J. Parasitol., 95(6), 2009, pp. 1408–1414

FIRST INTERMEDIATE HOST OF THE DIGENEAN TREMATODE *PROCTOECES LINTONI* (FELLODISTOMIDAE) IN CHILE

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ABSTRACT: The digenetic trematode, *Proctoeces lintoni*, is a parasite of the rocky intertidal ecosystems of the Chilean Pacific coastline. Although this species is relatively well known, the first intermediate host has not yet been described. In the present study, we used experimental protocols and field studies to identify the host that harbors the sporocysts and cercariae stages of the parasite. The first intermediate host was shown to be the dominant mussel of the mid-littoral zone, *Perumytilus purpuratus*.

The taxonomic state of *Proctoeces* sp. has been frequently questioned due to the difficulty in identifying diagnostic characters for the species (Bray and Gibson, 1980; Bray, 1983). There is apparently great variability in the form, body size, and internal structures, even among individuals of the same population that inhabit different hosts (Freeman, 1962). Fourteen *Proctoeces* spp. have been described; however, intraspecific morphologic variation and the inadequate recognition of taxonomic characteristics have raised questions regarding the validity of some of these putative species (Freeman and Llewellyn, 1958; Stunkard and Uzmann, 1959; Freeman, 1962; Manter and Pritchard, 1962; Prévot, 1965). As a result, Bray and Gibson (1980) reduced the number of species in the genus to 3 and, later, Bray (1983) considered them all to be synonymous of *Proctoeces maculatus*.

The great morphologic and morphometric variability of *P. maculatus* has been associated with its broad, worldwide distribution and the great plasticity of life histories that it presents (Bray, 1983). Several developmental stages of *P. maculatus* have been found along the coasts of Europe, South Africa, Japan, Australia, United States, and Chile (see review in Bray [1983], Wolf et al. [1987], and Lasiak [1989]), confirming the broad distribution of the genus. Within these areas of the world, *P. maculatus* displays diverse life cycle patterns in a broad variety of hosts (Bray, 1983). For example, the larval stages (sporocysts and cercariae) have been found in mytilid bivalves, i.e., several species of *Mytilus* and *Brachidontes* (= *Ischadium*) (Uzmann, 1953; Hopkins, 1954; Stunkard and Uzmann, 1959; Prévot, 1965; Canzonier, 1972; Wardle, 1980). The metacercariae have been described in a range of echinoderms, annelids, cephalopods, and molluscs (Bray, 1983). The adult stage has been reported in more than 50 species of marine teleost fishes (Freeman and Llewellyn, 1958; Prévot, 1965; Yamaguti, 1971; Wardle, 1980; Bray, 1983).

In Chile, one of the host–parasite systems that has received considerable attention involves the digenean trematode *P. lintoni* Siddiqi and Cable 1960 (Oliva and Huaquin, 2000; Balboa et al., 2001; Ponciano, 2001; Loot et al., 2005, 2008). *Proctoeces lintoni* is transmitted to the clingfish *Sicyases sanguineus* (Gobiesocidae), its definitive host, through consumption of keyhole limpets *Fissurella* spp. (Archaeogastropoda), the second intermediate host that harbors the metacercaria (Oliva, 1984; Oliva and Zegers, 1988; George-Nascimento et al., 1998; Balboa et al., 2001). However, the hosts for the larval stages of *P. lintoni* have not been described from Chilean waters.

On the Chilean coast, 3 species of mussels, i.e., *P. purpuratus* (Lamarck), *Semimytilus algosus*, and *Brachidontes granulata*, represent 30% of the sessile species of the middle intertidal rocky communities (Broitman et al., 2001). *Perumytilus purpuratus* is one of the most common rocky intertidal mussels along the Pacific coasts of South America, from Ecuador to the Strait of Magellan (Osorio and Bahamonde, 1968; Castilla, 1981). In Chile, it is commonly known as "chorito maico" and is the dominant sessile organism of the rocky middle intertidal shore (Castilla, 1981; Castilla and Durán, 1985; Paine et al., 1985). The mussel is able to competitively exclude other sessile species such as algae and barnacles (Paine et al., 1985; Navarrete and Castilla, 1990), forming dense, 3-dimensional matrices (Alvarado and Castilla, 1996).

