis. Numbers of true positive, true negative, false positive, and false negative were calculated. These figures were inserted in a functional tabulation below and used in calculating the sensitivity, specificity positive, and negative predictive values thusly:

\[
\begin{align*}
\text{Parasit +ve} & \quad \text{Parasit -ve} & \quad \text{Total} \\
\text{Quest +ve} & \quad a (TP) & \quad b (FP) & \quad a + b \\
\text{Quest -ve} & \quad c (FN) & \quad d (TN) & \quad c + d \\
\text{Total} & \quad a + c & \quad b + d &
\end{align*}
\]

Sensitivity = \[\frac{a}{a + c}\]  
Specificity = \[\frac{d}{a + c}\]  
Positive predictive value = \[\frac{a}{a + b}\]  
Negative predictive value = \[\frac{d}{d + c}\]

**RESULT**

Altogether 1,009 school children had their urine samples examined; 1,008 of the same children were interviewed with questionnaires. Of these, 90 (8.9%) were positive for *S. haematobium* infection via laboratory diagnosis, and 85 (8.4%) responded positively in the questionnaire (Table I).

Highest prevalences (20.4% and 18.5%) were recorded for school B in both parasitological and questionnaire tests, respectively, while lowest prevalence of infection were recorded in schools E (2.5%) for the parasitological test and J (2.3%) using the questionnaire method. The questionnaire method showed slight underreporting in all schools involved, except schools E, G, and H, where slight overreporting was shown. A correlation analysis of the school prevalence for the 2 methods was significant \(r = 2.9435, P < 0.05\) (Table I).

Gender-related infections (Table II) showed that 59 (10.6%) of the males examined parasitologically were infected, and 55 (10.6%) responded positive using the questionnaire method. A total of 31 (7.2%) of the females examined parasitologically tested positive, while 30 (6.9%) of them responded positively in the questionnaire. Highest prevalences, 16.3% and 13%, were recorded among the females in the parasitological and questionnaire methods, respectively, in schools B and I. Equal prevalences (23.1%) were recorded among males using the 2 methods in school B. Generally, prevalences were higher in males than in females using the 2 methods. Questionnaire results showed a higher precision in 6 schools among the males than females. Statistical analysis of the prevalences among males in the 2 methods was significant \(r = 1.0011, P < 0.5\). A slight overreporting was observed among the females in 3 schools for the questionnaire methods. Prevalences among the females using both parasitological and questionnaire methods were significantly correlated \(r = 1.574, P < 0.05\).

The highest prevalences, 12% and 11.5%, for the parasitological and questionnaire tests (Table III), respectively, occurred among pupils aged 10- and 11-yr-old, followed by the 12- and 13-yr-old group, with 10% and 9.7%, respectively. Lowest infections were found among the 8- and 9-yr-old group with the 2 methods. Analysis of the results for the 2 methods was statistically significant \(r = 0.9924, P < 0.05\).

A diagnostic performance analysis yielded a sensitivity of 93.4% and specificity of 99%. A positive predictive value of 96.6% and negative predictive value of 99.4% were also recorded (Table IV).

**DISCUSSION**

This study revealed a low endemic status of *S. haematobium* infection in this area. It also indicated the potentiality of the use of questionnaire as a diagnostic instrument in a community-wide screening for urinary schistosomiasis. There is, however, a general slight underreporting of the infection by the questionnaire method (8.4%) compared to the results of the parasitological method (8.9%). The underreporting shown in this work agrees with Savioli et al. (1989) and WHO (1995) in several African countries. The reasons for the observed underreporting could be due in part to lack of confidence in some of the pupils that their medical history would be handled with utmost confidentiality by the interviewing teachers. Second, there was suspicion by some pupils about the use of the exercise, and this affected their responses. Although slight overreporting was observed in 3 schools (E, G, and H), there was a significant correlation between the questionnaire and parasitological results \(r = 2.9435, P < 0.05\).
TABLE II. Sex-related prevalence in the parasitological and questionnaire methods.

<table>
<thead>
<tr>
<th>SCH</th>
<th>No. males</th>
<th>Parasitological</th>
<th></th>
<th>Questionnaire</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. +ve (%)</td>
<td>No. females</td>
<td>No. +ve (%)</td>
<td>No. females</td>
</tr>
<tr>
<td>A</td>
<td>64</td>
<td>7 (10.9)</td>
<td>42</td>
<td>3 (7.1)</td>
<td>64</td>
</tr>
<tr>
<td>B</td>
<td>65</td>
<td>15 (23.1)</td>
<td>43</td>
<td>7 (16.3)</td>
<td>65</td>
</tr>
<tr>
<td>C</td>
<td>45</td>
<td>4 (8.2)</td>
<td>45</td>
<td>2 (4.4)</td>
<td>45</td>
</tr>
<tr>
<td>D</td>
<td>101</td>
<td>8 (7.9)</td>
<td>65</td>
<td>4 (6.2)</td>
<td>101</td>
</tr>
<tr>
<td>E</td>
<td>41</td>
<td>1 (2.4)</td>
<td>38</td>
<td>1 (2.6)</td>
<td>41</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>6 (12.0)</td>
<td>45</td>
<td>3 (6.7)</td>
<td>50</td>
</tr>
<tr>
<td>G</td>
<td>49</td>
<td>3 (6.1)</td>
<td>32</td>
<td>2 (6.3)</td>
<td>49</td>
</tr>
<tr>
<td>H</td>
<td>55</td>
<td>3 (5.5)</td>
<td>41</td>
<td>2 (4.9)</td>
<td>55</td>
</tr>
<tr>
<td>I</td>
<td>56</td>
<td>10 (17.9)</td>
<td>46</td>
<td>6 (13.0)</td>
<td>56</td>
</tr>
<tr>
<td>J</td>
<td>50</td>
<td>2 (4.0)</td>
<td>36</td>
<td>1 (2.8)</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>576</td>
<td>59 (10.6)</td>
<td>433</td>
<td>31 (7.2)</td>
<td>574</td>
</tr>
</tbody>
</table>

In stratifying the schools for treatment priority, schools B and I, with the highest prevalences, require immediate therapeutic intervention, while other schools should follow later. Schools E and J (±5.0% prevalence) may be excluded in any intervention program to ensure prudent utilization of available resources.

Gender-related prevalence revealed that more males were infected than females in both the parasitological and questionnaire methods. This agrees with the work of Fajewonyomi et al. (1994), Nduka et al. (1995), and Ansel et al. (2001) and may be due to increased water contact activities like swimming, fishing, and playing in water, which are culturally gender-restrictive. The questionnaire result showed slight underreporting among males, the questionnaire results correlated significantly with the parasitological results. Similarly, among the females, the questionnaire results correlated significantly with the parasitological results (r = 1.574, P < 0.05).

The highest prevalence (12%) using the parasitological and 11.5% in the questionnaire methods in the 10- and 11-yr-old age groups was followed by 12- and 13-yr-old age groups, with 10% and 9.7%, respectively. A progressive decline in the prevalence was observed from the age of 14 yr and above. The lowest prevalence, among 8- to 9-yr-old children, may be due to the fact that they are still very young and not courageous enough to wade into large bodies of water for playing or swimming. Parental restrictions may prevent younger children from following the older ones to water-contact activities. From the age of 15 yr, there is reduced water contact among the children as they begin to groom for adult life and engage in more meaningful activities than swimming or playing in water. The methods showed high correlation between age and prevalence (r = 0.9924, P < 0.05).

Assessment of the diagnostic performance of the Rapid Assessment method (questionnaire) yielded a sensitivity of 93.4% and specificity of 99%. A positive predictive value of 96.6% and negative predictive value of 99.4% were also recorded. This indicates high potentials of the Rapid Assessment method in the rapid school/community-wide diagnosis of urinary schistosomiasis. The method should, however, be adopted with caution. It will be proper first to educate the people on the objectives and the need for accurate response. Those whose services would be used in the administration of the questionnaire should be remunerated to ensure greater diligence. When the package includes free treatment, it should not be presented as an attractant to the people lest it influence their responses.

TABLE III. Age-related prevalence in parasitological and questionnaire methods.

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Parasitological</th>
<th></th>
<th>Questionnaire</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. tested</td>
<td>No. +ve (%)</td>
<td>No. tested</td>
<td>No. +ve (%)</td>
</tr>
<tr>
<td>8-9</td>
<td>150</td>
<td>5 (3.3)</td>
<td>150</td>
<td>5 (3.3)</td>
</tr>
<tr>
<td>10-11</td>
<td>208</td>
<td>25 (12.0)</td>
<td>208</td>
<td>24 (11.5)</td>
</tr>
<tr>
<td>12-13</td>
<td>260</td>
<td>26 (10.0)</td>
<td>259</td>
<td>25 (9.7)</td>
</tr>
<tr>
<td>14-15</td>
<td>290</td>
<td>27 (9.3)</td>
<td>290</td>
<td>25 (8.6)</td>
</tr>
<tr>
<td>16-18</td>
<td>101</td>
<td>78 (6.9)</td>
<td>101</td>
<td>6 (5.9)</td>
</tr>
<tr>
<td>Total</td>
<td>1,009</td>
<td>90 (8.9)</td>
<td>1,008</td>
<td>85 (8.4)</td>
</tr>
</tbody>
</table>

results. Similarly, among the females, the questionnaire results correlated significantly with the parasitological results (r = 1.574, P < 0.05).

The highest prevalence (12%) using the parasitological and 11.5% in the questionnaire methods in the 10- and 11-yr-old age groups was followed by 12- and 13-yr-old age groups, with 10% and 9.7%, respectively. A progressive decline in the prevalence was observed from the age of 14 yr and above. The lowest prevalence, among 8- to 9-yr-old children, may be due to the fact that they are still very young and not courageous enough to wade into large bodies of water for playing or swimming. Parental restrictions may prevent younger children from following the older ones to water-contact activities. From the age of 15 yr, there is reduced water contact among the children as they begin to groom for adult life and engage in more meaningful activities than swimming or playing in water. The methods showed high correlation between age and prevalence (r = 0.9924, P < 0.05).

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TABLE IV. Diagnostic performance of the questionnaire.

<table>
<thead>
<tr>
<th>Predictive values</th>
<th>Para.</th>
<th>Quest.</th>
<th>Quest.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve (%)</td>
<td>-ve (%)</td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>90</td>
<td>8.5</td>
<td>922</td>
</tr>
<tr>
<td>TN</td>
<td>FP = 3</td>
<td>FN = 6</td>
<td></td>
</tr>
</tbody>
</table>

*Sensitivity = 93.4%, specificity = 99% (95% CI). TP = True Positive; TN = True Negative; FP = False Positive; FN = False Negative.*
ACKNOWLEDGMENT

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LITERATURE CITED


RESPONSE OF SCHISTOSOMA MANSONI ISOLATES HAVING DIFFERENT DRUG SENSITIVITY TO PRAZIQUANTEL OVER SEVERAL LIFE CYCLE PASSAGES WITH AND WITHOUT THERAPEUTIC PRESSURE

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ABSTRACT: The stability of praziquantel (PZQ)-insusceptible S. mansoni isolates and the possible selection of PZQ-insusceptible parasites upon applying therapeutic pressure were examined over several life cycle passages (snails to mice). To test isolate stability, 3 PZQ-susceptible and 7 PZQ-insusceptible isolates were used to establish infection in mice, and they were passaged each for 2–5 life cycles. After each passage, 6 groups of mice were used to assess the PZQ dose at which the worm burden was decreased by 50% (ED50). Five of them were treated with doses of PZQ (12.5, 25, 50, 100, and 200 mg/kg for 5 days) 7 wk after infection; the last group represented infected, but untreated, controls. Possible selection of PZQ-insusceptible parasites under therapeutic pressure was examined by subjecting 1 PZQ-susceptible and 1 PZQ-insusceptible S. mansoni isolate to therapeutic pressure by PZQ for 8 passages. After the final passage, PZQ ED50 was estimated. All PZQ-susceptible S. mansoni isolates showed stable susceptibility to PZQ (mean PZQ ED50 = 85 mg/kg) over all passages. Two of the 7 PZQ-insusceptible S. mansoni isolates (S47 and ER5) showed normal sensitivity to PZQ in 1–2 passages (although not the last passage, and without a declining ED50 profile), whereas the remaining passages kept a sustained insusceptibility to the drug (mean PZQ ED50 = 217 mg/kg). Worm maturity and sex were irrelevant to variability in drug ED50 within an individual isolate over different passages, revealing the heterogeneous nature of the parasite. Therapeutic pressure for limited life cycle passages did not result in a significant increase in drug ED50. The fact that reversion of some of the PZQ-insusceptible S. mansoni isolates to normal drug-sensitive state is not long lasting and that the therapeutic pressure by PZQ in the field is not comparable with that in the laboratory (unlimited), make monitoring the response of patients to the drug in the field an integral part of schistosomiasis control measures.

About 200 million people are affected with schistosomiasis worldwide, of which ~85% live on the African continent (Chitsulo et al., 2000). Chemotherapy of schistosomiasis is one of the most important methods of morbidity control of the disease in areas where it is endemic. Praziquantel (PZQ) will certainly remain the drug of choice over the next several years for treatment of schistosomiasis for several reasons, i.e., high efficacy against all schistosomiasis species, lack of serious short- and long-term side effects, administration as a single oral dose, competitive cost (Cioli et al., 1995; Colley et al., 2000).

The prospect of having a single drug available for a disease affecting 200 million people is quite alarming, and it may lead to the development of resistance (Cioli, 2000). Several reports in recent years have indicated the apparent failure of the recommended doses of PZQ to yield the expected cure rates in the human population in Kenya (Coles et al., 1987), Brazil (Katz et al., 1991), Senegal (Stelma et al., 1995; Fallon et al., 1997), and Egypt (Ismail et al., 1996). Most of the clinical trials that followed and examined the response of patients infected with S. japonicum, S. haematobium, or S. mansoni and treated with PZQ did not indicate resistance to PZQ (King et al., 2000; Yu et al., 2001). In Egypt, 2% of infected villagers were not cured after 3 doses of PZQ (Ismail et al., 1996). Adult worms recovered from these infections displayed significantly diminished responses to PZQ in vivo (Ismail et al., 1996; Cioli et al., 2004) and in vitro (Ismail et al., 1999; William, Botros et al., 2001; William and Botros, 2004). In contrast, in one focus showing potential resistance to PZQ a decade ago, data from the field by the same group revealed satisfactory responses to the drug.

The present study investigated how long PZQ-insusceptible S. mansoni isolates keep their insensitivity to the drug over several schistosome life cycle passages in the laboratory, a characteristic that may reflect on its impact in the field or on future endemic schistosome populations. The response of PZQ-insusceptible S. mansoni isolates collected from different geographical locations was examined by estimation of the dose at which the worm burden was decreased by 50% (ED50) over several life cycle passages (snail to mouse). The effect of worm sex and maturity on estimations of drug ED50 over the different passages was examined. In addition, the possible further selection of PZQ-insusceptible S. mansoni parasites in response to PZQ pressure was investigated.

MATERIALS AND METHODS

Animals

Swiss albino mice (CD-1), weighing 18–20 g, were used. Animals were kept under standard conditions at the Schistosome Biological Supply Center (SBSC) of the Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. Animal experiments were conducted in accordance with valid international animal ethics guidelines.

Praziquantel

Pure PZQ powder (Shin Poong Pharmaceutical Company, Seoul, South Korea) and the commercial brand Distocide (Egyptian International Pharmaceutical Industries Company, Cairo, Egypt) were used for studying the response of PZQ-susceptible and -insusceptible S. mansoni isolates with and without therapeutic pressure. The drug was freshly prepared before oral administration as a suspension in 2% Cremophor EL (Sigma-Aldrich, St. Louis, Missouri), and drug was given according to individual mouse body weight.

Infection of animals

Mice were infected with S. mansoni cercariae (80 ± 10 cercariae/mouse) using the body immersion technique (Liang et al., 1987).

Experiment I: S. mansoni isolates examined to test for response of PZQ-susceptible and -insusceptible S. mansoni isolates to PZQ over several life cycle passages without therapeutic pressure

Three PZQ-susceptible and 7 PZQ-insusceptible S. mansoni isolates were used. CD is an isolate that has never been exposed to PZQ. It has

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been maintained in the SBSC of the TBRI for >25 yr. MOC is an isolate established in 1996 with eggs from a patient living in the Nile Delta region of Egypt, who received a single, successful treatment of 40 mg/kg PZQ (Ismail et al., 1996). GP is a laboratory for a region of Egypt, who received a single, successful treatment of 40 mg/kg PZQ. This isolate had undergone >30 cycles (snail to mouse) without exposure to drug pressure. MB19 is an isolate established in 2002 in the laboratory of the University of Wales, with eggs excreted by a female from the Richard Toll area of Senegal, who was still excreting some eggs after receiving 2 treatments of 40 mg/kg PZQ. The isolate was kept for several passages without exposure to drug pressure in the laboratory. BANIL is an isolate generated by subjecting the GP isolate to subcurative doses of PZQ for 7 successive passages. Mice were infected with 100 cercariae and treated with 3 doses of 150 mg/kg PZQ on days 42, 45, and 47 postinfection (PI). Eggs that survived the treatment were used to infect snails. This isolate had been passaged under drug pressure >20 times, and it was then passaged 5 times without drug pressure (Fallon and Doenhoff, 1994). Cam231 is an isolate established in the laboratory of the University of Wales in Bangor, with eggs taken from a patient living in Cameroon, who was still infected after having 3 treatments of 40 mg/kg PZQ. This isolate had undergone >30 cycles without exposure to drugs. PZQ ED50 was examined for test isolates after 2-5 life cycle passages (snail to mouse) without therapeutic pressure.

