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MARITREMA ORENSENSE AND *MARITREMA BONAERENSE* (DIGENEA: MICROPHALLIDAE): DESCRIPTIONS, LIFE CYCLES, AND COMPARATIVE MORPHOMETRIC ANALYSES

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ABSTRACT: We elucidate the life cycle of *Maritrema orensense* for the first time and experimentally confirm that of the sympatric *Maritrema bonaerense*. In Argentinean estuaries, both species parasitize the cochliopid snail *Heleobia australis* as first intermediate host, the grapsid crabs *Neohelice granulata* and *Cyrtograpsus angulatus* as second intermediate hosts, and gulls as definitive hosts. Here, we describe the daughter sporocysts and cercariae of *M. orensense* and redescribe these stages for *M. bonaerense*. Sporocysts of *M. orensense* are shorter, with fewer developed cercariae than *M. bonaerense*. The cercariae of *M. orensense* have longer, larger, and more undulating cephalic glands than *M. bonaerense*. We redescribe metacercariae and adults of both species and compare them with the previous descriptions. Intestinal ceca length, vitellaria shape and extension, and egg size are the most relevant characteristics in metacercariae and adults for differentiating the species. Hence, the detailed morphological description and comparative analyses of morphometrics obtained from natural and experimental infections permit clear differentiation of *M. orensense* and *M. bonaerense* at each life stage.

Maritrema orensense Cremonte and Martorelli 1998 and *Maritrema bonaerense* Etchegoin and Martorelli 1997 are 2 microphallids described and reported as adults from gulls in Argentinean estuaries (Etchegoin and Martorelli, 1997; Cremonte and Martorelli, 1998; Cremonte et al., 1999; La Sala et al., 2009). These species are known to co-occur at least in the Olrog's gull *Larus atlanticus* Olrog 1958 (La Sala et al., 2009). The life cycle of *M. bonaerense* involves a cochliopid snail and grapsid crabs as first and second intermediate hosts (Etchegoin and Martorelli, 1997), but the life stages of *M. bonaerense* described by these authors differ in some regards with our present observations. The life cycle of the sympatric *M. orensense* has not been completely determined, nor has the first intermediate host been previously reported. Moreover, although grapsid crabs have been proposed as second intermediate hosts, this has not been experimentally confirmed (Alda et al., 2011). Thus, the life cycles and the life stages of both species are problematically, or incompletely, described.

Here, we describe the daughter sporocysts and cercariae of *M. orensense*, redescribe its metacercariae and adult stages, and compare these to the original description of Cremonte and Martorelli (1998) and Alda et al. (2011). We also provide emended descriptions for these life cycle stages for *M. bonaerense*, compare our descriptions with the original ones made by Etchegoin and Martorelli (1997), and perform morphological analyses to distinguish each species at all life stages. Finally, we use experimental infections in crabs and chicks to describe the life cycle of *M. orensense* and confirm that of *M. bonaerense*.

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MATERIALS AND METHODS

Field collections

We collected naturally infected first and second intermediate hosts at the innermost part of the Bahía Blanca Estuary, Puerto Cuatreros (38°44′S, 62°22′W). From July 2006 through July 2008, we collected 2,984 individuals of the intertidal snail *Heleobia australis* during low tide from mud at the intertidal fringe using a 1.19-mm mesh sieve. To collect the crabs, *Cyrtograpsus angulatus* Dana 1851 (n = 65) and *Neohelice granulata* (Dana 1851) (n = 36), we used lift nets and crab traps in March, April, July, September, and November 2008, and in February and April 2009.

Experimental infections

To perform experimental infections, we used uninfected crabs (*Cyrtograpsus angulatus*) collected at Mar del Plata Yacht Club (38°00′S, 57°33′W), where *Heleobia* spp. are not present. Previous dissections of 30 crabs yielded no metacercariae (Martorelli, data not shown). Samples were transported to the laboratory in La Plata City and kept alive in aquaria until used for experimentation.

From the 15 snail-crab experimental exposures, we obtained 10 *M. orensense* metacercariae in the gills of 1 crab 3 wk postinfection (PI). One day PI, we obtained 9 adults of *M. orensense* in the intestine of 1 chick fed with naturally infected crab gills of both *C. angulatus* and *Neohelice granulata*.