We hypothesize that the first intermediate host of *P. lintoni* in Chile is a mussel species, as has been reported in 16 other geographical zones worldwide for the mussels *Mytilus edulis*, *M. galloprovincialis*, and *Ischadium recurvum* (= *Brachidontes recurvis*) (Uzmann, 1953; Hopkins, 1954; Stunkard and Uzmann, 1959; Prévot, 1965; Canzonier, 1972; Wardle, 1980). In the present study, we have identified the mussel species that host the sporocysts and cercariae stages of *P. lintoni* in Chile.

MATERIALS AND METHODS

Natural and experimental infection of mytilids

Perumytilus purpuratus, *S. algosus*, and *B. granulata* mussels were collected from 8 localities along the coast of Chile: Carrizal Bajo (28°04′S, 71°09′W), Punta Choros (29°14′S, 71°28′W), "El Quisco" (33°23′S, 71°41′W), Fundación Chile (F. Chile) (33°28′S, 71°37′W), Las Cruces Sur (33°29′S, 71°37′W), Pelluhue (35°50′S, 72°36′W), Cocholgüe (36°35′S, 72°58′W), and Pucatrihue (40°31′S, 73°43′W). Samples were taken during summer (December 2002–March 2003) and winter (June–August 2003), with the exception of Carrizal Bajo and Pucatrihue, where sampling was done only in summer. At each location and season, we took samples of 600 individuals of each species except *B. granulata*, for which samples of only 100 individuals were collected due to its limited availability and the difficulty involved in accessing its microhabitat. Mussels were taken to the laboratory, where they were frozen (−20 C), then thawed and examined using a stereomicroscope to determine the presence of larval stages of *P. lintoni*.

Of the 3 species of mussels that we studied, only *P. purpuratus* harbored larval stages of the digenean (Table I). To confirm the identity of the parasite, 3 replicates of 300 individuals of *P. purpuratus* mussels of similar body size were exposed to eggs of *P. lintoni* obtained from clingfishes (*S. sanguineus*). Larval stages obtained experimentally were compared with those observed in naturally infected mussels. The clingfishes from intertidal and subtidal rocky shores in central Chile were killed with an overdose of ether, and adult parasites of *P. lintoni* were recovered from the

Received 3 January 2009; revised 22 April 2009; accepted 16 June 2009.
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DOI: 10.1645/GE-2004.1

TABLE I. Prevalence (%) of larval stages of digenetic trematodes in *P. purpuratus*, *S. algosus*, and *B. granulata*, at 8 localities along the coast of Chile, during summer and winter. Sample size is indicated in parentheses.

Locality (latitude S)	Species	Summer	Winter
Carrizal Bajo (28°04′S, 71°09′W)	*P. purpuratus*	2.83 (600)	—
	S. algosus	0 (600)	—
	B. granulata	0 (100)	—
Punta Choros (29°14′S, 71°28′W)	*P. purpuratus*	3.33 (600)	3.0 (600)
	S. algosus	0 (600)	0 (600)
	B. granulata	0 (100)	0 (100)
El Quisco (33°23′S, 71°41′W)	*P. purpuratus*	4.17 (600)	4.5 (600)
	S. algosus	0 (600)	0 (600)
	B. granulata	0 (100)	0 (100)
F. Chile (33°28′S, 71°37′W)	*P. purpuratus*	1.83 (600)	2.67 (600)
	S. algosus	0 (600)	0 (600)
	B. granulata	0 (100)	0 (100)
Las Cruces Sur (33°29′S, 71°37′W)	*P. purpuratus*	1.33 (600)	1.67 (600)
	S. algosus	0 (600)	0 (600)
	B. granulata	0 (100)	0 (100)
Pelluhue (35°50′S, 72°36′W)	*P. purpuratus*	1.83 (600)	2.67 (600)
	S. algosus	0 (600)	0 (600)
	B. granulata	0 (100)	0 (100)
Cocholgüe (36°35′S, 72°58′W)	*P. purpuratus*	2.83 (600)	3.17 (600)
	S. algosus	0 (600)	0 (600)
	B. granulata	0 (100)	0 (100)
Pucatrihue (40°31′S, 73°43′W)	*P. purpuratus*	2.0 (600)	—
	S. algosus	0 (600)	—
	B. granulata	0 (100)	—