Experiment II: Possible selection of PZQ-insusceptible *S. mansoni* parasites under therapeutic pressure

PZQ ED50 was examined for PZQ-susceptible (CD) and -insusceptible (ER5) *S. mansoni* isolates at the beginning of the experiment and after 8 life cycle passages with and without therapeutic pressure. Therapeutic pressure was applied by the administration of PZQ ED50 estimated for each isolate 7 wk PI for 8 cycles. Two months after each treatment, viable eggs (from livers and intestines) in each passage that survived the treatment were used to infect snails; 1 mo later, cercariae from these snails were used to infect mice.

Animal groups for experiments I and II

In experiment I, 8 and 21 batches (each batch in a separate passage) of mice infected with 3 PZQ-susceptible and 7 PZQ-insusceptible *S. mansoni* isolates were used, respectively. Animals in each batch were divided into 6 groups: 1 group was an untreated control and 5 groups were treated with PZQ in doses of 12.5, 25, 50, 100, and 200 mg/kg for 5 successive days beginning in week 7 after infection. Two weeks posttreatment, mice were killed, perfused, and PZQ ED50 was computed (Duvall and De Witt, 1967). In experiment II, 2 batches of Swiss albino mice were used. The first batch was infected with PZQ-susceptible (CD), and the second was infected with PZQ-insusceptible (ER5) *S. mansoni* isolates. Each of the 2 batches was divided into 2 groups. One group in each batch was treated 7 wk PI with PZQ ED50, and the second group was left without treatment. Treated groups were killed 2 mo posttreatment, and eggs from the livers and intestines of these mice were used to infect * Biomphalaria alexandrina* snails; 1 mo later, cercariae from these snails were used to infect mice. Isolates were subjected to therapeutic pressure using PZQ ED50 estimated for each of these isolates for 5 passages in snails and mice. At the end of the 8 cycles, mice were divided 7 wk PI into 5 groups each of 7 to 8 mice. Four of these groups received PZQ in rising doses (25, 50, 100, and 200 mg/kg) for 5 consecutive days, whereas the fifth group was left without treatment as controls. Two weeks post-PZQ treatment, mice were killed, perfused, and PZQ ED50 was computed. The 2 untreated groups were subjected to the same number of passages but without treatment.

Estimation of PZQ ED50

After being killed and perfused, the PZQ ED50 and the significance of different ED50 values were calculated using a computerized program Pharm/PCS, Version 4.2 (Pharmacologic Calculation System), by plotting the percentage of reduction in worm count (vs. infected untreated controls) against the amount of the drug administered.

**RESULTS**

**Response of PZQ-susceptible and -insusceptible *S. mansoni* isolates to PZQ without therapeutic pressure**

Comparison of PZQ ED50 estimates in mice infected with the PZQ-susceptible *S. mansoni* isolates (CD, MOC, and GP) did not reveal significant differences between them. All PZQ-susceptible *S. mansoni* isolates showed stable susceptibility to PZQ over the passages examined (8), with ED50 ranging from 72 to 117 mg/kg (x = 85 mg/kg). Of 7 PZQ-insusceptible *S. mansoni* isolates, and compared with ED50 value in CD infected mice, 5 (EE2, EE10, MB19, BANIL, and Cam 231) showed stable susceptibility to Praziquantel (71% of all PZQ-insusceptible isolates) over 2-5 passages for each isolate, with ED50 values ranging from 134 to 405 mg/kg (x = 217 mg/kg). The remaining 2 PZQ-insusceptible *S. mansoni* isolates (ER5 and S47) showed normal PZQ ED50 in 1 of 4 passages for the ER5 isolate (25% of all passages) and in 2 of 5 passages for the S47 (40% of all passages), with significantly higher PZQ ED50 in the remaining passages (Table I).

**Possible selection of PZQ-insusceptible *S. mansoni* parasites under therapeutic pressure**

**PZQ-susceptible *S. mansoni* isolate:** After therapeutic pressure for 8 life cycle passages, the PZQ-susceptible (CD) *S. mansoni* isolate showed a tendency to increase the ED50 of PZQ (133 versus 85 mg/kg at the beginning of the experiment), although the difference is not significant. The same isolate, which was also passaged for 8 life cycles but not subjected to therapeutic pressure, showed unchanged PZQ ED50 (73 versus 85 mg/kg).

**PZQ-insusceptible *S. mansoni* isolate:** In mice infected with the PZQ-insusceptible (ER5) *S. mansoni* isolate, the increase in PZQ ED50 (to 317 mg/kg) after therapeutic pressure for 8 passages was not significantly different from the drug ED50 value at the beginning of the experiment (252 mg/kg) or after 8 passages without therapeutic pressure (241 mg/kg) (Table II).

**Male/female ratio and percentage of immature worms**

Male/female ratio and percentage of immature worms did not affect PZQ ED50 estimation.

**DISCUSSION**

We have previously reported that some of the PZQ-insusceptible *S. mansoni* isolates, when passaged in the laboratory without therapeutic pressure, reverted to a PZQ-sensitive state (William, Sabra et al., 2001). In the present study encompassing a large number of *S. mansoni* isolates that have a diminished sensitivity to PZQ from areas other than Egypt, the same phenomenon was recorded. However, the recovery did not prove to be permanent when 7 PZQ-insusceptible *S. mansoni* isolates (EE2, EE10, ER5, S47, MB19, BANIL, and Cam 231) main-
tained in the laboratory were examined for their stable insensitivity to PZQ. To assess their stability, their ED_{so} values were compared with ED_{so} values in mice infected with PZQ-susceptible (CD) S. mansoni isolates. This is because the ED_{so} values in CD infected mice were not significantly different from those for the 2 PZQ-susceptible S. mansoni isolates. This is because the ED_{so} values in CD infected mice were not significantly different from those for the 2 PZQ-susceptible S. mansoni isolates (MOC and GP) used in this work and also because this isolate is the one that was collected before the introduction of PZQ. The majority (EE2, EE10, MB19, BANL, and Cam231) of PZQ-insusceptible S. mansoni isolates (5 of 7 [71%]) showed stable insusceptibility to PZQ when the drug ED_{so} was estimated for these isolates after 2–5 life cycle passages for each isolate without therapeutic pressure. The ED_{so} values ranged between 134 and 405 mg/kg (mean PZQ ED_{so} = 217 mg/kg). The remaining 2 (S47 and ER5), of 7 PZQ-insusceptible S. mansoni isolates (29% of the total isolates examined) showed stable insusceptibility to the drug in 60 and 75% of the passages for S47 and ER5, respectively, compared with a susceptible drug state in the rest of the passages (40 and 25%). Life cycle passages showing normal response to PZQ were not the last passages, but they were intermediate, indicating that it is not a matter of fading insensitivity. This is because when the drug ED_{so} of the same isolate was examined in a subsequent passage to that showing normal drug ED_{so}, an increased drug ED_{so}, i.e., a state of PZQ insusceptibility, was observed. Because it is well known that the sensitivity of S. mansoni to PZQ is dependent on the age of the worms (Gonnert and Andrews, 1977; Xiao et al., 1985) and that PZQ only kills mature worms, we have tried to relate the difference in the state of susceptibility to PZQ drug ED_{so} to possible changes in worm maturity. This was done by examining the number of immature worms in the infected, but untreated, control groups each time we estimated drug ED_{so}. Data revealed that such differences in response to the drug were not due to changes in worm maturity. This is because for the CD and GP (PZQ-susceptible isolates), we have had nearly the same small drug ED_{so} (76 and 74 mg/kg) over 2 passages, and the percentages of immature worms were 0 and 5.8, respectively. Moreover, we have had high (198 mg/kg) and low (108 mg/kg) drug ED_{so}, with 0% immature worms for the ER5 in passage numbers 2 and 4, respectively. Meanwhile, for the S47, no immature worms were present in passage number 5 with significantly increased drug ED_{so} (169 mg/kg), whereas 1 and 3.4% immature worms were recorded in passage numbers 1 and 4, respectively. The drug ED_{so} was significantly reduced to 78 and 80 mg/kg, respectively. Another possible factor affecting the varied PZQ ED_{so} for the same isolate over different life cycle passages was the ratio of male-to-female parasites. Females are known to be more resistant to PZQ than males (Mehlhorn et al., 1981; Andrews, 1985; Shaw, 1990). When we tried to relate such a difference in the drug ED_{so} to the possible dominance of either sex, we found that this was not the case. The male-to-female ratio was the same (1.3 and 1.3) for the CD (PZQ-susceptible isolate) in passage number 1 and EE2 (PZQ-insusceptible isolate) in passage number 2, whereas the drug ED_{so} values were 76 and 181 mg/kg, respectively. In addition, the ratio of males to females was nearly the same (1.0 and 1.2) for ER5 in passage numbers 2 and 4, whereas the drug ED_{so} values were 108 and 198 mg/kg, respectively.

The results of the present study reveal the irrelevance of worm maturity and sex on changing estimations of PZQ ED_{so} recorded for some of the PZQ-insusceptible S. mansoni isolates over different passages, suggesting that genetic heterogeneity of the parasite may be the underlying factor. This means that

Table I. Praziquantel ED_{so} in mice infected with PZQ-susceptible and -insusceptible S. mansoni isolates over several schistosome life cycle passages without drug pressure.

<table>
<thead>
<tr>
<th>S. mansoni isolates</th>
<th>Upper limit of PZQ ED_{so} (mg/kg)</th>
<th>Lower limit of PZQ ED_{so}</th>
<th>Male/female ratio</th>
<th>% Immature worms</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD (1)</td>
<td>124</td>
<td>76</td>
<td>46</td>
<td>1.3</td>
</tr>
<tr>
<td>CD (2)</td>
<td>155</td>
<td>98</td>
<td>63</td>
<td>1.1</td>
</tr>
<tr>
<td>CD (3)</td>
<td>116</td>
<td>73</td>
<td>46</td>
<td>1.6</td>
</tr>
<tr>
<td>MOC (1)</td>
<td>131</td>
<td>82</td>
<td>52</td>
<td>1.4</td>
</tr>
<tr>
<td>MOC (2)</td>
<td>142</td>
<td>88</td>
<td>54</td>
<td>0.8</td>
</tr>
<tr>
<td>MOC (3)</td>
<td>166</td>
<td>117</td>
<td>82</td>
<td>2.9</td>
</tr>
<tr>
<td>GP (1)</td>
<td>155</td>
<td>72</td>
<td>33</td>
<td>0.8</td>
</tr>
<tr>
<td>GP (2)</td>
<td>116</td>
<td>74</td>
<td>47</td>
<td>0.8</td>
</tr>
<tr>
<td>EE10 (1)</td>
<td>273</td>
<td>171</td>
<td>107</td>
<td>0.9</td>
</tr>
<tr>
<td>EE10 (2)</td>
<td>333</td>
<td>244</td>
<td>179</td>
<td>0.9</td>
</tr>
<tr>
<td>EE10 (3)</td>
<td>573</td>
<td>405</td>
<td>286</td>
<td>1.0</td>
</tr>
<tr>
<td>ER5 (1)</td>
<td>354</td>
<td>218</td>
<td>134</td>
<td>0.6</td>
</tr>
<tr>
<td>ER5 (2)</td>
<td>161</td>
<td>108*</td>
<td>73</td>
<td>1.0</td>
</tr>
<tr>
<td>ER5 (3)</td>
<td>302</td>
<td>196</td>
<td>127</td>
<td>1.3</td>
</tr>
<tr>
<td>ER5 (4)</td>
<td>302</td>
<td>198</td>
<td>129</td>
<td>1.2</td>
</tr>
<tr>
<td>EE2 (1)</td>
<td>283</td>
<td>200</td>
<td>142</td>
<td>2.3</td>
</tr>
<tr>
<td>EE2 (2)</td>
<td>273</td>
<td>181</td>
<td>120</td>
<td>1.3</td>
</tr>
<tr>
<td>S47 (1)</td>
<td>195</td>
<td>78*</td>
<td>31</td>
<td>1.6</td>
</tr>
<tr>
<td>S47 (2)</td>
<td>354</td>
<td>270*</td>
<td>206</td>
<td>1.8</td>
</tr>
<tr>
<td>S47 (3)</td>
<td>232</td>
<td>137</td>
<td>81</td>
<td>1.2</td>
</tr>
<tr>
<td>S47 (4)</td>
<td>124</td>
<td>80*</td>
<td>52</td>
<td>1.7</td>
</tr>
<tr>
<td>S47 (5)</td>
<td>245</td>
<td>169</td>
<td>117</td>
<td>1.2</td>
</tr>
<tr>
<td>BANL (1)</td>
<td>243</td>
<td>169</td>
<td>118</td>
<td>1.3</td>
</tr>
<tr>
<td>BANL (2)</td>
<td>241</td>
<td>167</td>
<td>116</td>
<td>0.9</td>
</tr>
<tr>
<td>BANL (3)</td>
<td>364</td>
<td>269</td>
<td>199</td>
<td>5.6</td>
</tr>
<tr>
<td>MB19 (1)</td>
<td>209</td>
<td>134</td>
<td>85</td>
<td>1.9</td>
</tr>
<tr>
<td>MB19 (2)</td>
<td>311</td>
<td>162</td>
<td>85</td>
<td>0.6</td>
</tr>
<tr>
<td>Cam 231 (1)</td>
<td>358</td>
<td>307</td>
<td>262</td>
<td>4.4</td>
</tr>
<tr>
<td>Cam 231 (2)</td>
<td>309</td>
<td>191</td>
<td>119</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Number of schistosome life cycle passages. * No significant difference versus CD.

Table II. Praziquantel ED_{so} in mice infected with PZQ-susceptible and -insusceptible S. mansoni isolates subjected to therapeutic pressure for eight successive schistosome life cycle passages.

<table>
<thead>
<tr>
<th>S. mansoni isolates</th>
<th>PZQ ED_{so} (mg/kg) PCS</th>
<th>After 8 life cycle passages</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>85</td>
<td>73</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>% Immature worms</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ER5</td>
<td>252*</td>
<td>241*</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>1.3</td>
<td>0.4</td>
</tr>
<tr>
<td>% Immature worms</td>
<td>1.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Significant difference versus CD.
in spite of the PZQ-insusceptible \textit{S. mansoni} isolates being collected from a single patient, which should ensure that they are clonal, the fact remains that they are not clonal because each is the product of a heterogeneous cercaria population. This may generate differences in sensitivity to the drug, depending on the preponderant subtypes of schistosome populations in the field, i.e., sensitive versus insensitive, reflecting on final sensitivity to the drug. This heterogeneity is likely to be affected by the system adopted for control of schistosomiasis in different countries where it is endemic, and by changing ecological conditions. In Egypt, hybrids of \textit{B. alexandrina} and \\textit{ Biomphalaria glabrata} have now been recorded at Giza, Qalyoubia, and Kafr el-Sheikh governorates with different biological characteristics, e.g., greater longevity for cercariae shed from hybrid snails. Infectivity of these cercariae to the definitive host and response to chemotherapy may not be the same as for the appropriate snail intermediate hosts in Egypt, i.e., \textit{B. alexandrina} (Youssif, Haroun et al., 1996, Youssif, Ibrihim, and Bardicy, 1998).

It has been reported that if \textit{S. mansoni} isolates are subjected to subcurative doses of PZQ, resistance to the drug will develop in the following generation (Fallon and Doenhoff, 1994), even if the \textit{S. mansoni} isolate was PZQ-susceptible (Ismail et al., 2002). In the present study, we subjected 1 PZQ-susceptible and 1 PZQ-insusceptible \textit{S. mansoni} isolate to therapeutic pressure (for 8 cycles) using the ED_{50} of PZQ estimated for each of the isolates. The imposed pressure did not result in a further increase of drug ED_{50}. PZQ ED_{50} estimated for mice infected with PZQ-susceptible and -insusceptible \textit{S. mansoni} isolates were used to apply therapeutic pressure to allow for the presence of a comparable parasite burden (50%) in animals infected with either of these isolates. Applying therapeutic pressure with a common sub-curative drug dose will ultimately leave more parasites in the host infected with PZQ-insusceptible \textit{S. mansoni} isolates than in the host infected with the PZQ-susceptible \textit{S. mansoni} isolates. Consequently, this may reflect on final PZQ ED_{50} estimates. The only recorded finding upon applying therapeutic pressure was a slight tendency for an increase in drug ED_{50} for both the PZQ-susceptible and -insusceptible \textit{S. mansoni} isolates, which were not significantly different from estimates of drug ED_{50} for each of the isolates at the beginning of the experiment. It is worth noting that, in the field, therapeutic pressure for a decade in an area where the potential for resistance to PZQ was recorded in Egypt (Botros et al., 2005) did not reveal any increase in drug resistance. In view of the lower survival efficiency recorded for some of the Egyptian isolates showing diminished sensitivity to PZQ (William, Sabra et al., 2001), it would obviously be difficult for the resistant strains to establish themselves under limited selection pressure.

In the field, selection pressure is unlimited, especially in high transmission areas, and it is not comparable with that in the laboratory, i.e., the recovery of parasites showing diminished sensitivity to PZQ to a normal sensitive state did not prove to be long-lived. It may be a result of the heterogeneous nature of the parasite that the lower survival efficiency recorded for the Egyptian PZQ-insensitive isolates limiting their impact in the field may not apply to other strains. Monitoring the response of patients to PZQ at regular intervals should be an integral part of schistosomiasis control measures.