From the 15 snail-crab experimental exposures, we obtained more than 100 metacercariae of *M. bonaerense* in gills, coelom, and muscle of the thorax and chelipeds of 1 crab maintained during 5 wk with the specimen of *H. australis* parasitized with *M. bonaerense*. One *M. bonaerense* adult was recovered 3 days PI in the intestine of 1 chick fed infected gills of both crab species *C. angulatus* and *N. granulata*.

Laboratory procedures

For experimental infections, we isolated snails in 2-ml vials for 8 hr over each of 3 consecutive days at room temperature and under constant illumination to promote cercariae shedding. Each snail that shed microphallid cercariae was placed in a 750-ml plastic jar with an uninfected crab (*C. angulatus*). We performed 15 experiments where an infected *H. australis* individual cohabited with an uninfected crab. One to 5 weeks PI, we ended the experiments (3 per wk), killed the crabs, and isolated the snails. Crabs were killed by freezing (–20 C) for 20 min and

checked for infection, and metacercariae were recovered. We isolated and shed cercariae from those snails that cohabited with crabs experimentally infected with *M. orensense* and *M. bonaerense* to study live cercariae (neutral red stained). We dissected snails to examine daughter sporocysts. Mother sporocysts were never observed. Cysts, excysted metacercariae, cercariae, and sporocysts were fixed in hot 10% formalin without pressure for drawings and measurements.

Naturally infected crabs were also killed by freezing. We removed and examined gills, foregut, hepatopancreas, gonads, and the muscle of the thorax and chelipeds for the presence of metacercariae using a stereomicroscope. We identified parasites following Alda et al. (2011). To assess for metacercaria excystation, we placed encysted metacercariae in culture plates with physiological saline solution (0.85% NaCl) and incubated them at 38 C for 24–48 hr. Cysts and excysted metacercariae were fixed in hot 10% formalin without pressure for drawings and measurements. Additional excysted metacercariae were killed with flame using coverslip pressure, fixed in Bouin's fixative, stored in 70% ethanol, stained with carmine, mounted in Canada balsam, and studied using compound microscopy.

Metacercariae of *M. orensense* and *M. bonaerense* from naturally infected crabs of both species were fed to 8, 1-day-old chicks *Gallus gallus domesticus* (Linnaeus 1758). Some metacercariae that were encysted in gills were fed to chicks using forceps, others were separated from gills, muscle, and hepatopancreas, put in saline solution (0.85% NaCl), and fed to chicks using pipettes (~100 cysts of each *Maritrema* spp. per chick). Chicks were dissected 1 to 18 days PI: 4 chicks, 1 day PI; 1 chick, 3 days PI; 2 chicks, 7 days PI; and 1 chick, 18 days PI. Adult digeneans were fixed in hot 10% formalin without pressure.

To locate the site of infection within the snail and crabs, snail tissue and gill, muscle, and hepatopancreas of crabs were fixed in Bouin's fixative for at least 10 days. Tissues were embedded in paraffin and cut at a thickness of 4 μm. Serial sections were stained with hematoxylin and eosin (H&E).

For scanning electron microscopy (SEM), larval digeneans were fixed in hot 10% formalin without pressure, dehydrated through an ethanol series, immersed in hexamethyldisilazane for 1 min, and air dried at room temperature. Specimens were mounted and coated with gold. Photographs were obtained with a Philips SEM 505.

Specimens of cercariae, sporocysts, metacercariae, and adults of *M. orensense* and *M. bonaerense* were deposited in the Helminthological Collection of La Plata Museum (MPHC), La Plata, Argentina. In addition, the following material was studied for comparison: *M. orensense* holotype and paratypes nos. 3955–3956 deposited in the MPHC; and *M. bonaerense* holotype and paratypes nos. 3661a–b deposited in the MPHC.

Morphometric and statistical analyses

We measured and drew sporocysts, cercariae, and metacercariae from natural infections and metacercariae and adults from experimental infections of *M. orensense* and *M. bonaerense* for comparison with those described by Etchegoin and Martorelli (1997), Cremonte and Martorelli (1998), and Alda et al. (2011). We obtained measurements from drawings made with the aid of a drawing tube. Measurements are presented in mm. To compare morphometrics of the 2 species, we used 2-tail *t*-tests. To assess the contribution of each variable in the separation of different groups, we applied a standard discriminant analysis for quantitative data (Hair et al., 1999). Data were ln- and squared-root transformed in each case to meet assumptions of normality and homoscedasticity (Hair et al., 1999).