intestinal tract of the fishes. Eggs released from the uterus of adult parasites, spontaneously or with the help of needles, were stored in Petri dishes with sea water. The 3 replicates were exposed for 3 consecutive days to the eggs extracted from 10 *P. lintoni*. For controls, we used the same number of replicates and individuals under the same conditions of water circulation and constant aeration, but without exposure to parasite eggs. The maximum shell length of the mussels was measured using a Bull Tools vernier caliper 6"/150 mm (Bull Tools, Ningbo City, China). Control and experimental mussels were obtained from Las Cruces, a location that exhibited the smallest prevalence of natural infection in central Chile.

Ten weeks after the infection event, all mussels were measured, dissected, and examined using an Olympus SZ51 stereomicroscope (Olympus Corporation, Tokyo, Japan) to verify the presence of sporocysts and cercariae. The mussels that died during the development of the experiment were also necropsied. The success of the experimental infections was statistically assessed using a chi-square goodness-of-fit test. Homogeneity among replicates was analyzed by means of a heterogeneity chi-square test (Sokal and Rohlf, 1981; Zar, 1984). The similarity of maximum shell length of mussels between treatments was tested using analysis of variance (ANOVA). Prior to the analysis, shell length data were log-transformed to meet the assumptions of parametric tests.

TABLE III. Morphometric variables considered in the principal component analysis and their contribution to the first 2 derivative axes.

	First component	Second component
Cercariae width	−0.62	−0.58
Oral sucker length	−0.84	0.46
Oral sucker width	−0.90	0.09
Oral sucker diameter	−0.91	0.29
Pre-pharynx length	−0.79	−0.30
Pharynx length	−0.84	0.38
Pharynx width	−0.91	0.20
Acetabulum length	−0.88	−0.15
Acetabulum width	−0.90	−0.32
Acetabulum diameter	−0.93	−0.22

Description of larval stages

To confirm the taxonomic identity of the parasites in *P. purpuratus*, diagnostic characters for the species were estimated (Yamaguti, 1971; Shimura and Egusa, 1979a; Bray and Gibson, 1980), using the program Imagen Pro Plus. The morphometric observations of cercariae were performed using live specimens stained by neutral red, under light cover glass pressure (Wardle, 1980). The parasites in naturally infected mussels were examined morphometrically in winter and summer to consider the possible variations due to parasite development (Stunkard and Uzmann, 1959; Cheng, 1967; Lang and Dennis, 1976). The values were divided by the length of the cercariae to estimate the relative size of the structures, instead of absolute size, in order to eliminate any size effect in the data set when comparing cercariae of different development stages. Variation should be attributable to body shape differences, and not be related to the relative size of the cercariae (Zelditch et al., 2004).

The morphometric study of natural and experimental cercariae was performed using a principal components analysis based on the correlation matrix between 10 morphometric traits. The effect of larval source (winter natural, summer natural, or experimental) on the morphometric measurements was tested using an ANOVA over the score of the component that explained a larger proportion of the variance. We used a Tukey multiple comparison test to establish differences among groups.