**ACKNOWLEDGMENTS**

Part of this investigation received financial support from the INCO-II Programme of the European Commission (contract ICA4-CT-2001-10079). We are grateful to Dr. Amadou Mbaye and Dr. Albert Tchuem Tchuente for collecting the \textit{S. mansoni} isolates (S47, MB19, and Cam 231) from the field in Senegal and Cameroon. Also we are grateful to Prof. Michael Doenhoff for supplying the isolates kept at the laboratory of the University of Wales.

**LITERATURE CITED**


The Effect of *Echinorhynchus borealis* (Acanthocephala) Infection on the Anti-Predator Behavior of a Benthic Amphipod

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**ABSTRACT:** In benthic habitats, predators can generally not be detected visually, so olfaction may be particularly important for inducing anti-predation behaviors in prey organisms. Manipulative parasites infecting benthic hosts could suppress these responses so as to increase the probability of predation and thus trophic transmission. We studied how infection with the acanthocephalan *Echinorhynchus borealis* affects the response of the amphipod *Pallasea quadrispinosa* to a conditioned by burbot (*Lota lota*), the parasite’s definitive host. In normal lake water, refuge use by infected and uninfected amphipods was similar, but when exposed to burbot-conditioned water, uninfected amphipods spent much more time hiding than infected amphipods. Thus, rather than affecting ambient hiding behavior, *E. borealis* infection seems to alter the host response to a predator. A group of amphipods sampled from a postglacial spring that is devoid of fish predators exhibited a weak response to burbot-conditioned water, perhaps suggesting these anti-predator behaviors are costly to maintain. The hiding behavior of spring and infected amphipods was very similar. If the reduced refuge use by the spring amphipods reflects adaptation to a predator-free environment, this indicates that *E. borealis* severely weakens its host’s anti-predator behavior. Presumably this increases the likelihood of parasite transmission.

Predation is a cosmopolitan selective force acting on animals, and, as a result, anti-predator adaptations are common. Frequently these adaptations are behavioral; prey organisms detect stimuli indicative of a predator and behave in a way that limits the likelihood of being eaten. For example, many aquatic animals reduce activity levels and/or increase refuge use when exposed to chemical signals, i.e., kairomones, indicative of elevated predation risk (reviewed by Kats and Dill, 1998), thereby decreasing their predation susceptibility (e.g., Wisenden et al., 1999). In aquatic ecosystems, benthic organisms may be particularly favored to use olfaction for predator detection, because visual recognition of predators is difficult or impossible (Wisenden, 2000). Trophically transmitted parasites, in contrast to their hosts, are under pressure to increase the likelihood of predation events and thus their own transmission (Holmes and Bethel, 1972). Presumably as a consequence, many parasites alter their host’s phenotype in ways that seem to increase host susceptibility to predation (reviewed by Moore, 2002). In particular, the suppression or inversion of host anti-predator behaviors may be an excellent way for manipulative parasites to increase their transmission success (Perrot-Minnot et al., 2007). Some parasites modify the response of their hosts to predator kairomones (Jakobsen and Wedekind, 1999; Deetz'uli et al., 2003; Wellnitz et al., 2003; Baldauf et al., 2007; Perrot-Minnot et al., 2007), but this type of behavioral alteration has not been examined in a benthic system, where it may be especially relevant for parasite transmission.

The benthic system studied here was the “glacial relict” amphipod species *Pallasea quadrispinosa* infected with an acanthocephalan *Echi­norhynchus borealis*. The definitive host of *E. borealis* is primarily burbot (*Lota lota*) (Grabda-Kazubaska and Ejsymont, 1969), which seems well adapted to hunting in the dark, cold conditions of lake bottoms (Pääkkönen, 2000). In Finland, *P. quadrispinosa* is a common prey item in this habitat. Adult *E. borealis* mate in the gastrointestinal tract of burbot, and eggs are released into the environment with the feces. Am­hippods become infected by eating eggs, and parasites develop in the amphipod’s hemocoel to an infective, cystacanth stage. The life cycle is completed when an amphipod harboring a cystacanth is ingested by an appropriate definitive host. Though *P. quadrispinosa* is normally found in the deepest parts of oligotrophic lakes, populations in postglacial springs are also known. These springs generally lack inlets and outlets, and they have sometimes been isolated for thousands of years. Often, if no stocking has occurred, these springs are devoid of fish. Thus, spring populations provide an opportunity to examine whether anti-predator behaviors occur even in the absence of any threat of fish predation.

The aims of this study were to (1) evaluate how benthic amphipods change their hiding behavior in response to chemical cues from predators, (2) assess whether this response is affected by *E. borealis* infection, and (3) compare the anti-predator behavior of spring and lake amphipod populations.

Most of the amphipods used in the experiment were collected in November 2005 from Lake Pääjärv, southern Finland (61°04’N, 25°05’E), an oligotrophic lake approximately 10 km by 3.8 km, with several outlets and a maximum depth of 87 m (Ruuhijärvi, 1974). Triangular nets, with an opening of approximately 40 × 40 cm, were baited with a small amount of fish meat and lowered to a depth of approximately 75 m. The nets were left on the lake bottom overnight and brought up the next day. Some amphipods were also collected in a similar manner from Lake Leppävesi, central Finland (62°18’N, 25°56’E). The *P. quadrispinosa*- *E. borealis* system is also present in this lake, and the lake characteristics are similar to those of Lake Pääjärv. An isolated population of *P. quadrispinosa* was also sampled from a postglacial spring that lacks fish. The spring (61°00’N, 25°11’E) is located approximately 5.5 km southeast from the nearest shoreline of Lake Pääjärv. It was likely isolated from other water bodies approximately 10,000 yr ago when the Baltic Ice Lake formed an outlet into the Atlantic Ocean and its water level dropped 26–29 m (Winterhalter et al., 1981). Amphipods from this population were collected with a dip net. After being transported to the laboratory, amphipods were sorted into 10-L tanks in groups of approximately 30 individuals and kept at 4°C. Over the course of a week, amphipods were acclimated to 13°C, the temperature at which the experiment took place. During the acclimation period, amphipods were fed frozen chironomids (*Chironomus rossio*, Ruto Frozen Fish Food, Guisborough, U.K.).

Three burbot (mean length = 34 cm ± 2 SD) were captured from Lake Leppävesi. These fish were kept in a large, plastic tank (70 L) with a flow-through rate of approximately 0.75 L/hr, and were fed frozen pieces of perch (*Perca fluviatilis*) every 2 or 3 days.

The day before the experiment, amphipods were individually isolated in plastic containers (10 × 15 × 5 cm), given a large chironomid to eat, and allowed to feed overnight. This step was intended to limit the potential effects of hunger on amphipod behavior during the experiment. The next day amphipods were taken from their containers and placed individually in small tanks (16 × 15 × 9 cm) filled with 1.2 L of lake water. This lake water was taken from Lake Jyväsjärvi (62°14’N, 25°44’E), located near the University of Jyväskylä campus. There are fish in this lake, so there may be a variety of fish kairomones in the collected water. Before being used though, the water was left to stand for at least a day to adjust its temperature to lab conditions. Presumably this also allowed the concentration of any kairomones present to decrease via decomposition so that the utilized lake water had only a very small concentration of kairomones relative to that in the predator-conditioned water used in the experiment. Each of the experimental tanks contained a shelter for the amphipod. The shelters were pieces of black plastic 3.3 cm long by 8.5 cm wide, and they were propped up with 2 screws drilled through the front corners. This provided a triangular hiding space for the amphipods with an entrance gap of approximately 5 mm. The shelters were placed on one side of the experimental tanks and held in place with a small stone that prevented them from floating.
The experiment was conducted between the hours of 1000 and 1800 in dim, red light. Within the visible spectrum, red wavelengths are probably weakly perceived by crustaceans (Shaw and Stowe, 1982), so these light conditions were considered the least likely to affect amphipod behavior. Amphipods were allowed 10 min to acclimate after introduction into the experimental tanks. Individuals were then noted as being under the shelter or exposed in the water column every 5 min for 1 hr. After observing each individual for 1 hr, 500 ml of burbot-conditioned water was poured into every tank. This water was “conditioned” by stirring the water for 2 hr through the 70-L tank containing the 3 burbot for 2 hr, i.e., 1 hr before the initial behavioral observations on amphipods in normal lake water began. Each day the experiment was conducted, 7 to 12 individuals were observed concurrently. The amphipods observed on any given day thus received conditioned water at the same time. As this water was “conditioned” for the same length of time at a consistent volume, the “quality” of the conditioned water received by each amphipod was likely very similar. The conditioning procedure was identical each day the experiment was conducted; accordingly, between-day variation in kairrome concentrations in the conditioned water was presumably minimized. Once the conditioned water was added to the tanks, amphipods were allowed to acclimate for 10 min before hiding behavior was monitored in the same manner, i.e., recordings every 5 min for 1 hr. All the turbulence created by adding the conditioned water was gone by the end of the 10 min acclimation period. At the end of the experiment, the length of amphipods, from the rostrum to the end of the 11th segment, was measured to the nearest 0.5 mm. All amphipods were dissected to determine if they were infected with *E. borealis*; any parasites found were counted and their developmental stage noted.

In total, 121 amphipods were observed; they were classified into 3 groups, i.e., uninfected, lake amphipods (n = 45), infected, lake amphipods (n = 43, all harbored *E. borealis* cystacanths), and amphipods from the fishless spring (n = 31). The behavior of amphipods was summarized as the proportion of time an individual was observed hiding under the shelter before, and after, the addition of burbot-conditioned water. In both cases data were not normally distributed, and various transformations failed to normalize the data’s distribution. Furthermore, variances were heterogeneous among the treatment groups (uninfected, infected, and spring amphipods). Thus, only nonparametric tests could be used. Within each treatment, hiding behavior in unconditioned water was compared to that in burbot-conditioned water with Wilcoxon signed rank tests. Comparisons among the treatments, in both unconditioned and burbot-conditioned water, were conducted using Kruskal-Wallis tests. Post hoc tests were used to assess pairwise differences between treatments. For these multiple comparisons, the level of alpha was Bonferroni adjusted (post hoc procedure in Siegel and Castellan, 1988).

The uninfected and infected lake amphipods came from 2 sources, Lakes Bethel (n = 65) and Lake Leppavesi (n = 25). When Leppävesi amphipods were removed from the analyses, the results were qualitatively unchanged, so pooling the data from these 2 lakes was considered justified. Several infected amphipods harbored multiple cystacanths (n = 9, intensity = 3.67, range 2–7). So, if parasites cooperate to manipulate host behavior (e.g., Poulin et al., 2003), these individuals could be a source of bias. Exclusion of these individuals from the data, however, had no effect on the results; therefore, the multiply-infected amphipods were also included in the analysis. Additionally, for each treatment Spearman correlations were used to evaluate whether amphipod size affects hiding behavior. Correlations were conducted separately for hiding behavior before, and after, the addition of conditioned water.

Before the addition of burbot-conditioned water, refuge use by uninfected, infected, and spring amphipods was relatively similar (Kruskal-Wallis test, H = 3.28, P = 0.18; Fig. 1), and post hoc tests did not reveal any significant differences between the groups (all P > 0.05). Amphipods in all 3 treatments responded to the addition of burbot-conditioned water by increasing the amount of time spent under the shelter (Wilcoxon tests, all Z < −3.29, all P < 0.001). The magnitude of this increase, however, differed between treatments (Kruskal-Wallis test, H = 19.95, P < 0.001; Fig. 1). In burbot-conditioned water, uninfected amphipods spent more time hiding and had no effect on the results; therefore, the multiply-infected amphipods (post hoc comparisons, both P < 0.05; Fig. 1). The behavior of infected and spring amphipods in conditioned water did not differ (post hoc comparison, P > 0.05). None of the 6 Spearman correlations between amphipod size and hiding behavior was significant after a Bonferroni correction to the alpha level (for infected amphipods in unconditioned water the correlation was significant before Bonferroni adjustment, r = −0.35, P = 0.03, otherwise all r < 0.18, all P > 0.32).

All groups of amphipods spent more time under the shelter in burbot-conditioned water than in unconditioned water. Because amphipods were always observed in conditioned water after being observed in unconditioned water, increasing refuge use by amphipods over time could have been partially responsible for this result. However, this is unlikely to explain the clear behavioral differences between uninfected, parasitized, and spring amphipods when confronted with chemical cues from a potential predator. After the addition of burbot-conditioned water, the proportion of time uninfected amphipods spent under the shelter increased from about 20% to over 60%. This increased refuge use in response to chemical stimuli may be an especially important anti-predator behavior in a benthic habitat. Moreover, this behavior is consistent with the response of other amphipod species to chemicals from burbot (Baumgartner et al., 2002) or other fish species (Anderson et al., 1986; Holomuzki and Hoyle, 1990; Wudekevich et al., 1997; Wooster, 1998; Abjörnsson et al., 2000; Dahl et al., 2000). In contrast to the uninfected amphipods, infected and spring amphipods did not increase shelter use in response to conditioned water nearly as much. The weaker reaction to predator cues in these 2 groups likely stems from different sources, parasitic manipulation and eased predation pressure.

The reduced hiding of infected amphipods in the presence of burbot “odour,” relative to uninfected amphipods, likely increases their exposure to predators and, consequently, the probability of parasite transmission. Thus, as in other acanthocephalan-amphipod systems (e.g., Bethel and Holmes, 1977; Bakker et al., 1997; Perrot-Minnot et al., 2007), the behavioral alterations associated with *E. borealis* infection presumably reflect an adaptive parasite strategy. Alternative explanations for the altered behavior of infected animals are that they are by-products of infection or they reflect adaptive host strategies (Poulin, 1995; Thomas et al., 2005). For instance, the reduced hiding of infected amphipods may be a by-product of the host’s need to increase foraging (Jákobsen and Wedekind, 1998). Although this cannot be ruled out, it seems unlikely because amphipods were well fed prior to the experiment. Additionally, the metabolic activity of *E. borealis* cystacanths seems to be rather low (Benesh and Valtonen, 2007), so infection may not substantially increase the host’s energetic needs. It also seems inappropriate to label the behavior of infected amphipods an adaptive host strategy because it is difficult to envision how reduced hiding in the presence of a predator will increase host fitness.

**Figure 1.** The proportion of time amphipods spent hiding under a shelter during 1 hr of observation in unconditioned water and water conditioned by burbot. Three groups of amphipods were observed: (1) uninfected amphipods, (2) amphipods infected with *Echinorhynchus borealis*, and (3) amphipods from a postglacial spring that lacks fish. Bars are SE.
In several previous studies on the response of parasitized animals to chemical cues, infected animals exhibited modified behavior even in the absence of predator-derived chemicals (Jakobsen and Wedekind, 1998; Dezfuli et al., 2003). Indeed, most documented examples of parasitoid-induced altered host behavior come from experiments that did not include a predator. The behavior of uninfected and infected amphipods, however, was similar in unconditioned water; significant differences in refuge use occurred only in burbot-conditioned water. Thus, infection with E. borealis seems to impair the anti-predator response of amphipods to chemical stimuli, rather than ambient hiding behavior. Similarly, the acanthocephalan Pomphorhynchus terecticollis does not seem to affect the refuge use of its amphipod intermediate host, in general, but, instead, refuge use in the presence of a predator’s odor is altered (Perrot-Minnot et al., 2007). Thus, the behavioral alterations induced by some parasites can be rather subtle and easily overlooked if hosts are only observed in the absence of a predator.

The spring amphipods have not experienced any selection from fish predation for thousands of years, so, if predator avoidance behaviors are costly, then the ability to detect and respond to fish predators may be lost. The reduced response of spring amphipods to predator stimuli, relative to uninfected, lake amphipods, seems to corroborate this expectation. Life history tradeoffs associated with anti-predator traits have been demonstrated in a variety of other systems (e.g., Lively, 1986; Skelton, 1990; Ball and Baker, 1996; McCollum and Van Buskirk, 1996; Brodie and Brodie, 1999), but the actual loss of predator avoidance behaviors is less well established (O’Stein et al., 2002). In some cases the evolutionary loss of anti-predator traits may be quite rapid, suggesting their maintenance costs are substantial (O’Stein et al., 2002). Alternatively, rather than an adaptive evolutionary change, the decreased response of spring amphipods to burbot kairomones could reflect a plastic response to developing in a predator-free environment. Chemically mediated behavior seems age-dependent in some amphipods (Wisenden et al., 2001), so it is possible that spring P. quadrispinosa are simply not exposed to some necessary stimuli for developing a response to burbot kairomones. Interestingly, the hiding behavior of spring and infected amphipods was very similar. Assuming that the reduced refuge use of the spring amphipods actually reflects adaptation to a predator-free environment, then the very similar behavior of the infected amphipods suggests that E. borealis severely reduces the anti-predator behavior of P. quadrispinosa.

We thank Kalevi Salonen for helping to collect amphipods and Arto Tuomainen for catching the burbot. D.P.B. was supported by the Biological Interactions Graduate School at the University of Turku.

LITERATURE CITED


Genotypic Characterization of an Epithelial Cell Line for the Study of Parasite–Epithelial Interactions

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ABSTRACT: The findings discussed in the present research note report the extensive genotypic characterization of an intestinal epithelial cell line originally obtained from a human patient. Although the line exhibits karyotypic anomalies, with 76 modal chromosomes, its immunological, biochemical, and physiological phenotype make it a well-suited model to study intestinal epithelial processes, including those involved during intestinal parasitism. Polymerase chain reaction (PCR), isoenzyme analysis, and PCR gene product sequencing ultimately revealed that SCBN epithelial cells express a canine genotype. The observations held true for one of the early cell stocks obtained directly from the laboratory where the cell line was first isolated. Since no canine cells were used in that laboratory at that time, and in view of the normal canine modal chromosomal number of 78, the canine genotype of SCBN cells cannot be explained through simple laboratory contamination. The various characteristics of SCBN nonetheless make it a useful tool for research in epithelial biology, as well as in parasite–epithelial interactions. Its newly discovered genotypic characteristics are of significant relevance to researchers using this cell line.