DESCRIPTION

Maritrema orensense Cremonte and Martorelli, 1998

Diagnosis: Daughter sporocysts brownish, elongated, thin-walled sacs filled with 0–3 late developmental stage cercariae and more numerous embryos and germinal balls (Table I; Figs. 1A, 2A). Birth pore not discernible. Daughter sporocysts (around 50) appear to completely displace gonadal tissues and also reside in part of digestive gland region.

Cercariae small, monostome, with stylets (Table I; Figs. 1C, 2A, 3A). Body oval to elongate with truncated posterior end. Tegument with numerous minute pointed spines (~0.7 μm) arranged regularly over dorsal and ventral body surfaces. Tail with transverse tegumental striations, inserted postero-ventrally into small tail socket. Oral sucker subterminal. Stylet sclerotized, deeply attached to center of oral sucker with pointed anterior that almost reaches anterior body margin; stylet point about 1/3 stylet total length, with thickened and slightly projecting base; stylet shaft slightly concave in outline, widest at anterior and posterior ends. Ceca not observed. Ventral sucker absent. Cephalic glands in 4 pairs, with anterior extent equatorial and posterior extent just over 3/4 way from anterior to posterior body margin, with slightly undulating margins, filled with fine granular inclusions, with no discernible nuclei; antero-ventral pairs shorter than postero-dorsal pairs and with ducts more undulating; dorsal and ventral cephalic gland ducts separated into 2 bundles of 2 ducts each just posterior to oral sucker, dorsal pair of ducts continues laterally and opens as small pores just lateral to anterior end of stylet, while ventral pair undulates more markedly and opens posteriorly to dorsal ducts; undulations of ventral pair of ducts more pronounced when cercaria contracted. Excretory bladder V-shaped, opening at posterior body extremity. Flame cell formula $2([2+2]+[2+2])=16$.

Metacercariae oval, small, translucent, and with 2 hyaline layers, deeply encysted in primary filament of crab's gill and hard to separate from crab's tissue (Table I; Figs. 4A, C, E, 5A, C). First third of body folded inside cyst. Morphology of excysted metacercariae closely resembles adults except for the absence of eggs. Excysted body narrowly ovate to oval. Tegument covered with numerous spines situated always less than 1 spine width to each other, distributed throughout the entire body surface, more dense on ventral surface; spines ~0.7 μm long, hand-shaped with 7–9 fingers projecting posteriorly (fingers only visible with SEM). Oral sucker round and subterminal. Digestive system formed by prepharynx almost as long as esophagus, muscular pharynx, esophagus, and 2 intestinal ceca that terminate before anterior of ventral sucker. Ventral sucker small and round. Cirrus sac curved. Ovary middle-dextral. Testes oval, symmetrical, posterior to ovary, enclosed between lateral and anterior branches of vitellarium. Vitellarium with oval follicles arranged in an inverted U-shaped incomplete ring between testes and ovary. Excretory vesicle V-shaped; flame bulb formula $2([2+2]+[2+2])=16$.

Adults narrowly ovate distomes, covered with spines (Table II; Fig. 6A). Oral sucker round and subterminal. Digestive system formed by prepharynx almost as long as esophagus, pharynx muscular and relatively large, esophagus, and 2 short and thick intestinal ceca extending laterally along the anterior edge of the cirrus sac almost to the lateral body wall, terminating before ventral sucker anterior; posterior half of pharynx, esophagus, and intestinal ceca with thick epithelium. Cirrus sac non-muscular, curved, transverse, thin-walled, and located between intestinal ceca and ventral sucker anterior. Seminal vesicle oval, occupying 1/4 to 1/3 of the cirrus sac. Ductus ejaculatorius with 1 or 2 curves. Cirrus without spines, always observed invaginated. Prostatic cells numerous, arranged in row located anterior to cirrus near gonopore. Pars prostatica not seen. Genital pore sinistral to ventral sucker. Ovary middle-dextral, with anterior border overlapping ventral sucker. Ovary in adults with few eggs, subspherical to oval, sometimes slightly lobed, but ovary in adults with many eggs, triangular. Laurer's canal and seminal receptacle not seen. Testes oval, slightly irregular rounded, lateral, and posterior to ovary. Vitellarium with oval follicles arranged in an inverted U-shaped incomplete ring between testes and ovary. Short, wide, and muscular metraterm; uterus extends from posterior end of cirrus sac to posterior end of body. Eggs operculated, light yellow to brown. Excretory vesicle V-shaped; flame bulb formula $2([2+2]+[2+2])=16$.