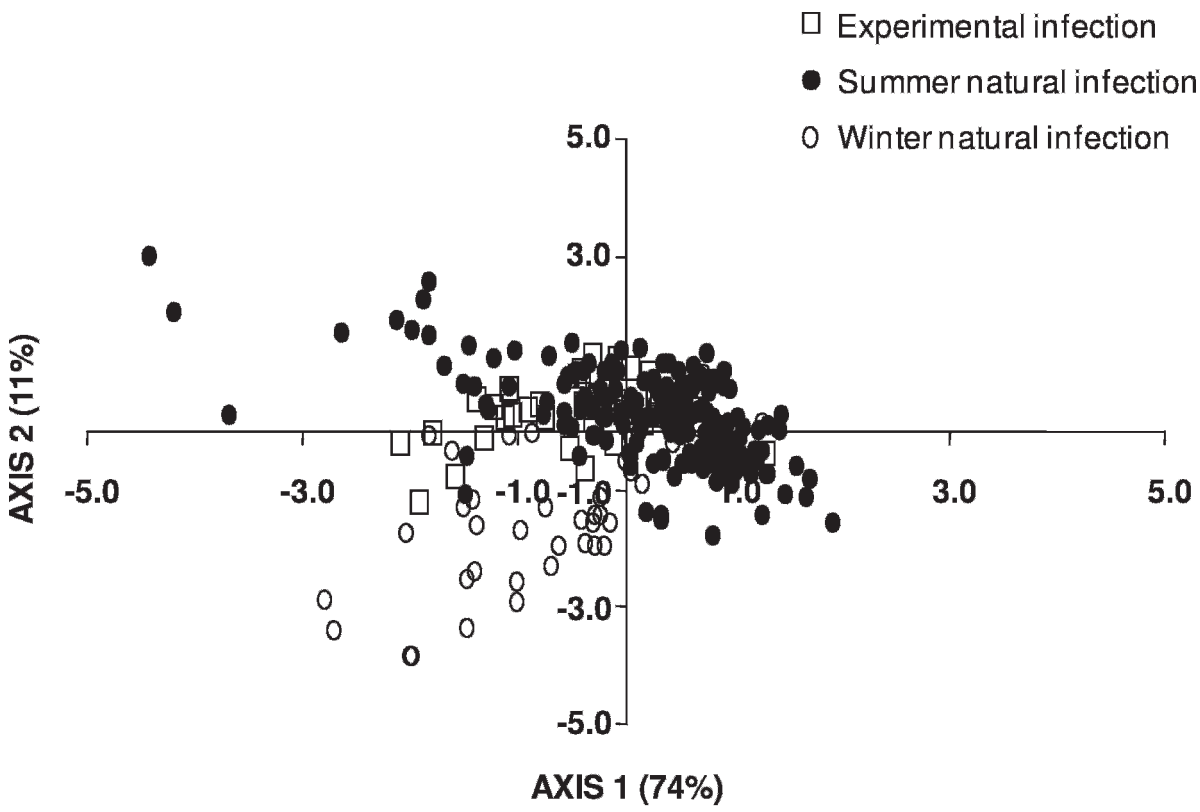
RESULTS

Natural and experimental infection of mytilids

In every location, observations showed that digenean larval stages occurred only in *P. purpuratus* (Table I). Additionally, results of experimental infections revealed a greater frequency of infection of *P. purpuratus* in replicates exposed to parasite eggs than in the controls (Table II). Heterogeneity chi-square tests

TABLE II. Results of experimental infection of *P. purpuratus* exposed to eggs of *P. lintoni* from clingfishes *S. sanguineus*. Prevalence of parasites (%) and mean total length of mussels (± SD), are indicated in each treatment and replicate (n per group = 300 mussels). Significant P values ($P < 0.05$) are indicated with an asterisk (*).

Replicates	Variable	Infected	Control	Analysis
1	Prevalence	4.67	1.33	$\chi^2 = 5.54$; g.l. = 1; $P = 0.018$*
	Total length	23.37 ± 3.69	23.73 ± 2.94	$F_{(1,\ 598)} = 2.92$; $P = 0.088$
2	Prevalence	4	1	$\chi^2 = 4.0$; g.l.= 1; $P = 0.046$*
	Total length	23.47 ± 2.87	23.26 ± 2.78	$F_{(1,\ 598)} = 0.79$; $P = 0.376$
3	Prevalence	5	1.33	$\chi^2 = 6.37$; g.l.= 1; $P = 0.012$*
	Total length	23.25 ± 3.01	23.05 ± 2.86	$F_{(1,\ 598)} = 0.69$; $P = 0.406$
Total	Prevalence	4.1	1.1	$\chi^2 = 17.31$; g.l. = 1; $P < 0.001$*
Heterogeneity between replicates				$\chi^2 = 1.38$; g.l. = 2; $P = 0.502$