Diarrheal disease is the leading cause of morbidity and mortality in children worldwide and represents a ubiquitous burden to the adult population. Intestinal parasites are major contributors to this health concern. Infections with *Giardia duodenalis* (syn. *lamblia, intestinalis*) are the most common cause of waterborne disease in North America, and giardiasis was recently included in the list of the World Health Organization’s “Neglected Diseases Initiative” (Savijärvi et al., 2006). A better understanding of the basic biological processes through which enteropathogens cause disease is sorely needed, as it may shed light on novel therapeutic targets. In an attempt to unravel the mechanisms by which *Giardia* sp. and other enteropathogens exert their clinical effects, researchers have relied on a variety of cell systems and animal models. Studies on pathophysiological processes in the gastrointestine may make extensive use of cell lines as model systems. Unfortunately, to model the confluent human intestinal epithelium, researchers must rely on cancer cell lines. Therefore, the establishment of a human nontumorigenic intestinal cell line growing into confluent monolayers with functional tight junctions would represent a significant advancement in this field.

Pang et al. (1996) reported the isolation of 3 nontumorigenic intestinal epithelial cell lines from 3 different human patients. One of the cell lines, originally derived from patient BN, was used for a first set of experiments on *Giardia* sp.–epithelial interactions (Teoh et al., 2000).

This line has since been named SCBN. Using the SCBN cell line and a variety of other model systems, recent research has been able to establish that the mechanisms by which *Giardia* sp. causes diarrhea is multifactorial, and includes parasite-induced chloride hypersecretion, epithelial apoptosis, and loss of barrier function that lead to lymphocyte-mediated reduction of absorptive surface area, which ultimately causes malabsorption of nutrients, sodium, and water (Buret et al., 1992, 2002; Chin et al., 2002; Scott et al., 2002, 2004; Mueller and von Allmen, 2005; Gascon, 2006; Troeger et al., 2007). The SCBN cell line has been used by a number of laboratories and was made available to other research organizations, such as Kinetana Group Inc. (Buresi et al., 2001, 2002; Buret et al., 2002, 2003; Chin et al., 2002, 2003; Scott et al., 2002, 2004; Chang and Peperman, 2003). The SCBN cell line grows into tight polarized confluent monolayers; possesses cytokeratins, mucus, and sucrase-isomaltase antigens, mRNA for epidermal growth factor, interleukin-6, vascular cellular adhesion molecule-1, responsive cytoskeletal and tight junctional proteins, calcium-dependent chloride secretion; and expresses functional protein kinases and protease-activated receptors. The cells proved a useful tool to investigate mechanisms of epithelial responses to eukaryotic parasites and bacteria (Teoh et al., 2000; Buret et al., 2002; Chin et al., 2002, 2003 2006; Scott et al., 2002; Fedwick et al., 2005). All recent publications using SCBN refer to the genotypic observations discussed herein (Fedwick et al., 2005; Chin et al., 2006; Smith et al., 2006). However, the present research note offers a detailed description of how its unexpected genotype was revealed.

The cell line was derived from the duodenum of a human patient (BN) with diarrhea of unknown etiology (Pang et al., 1996). Research findings suggested that SCBN was of a crypt-like intestinal epithelial phenotype, and was nontumorigenic (Pang et al., 1996). The original paper describing SCBN also highlighted that the line exhibited karyotypic anomalies, with 76 modal chromosomes (Pang et al., 1996). Between 2000 and 2002, experimental difficulties were encountered during PCR-based experiments in the cells, which showed lower than expected homology with human gene probes. In an attempt to clarify these observations, the cell line was sent out for isoenzyme analysis in 2002 (Cell Culture Laboratory, Children’s Hospital of Michigan, Detroit, Michigan). Electrophoretic mobilities of enzymes lactate dehydrogenase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, and nucleotide phosphatase were more similar to those of canine cells than to those of human or mouse cells. The results suggested that the cells’ isoenzyme pattern expressed canine antigens. We then sequenced the *CFTR* gene of SCBN and carried out a blast search. The results yielded...
83% homology with the human gene. At that time, several species, i.e., cat, horse, pig, chimpanzee, and fruit-eating bat, showed slightly better matches than human, and 1 species, canine, which matched all but one of 320 nucleotides in the sequence. In May 2004, Kinetana Group Inc. finished sequencing PCR products of 9 genes from SCBN cells for the purpose of genotypic characterization. Kinetana Group Inc. was not able to amplify DNA sequences from SCBN cells using primers specific for canine genes (Table I). All of the DNA sequences from 9 genes matched sequences of canine genes almost perfectly through a BLAST search (Appendix A–I). The DNA sequencing results led us to conclude that SCBN cells are of canine, not human, genotype. Our observations hold true for one of the early cell stocks (passage 4) obtained directly from the Australian laboratory where the cell line was first isolated. In the original studies, epithelial cells were obtained after 1–2 passages following purification, and the experiments that were performed could not shed light on the cells’ genotypic characteristics (Pang et al., 1996). Since no canine cells were used in that laboratory at that time, and in view of the normal canine modal chromosomal number of 78, the canine genotype of SCBN cells cannot be explained through simple laboratory contamination. The genotypic characteristics of SCBN cells are of great relevance to ongoing and future studies investigating parasite–epithelial interactions.

**LITERATURE CITED**


### APPENDIX—SEQUENCES OF 9 PCR PRODUCTS FROM DNA OF SCB CELLS

A. Sequence of PCR product using PPAR canine-specific primer: 100% match to *Canis familiaris* PPAR alpha (Accession NM_001003093).

B. Sequence of PCR product using PPAR canine-specific primer: 100% match to *Canis familiaris* PPAR alpha (Accession NM_001003093).

C. Sequence of PCR product using beta-actin, A2 canine-specific primer: 97% match to *C. familiaris* beta-actin (Accession NM_001003349).

D. Sequence of PCR product using beta tubulin vertebrate-common primer: 99% match to *C. familiaris* similar to tubulin, beta 5 (Accession XM_842460); 88% match to *Homo sapiens* tubulin, beta (Accession NM_178014).

E. Sequence of PCR product using GAPDH vertebrate-common primer: 98% match to *C. familiaris* GAPDH (Accession NM_001003142); 89% match to *Homo sapiens* GAPDH (Accession NM_002046).
Metazoan Parasites of Lesser Yellowlegs, *Tringa flavipes* (Charadriiformes) From Southwestern United States and Alaska with a Checklist of Parasites Reported From This Host

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**ABSTRACT:** In total, 30 lesser yellowlegs, *Tringa flavipes* (Charadriiformes), from 24 southwestern states and 6 from Alaska, were examined for metazoan parasites. Ten species of helminths (4 cestodes, 4 trematodes, and 2 nematodes), and 5 species of ectoparasites were collected. Cestodes were the most prevalent and abundant taxon. The dominant cestode was an undescribed species of *Choanotaenia*. The cestode *Kowalewskia totani* was the only helminth recovered from Alaskan hosts, and it was also present in birds from the southwest. It appears to be a specialist in lesser yellowlegs. The mallophags *Quadraiceps falcigerus* and *Actornithophilus totani* and the nasal mite *Colionyssus cabanensis* were common to birds from both geographic regions. Characteristics of the helminth community from the southwest and ectoparasite communities of this region and Alaska were low mean species richness, low mean abundance, medium diversity, and uneven distribution of parasites.

The lesser yellowlegs, *T. flavipes*, is a charadriid that breeds only in North America, from northwestern Alaska to central Québec. Most migrate to wintering areas in the southern United States, Mexico, Central and South America, and the West Indies (Tibbitts and Moskoff, 1999). A few, usually single, birds migrate along the Rio Grande Valley, near El Paso, Texas, and are present for short periods of time during the fall and spring migration.

Previous authors have reported 21 species of helminths and 10 species of ectoparasites from this host. All reports were about 1 or 2 species
of parasites, except for Euzeby and Graber (1975) and Graber and Euzeby (1976a, 1976b) who reported 5 species of cestodes and 3 species of trematodes in lesser yellowlegs from Guadeloupe.

The purpose of the present study is to present new information on the metazoan parasite composition and structure in migrating T. flavipes from the southwestern United States, and from a summer population from Cooke Inlet, Alaska.

Twenty-four lesser yellowlegs, T. flavipes, were collected from ephemeral wetlands in the Rio Grande Valley from Hudspeth County, Texas, to Dona Ana County, New Mexico (sample referred to as NMTX), between April 1984 and July 1988, and 6 additional specimens were collected at Cook Inlet, Alaska (sample referred to as AK), on 22 and 23 July 1985. Birds were killed with a shotgun, placed in individual plastic bags, and frozen for later examination. All internal organs were examined for helminth parasites. Feathers were combed and examined for ectoparasites. Cestodes and trematodes were fixed and preserved in AFA (alcohol–formalin–acetic acid), stained in Semichon’s acid carmine, and mounted in neutral balsam. Nematodes were fixed in 70% ethanol and placed in temporary lactophenol mounts. Ectoparasites were fixed in 70% ethanol, cleared in 10% potassium hydroxide, and mounted in Hoyer’s medium.

The following statistical procedures were used: Kruskal-Wallis multiple-comparison test, combined with the Bonferroni test for significance of differences among samples (Z value for significance was >1.96); Simpson’s index of diversity (to emphasize the more common species—ranges from low of 0 to high near 1); Smith and Wilson’s index of evenness (independent of species richness, sensitive to both rare and common species); and the interspecific associations test (χ² statistic with Cole’s coefficient) between appropriately prevalent helminth species.

Voucher specimens were deposited in the National Parasite Collection, Beltsville, Maryland, under accession numbers 081183–081192.

In total, 495 helminths, 75 mallophagans, and 62 nasal mites was collected from lesser yellowlegs, T. flavipes, from NMTX. Ten species of helminths, 4 of Mallophaga, and 1 species of nasal mite were recorded. Seventy-nine percent of the hosts were infected with at least 1 species of helminth, 4 of Mallophaga, and 1 species of nasal mite were recorded. Seventy-nine percent of the hosts were infected with at least 1 species of helminth, 4 of Mallophaga, and 1 species of nasal mite were recorded.

Voucher specimens were deposited in the National Parasite Collection, Beltsville, Maryland, under accession numbers 081183–081192.

Table I. Metazoan parasites of the lesser yellowlegs, Tringa flavipes, from Dona Ana County, New Mexico, and El Paso and Hudspeth Counties, Texas (n = 24), and Cook Inlet, Alaska (n = 6).*

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Prevalence (%)</th>
<th>Mean abundance</th>
<th>±SE</th>
<th>Range</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMTX</td>
<td>AK</td>
<td>NMTX</td>
<td>AK</td>
<td>NMTX</td>
</tr>
<tr>
<td>Cestoda (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choanotaenia sp.</td>
<td>42</td>
<td>6.79</td>
<td>2.59</td>
<td>1–49</td>
<td>163</td>
</tr>
<tr>
<td>Choanotaenia dispar</td>
<td>21</td>
<td>6.29</td>
<td>4.11</td>
<td>5–97</td>
<td>151</td>
</tr>
<tr>
<td>Kowalewksiella totani</td>
<td>25</td>
<td>5.46</td>
<td>1.63</td>
<td>2–67</td>
<td>131</td>
</tr>
<tr>
<td>Aploparaksis sp.</td>
<td>33</td>
<td>1.66</td>
<td>0.77</td>
<td>1–16</td>
<td>40</td>
</tr>
<tr>
<td>Trematoda (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucochloridium cyanocittae</td>
<td>4</td>
<td>0.13</td>
<td>0.13</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Selfcoleum brasiliunum</td>
<td>4</td>
<td>0.08</td>
<td>0.08</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Paramonostomum histrionici</td>
<td>4</td>
<td>0.04</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notocotylus attenuatus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nematoda (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nematode sp.</td>
<td>18</td>
<td>0.08</td>
<td>0.06</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Echinuria uncinata</td>
<td>4</td>
<td>0.04</td>
<td>0.04</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Mallophaga (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadriceps falcigerus</td>
<td>58</td>
<td>3.33</td>
<td>2.13</td>
<td>4.34</td>
<td>51</td>
</tr>
<tr>
<td>Actornithophilus totani</td>
<td>29</td>
<td>3.33</td>
<td>0.96</td>
<td>1.50</td>
<td>4.31</td>
</tr>
<tr>
<td>Trinoton sp.</td>
<td>4</td>
<td>0.04</td>
<td>0.04</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Austromenopon sp.</td>
<td></td>
<td>0.33</td>
<td>0.33</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Acarina (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colinoptes cubanensis</td>
<td>33</td>
<td>2.58</td>
<td>0.50</td>
<td>1.12</td>
<td>1.23</td>
</tr>
</tbody>
</table>

* NM = New Mexico; TX = Texas; AK = Alaska.

Table II. Species richness, helminth abundance, diversity, and evenness data for metazoan parasites of lesser yellowlegs Tringa flavipes (Charadrii) from southwestern United States* (n = 24) and Alaska (n = 6).*

<table>
<thead>
<tr>
<th>Region</th>
<th>Helminth</th>
<th>Ectoparasite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parasite</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMTX</td>
<td>AK</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species richness</td>
<td>(1.5, 0.2, 1)†</td>
<td>(1.4, 0.2, 1)</td>
</tr>
<tr>
<td>Helminth abundance</td>
<td>(49.5, 22.5, 2.5)†</td>
<td>(27.4, 12.7, 23)</td>
</tr>
<tr>
<td>Diversity</td>
<td>0.72</td>
<td>0.63</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.14</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Mean ± standard error, median.
† NM = New Mexico; TX = Texas; AK = Alaska.

Among helminths, cestodes were the most prevalent (75%) and abundant (mean = 20.2). Prevalence for both trematodes and nematodes was 13%, with a mean abundance of 0.3 and 0.1, respectively. The cestode, Choanotaenia sp., an undescribed species, was the dominant helminth with the largest prevalence and abundance (Table I). There was 1 significant association, a positive one, between Choanotaenia sp. and K. totani (χ² = 4.17; P < 0.05). Among ectoparasites, the mallophagan Q. falcigerus was the most abundant, and the nasal mite C. cubanensis was the most prevalent (Table I).

Characteristics of the helminth community from NMTX and ectoparasite communities from both NMTX and AK included low mean species richness, low mean abundance, medium diversity, and an uneven parasite distribution (Table II). There was no significant difference for either species richness (Z = 1.06) or abundance (Z = 0.63) for
Table III. Checklist of metazoan parasites of the lesser yellowlegs, *Tringa flavipes*.*

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Locality</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cestoda (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aploparaksis sp.</td>
<td>NMTX</td>
<td>This study</td>
</tr>
<tr>
<td>Choanotaenia dispar</td>
<td>NMTX</td>
<td>This study</td>
</tr>
<tr>
<td>Echinoctyle flavipesis</td>
<td>Paraguay</td>
<td>Deblock and Vaucher (1995)</td>
</tr>
<tr>
<td>Hymenolepis amphitricha</td>
<td>Guadeloupe</td>
<td>Graber and Euzebey (1976a)</td>
</tr>
<tr>
<td>Hymenolepis tenuis</td>
<td>Guadeloupe</td>
<td>Graber and Euzebey (1976a)</td>
</tr>
<tr>
<td>Kowalewskia cingulifera</td>
<td>Guadeloupe</td>
<td>Graber and Euzebey (1976a)</td>
</tr>
<tr>
<td>Kowalewskia totani</td>
<td>Kansas</td>
<td>Self and Janovy (1965)</td>
</tr>
<tr>
<td>Shipleya inermis</td>
<td>Guadeloupe</td>
<td>Graber and Euzebey (1976a)</td>
</tr>
<tr>
<td>Trichocephaloides beauporti</td>
<td>Leeward Islands</td>
<td>Graber et al. (1977)</td>
</tr>
<tr>
<td>Trematoda (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corpopyrum brasilianum</td>
<td>Guadeloupe</td>
<td>Euzebey and Graber (1975)</td>
</tr>
<tr>
<td>Cyclocoelum vanelli</td>
<td>Brazil</td>
<td>Fernandes (1976)</td>
</tr>
<tr>
<td>Diplostomum marshalli</td>
<td>Minnesota</td>
<td>Chandler (1954)</td>
</tr>
<tr>
<td>Echinoctyle flavipesis</td>
<td>Paraguay</td>
<td>Deblock and Vaucher (1995)</td>
</tr>
<tr>
<td>Harraium halli</td>
<td>Guadeloupe</td>
<td>Euzebey and Graber (1975)</td>
</tr>
<tr>
<td>Himasthla alincia</td>
<td>Louisiana</td>
<td>Lumsden (1962)</td>
</tr>
<tr>
<td>Leucochloridium cyanocittae</td>
<td>NMTX</td>
<td>This study</td>
</tr>
<tr>
<td>Notocotylus atlanticus</td>
<td>NMTX</td>
<td>This study</td>
</tr>
<tr>
<td>Ornithobilharzia sp.</td>
<td>Guadeloupe</td>
<td>Euzebey and Graber (1975)</td>
</tr>
<tr>
<td>Paramonostomum histrionicci</td>
<td>NMTX</td>
<td>This study</td>
</tr>
<tr>
<td>Selfcoelum brasilianum</td>
<td>Iowa, Wisconsin, Brazil</td>
<td>Taft (1975), Fernandes (1976)</td>
</tr>
<tr>
<td>Nematoda (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echiuria uncinata</td>
<td>NMTX</td>
<td>This study</td>
</tr>
<tr>
<td>Mallophaga (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actornithophilus totani</td>
<td>NMTX, AK</td>
<td>This study</td>
</tr>
<tr>
<td>Austromenopon sp.</td>
<td>NMTX, AK</td>
<td>This study</td>
</tr>
<tr>
<td>Quadraceps falcigerus</td>
<td>Ontario; South Pacific</td>
<td>Eveleigh and Amano (1977)</td>
</tr>
<tr>
<td>Quadraceps sp.</td>
<td>NMTX</td>
<td>Amerson and Emerson (1971)</td>
</tr>
<tr>
<td>Acarina (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colinoptes cubanensis</td>
<td>NMTX, AK</td>
<td>This study</td>
</tr>
<tr>
<td>Logipedia tricalcarata</td>
<td>USA</td>
<td>Dabert (1992)</td>
</tr>
<tr>
<td>Neoboydalia philomachi</td>
<td>Texas</td>
<td>Clark (1964)</td>
</tr>
<tr>
<td>Pilochaeta rafalshki</td>
<td>USA</td>
<td>Dabert (1997)</td>
</tr>
<tr>
<td>Pilochaeta tringae</td>
<td>Colombia, Paraguay</td>
<td>Dabert (1997)</td>
</tr>
<tr>
<td>Rhinonyssus conventris</td>
<td>Louisiana</td>
<td>Pence (1972)</td>
</tr>
</tbody>
</table>

* NM = New Mexico, TX = Texas, AK = Alaska.

ectoparasites between the 2 geographical regions, and they were very similar (95%).