Taxonomic summary

Natural definitive host: Larus dominicanus Lichtenstein 1823 and *Larus atlanticus* (see Cremonte and Martorelli, 1998; La Sala et al., 2009).

Experimental definitive host: Gallus gallus domesticus.

Site of infection: Intestine.

TABLE I. Descriptive statistics for larval stages of *Maritrema orensense* and *Maritrema bonaerense* obtained from snails and gill's crabs naturally infected. SD: standard deviation; CV: coefficient of variation; N: number of specimens observed; (*): $p < 0.05$; (†): $p < 0.01$; ns: nonsignificant; BL: body length; BW: body width; Nc: number of cercariae per sporocyst; OSL: oral sucker length; OSW: oral sucker width; TL: tail length; TW: tail width; SL: stylet length; SW: stylet width; CL: cyst length; CW: cyst width; OT: outermost wall thickness; IT: innermost wall thickness; PpL: prepharynx length; PL: pharynx length; PW: pharynx width; EL: esophagus length; IL: intestinal ceca length; IW: intestinal ceca width; VSL: ventral sucker length; VSW: ventral sucker width; CiSL: cirrus sac length; CiSW: cirrus sac width; OvL: ovary length; OvW: ovary width; TeL: testes length; TeW: testes width; CiL: cirrus length; ND: no data.

		Maritrema orensense				*Maritrema bonaerense*				
Stage	Variables	Mean ± SD	Range	CV	N	Mean ± SD	Range	CV	N	Significance level
Sporocyst	BL	0.542 ± 0.139	[0.383–0.787]	0.26	11	0.725 ± 0.145	[0.464–0.950]	0.20	11	†
	BW	0.154 ± 0.036	[0.103–0.217]	0.23	11	0.153 ± 0.029	[0.121–0.221]	0.19	11	ns
	BL/BW	3.62 ± 1.05	[2.31–6.00]	0.28	11	4.89 ± 1.10	[2.10–6.05]	0.21	11	*
	Nc	2 ± 1	[0–3]	0.50	11	7 ± 3	[4–12]	0.43	11	†
Cercariae	BL	0.103 ± 0.016	[0.079–0.130]	0.15	20	0.115 ± 0.014	[0.088–0.139]	0.13	22	*
	BW	0.045 ± 0.005	[0.037–0.058]	0.11	20	0.042 ± 0.007	[0.029–0.054]	0.16	22	ns
	BL/BW	2.33 ± 0.53	[1.36–3.43]	0.23	20	2.83 ± 0.83	[1.79–4.41]	0.29	22	*
	OSL	0.031 ± 0.003	[0.026–0.035]	0.10	20	0.035 ± 0.003	[0.027–0.039]	0.08	22	†
	OSW	0.029 ± 0.003	[0.025–0.037]	0.11	20	0.025 ± 0.003	[0.019–0.032]	0.12	22	†
	TL	0.115 ± 0.017	[0.086–0.139]	0.15	20	0.133 ± 0.028	[0.088–0.181]	0.21	22	*
	TW	0.011 ± 0.001	[0.009–0.014]	0.12	20	0.012 ± 0.002	[0.008–0.017]	0.18	22	*
	SL	0.019 ± 0.002	[0.014–0.021]	0.10	18	0.021 ± 0.002	[0.019–0.025]	0.10	20	†
	SW	0.004 ± 0.001	[0.004–0.005]	0.18	18	0.004 ± 0.001	[0.002–0.005]	0.26	19	ns
Encysted metacercariae	CL	0.290 ± 0.014	[0.270–0.307]	0.05	10	0.308 ± 0.024	[0.277–0.343]	0.08	8	ns
	CW	0.217 ± 0.025	[0.180–0.247]	0.12	10	0.271 ± 0.034	[0.230–0.320]	0.13	8	†
	CL/CW	1.35 ± 0.13	[1.23–1.60]	0.10	10	1.15 ± 0.15	[1–1.43]	0.13	8	†
	OT	0.005 ± 0.002	[0.003–0.007]	0.40	10	0.008 ± 0.002	[0.007–0.010]	0.25	8	†
	IT	0.007 ± 0.002	[0.003–0.010]	0.29	10	0.009 ± 0.002	[0.003–0.010]	0.22	8	*
Excysted metacercariae	BL	0.310 ± 0.026	[0.275–0.366]	0.08	10	0.591 ± 0.091	[0.380–0.700]	0.15	10	†
	BW	0.162 ± 0.019	[0.131–0.198]	0.12	10	0.320 ± 0.026	[0.277–0.350]	0.08	10	†
	BL/BW	1.92 ± 0.15	[1.57–2.10]	0.08	10	1.85 ± 0.27	[1.31- 2.23]	0.15	10	ns
	OSL	0.037 ± 0.003	[0.032–0.041]	0.08	10	0.057 ± 0.006	[0.047–0.067]	0.11	9	†
	OSW	0.034 ± 0.006	[0.027–0.044]	0.18	10	0.064 ± 0.008	[0.050–0.077]	0.13	9	†
	PpL	0.025 ± 0.006	[0.027–0.034]	0.24	10	0.019 ± 0.016	[0.007–0.060]	0.84	9	ns
	PL	0.023 ± 0.003	[0.012–0.027]	0.13	10	0.029 ± 0.004	[0.023–0.033]	0.14	10	†
	PW	0.014 ± 0.002	[0.017–0.019]	0.14	10	0.023 ± 0.003	[0.017–0.027]	0.13	10	†
	EL	0.023 ± 0.006	[0.014–0.034]	0.26	10	0.086 ± 0.020	[0.060–0.120]	0.23	10	†
	IL	0.074 ± 0.011	[0.064–0.095]	0.15	9	0.257 ± 0.024	[0.207–0.283]	0.09	10	†
	IW	0.010 ± 0.001	[0.008–0.013]	0.10	10	0.012 ± 0.003	[0.007–0.017]	0.25	10	ns
	VSL	0.024 ± 0.003	[0.020–0.029]	0.13	10	0.061 ± 0.004	[0.057–0.067]	0.07	7	†
	VSW	0.026 ± 0.004	[0.022–0.036]	0.15	10	0.064 ± 0.004	[0.057–0.067]	0.06	7	†
	CiSL	0.097 ± 0.018	[0.083–0.136]	0.19	8	0.204 ± 0.024	[0.167–0.233]	0.12	10	†
	CiSW	0.023 ± 0.004	[0.015–0.029]	0.17	8	0.035 ± 0.009	[0.017–0.047]	0.26	10	†
	OvL	0.023 ± 0.003	[0.021–0.029]	0.13	6	0.075 ± 0.013	[0.053–0.097]	0.17	10	†
	OvW	0.032 ± 0.016	[0.013–0.053]	0.50	6	0.087 ± 0.019	[0.040–0.107]	0.22	10	†
	TeL	0.033 ± 0.001	[0.032–0.034]	0.03	3	0.075 ± 0.013	[0.050–0.097]	0.17	10	ND
	TeW	0.047 ± 0.013	[0.033–0.058]	0.28	3	0.093 ± 0.008	[0.075–0.102]	0.09	10	ND
	CiL	ND	ND	ND	ND	0.106 ± 0.012	[0.093–0.120]	0.11	5	ND
	CiW	ND	ND	ND	ND	0.017	ND	ND	1	ND