AXIS 1

		{1}	{2}	{3}
		-0.42766	-0.546685	0.2265938
Experimental	{1}		0.843363	0.006341
Winter field infection	{2}	0.843363		0.000232
Summer field infection	{3}	0.006341	0.000232	

FIGURE 1. Graphic result of principal component analysis (PCA). Scores for 2 axes on 10 morphometric variables obtained from experimental and natural (winter and summer) infections of *P. purpuratus* with *P. lintoni* cercariae. The box below shows the results of the Tukey's multiple comparison tests to evaluate the statistical significance of differences among groups along the first principal component.

revealed homogeneity of variance across replicates; therefore, they can be grouped (Table II). The maximum shell length of the mussels did not vary significantly between treatments (Table II).

Principal component analysis demonstrated that 85% of the cumulative variance in the morphology of cercariae is explained by components 1 and 2, and 74% is explained by the first component (Table III; Fig. 1). Graphic representation of the morphometric measurements of natural and experimental cercariae showed great overlap (Fig.1). The ANOVA over the first principal component indicated that the experimental cercariae did not differ from the ones obtained naturally during summer. However, both groups were significantly different from winter cercariae (Fig. 1). The morphometric variables that were best correlated with the first component included: width and diameter of the oral sucker, width and diameter of the acetabulum, and pharynx width (Table III). The oral sucker diameter and pharynx width were not significantly different among experimental cercariae, summer naturals, and winter naturals (sucker diameter: $F_{(2,\ 236)} = 1.74$, $P = 0.177$; pharynx width: $F_{(2,\ 242)} = 2.12$, $P = 0.123$) (Fig. 2). However, the oral sucker width, as well as width and diameter of the acetabulum of natural winter cercariae, were significantly greater than those of natural summer cercariae and/or experimental cercariae (sucker diameter: $F_{(2,\ 245)} = 4.51$, $P < 0.012$; acetabulum width: $F_{(2,\ 245)} = 17.66$, $P < 0.001$; acetabulum diameter: $F_{(2,\ 239)} = 12.57$, $P < 0.001$); however, no significant differences were observed between these latter groups (Fig. 2). Additionally, natural summer cercariae were longer than natural winter cercariae, while experimental cercariae presented interme-