The cestode *K. totani* appears to be a specialist in the lesser yellowlegs and its congener, the greater yellowlegs, *Tringa melanoleuca*. It was originally described in lesser yellowlegs from Kansas (Self and Janovy, 1965), and is reported herein from the NMTX and AK. It was also recovered from greater yellowlegs from NMTX (Secord and Canaris, 1993). The positive association between *Choanotaenia* sp. and *K. totani* may be the result of both infecting the same intermediate host, or both being available in a feeding locality.

In general, helminth species richness in the lesser yellowlegs from the NMTX is similar to the greater yellowlegs from the same region (10 vs. 9), but the mean abundance is much larger in the lesser yellowlegs (50 vs. 23). Only 2 helminth species were common to both species of host, *K. totani* and *Selfcoelum brasilianum*. None of the ectoparasites was in common, including the *Austromenopon* species obtained from both species of host. The parasites in the lesser yellowlegs were also absent from the 5 other species of shorebirds examined from this region (Secord and Canaris, 1993).

Apparently the cestode *K. totani* is a specialist in the lesser yellowlegs with a wide distribution, from Alaska to Kansas, New Mexico, and Texas. It appears, at least from migrating lesser yellowlegs from NMTX, that this host has a suite of parasites common to it, with little circulation among other charadriids from NMTX.

A checklist of metazoan parasites of the lesser yellowlegs is included (Table III).

We wish to thank Gay J. Canaris and John M. Kinsella for their suggestions.

**LITERATURE CITED**


---. 2002; Owens, 2002; Morales-Montor et al., 2004). A very conspicuous exception is that BALB/cAnN female mice are more frequently infected with Taenia crassiceps cysticerci (ORFfast line) and carry greater individual parasite loads (PLs) than male mice (Larralde et al., 1989; Scitto et al., 1991). Curious in assessing the general validity for mice of this one exception to the female supremacy paradigm, and in search of the biological factors involved, we examined our 14-yr records in experimental murine cysticercosis in search of sexual differences to infection among several laboratory strains of mice.

In total, 1,198 mice (604 females and 594 males), from 17 different inbred strains of 4 different genetic backgrounds (Table I), were included in the retrospective analysis of records dating from 1990 to 2004. The strains studied are known to vary in terms of their immunology, endocrinology, and pathology, which could collectively influence the host’s response to infection (Spinedi et al., 1992, 1997; Frederic et al., 1993; Mucci et al., 2005). The mice included had all been used as “untreated controls,” and they had been similarly infected as the experimental mice in studies originally designed to test other questions related to infection, immunity, and genetics (Scitto et al., 1990, 1991; Fragoso et al., 1996, 1998). The cysticerci used came from 3 different lines, which differ in their rate of reproduction and capacity to develop into adult tapeworms: HYGlow and WFUlow lines are capable of full development, whereas ORFslow is incapable (Freeman, 1962; Smith et al., 1972; Chau and Freeman, 1976; Everhart et al., 2004). Because of its fast reproduction, cysticerci from the ORFfast line were mostly used in experiments, but all lines were kept in the Institute’s animal facility intraperitoneally with 10 small (2-mm-diameter) nonbudding cysticerci.

Preferential Growth of Taenia crassiceps Cysticerci in Female Mice Holds Across Several Laboratory Mice Strains and Parasite Lines

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ABSTRACT: A retrospective study of our 14-yr records on experimental Taenia crassiceps (ORFfast line) cysticercosis (n = 1,198) shows that in 16 of 17 different mouse strains, female mice are more frequently infected and carry larger individual parasite loads than males. However, sexual differences in parasite loads significantly varies between strains in relation to their different genetic backgrounds (BALB > C57Bl = OTH-ERS > C3H). The coefficient of variation in all female mice is significantly smaller than that of all males, an indication of males’ more potent, but erratically effective, restraint of cysticercus growth. Similarly positive growth bias for female mice is shown by other lines of cysticerci, i.e., HYGlow and WFUlow. These results contraven the usual expectation of female hosts being more resistant than males to parasite infections, and they point to the multiple factors that combined determine sex related differences of mice to experimental cysticercosis infection.

It is generally held that female vertebrates are more resistant than males to parasite infections, despite the many shortcomings of such a “female supremacy paradigm” (Addis, 1946; Zack and McKeen, 1996; Brey and Fish, 2002; Owens, 2002; Morales-Montor et al., 2004). A very conspicuous exception is that BALB/cAnN female mice are more frequently experimentally infected with Taenia crassiceps cysticerci (ORFfast) and carry greater individual parasite loads (PLs) than male mice (Larralde et al., 1989; Scitto et al., 1991). Curious in assessing the general validity for mice of this one exception to the female supremacy paradigm, and in search of the biological factors involved, we examined our 14-yr records in experimental murine cysticercosis in search of sexual differences to infection among several laboratory strains of mice.

In total, 1,198 mice (604 females and 594 males), from 17 different inbred strains of 4 different genetic backgrounds (Table I), were included in the retrospective analysis of records dating from 1990 to 2004. The strains studied are known to vary in terms of their immunology, endocrinology, and pathology, which could collectively influence the host’s response to infection (Spinedi et al., 1992, 1997; Frederic et al., 1993; Mucci et al., 2005). The mice included had all been used as "untreated controls," and they had been similarly infected as the experimental mice in studies originally designed to test other questions related to infection, immunity, and genetics (Scitto et al., 1990, 1991; Fragoso et al., 1996, 1998). The cysticerci used came from 3 different lines, which differ in their rate of reproduction and capacity to develop into adult tapeworms: HYGlow and WFUlow lines are capable of full development, whereas ORFslow is incapable (Freeman, 1962; Smith et al., 1972; Chau and Freeman, 1976; Everhart et al., 2004). Because of its fast reproduction, cysticerci from the ORFfast line were mostly used in experiments, but all lines were kept in the Institute's animal facility by serial passage in donor BALB/cAnN female mice, as described previously (Scitto et al., 1990, 1991). Parasites for infection were harvested from the peritoneal cavities of the BALB/cAnN female donors, 1 to 3 mo after infection. To evaluate the intensity (I) in each individual mouse from all the mouse strains, 10 mice from each sex were injected intraperitoneally with 10 small (2-mm-diameter) nonbudding cysticerci, suspended in phosphate-buffered saline. Thirty days after infection, mice were killed following the principles set forth in the Guide for the
Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC), and their individual intraperitoneal ISs were counted macroscopically, as described previously (Sciutto et al., 1991).

Table I shows the effects of sex and genetic background of mice upon the ISs of the individual mice and upon the proportion (R = n/n) of totally resistant mice (n being the number of mice with zero parasites) in each group of n mice, 30 days after infection, expressed as the means, standard deviations in all the 17 mouse strains studied.

In 16 of the 17 mouse strains infected with ORF of T. crassiceps, some significant genes apparently located in the Y chromosome.

Notwithstanding the general validity of a positive biased growth of T. crassiceps in female mice, the significant variation in the magnitude of sexual differences among the different mouse strains presages other profiles of the sex-related differences may be found in different experimental conditions, i.e., different times after infection, or in other organic compartments, or when measured in ways other than intensity, i.e., prevalence, severity, or mortality. Strain variation in sexual differences could underlie in the discrepancy found between the changes in endocrinological profiles reported for the infected male BALB/cAnN mice, which become estrogenized and deandrogenized in late infections of T. crassiceps cysticercosis, at least, the host-parasite relationship involves a complex network that includes the endocrine, immune, and nervous systems of the host, in association with the reproductive system of the parasite (Morales-Montor and Larralde, 2005; Morales-Montor and Larrañaga, 2005; Aldridge et al., 2007), and those apparently stable endocrinological profiles of the more resistant BALB/c mice, found at earlier times after infection (Aldridge et al., 2007). These findings confirm that female mice are more permissive to the intraperitoneal growth of T. crassiceps than male mice in a variety of laboratory mice through the mediation of complex mechanisms involving the genetic background of the host.

How the role of hosts’ sex in each parasite infection operates in cellular and molecular detail is fascinating and largely unexplored in many different parasite infections (Zuk and McKean, 1996). In experimental murine T. crassiceps cysticercosis, at least, the host–parasite relationship involves a complex network that includes the endocrine, immune, and nervous systems of the host, in association with the reproductive system of the parasite (Morales-Montor et al., 2004). These systems regulate cellular differentiation, reproduction, and apoptosis.

Table I. Effects of the host’s sex and genetic background upon individual I and proportion of totally resistant mice (R = n/n) after 30 days of infection with the ORF of T. crassiceps. All the 17 strains tested are shown ordered by genetic background families (BALB, C57BL, C3H, and Others) and strain denomination (cAnN, B, K, and so on), along with the corresponding means.

<table>
<thead>
<tr>
<th>Family and strain</th>
<th>Females</th>
<th>Males</th>
<th>Probability of equalities between sexes in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n*</td>
<td>PL (± SD)</td>
<td>n</td>
</tr>
<tr>
<td>BALB/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAnN</td>
<td>145</td>
<td>115 ± 8</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>58</td>
<td>23 ± 4</td>
<td>3</td>
</tr>
<tr>
<td>K</td>
<td>35</td>
<td>36 ± 11</td>
<td>5</td>
</tr>
<tr>
<td>cByJ</td>
<td>20</td>
<td>31 ± 5</td>
<td>4</td>
</tr>
<tr>
<td>cJ</td>
<td>30</td>
<td>4 ± 0.5</td>
<td>7</td>
</tr>
<tr>
<td>Totals</td>
<td>288</td>
<td>209 ± 28</td>
<td>19</td>
</tr>
<tr>
<td>C57BL/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6J</td>
<td>48</td>
<td>7 ± 3</td>
<td>15</td>
</tr>
<tr>
<td>B6.Kl</td>
<td>18</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>10J</td>
<td>20</td>
<td>23 ± 6</td>
<td>0</td>
</tr>
<tr>
<td>B10.D2</td>
<td>20</td>
<td>16 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>B10.D2(R103)</td>
<td>20</td>
<td>18 ± 3</td>
<td>15</td>
</tr>
<tr>
<td>B10.D2(R107)</td>
<td>20</td>
<td>22 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>B10.A2R</td>
<td>20</td>
<td>18 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>166</td>
<td>104 ± 24</td>
<td>48</td>
</tr>
<tr>
<td>C3H/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeJ</td>
<td>15</td>
<td>22 ± 5</td>
<td>3</td>
</tr>
<tr>
<td>HeB/FeJ</td>
<td>10</td>
<td>6 ± 2</td>
<td>4</td>
</tr>
<tr>
<td>Totals</td>
<td>25</td>
<td>28 ± 7</td>
<td>7</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg1</td>
<td>14</td>
<td>28 ± 18</td>
<td>0</td>
</tr>
<tr>
<td>Tg2</td>
<td>19</td>
<td>16 ± 15</td>
<td>1</td>
</tr>
<tr>
<td>A/J</td>
<td>92</td>
<td>111 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>125</td>
<td>155 ± 38</td>
<td>1</td>
</tr>
<tr>
<td>Totals</td>
<td>604</td>
<td>496 ± 97</td>
<td>75</td>
</tr>
</tbody>
</table>

* n, number of mice.
† n, number of resistant mice.
through genes, hormones, cytokines, antigens, receptors, antibodies, and metabolic pathways of both the host and the parasite (Morales-Montor and Larralde, 2005); thus, they modulate the outcome of infection. As a consequence of such complexity, the sexually dimorphic profile in a given parasite infection may also vary in its form of expression (i.e., prevalence, intensity, severity, or mortality), and with the time and anatomical location of the infection, the developmental stage of the infecting parasite, the effectiveness of the host immune response, and environmental factors (e.g., stress, concomitant infections, nutrient availability, or predators) acting upon both host and parasite in laboratory or natural conditions (Klein, 2004; Morales-Montor and Larralde, 2005).

We are confident that other host–parasite interactions are governed by similar factors and in different ways, but with comparable complexity. If so, claims of an inflexibly biased sexual dimorphism affecting all host–parasite interactions should be narrowed to the particular infection under consideration.

**LITERATURE CITED**


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**The Association of Zygocotyle lunata and Echinostoma trivolvis with Chaetogaster limnaei, an Ectosymbiont of Helisoma trivolvis**

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**ABSTRACT:** Helisoma trivolvis snails collected from a lake in Warren County, New Jersey, in June 2007 possessed the ectosymbiont Chaetogaster limnaei (Amnellida). Some of these snails were also infected with larval stages of Zygocotyle lunata and Echinostoma trivolvis. Chaetogaster limnaei associated with the infected snails fed on the cercariae of both digeneans. Zygocotyle lunata cercariae were observed in the stomach of C. limnaei and whole cercariae were loosely attached to the ventral surface of the chaetogasters. Cercariae in the stomachs were digested within 48 hr and probably served as a source of nutrient for the annelids. Whole cercariae and 1 noviable metacercaaria of E. trivolvis were seen in the stomachs of the chaetogasters. The protective action of the chaetogasters on the transmission of the cercariae of E. trivolvis and Z. lunata to second intermediate hosts in the wild awaits further study.
The purpose of the present paper is to describe our observations on the association of Zygocotyle lunata and Echinostoma trivolvis cercariae with Chaetogaster limnaei, an ectosymbiont of Helisoma trivolvis. On 23 June 2007, 100 H. trivolvis snails, 10–20 mm in shell diameter, were collected at Oxford Furnace Lake, Warren County, New Jersey (40°47′46.5″N, 75°00′44.9″W), as described by Klockars et al. (2007). The snails were each isolated in individual wells (3.5 cm in diameter) of a multiwell chamber. Examination of the wells water prior to snail isolation showed that they were free of aquatic invertebrates and larval trematodes. Examination of the snails for cercariae release at 2 hr postisolation showed that 5 were infected with E. trivolvis. Two other snails released cercariae of Z. lunata into the water. The remaining 95 snails were negative for larval trematodes based on this initial observation.

Examination of the wells with a dissecting scope at 2 hr postisolation revealed the presence of C. limnaei, an ectosymbiont of H. trivolvis, in most of the cultures. Chaetogaster limnaei did not consume E. trivolvis cercariae. However, cercariae of Z. lunata were observed in the stomachs of C. limnaei; some cercariae were also attached to the anterior ventral surface of the chaetogasters in wells containing snails releasing Z. lunata cercariae.

Approximately 10 chaetogasters were present in wells in which Z. lunata cercariae were seen. Six of the C. limnaei in one well each contained 1 cercaria, and 4 each contained a cercaria (body and tail) attached to the anterior ventral surface. Penetration of the cercaria into the body of the chaetogaster did not occur. In the other well, 5 of 10 C. limnaei each contained a single cercaria in the stomach. Several C. limnaei that had ingested cercariae of Z. lunata were fixed in hot 5% neutral buffered formalin and mounted on glass microscope slides in glycerin jelly. The cercaria in the stomach distended the anterior region of the chaetogaster. The cercaria was heavily pigmented with melanin and appeared as a dark body at low magnification. Under higher magnification, some details of the cercaria were apparent, including the eyespots, excretory ducts, and dispersed pigment granules. The cercaria morphology conformed to earlier descriptions of this larval stage by Willey (1936, 1941). No release of cystogenous material was observed at the junction of the stomach and the cercaria, indicating the inability of the larval stage to encyst in this site.

For 48 hr, we observed a C. limnaei that had ingested a Z. lunata cercaria to see if the parasite would be digested. At 24 hr, there was clear-cut lysis of the cercaria and, by 48 hr, digestion was complete and minimal residue of the body was seen. Our observations indicated that the cercaria was digested and absorbed in the gut of the chaetogaster, thereby serving as a nutrient source for the oligochaete.

We attempted to see if chaetogasters isolated from snails would also feed on Z. lunata cercariae. To accomplish this objective, Z. lunata cercariae were pipetted into a 3.5-cm diameter petri dish containing artificial spring water (ASW) (Ulmer, 1970), along with 2–4 chaetogasters. No evidence of cercaria ingestion was seen at 2 hr, but some cercariae had formed a loose association with the ventral surface of the annelids. By 4 hr, the attached cercariae detached and became motionless at the bottom of the dish. Whether the surface of the chaetogaster serves as an attachment site for encystment and allows for possible transmission of this trematode to a vertebrate host remains to be determined.

The snails infected with Z. lunata were removed to a mason jar containing 1 L of ASW in an attempt to collect cercariae. Unfortunately, the 2 snails infected with Z. lunata died within 24 hr postisolation and released less than 10 cercariae into the cultures. Further attempts to continue work with Z. lunata larvae were abandoned.

Zygocotyle lunata is mainly a North American digenean that uses H. trivolvis and H. anceps snails as first intermediate hosts; cercariae released from these snails encyst on vegetation or mollusk shells and perhaps other invertebrates, and are then transferred as a foodborne parasite to avian and ruminant final hosts. The role of Z. lunata as a possible pathogen for humans has not been determined. Recent work has reported this parasite in South America associated with Biomphalaria tenagophila snails (De Nunez et al., 2003).

When the Z. lunata studies were completed, we reexamined the association between E. trivolvis cercariae and C. limnaei. The 5 snails releasing cercariae of E. trivolvis on 23 June 2007 had been maintained in a mason jar with 1 L of ASW and juvenile Biomphalaria glabrata snails to obtain metacercariae of E. trivolvis as described by Schmidt and Fried (1996). At 5 days postisolation, 4 of the 5 H. trivolvis snails infected with E. trivolvis were still alive. These snails were again placed in multiwell chambers to reexamine the C. limnaei-E. trivolvis relationship. Removal of C. limnaei to a depression slide and examination of the preparation at ×100 and ×200 with a compound microscope clearly showed the cercariaphagic activity of C. limnaei. Of 20 C. limnaei examined from all the isolates, 10 had 1 or 2 cercariae per C. limnaei stomach. In most cases, the cercariae had retained their tails. Some cercariae showed minimal activity and most had become pigmented and exhibited evidence of initial lysis. One metacercaria was observed in the stomach of an oligochaete. The metacercaria was granular and obviously undergoing lysis. Our findings thus suggested that the E. trivolvis cercariae served as a nutrient source for the chaetogaster, and that transmission of metacercariae to vertebrate hosts by C. limnaei was not a likely route.

The protective role of C. limnaei in regard to ingesting miracidia and cercariae of schistosomes is well known (Michelson, 1964). Some information exists on the role of chaetogasters in ingesting uneconomically important larval digeneans. For instance, Fernandez et al. (1991) reported that C. limnaei in association with H. anceps preyed on the nonmobile cercariae of the hemiurid trematode Halipegus occidualis. Studies have also been conducted on cercariae that encyst on vegetation, such as those of the paramphistomids or fascioloids (Rajasekaraiah, 1978), and have shown that C. limnaei associated with Lymnaea truncatula preyed upon the cercariae of Fasciola hepatica. The same investigator suggested that such predation could be exploited as a control measure against economically important liver fluke disease.

There is no indication that Z. lunata, E. trivolvis, or other species of trematodes are selectively pursued as food sources by C. limnaei, or whether the oligochaete ingests them simply because of their size, proximity, availability, and possible indiscriminate feeding behavior of C. limnaei. Our observations suggested that Z. lunata and E. trivolvis cercariae released from H. trivolvis were ingested by C. limnaei, an ectosymbiont of the snail. The effect of such predation in terms of the protective action of the chaetogasters on the transmission of these parasites in the wild awaits further study.

LITERATURE CITED


Evaluation of the Mood-Stabilizing Agent Valproic Acid as a Preventative for Toxoplasmosis In Mice and Activity Against Tissue Cysts in Mice

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ABSTRACT: Toxoplasma gondii is a common intracellular protozoan infection of a wide range of animals. Severe disease in compromised individuals and the in the fetuses of nonimmune pregnant women. Chronic infection is associated with vision and hearing problems, and functional mental alterations, including schizophrenia. The mood-stabilizing agent valproic acid has been shown to inhibit the development of T. gondii in vitro at dosages that are normally achieved in the serum and cerebral spinal fluid of human patients and to have positive effects on the behavior of rats chronically infected with T. gondii. The present study was done to examine the in vivo activity of valproic acid against acute toxoplasmosis in mice. Two studies were done with valproic acid given in the drinking water at concentrations of 1.5 mg/ml (Experiment 1) or 3.0 mg/ml (Experiment 2). In a third experiment (Experiment 3), valproic acid was injected intraperitoneally (i.p.) at doses of 200 or 300 mg/kg every 12 hr. Valproic acid was not effective in preventing acute toxoplasmosis. All mice treated with valproic acid died or were killed and did not (P > 0.05) live significantly longer than the controls. Tachyzoites were demonstrated in the tissues of infected valproic-acid–treated mice. A fourth study was done to determine if valproic acid has activity against T. gondii tachyzoites in chronically infected mice. Mice were chronically infected with the ME-49 strain of T. gondii for 8 wk and then treated orally with valproic acid at approximately 6.6 mg/ml (800 mg/kg/day) in the drinking water for 10 wk (amount was varied due to increasing mouse weights). No significant differences (P > 0.05) were present in tissue cyst numbers in valproic-acid–treated T. gondii chronically infected mice and in mice chronically infected with T. gondii but not given valproic acid. Our results indicate that valproic acid, although effective in vitro against T. gondii tachyzoites, is not effective as a preventative in mice inoculated with T. gondii tachyzoites. Additionally, no activity against tissue cysts was observed in chronically T. gondii-infected valproic-acid–treated mice.

Valproic acid is a mood stabilizer used in the treatment of mental illness, including bipolar disorder and schizophrenia (see Bowden, 2007). It has also been shown to inhibit T. gondii reproduction in human fetal lung cell cultures (Jones-Brando et al., 2002; Strobl et al., 2007). The IC_{50} of valproic acid (sodium salt) in a tachyzoite production assay was 266 µg/ml (Strobl et al., 2007), whereas in an ELISA-based assay the IC_{50} of valproic acid (free acid) was 4.7 µg/ml and the IC_{50} of valproic acid (sodium salt) was 4.1 µg/ml (Jones-Brando et al., 2003). These studies indicate that this drug is active against tachyzoites of T. gondii.

Rats chronically infected with T. gondii lose their innate fear of cat odor (Berdoy et al., 2000; Vyas et al., 2007). This makes them easier prey for cats and enhances the transmission of the parasite. The effects of valproic acid on the feline avoidance behavior of chronically infected rats was examined by Webster et al. (2006) and it was shown to help treated T. gondii–infected rats (40 mg/kg valproic acid/day orally) retain their innate avoidance of cat smell.

The present study was done to examine the anti-T. gondii activity of valproic acid in the prevention of acute toxoplasmosis in mice or activity against the tissue cyst stage in chronically infected mice. Female ICR mice were housed in groups of 5 mice per cage (Experiments 1–3) or 3–4 mice per cage (Experiment 4). Mice in Experiments 1–3 were inoculated subcutaneously with 5 × 10^5 tachyzoites of the RH strain of T. gondii on Day 0. Valproic acid was given in the drinking water 1 day prior to subcutaneous inoculation of mice in Experiments 1 and 2. Valproic acid–containing water in lightproof water bottles was provided ad libitum for the remainder of the study. Fresh valproic-acid–containing water was provided every 2 days. For dosing considerations, we assumed that each mouse would drink 4 ml of water each day. Saccharin was added at 0.2% (w/v) to mask the flavor of the valproic-acid–treated water.

Experiment 1 contained 10 mice treated with 1.5 mg/ml valproic acid (Groups 1 and 2) in the drinking water supplemented with 0.2% (w/v) saccharin and 10 mice not treated with valproic acid (Groups 3 and 4) (Table I). Saccharin was added at 0.2% (w/v) to 1 group of 5 of these mice (Group 3) given water without valproic acid. Experiment 2 contained 5 mice treated with 3 mg/ml valproic acid in the drinking water (Group 5) and 5 mice not treated with valproic acid (Group 6) (Table II). The mean weight of valproic-acid–treated mice in Experiment 1 was 24 g and in Experiment 2 the mean weight was 22 g. Valproic-acid concentrations in the drinking water provided doses of 250 mg/kg/day (Experiment 1) and 545 mg/kg/day (Experiment 2). These dosages translate to 300 mg/kg and 600 mg/kg for a 20-g mouse, respectively.

Experiment 3 was done to evaluate valproic acid administered intraperitoneally (i.p.) at doses of 200 or 300 mg/kg every 12 hr (Table III). The mean weight of valproic-acid–treated mice was 23 g. Valproic acid was dissolved in sterile saline (0.14 M NaCl solution) and i.p. injections were started 2 days prior to RH strain T. gondii infection with 5 × 10^5 tachyzoites s.c. There were 2 groups (Groups 7 and 8) of 5 mice each that received the 400-mg/kg/day total dose and 2 groups (Groups 9 and 10) of 5 mice each that received the 600-mg/kg/day total dose. A group of 5 mice (Group 11) were infected controls and treated every 12 hr with i.p. sterile saline only.

Experiment 4 (Table IV) was conducted to determine if valproic acid has activity against T. gondii tissue cysts in vivo. Eight mice (4 mice in Group 12 and 4 mice in Group 13) were s.c. infected with 1 × 10^5 tachyzoites of the ME49 strain of T. gondii in 0.5 ml HBSS and left untreated for 8 wk. Three mice (Group 14) were s.c. inoculated with 0.5 ml HBSS and treated similarly. After 8 wk, mice in Groups 12 and 14 were provided drinking water containing 0.2% saccharin and approximately 6.6 mg/ml (amount varied because of mouse weights), which changed during the study) valproic acid for 10 wk to deliver a daily dose of 800 mg/kg. Mice in Groups 12–14 were killed 10 wk after valproic-acid treatment, and their brains were removed. The left half of the brain was homogenized in 2 ml HBSS for 2 min with the use of a stomacher machine (Seward Lab Blender Stomachor 80, London, England). The numbers of tissue cysts in a 50-µl sample of the homogenized brain was determined with the use of light microscopy.
TABLE I. Protocol and results of Experiment 1 on 1.5-mg/ml dose of valproic acid given in the drinking water on acute toxoplasmosis in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse number</th>
<th>Dose of valproic acid*</th>
<th>Day post inoculation died/killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1.5 mg/ml</td>
<td>Died 9</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>1.5 mg/ml</td>
<td>Died 10</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>1.5 mg/ml</td>
<td>Died 11</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>1.5 mg/ml</td>
<td>Died 11</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>1.5 mg/ml</td>
<td>Died 12</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>1.5 mg/ml</td>
<td>Died 12</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>1.5 mg/ml</td>
<td>Died 12</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>1.5 mg/ml</td>
<td>Died 12</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>1.5 mg/ml</td>
<td>Died 12</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1.5 mg/ml</td>
<td>Died 13</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>None</td>
<td>Died 10</td>
</tr>
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<td>None</td>
<td>Died 11</td>
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<td>14</td>
<td>None</td>
<td>Died 11</td>
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<td>3</td>
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<td>None</td>
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<td>None</td>
<td>Died 10</td>
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<td>None</td>
<td>Died 11</td>
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<td>4</td>
<td>18</td>
<td>None</td>
<td>Died 11</td>
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<tr>
<td>4</td>
<td>19</td>
<td>None</td>
<td>Died 11</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>None</td>
<td>Died 12</td>
</tr>
</tbody>
</table>

* Provided continuously in water in lightproof drinking bottle to provide an estimated total dose of 250 mg/kg/day.

The right half of the brain was fixed in 10% neutral buffered formalin solution and processed routinely for histological examination following staining with hematoxylin and eosin.

Impression smears were made from the livers or lungs of mice that died or were killed during the study. They were examined unstained with interference contrast optics.

Kaplan–Meier survival analysis was performed with the use of PrismGraphpad version 4.0 on mice in Experiments 1–3. The data were analyzed for statistical significance with the use of the chi-square and log-rank tests with a P value of 0.05. Tissue cyst counts from mice in Experiment 4 were examined with the use of a 1-way ANOVA and an unpaired t-test with a P value of 0.05.

Acute toxoplasmosis occurred in all mice given the RH strain (Tables I-III). Neither oral (Experiments 1 and 2) nor i.p. (Experiment 3) treatment with valproic acid was effective. Deaths occurred in treated mice from 8 to 13 days after inoculation with tachyzoites and in untreated mice 9–12 days after tachyzoite inoculation (Tables I-III). There was no significant positive effect of valproic-acid treatment on mouse survival (P > 0.05). Tachyzoites were seen in tissues of all mice given the RH strain of T. gondii.

None of the mice inoculated with tachyzoites of the ME49 strain of T. gondii died during the study (Groups 12 and 13). None of the mice inoculated with the RH strain developed tissue cysts.

TABLE II. Protocol and results of Experiment 2 on 3-mg/ml dose of valproic acid given in the drinking water on acute toxoplasmosis in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse number</th>
<th>Dose of valproic acid*</th>
<th>Day post inoculation died/killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>21</td>
<td>3.0 mg/ml</td>
<td>Died 11</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>3.0 mg/ml</td>
<td>Died 11</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>3.0 mg/ml</td>
<td>Died 12</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>3.0 mg/ml</td>
<td>Died 12</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>3.0 mg/ml</td>
<td>Died 12</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>None</td>
<td>Killed 10</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>None</td>
<td>Killed 10</td>
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<tr>
<td>6</td>
<td>28</td>
<td>None</td>
<td>Died 10</td>
</tr>
<tr>
<td>6</td>
<td>29</td>
<td>None</td>
<td>Killed 11</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>None</td>
<td>Killed 11</td>
</tr>
</tbody>
</table>

* Provided continuously in water in lightproof drinking bottle to provide an estimated total dose of 545 mg/kg/day.

TABLE III. Protocol and results of Experiment 3 examining 2 doses of valproic acid given intraperitoneally every 12 hr on acute toxoplasmosis in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse number</th>
<th>Total dose of valproic acid*</th>
<th>Day post inoculation died/killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>31</td>
<td>400 mg/kg</td>
<td>Died 9</td>
</tr>
<tr>
<td>7</td>
<td>32</td>
<td>400 mg/kg</td>
<td>Died 9</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
<td>400 mg/kg</td>
<td>Died 10</td>
</tr>
<tr>
<td>7</td>
<td>34</td>
<td>400 mg/kg</td>
<td>Died 10</td>
</tr>
<tr>
<td>7</td>
<td>35</td>
<td>400 mg/kg</td>
<td>Died 10</td>
</tr>
<tr>
<td>8</td>
<td>36</td>
<td>400 mg/kg</td>
<td>Died 8</td>
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<td>8</td>
<td>37</td>
<td>400 mg/kg</td>
<td>Died 9</td>
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<td>8</td>
<td>38</td>
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<td>Died 10</td>
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<tr>
<td>11</td>
<td>51</td>
<td>None†</td>
<td>Died 9</td>
</tr>
<tr>
<td>11</td>
<td>52</td>
<td>None†</td>
<td>Died 10</td>
</tr>
<tr>
<td>11</td>
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<td>Died 10</td>
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<td>Died 10</td>
</tr>
<tr>
<td>11</td>
<td>55</td>
<td>None†</td>
<td>Killed 11</td>
</tr>
</tbody>
</table>

* Given intraperitoneally as 200 or 300 mg/kg every 12 hr. † Sterile saline given intraperitoneally every 12 hr.

from 8 to 13 days after inoculation with tachyzoites and in untreated mice 9–12 days after tachyzoite inoculation (Tables I-III). There was no significant positive effect of valproic-acid treatment on mouse survival (P > 0.05). Tachyzoites were seen in tissues of all mice given the RH strain of T. gondii.

None of the mice inoculated with tachyzoites of the ME49 strain of T. gondii died during the study (Groups 12 and 13). None of the mice inoculated with the RH strain developed tissue cysts.

TABLE IV. Protocol and results of Experiment 4 on 6.6-mg/ml dose of valproic acid given in the drinking water* for 10 wk on chronic toxoplasmosis in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse number</th>
<th>Dose of valproic acid with Toxoplasma gondii</th>
<th>Number of tissue cysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>56</td>
<td>3.0 mg/ml</td>
<td>Yes</td>
</tr>
<tr>
<td>12</td>
<td>57</td>
<td>3.0 mg/ml</td>
<td>Yes</td>
</tr>
<tr>
<td>12</td>
<td>58</td>
<td>3.0 mg/ml</td>
<td>Yes</td>
</tr>
<tr>
<td>12</td>
<td>59</td>
<td>3.0 mg/ml</td>
<td>Yes</td>
</tr>
<tr>
<td>13</td>
<td>60</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>13</td>
<td>61</td>
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</tr>
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<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>14</td>
<td>64</td>
<td>3.0 mg/ml</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>65</td>
<td>3.0 mg/ml</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>66</td>
<td>3.0 mg/ml</td>
<td>No</td>
</tr>
</tbody>
</table>

* Provided continuously in water in lightproof drinking bottle to provide an estimated total dose of 800 mg/kg/day.
given only valproic acid (Group 14) died during the study. Tissue cysts were structurally normal when viewed as fresh preparations with light microscopy. They were also normal when viewed in stained histological sections. The mean number of tissue cysts in 50 μl was 1.3 ± 1.3 (range, 0–3) for mice in Group 12 and 2.0 ± 1.8 (range, 0–4) for mice in Group 13. No tissue cysts were seen in the brains of mice in Group 14. There were no significant differences (P > 0.05) in tissue cyst counts in mice infected with the ME49 strain of *T. gondii* and treated with valproic acid (Group 12) and those infected but not treated with valproic acid (Group 13).

Valproic acid is active against tachyzoites of *T. gondii* in 2 different cell-culture-based assays (Jones-Brando et al., 2003; Strobl et al., 2007). The studies of Jones-Brando et al. (2003) indicate that valproic acid is active at lower concentrations than reported by Strobl et al. (2007), but both indicate that it is active and the differences are probably due to different test systems used to determine activity.