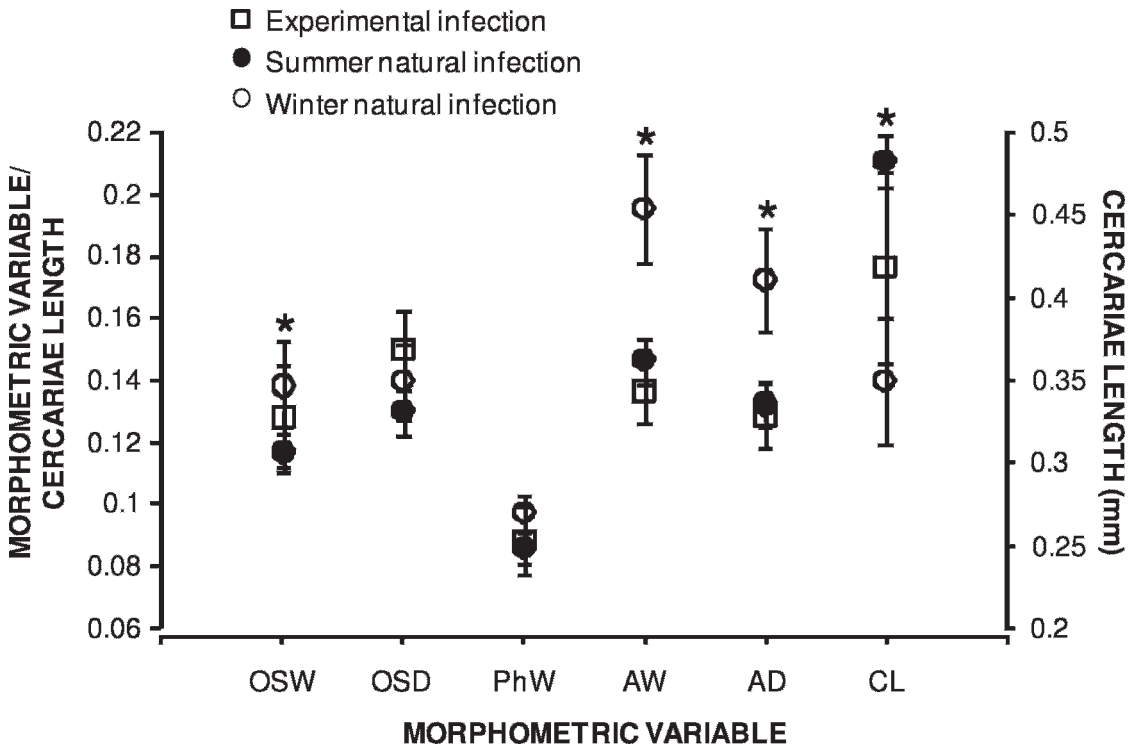


FIGURE 2. Morphometric variables (average ± 2 EE) correlated with the first principal component score evaluated in experimental and natural *P. lintoni* cercariae. OSW, oral sucker width; OSD, oral sucker diameter; PhW, pharynx width; AW, acetabulum width; AD, acetabulum diameter; CL, cercariae length. Means that are significantly different from each other are indicated with an asterisk (*).

diate values between both groups ($F_{(2,\ 274)} = 23.59$, $P < 0.001$; Tukey test, $P < 0.003$) (Fig. 2).

Description of larval stages

The larval stages are regularly distributed inside the visceral mass adjacent to the surface of the mussel mantle, especially in blood sinuses and lymph spaces of the gonad and digestive glands, which can be completely replaced. The number of sporocysts found in *P. purpuratus* individuals ranged from 6 to 4,374, while their lengths ranged from 0.1 to 2.3 mm. Sporocysts are characterized by a light orange pigmentation that extends into the host gonadal tissue, which is normally light yellow (in females) or dark brown (in males), and by the presence of a pore at one end. In each sporocyst, approximately 20–30 cercariae in various stages of development could be seen, but only 2–5 appeared to be mature (Fig. 3).

The cercariae are elongated to oval, and are thinner toward the anterior and posterior ends. Structures most evident were the oral and ventral (acetabulum) suckers, the pharynx, the excretory bladder, and the intestinal caeca (Fig. 4). The oral sucker is found

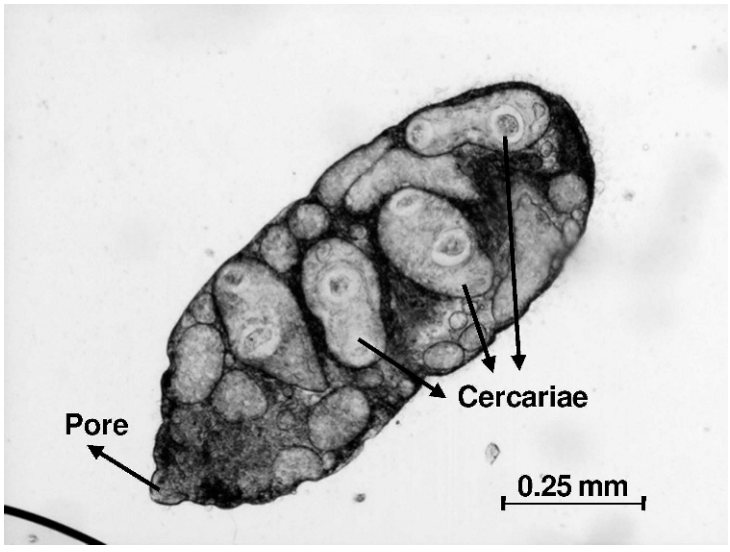


FIGURE 3. *Proctoeces lintoni* sporocysts obtained from *P. purpuratus*.

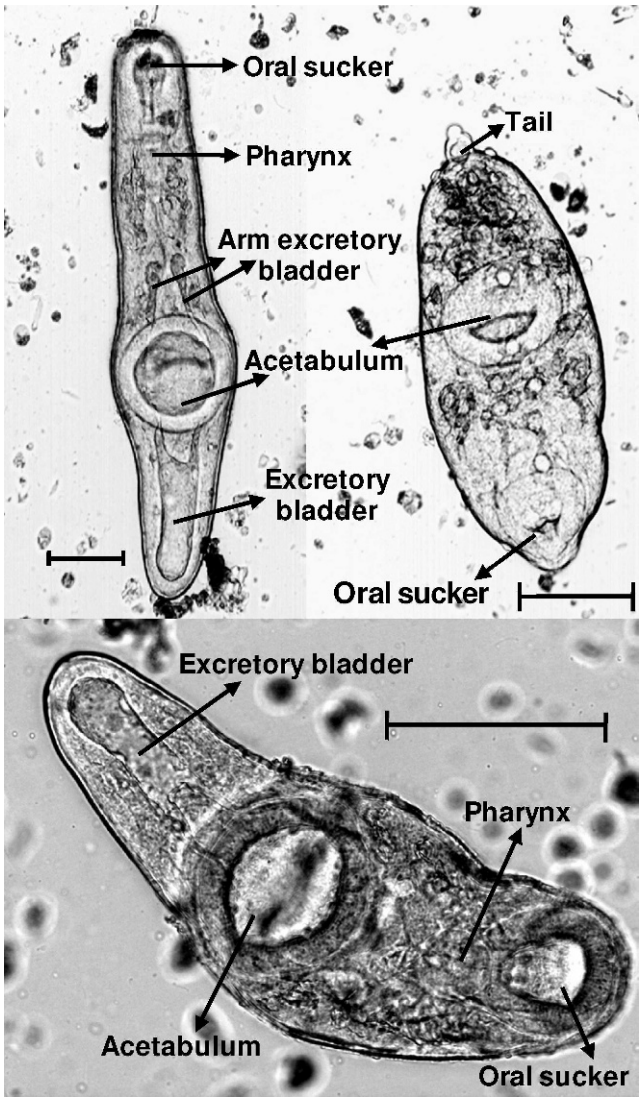


FIGURE 4. Adult cercariae of *P. lintoni* obtained from *P. purpuratus*. Scale bar = 0.05 mm.

at the anterior end and the acetabulum is located near the body midpoint (Fig. 4). A pre-pharynx may or may not be present; the pharynx is pyriform or sub-cylindrical (Fig. 4). Three pairs of cephalic glands are distinguished at each side of the pharynx, with ducts extending to the oral sucker. The intestine bifurcates before it reaches the acetabulum, and the intestinal caeca extend to the posterior end of the body, near the bifurcation point of the excretory bladder. The excretory bladder is oval, with arms extending laterally from the anterior lateral margin of the bladder to the bifurcation of the intestine. The body of the cercaria possesses large unicellular cystogenous glands, which makes visualization of internal anatomy difficult; thus, the flame cell formula could not be determined (Uzmann, 1953; Cheng, 1967; Canzonier, 1972) (Fig. 4). Some cercariae have small, spherical tails attached to slight invaginations at the posterior end of the body.

DISCUSSION

The experimental, morphometric, morphologic, and ecological evidence presented in this paper suggests that larval stages found in *P. purpuratus* correspond to sporocysts and cercariae of *P. lintoni*. Of the 3 species of intertidal mussels studied in 8 locations along the coast of Chile, covering an extension of 12 degrees of latitude, infections were observed only in *P. purpuratus* (Table I). The ecological factors that affect the probability of encounter between parasites and hosts are some of the main determinants for parasite transmission (Kennedy, 1975; Kuris et al., 1980; Price, 1980; Poulin, 1998). In this context, the abundance and body size of the mussels, as well as the microhabitat and activity pattern of host species, could be relevant in the recruitment of *P. lintoni*. *Perumytilus purpuratus* mussels are the dominant organisms in the mid intertidal, excluding other sessile organisms such as barnacles, algae, and even other mussels (Castilla and Durán, 1985; Paine et al., 1985; Castilla et al., 1989; Durán and Castilla, 1989; Navarrete and Castilla, 1990) and larger than *S. algosus*, which does not stay fixed to the substrate, showing daily movement rhythms that have a great impact on its physiology (Abades et al., 2000). We hypothesize that the larger size, greater density, and degree of spatial aggregation of *P. purpuratus* make this species more susceptible to parasitism by *P. lintoni*.