The results of the present study indicate that valproic acid is not active in preventing acute toxoplasmosis in mice. The highest oral daily dose of 800 mg/kg/day we tested is approaching the oral LD₅₀ dose of this agent in mice (1,098 mg/kg); the highest i.p. dose of valproic acid tested, 600 mg/kg/day, is greater than the LD₅₀ for mice for this route of administration (470 mg/kg) (American Pharmaceutical Partners, Inc., Bedford Labs, Bedford, Ohio). Therefore, further increasing the dose of valproic acid is impractical. In contrast, Webster et al. (2006) demonstrated that oral treatment of chronically *T. gondii*-infected rats with 40 mg/kg/day of valproic acid was beneficial in their study system. Treated *T. gondii*-infected rats retained their innate avoidance of feline smell (cat urine). The mode of action of valproic acid, including which stage of *T. gondii* is affected by valproic acid in the rats, is not known. Because the rats were chronically infected, it is possible that valproic acid acted on the tissue cyst/bradyzoite stages of *T. gondii*. Valproic acid might also influence the bradyzoite-to-tachyzoite or tachyzoite-to-bradyzoite stage conversion. We did not demonstrate a significant (P > 0.05) effect of valproic acid on the numbers of tissue cysts in treated mice versus controls. Additional study is needed to examine the activity of valproic acid and similar agents against the tissue cysts/bradyzoites of *T. gondii*.

The study was made possible by funds from a joint research program between the Carillon Biomedical Institute, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, Virginia, and the University of Virginia, Charlottesville, Virginia, to J.S. and D.S.L.

LITERATURE CITED


**Molecular Characterization of Babesia kiwiensis From the Brown Kiwi (Apteryx mantelli)**

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ABSTRACT: To further investigate the recently described avian piroplasm, *Babesia kiwiensis*, blood samples were collected from 13 wild-caught and 8 zoocaptive brown kiwi (*Apteryx mantelli*) and screened for the presence of piroplasm DNA using a nested-polymerase chain reaction (PCR) targeting the 18S rRNA gene of most members of Piroplasmodia. All captive birds gave a negative PCR result, while 12 wild-caught birds were PCR positive. The nearly full-length 18S RNA gene for *B. kiwiensis* was sequenced. Upon phylogenetic analysis, it was found to belong to the babesid group of piroplasms and was ancestral, yet genetically similar, to the *Babesia canis*-related species. An insight into the current taxonomy of the avian piroplasms is also given. An *Iodes anatis* tick collected from 1 of the North Island brown kiwi was also screened using PCR and was found to be positive for *B. kiwiensis* DNA.

The avian piroplasms (*Piroplasmodia*) remain an understudied group of protozoans. In comparison to many of the mammalian species of
Babesia and Theileria, very little is known about the life cycle or biology of any of the avian species (Peirce, 1975, 2000). To date, approximately 18 species of Babesia have been described from birds, although much confusion exists over the correct taxonomic classification of each of these (Peirce, 2000; Peirce et al., 2003; Peirce, 2005).

The introduction of molecular-based analysis is likely to provide greater insights into the biology of these piroplasms, in addition to offering improved methods of detection, differentiation, and solutions to current taxonomic idiosyncrasies. An avian species of piroplasm, Babesia kiwiensis, was recently described on the basis of morphology alone in the brown kiwi (Apteryx mantelli) of the North Island of New Zealand (Peirce et al., 2003). The kiwi is the smallest member of the ratites (Struthioniformes) and is one of the most primitive birds alive today (Heather and Robertson, 2005). All kiwi species are currently threatened with extinction and there are only five species and subspecies recognized (Patterson et al., 2000), with the key factor contributing to their decline being predation by introduced mammals (McLennan et al., 1996). The pathogenicity of B. kiwiensis is currently unknown, as is much of the life cycle and biology of this species.

The present study aimed to identify kiwi infected with Babesia sp. by using PCR, to characterize B. kiwiensis on the basis of the 18S rRNA gene sequence, and to phylogenetically compare this species to other members of Babesia.

Blood samples used in this study were collected by venipuncture from 13 wild-caught juvenile North Island brown kiwi at 3 locations (Rarewarea, Riponui, and Purua) in Northland, New Zealand, during a field trip in September and October 2003. For a description of the study sites, see Robertson et al. (1999). Additional blood samples were collected from 8 wild-caught kiwi at a semi-engorged stage at Backland Zoo. The thin blood films were made immediately; blood was mixed into EDTA anticoagulant and stored at −20°C until PCR analysis. Ticks were also collected from the wild-caught birds and preserved in 70% ethanol. Both blood and tick samples were transported on ice to Australia for analysis.

Thin blood films were stained with Wright-Giemsa stain (Hematek Stain Pak, Bayer Diagnostics Europe, Ltd., Dublin, Ireland) and examined using an Olympus BX50 microscope (Olympus, Tokyo, Japan) using X100 magnification and oil immersion. A relatively uniform region of the blood film was viewed and approximately 2,000 erythrocytes were examined for the presence of parasites. Individual ticks were morphologically identified to the species level, then macerated with a sterile scalpel blade before DNA was extracted using a QIAamp tissue kit (QIAGEN, Berlin, Germany). DNA was purified from the blood samples using the QIAamp blood mini kit (QIAGEN) following the manufacturer’s instructions.

All samples were initially screened for piroplasm species using a nested PCR capable of detecting most members of the Piroplasmida (Jefferys et al., 2007) that amplified an approximately 800 bp region of the 18S rRNA gene. Amplification of the near-complete 18S ribosomal RNA gene was performed using primers previously described by Criadó-Fornelio et al. (2003a). PCR assays were performed in a final reaction volume of 25 μl that consisted of the following: 10× polymerase buffer, 2.5 mM MgCl2, 0.4 mM dNTPs, 0.5 μM of each primer (12.5 ng/ml), 0.5 U Tth DNA polymerase and 2 μl extracted template DNA. PCR products were electrophoresed on a 1% agarose gel and visualised using ethidium bromide and UV illumination. Amplified products were purified using an UltraClean Gelspin DNA Purification Kit (MO Bio Laboratories Inc., Solihull, B78, England). Sequencing reactions were performed using an ABI Prism Dye Terminator Cycle Sequencing Core kit (Applied Biosystems, Foster City, California) and sequence data was analysed using 4peaks analysing software (v 1.7.1 for Mac OS X, A. Griebspoor and Tom Groothuis, mkentosj.com).

Using ClustalW, a 1,472 bp region of the 18S rRNA gene of B. kiwiensis was aligned with sequences from 55 other Babesia and selected other piroplasm species (GenBank accession numbers are given in Fig. 1) obtained from the GenBank database. Hepatozoon canis (GenBank Accession Number AJ789075) was also included in analyses as an outgroup species. Phylogenetic relationships were determined using TREECON v1.3b (using the Gauvilar and Goye distance method and neighbor-joining method algorithms) (Van de Peer and De Wachter, 1993) and MEGA v2.1 softwares (minimum evolution) (Kumar et al., 2001). Statistical support was provided by bootstrapping 1,000 replicates. Bootstrap values greater than 70% were considered statistically significant.

In total, 8 blood films, corresponding to 8 of the 13 wild-caught birds, were available for microscopic examination. Intra-erythrocytic piroplasms similar to those described by Peirce et al. (2003), including a tetrad (‘Maltese cross’) form, were observed in 6 of the 8 blood films. An approximately 800 bp region of the 18S rRNA gene was amplified by nested PCR from blood in 12 of 13 wild-caught birds. No DNA amplification was observed for any of the captive kiwi. A tick, identified as Ixodes anatis, was collected from 1 of the captive kiwi and subjected to DNA extraction; an approximately 700 bp product was also amplified by nested-PCR. All PCR products were sequenced and found to be identical.

The near-complete 18S rRNA gene was then amplified and 1,510 bp were sequenced from 3 of the infected kiwi, then BLAST searched and found to be most homologous to Babesia canis vogeli (GenBank ABO83374), sharing 94% sequence identity. All 3 kiwi sequences were identical and submitted to the GenBank database (GenBank EF551335). Distance-based and neighbor-joining phylogenetic analysis on the basis of the 18S rRNA gene (1,472 of the total 1,510 bp; 531 informative sites) revealed 3 distinct groups within Babesia (Fig. 1). Each cluster was classified in accordance to groups proposed by Criadó-Fornelio et al. (2003b).

The present study successfully characterized B. kiwiensis on the basis of the 18S rRNA gene, adding to only 2 previously genetically-described avian species. Interestingly, this species is not closely related to B. poeleti from the brown booby, Sala leucogaster (Work and Ramirez, 1997; Yabsley et al., 2006) or B. bennetti from the yellow-legged gull, Larus cachinnans (Merino, 1998; Criadó et al., 2006). It is more genetically similar, yet ancestral, to the Babesia canis-related species within the babesiid group described by Criadó-Fornelio et al. (2003b).

Indeed, the unique branch separation of B. kiwiensis suggests that this species may be representative of an as yet uncharacterized group of piroplasms that may have co-evolved with the palaegnaths (rattites and tinamous), which are themselves taxonomically separated from all other living birds (neognaths) (Cooper et al., 2001). Identification of these piroplasm species within other members of the Struthioniformes, such as the emu and ostrich, may also give further insight into the evolutionary origins of B. kiwiensis. Alternatively, B. kiwiensis may have originated in a mammalian host, introduced to New Zealand with the animals accompanying Polynesians (dogs and Pacific rats) or European settlers (including dogs, cats, rats, mustelids, pigs, and brush-tailed possums), none of which has been screened for B. kiwiensis to the best of the authors’ knowledge. However, with the exception of B. kiwiensis, New Zealand is considered free of Babesia spp. (Bevan, 2003).

The kiwi itself, being both flightless and ground dwelling, is also susceptible to tick infestation, principally by Ixodes anatis, which is found in considerable numbers in kiwi nests and engorged on kiwi down in New Zealand (Peirce et al., 2003). The identification of B. kiwiensis DNA in a semi-engorged I. anatis tick taken from an infected kiwi chick raises the possibility, originally suggested by Peirce et al. (2003), that this tick species is a vector candidate. It is important to note, however, that detection of DNA in the tick does not imply a life cycle contribution, as all hematophagous arthropods can potentially contain the DNA of blood pathogens. Further research is, therefore, necessary before a definitive role can be attributed.
(Peirce, 2000). One of the greatest points of contention surrounding the classification of the avian piroplasms within Babesia is that a majority of avian species develop cruciform tetrad schizonts in typical ‘Maltese cross’ formations and some may develop exoerythrocytic schizont life cycle stages, although this has been disputed (Peirce, 2000). The latter of these life cycle stages has previously been considered to be characteristic of Theileria (Zahler et al., 2000). Analyses carried out by Yabsley et al. (2006) and the present study found B. poelea to be phylogenetically similar to B. duncani and B. conradae (the Western clade piroplasms-prototheilerids), which also exhibit tetrad schizonts (Conrad et al., 2006; Kjemtrup et al., 2006) as do many of the species within the archaeopiroploasmid group (Zahler et al., 2000; Yokoyama et al., 2003) and could, therefore, be an ancestral characteristic among the piroplasms. It is possible that B. kiwiensis has simply retained this ancestral trait not observed in the other babesid group species and this warrants further study.

The current taxonomic paraphyly within the Piroplasmida (Reichard et al., 2005; Allsopp and Allsopp, 2006) is also exposed by the present study, with members of Babesia represented in all 3 phylogenetically distinct clades. While molecular-based analysis of these species will hopefully allow for a greater understanding of piroplasm biology, the taxonomic confusion that exists within Babesia and the Piroplasmida needs to be addressed urgently.

The current study has also shown that PCR offers an effective method of rapidly and accurately identifying kiwi infected with B. kiwiensis and is more sensitive than microscopic-based diagnosis in accordance with numerous other studies (Almeria et al., 2001; Ano et al., 2001). This detection method can, therefore, potentially be used to identify infected kiwi and to assess the pathogenicity of this infection in future studies aiming to conserve these threatened bird species.

The authors are grateful to Ngati Hine for supporting the collection and export of the samples from their traditional lands for this study. The samples were obtained from kiwi being studied as part of a Department of Conservation and Bank of New Zealand Save the Kiwi.

**Figure 1.** Phylogenetic tree constructed using partial 18S rRNA gene sequences based on distance (Gaultier and Gouy) and neighbor-joining analyses. Numbers above branches represent bootstrap percentages of 1,000 replicates. Avian species are shown in capitals and the novel sequence is indicated with an asterisk.
Trust project. We also wish to acknowledge Russ Hobbs, Parasitology Department at Murdoch University, for identification of the tick species. Mr. Down’s Honours Project was supported by a scholarship provided by Murdoch University.

LITERATURE CITED


Ticks, Amblyomma rotundatum (Acari: Ixodidae), on Toads, Chaunus schneideri and Chaunus granulosus (Anura: Bufonidae), in Northern Argentina

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ABSTRACT: This communication provides notes on 2 species of toads, Chaunus schneideri and Chaunus granulosus, infested with ixodid ticks, Amblyomma rotundatum, from the provinces of Corrientes and Formosa in northern Argentina. Chaunus schneideri is a new amphibian host record for A. rotundatum, a species previously reported to parasitize other anurans and also reptiles. We examined 74 ticks on 5 toads. All ticks were A. rotundatum; all adults were females, and all developmental stages were randomly attached to host body parts. Ticks remained
attached to one of the toads for from 7 to 17 days after the host was captured. One toad, encumbered with 33 ticks, was moribund when found and died shortly thereafter.

On the evening of 9 November 2006, we captured 4 male bunofid toads, Chaunus schneideri (Werner 1894) (formerly Byko paracematis Lutz 1925), on the 8-ha compound of the Centro de Ecologia Aplicada del Litoral (CECOAL), a field station in the suburbs of the city of Corrientes, in Corrientes Province (27°30’S, 58°45’W). CECOAL is operated by Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET) of Argentina. Chaunus schneideri is a giant toad, the largest species in Argentina. Weights and snout vent length (SVL) of the 4 toads were as follows: 654 g, 185.5 mm; 474 g, 154.4 mm; 546 g, 186.6 mm; and 516 g, 160.7 mm. We noticed that the first of these toads that we encountered feeding on insects (mostly beetles) at an insect light trap was conspicuously infested with hard ticks (Ixodidae). This prompted us to search the CECOAL compound for additional toads. We found 3 more males, and all 3 males carried ticks. We encountered no female C. schneideri on the compound. Ticks on male toads represented larvae (seed ticks) and engorged nymphs or adults. Five, 6, 9, and 18 were the tick counts for the 4 toads.

On 11 November 2006, we noted the locations of ticks attached to the toads and we used ice to release the ticks, which were removed from 3 of the toads with watchmakers’ forceps. We observed that each site of tick attachment to the toads’ skin showed a small, 2–4 mm reddened spot of inflammation. After we removed the ticks, we released the toads back into the CECOAL compound. The toads were robust, active, and none seemed negatively affected by their tick loads or by having had the ticks removed. Ticks were attached to the toads’ legs, feet, throat, abdomen (venter), back (dorsum), posterior quarter, and on the head. The points of attachment seemed random and evidenced no pattern to suggest that they had preferred feeding locations on the toads. However, we observed that no ticks were attached to these bufonids’ large parotid glands, which may contain high concentrations of the toxins bufobolin/bufoalbin. We removed ticks from the toads, and we killed the ticks in 100% ethanol; alcohol was changed after approximately 1 hr. We used a computer based video microscope system (ProScope®; Bodelin Technologies, Lake Oswego, Oregon) with 10 and 50× lenses to produce scaled images of each tick, and we used the NIH public domain ImageJ software to measure these images. We removed 29 ticks from 3 toads. The ticks ranged in size (length × width) from 1.03 × 0.78 mm to 9.28 × 5.33 mm. The range in ratios of length to width (a possible index of degree of engorgement) was 1.17 to 1.55. We held one of the infested toads in a laboratory basin, and we observed it once a day to determine when its ticks would voluntarily abandon their host. Abandonment for 4 ticks occurred at 7, 11, 12, and 17 days from the day of host capture. All of the ticks were Amblyomma rotundatum (Koch 1844) (Keirans and Oliver, 1993; and independently determined by Dr. Lorenza Beati).

On 6 December 2006, 1 of us (E.F.S.) captured an adult male Chaunus granulosus (Spix, 1824) at La Maravilla, 60 km southwest of Ingeniero G. Juárez, Formosa Province (23°54’S, 61°51’W). This specimen of a very small species was just 60 mm SVL and weighed only 16.2 g. It was infested with 3 A. rotundatum (confirmed by Dr. Lorenza Beati), each attached to a different leg. Chaunus granulosus has previously been recognized as a host of A. rotundatum (Burridge and Simons, 2003, and references therein). It would seem that this relatively tiny toad species might be especially vulnerable to a critical loss of blood taken by feeding ticks.

Years earlier, on 26 December 1998, 1 of us (A.L.K.) found a heavily infested male C. schneideri on the CECOAL compound. This individual contained 33 ticks on its legs, feet, and body. The toad was 137.8 mm long (SVL) and emaciated, weighing a slight 365 g. Remarkably, this toad was found during the day walking very slowly and in a labored manner. The air temperature at the time was >30°C. Chaunus schneideri is rarely seen moving during the daylight hours and especially not when the air temperature is high. Each time the toad had advanced approximately 40–50 cm, the animalarched its back with nose and posterior pointed high creating a contorted and aberrant concave posture that it held for several seconds. This pattern of behavior was observed repeatedly before the toad was taken to the laboratory, where it flattened itself against the floor and remained motionless until it died <30 min later. We attribute the morbidity and mortality of this toad to exsanguination (see Keirans and Durden, 1998) by its extraordinary load of ticks. The toad with its attached ticks was preserved in formaldehyde shortly after it died. Recently, we removed 9 ticks from the preserved specimen, and we measured them with calipers. These ticks ranged in size from 5.6 × 4.4 to 15.8 × 11.4 mm (length × width). The 9 randomly removed ticks were all A. rotundatum (determined by Dr. Lorenza Beati).