The higher prevalence of infection in mussels exposed to parasite eggs versus those in the controls (Table II) indicates a successful experimental infection of *P. purpuratus* by *P. lintoni*, since the eggs were coming from parasites extracted from clingfish *S. sanguineus*, the definitive host of *P. lintoni* (Oliva, 1984; Oliva and Zegers, 1988; George-Nascimento et al., 1998). Shorter (45 days) experimental trials using *S. algosus* did not result in infection events. Comparison of morphometric variables of cercariae obtained experimentally and naturally showed large overlapping of values, especially between experimental and natural summer cercariae (Figs. 1, 2). This suggests that at least naturally obtained cercariae during the summer effectively correspond to larval stages of *P. lintoni*.

The morphometric differences between natural cercariae in winter and summer (Figs. 1, 2) could be attributed to the differences in the developmental stage of the larvae in *P. purpuratus*. Morphological and prevalence data for *Proctoeces maculatus* in *Mytilus edulis* indicated a seasonal development of sporocysts from which cercariae would be released in late winter and during spring, due to the influence of temperature on larval physiology (Uzmann, 1953; Stunkard and Uzmann, 1959; Cheng, 1967; Lang and Dennis, 1976). In the present study, cercariae obtained in the winter possessed proportionately larger suckers compared to those obtained in summer and in experimental infections (Figs. 1, 2). Larger cercariae with smaller structures, i.e., suckers, may represent intermediate states of larval maturation associated with the development of the parasite in mussels following their arrival as miracidia (Shimura and Egusa, 1979b). Hence, the differences between natural cercariae in winter and summer can be interpreted as cercariae in different developmental stages.

The large morphologic and morphometric variability in the larvae and adults of species of *Proctoeces*, partly a consequence of the broad spectrum of hosts that it uses, has suggested that most described species in the genus correspond to *P. maculatus* (Bray and Gibson, 1980; Bray, 1983). Based on the morphology and development of specimens from different locations, or hosts, or both, 3 types of cercariae have been described as the larval stage of *P. maculatus* (see Table A in Aldana, 2007), i.e., *Cercaria tenuans* from England (Cole, 1935) and *C. milfordensis* from Connecticut and Massachusetts (Uzmann, 1953; Stunkard and Uzmann, 1959) in *M. edulis*, and *C. brachidontis* from Louisiana

(Hopkins, 1954) in the mussel *B. recurvus*. Subsequent studies have indicated that the differences among these cercariae appear insignificant in view of the wide morphological variation seen between metacercariae and adult *P. maculatus* (Wardle, 1980). Therefore, it is considered that *C. tenuans*, *C. milfordensis*, and *C. brachidontis* are conspecific and are the cercaria stage of *P. maculatus* (Stunkard and Uzmann, 1959; Wardle, 1980).

In the present study, morphometric differences between cercariae of *P. lintoni* and *P. maculatus* were not statistically analyzed. However, most measurements of *P. lintoni* cercariae were found inside the range described for *P. maculatus* (see Tables 3 and A in Aldana, 2007). Cercariae morphology has often been considered a more reliable indicator of phylogenetic relationships (at least among families) than the morphology of adult digeneans (Roberts and Janovy, 2009). Hence, morphometry of studied cercariae constitutes new evidence suggesting that *P. lintoni* is a synonym of *P. maculatus*.

In conclusion, the evidence provided here suggests that the larval stages found in *P. purpuratus* correspond to *P. lintoni* and, therefore, *P. purpuratus* is the first intermediate host of the digenean *P. lintoni*. However, additional DNA analyses are required to confirm the specific status of these larval stages (Criscione et al., 2005).

ACKNOWLEDGMENTS

We are grateful to Dr. Juan Correa for granting us access to the program Imagen Pro Plus. M. Aldana acknowledges a doctoral fellowship from Conicyt-Chile. P.A.M. acknowledges support from grant FONDAP-FONDECYT 1501-0001, ICM P05-002 and PFB-23. J.P.A. acknowledges DI 05-08 Universidad Andrés Bello.

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