This toad’s morbidity and death reinforces previous assertions that ticks may play an important role in regulating natural toad populations in tropical and subtropical regions of the New World (Oba and Schumaker, 1983; Lampo and Bayless, 1996). If this is true, ticks could have value as a biological control agent for exotic “giant toads” that have been introduced into Australia and the United States from Central and South America. Unregulated populations of these enormous exotic toads have expanded dramatically, poisoning pets and predators, and they are threatening native species by both indirect and direct competition. Amblyomma rotundatum has been reported on exotic giant toads, Chaunus marinus (Linnaeus, 1758) (formerly Byko marinus), from the greater Miami, Florida area (Oliver et al., 1993), but the impact of the ticks on the introduced U.S. C. marinus population has not been evaluated.

All of the ticks we recovered from the 6 toads we examined were males. Populations of A. rotundatum from within its historic range in Central and South America have long been known to be parthenogenetic (Robinson, 1926), but 1 laboratory-reared male (Keirans and Oliver, 1993) and 1 male from a field-collected host (Labruna et al., 2005) have been found. Parthenogenesis may have evolved in these ticks of reptiles and amphibians because of the difficulty of movement on reptilian and amphibian skin (compared with feathered or furred skin) by adult males to find and mate with adult females. If the parent species to A. rotundatum was a sexually reproducing parasite of mammals, birds, or both, and in the course of its speciation it underwent a host shift to amphibians and reptiles, we can imagine that a proportion of females of the new species might have gone unmasked owing to the relative difficulty of male movement to access females on the slippery amphibian and reptilian skin. Failure to be mated could have created intense selection on females to produce asexual offspring. Molecular phylogenetics data indicate that the hypothesized host shift from mammals or birds to amphibians and reptiles within species of Amblyomma is probable, suggesting that the taste for amphibian and reptilian blood is derived (Klampen et al., 1996).

Dr. Lorenza Beati, U.S. National Tick Collection, Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, Georgia, kindly examined and identified all of tick specimens and helped enlighten R.L.S. about tick evolution. All of the tick specimens have been contributed to the U.S. National Tick Collection and received accession numbers 124001–124010. R.L.S. and J.A.S. are grateful to Dr. Lorenza Beati, Alicia Pot de Neiff for providing living quarters, assistance, congenial company, and generous hospitality during their stay at the CECOAL in Corrientes.

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A Cautionary Note on the Use of Nested PCR for Parasite Screening—An Example From Avian Blood Parasites

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ABSTRACT: The use of new powerful nested polymerase chain reaction (PCR) techniques to identify and screen for prevalence of parasites has a huge potential. It allows for the detection and identification of low-intensity infections, but its high sensitivity and technical setup may also induce problems. Here, we report a cautionary note regarding misleading amplification of avian malaria species (Haemoproteus and Plasmodium) during Leucocytozoon spp. detection. We used a previously described nested PCR method for the molecular detection of avian malaria and Leucocytozoon spp. In the first step of the PCR protocol, these parasites are detected simultaneously; in the second PCR, Haemoproteus and Plasmodium spp. are separated from Leucocytozoon spp. However, in certain cases when a bird was infected with avian malaria, we obtained a slightly longer PCR product during the detection of Leucocytozoon spp. Our data imply that these “false” Leucocytozoon fragments are the consequences of strong amplification of certain malaria lineages in the first PCR, which can also be detected after the second PCR amplification that is specific to Leucocytozoon spp. parasites. Because these “false” Leucocytozoon fragments are slightly longer than the normal Leucocytozoon fragments, we suggest the use of well-separating agarose gels, several positive controls, and molecular standards to facilitate their separation. If one obtains a fragment that differs in length from the one expected for Leucocytozoon spp., sequencing is essential. More generally, in order to limit this type of problem with nested PCR protocols, we suggest that the first and the second primer pair be chosen so that they have different annealing temperatures.

Using molecular methods for detecting microorganisms requires high sensitivity because these organisms often occur in low numbers (intensities) in their hosts or the environment. One way of increasing the sensitivity of a polymerase chain reaction (PCR) is to apply a nested approach, where the screening is conducted with the use of two PCRs that are performed sequentially. However, this approach is more costly and takes additional time. In addition, along with the increase in sensitivity comes the risk of contaminations and amplification of nonspecific genes, i.e., genes for which the primers were not designed (reviewed in Burkard, 2000; Freed and Cann, 2006). To ensure that the correct target gene has been amplified, most studies also sequence the PCR product. However, as the sample sizes in data sets used for molecular, biological, and ecological studies steadily increase, combined with a decrease in the cost of running PCRs, large-scale ecological and biological studies may use nested PCR protocols just to screen samples for positive or negative amplifications for a group of parasites or microorganisms. To ensure the validity of such studies, it is therefore of importance to investigate and note any shortcomings or pitfalls that occur when nested PCR methods are used to screen for microorganisms.

The study of avian haemosporidian parasites, i.e., Haemoproteus, Plasmodium, and Leucocytozoon spp., is one area that has greatly benefited from the use of different PCR methods in the detection of parasites from blood samples (Li et al., 1995; Bensch et al., 2000; Fallon, Ricklefs et al., 2003). Thus, several studies have demonstrated that PCR-based methods have higher sensitivity at low levels of parasitemia compared to the traditional microscopic examination of blood smears (Richard et al., 2002; Waldenström et al., 2004), though they are not flawless (Cosgrove et al., 2006; Valkiūnas et al., 2006). Comparing different molecular methods further showed that nested PCRs have the ability of detecting lower degrees of parasitemia compared to single PCRs (Waldenström et al., 2004). Thanks to these new methods for detecting blood parasites, there has been a recent boom in studies on the distribution and prevalence of avian haemosporidian parasites all over the world (Perkins and Schall, 2002; Waldenström et al., 2002; Fallon, Berningham, and Ricklefs, 2003; larvi et al., 2003; Beadell et al., 2004; Kimura et al., 2006; Hellgren et al., 2007).

Here, we report a cautionary note regarding misleading amplifications due to a carryover effect of the first PCR round primers in the second reaction, when a nested PCR protocol is being used. The procedure was designed by Hellgren et al. (2004) for the simultaneous detection of Haemoproteus, Plasmodium, and Leucocytozoon spp. We applied it to 495 blood samples collected from different bird species, including both European migrant and African resident species. In short, the blood samples were collected in SET buffer (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0), kept at −20°C until analysis, and extracted using the standard ammonium–acetate protocol (Nicholls et al., 2000). The concentration of genomic DNA was set to 25 ng/µl; a nested PCR was performed. In the first PCR, 2 µl of the host DNA was used. The first primers were targeted at the mtDNA of the parasites, amplifying a 570-bp-long fragment (617-bp fragment including primers) of the cytochrome b gene from species of Haemoproteus, Plasmodium, and Leucocytozoon. In the second PCR, the detection of avian malaria parasites (Haemoproteus and Plasmodium, sensu Pérez-Tris et al., 2005) is separated from the detection of Leucocytozoon spp. by the use of different and more specific primers. In these reactions, 2 µl of 1 µl PCR product from the first reaction was used (for Leucocytozoon spp. and avian malaria, respectively). The second primer pairs bind to the fragment amplified in the first reaction, producing smaller, 478- and 480-bp-long fragments (526 and 527 bp with primers), for Leucocytozoon spp. and avian malaria, respectively (Hellgren et al., 2004). All reactions were performed in 25-µl volumes and both negative (ddH₂O) and positive controls (samples from birds that were previously confirmed to be infected) were applied to control for possible contamination or failures during PCRs. Amplified PCR products were sequenced with the use of the AmpliCycle® sequence kit on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, California), in accordance with the manufacturer’s recommendations. Sequences were edited and aligned with the use of the program BioEdit (Hall, 1999) and identified to genus level based on their position in the phylogenetic tree of previously identified parasites (Hellgren et al., 2007) with the use of the software MEGA2 (Kumar et al., 2001).

In the second PCR, specific for Leucocytozoon spp., 123 of the 495 samples produced a PCR product. However, in 23 cases, the fragments seen on agarose gels were slightly longer than the usual 526-bp-long Leucocytozoon spp. species–specific fragment, including primers (Hellgren et al., 2004) (Fig. 1). Despite several trials, we were not able to se-
our 181 malaria-positive samples, only 9 produced the 617-bp-long distance: lineages in our database (mean Jukes-Cantor distance: 0.055 ± 0.006 SE; Plasmodium spp.: 0.058 ± 0.006 SE). This indicates that the 9 lineages do not group into a closely related clade of avian haemosporidians and the strong amplification might be a result of high parasite intensity, or analogous mutations in the primer binding sites that increase the amplification success. The latter notion is supported by the study of Valkíñnas et al. (2006), who found that in case of mixed infections of avian malaria parasites, some lineages were detected preferentially, but that this was not related to the level of parasitemia of different lineages in the blood. In addition, Sowmya et al. (2006) showed that differences in the primer-binding sites can affect the amplification success.

Based on our results, we suggest the necessity to apply molecular standards and positive controls in each gel during the detection of Leucocytozoon spp. parasites and run PCR products in a well-separating agarose gel for a period long enough to be able to detect differences between fragment lengths. If deviations from the standard Leucocytozoon species fragment length are detected, then sequencing is essential to avoid the risk of considering “false” detections as normal Leucocytozoon spp. infection. More generally, to avoid the problems that primer pairs from the first reaction continue to amplify also in the second PCR when a nested approach is used, we suggest the following. First, primer pairs should be designed so that the optimal annealing temperatures for the first and second primer pairs, if possible, are different. Second, the amount of primers used in the first reaction should be optimized so that the amount of leftover is reduced without affecting the outcome of the results.

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**LITERATURE CITED**


Helminth Fauna of the Nine-Banded Armadillo (Dasypus novemcinctus) in North-Central Florida

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ABSTRACT: The gastrointestinal tracts of 32 free-ranging, 9-banded armadillos (Dasypus novemcinctus) from north-central Florida were examined for the presence of helminths during July 1991 to November 1993. Aspidodera sp. (Nematoda: Aspidoderidae), most closely resembling Aspidodera sogandaresi, were recovered from 20 of 32 armadillos examined. Total numbers of A. sogandaresi ranged from 1 to 1,021 per infected animal, and followed an inverse correlation to body condition index for those animals. The cystacanth stage of 1 acothophalan, Macracanthorhynchus ingens, was present in 1 armadillo, and is the first report of M. ingens in the 9-banded armadillo. The present study contributes to the known natural history of the 9-banded armadillo, an important animal research model.

Armadillos (Dasypus novemcinctus) are an important animal model with unique research potential that predominantly reflects their tendency to produce monozygous quadruplets and an unusual susceptibility to Mycobacterium leprae and other human disease agents (Storrs, 1971). Attempts to breed these animals in captivity have been largely unsuccessful thus far, prompting most research programs to utilize wild-caught armadillos (Storrs, 1987). However, limited epidemiological studies have shown that 5–10% of wild-caught armadillos from Texas and Louisiana have leprosy (Walsh et al., 1975; Meyers et al., 1978; Walsh et al., 1986; Storrs, 1987). Because wild armadillos in peninsular Florida are free of leprosy, these are the preferred choice for use in research (Storrs, 1987). Consequently, it is desirable to gather data concerning factors that might impact the general health of wild armadillos in peninsular Florida. In the present study, the prevalence and abundance of helminths in free-ranging armadillos from north-central Florida are described.

In 1991–1993, 32 9-banded armadillos were collected in Alachua County, Florida. Armadillos were either the victims of automobile impact or had been shot as pests. Upon collection, the gastrointestinal (GI) tracts were dissected; the stomach, small intestine, and large intestine were examined separately for the presence of helminths. The mucosa of each section of the GI tract was scraped and combined with gut contents from that section, then processed by sedimentation. The resultant sediment was examined in a backlit box. Helminths were identified using a body condition index (BCI) calculated for 25 animals with the use of the following equation:

\[ BCI = \frac{\text{actual body mass (kg)}}{\text{expected body mass (kg)}} \times 100 \]

where:

- expected body mass = \( \frac{\text{pectoral length (cm)}}{0.22725} \times 100 \)
- pelvic length (cm)\]

The equation used to calculate expected body weight was determined by regressing body weight against the cubed sum of pelvic length and pectoral length for a large number of adult armadillos (L. H. Herbst, pers. comm.). Thus, a BCI of 1.0 indicates an animal of “average” condition, and deviations above and below reflect those animals that weigh more or less than expected, respectively.

Aspidodera species were recovered from the large intestines from 20 of the 32 armadillos, and were also present in the small intestines of 10 of these 20 infected animals. Total numbers were counted in 12 of the 20 armadillos, and ranged from 1 to 1,021 per infected host. In the remaining 8 armadillos, the number of Aspidodera sp. present was estimated to be either greater than or less than 100 nematodes. For purposes of this study, intensities were defined as heavy (greater than 100 nematodes); 14 and 18 animals had heavy and light infections, respectively.

The expected body weight and condition index were calculated for 25 animals. When the relationship between body condition and the total number of Aspidodera sp. was examined for 12 animals, a significant inverse correlation between body condition and infection status was observed (Fig. 1). We used a Student’s t-test to compare average body condition for 10 animals with greater than 100 Aspidodera sp. nematodes (high intensity) to animals with fewer than 100 Aspidodera sp.
(light load). Animals with light intensities had a significantly higher \( P < 0.05 \) BCI than animals with heavy intensities. This inverse relationship was also significant when analyzed by season in which animals were collected (Fig. 2).

Of the 10 species of *Aspidodera* that have been described, 7 are found in armadillos. All 7 species occur in the Neotropical region; only 2, *Aspidodera fasciata* and *A. sogandaresi*, occur both in the Neotropical and Nearctic regions. A characteristic feature of species of *Aspidodera* is the presence of cephalic cords that form usually 6–9 longitudinal loops. In the present study, a total of 10 females and 13 males of *Aspidodera* sp. were examined under a compound microscope after being cleared in lactophenol. In these specimens, 6 cordons were observed, the same number as both *A. fasciata* and *A. sogandaresi*. However, the average length of all specimens was 5.97 mm (5.39–6.44 mm) for females and 6.50 mm (4.78–7.31 mm) for males, lengths that are more consistent with *A. sogandaresi*. In addition, egg dimensions from ova examined in utero in specimens from this study were more oval (mean 49.7 \( \times \) 72.6 \( \mu \)m) compared to *A. fasciata* (40 \( \times \) 57 \( \mu \)m) and dimensions were more similar to *A. sogandaresi* (50 \( \times \) 71 \( \mu \)m) (Santos et al., 1990; Jimenez-Ruiz et al., 2006). Some measurements, however, were not consistent with *A. sogandaresi* or *A. fasciata*. For example, mean distance from vulva to cephalic end for these *Aspidodera* sp. was 3.528 \( \mu \)m, whereas mean distances for *A. fasciata* and *A. sogandaresi* were 2,265 and 2,900 \( \mu \)m, respectively, although values were not significantly different statistically. Mean esophagus lengths were also somewhat different, but fell within the range of both *A. sogandaresi* and *A. fasciata*. Based on the most morphological similarities, the *Aspidodera* sp. recovered from armadillos in the present study most closely resemble *A. sogandaresi*.

In addition to *A. sogandaresi*, a cystacanth stage of the acanthocephalan *M. ingens* was recovered from mesenteries of the small intestine of an armadillo heavily infected with the *Aspidodera* species. Other unidentified nematodes were also collected. An unidentified nematode and 3 unidentified pinworms were found in the stomachs of 2 individual armadillos, and an animal heavily infected with *A. sogandaresi* was coinfected with 1 pinworm in the small intestine and another unidentified nematode in the large intestine. No helminth ova were detected in the feces of the 21 fecal samples examined, although 10 of the 21 armadillos were heavily infected with *A. sogandaresi*.

The range of the 9-banded armadillo encompasses much of the Neotropical region; of the 20 species of armadillos, it is the only one to have extended its range into the United States. Its rapid expansion into the United States began entering into the western border of Florida (Humphrey, 1974; Storrs, 1987). Prior to this study, 10 helminths had been reported from 9-banded armadillos in the United States. These include the trematode *Brachylaemus virginiensis*; a cestode, *Oochoristica* sp.; 2 acanthocephalans, *Hamannella* sp. and *Onicola canis*; and 7 nematodes, *Dipetalonema averyi*, *Aspidodera fasciata*, *A. sogandaresi*, *Physcocephalus* sp., *Ascarops* sp., *Physalotera* sp., and *Gnathostoma* sp., with the latter 3 using armadillos as a paratenic host (Chandler, 1946, 1954; Storrs, 1971; Eberhard, 1982; Cockman-Thomas et al., 1993; Jimenez-Ruiz et al., 2006). The present study reports the presence of an *Aspidodera* species that is most similar to *A. sogandaresi* and reports, for the first time, *M. ingens* in the 9-banded armadillo. Because there was a negative correlation between *Aspidodera* sp. numbers and BCI, these nematodes appear to have an adverse influence on the armadillo. Consequently, determining the helminth fauna and prevalence in 9-banded armadillos is critical for a comprehensive understanding of the animal’s natural history, particularly when wild populations are to be used as models for human research.

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**LITERATURE CITED**


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