First intermediate host: Heleobia australis (d'Orbigny 1835).

Second intermediate host: Cyrtograpsus angulatus and *Neohelice granulata.*

Locality: Bahía Blanca Estuary, Argentina.

Specimens deposited: Shedding cercariae from *Heleobia australis* (no. 6522), metacercariae from *Neohelice granulata* (no. 6523), and adults experimentally obtained in chicks (no. 6524) deposited in the MPHC.

Maritrema bonaerense **Etchegoin and Martorelli, 1997**

Diagnosis: Daughter sporocysts brownish, elongated, sometimes with constrictions, thin-walled sacs, with 4–12 cercariae in late developmental stages and with some germ balls (Table I; Figs. 1B, 2B). Birth pore not discernible. Daughter sporocysts (around 50) completely displace gonadal tissues and digestive gland region.

Cercariae small, monostome, with stylets (Table I; Figs. 1D, 2B, 3B). Body oval to elongate with a truncated posterior end. Tegument with numerous minute pointed spines (~0.9 μm) arranged regularly over dorsal and ventral body surfaces. Tail simple, with transverse cuticular striations, inserted postero-ventrally into small socket. Oral sucker subterminal. Stylet sclerotized with pointed anterior that almost reaches anterior body margin; stylet point about 1/3 stylet total length, with thickened and slightly projecting base; stylet shaft slightly concave in outline, widest at anterior and posterior ends. Ceca not observed. Ventral sucker absent. Cephalic glands in 4 pairs, with anterior extent equatorial and posterior

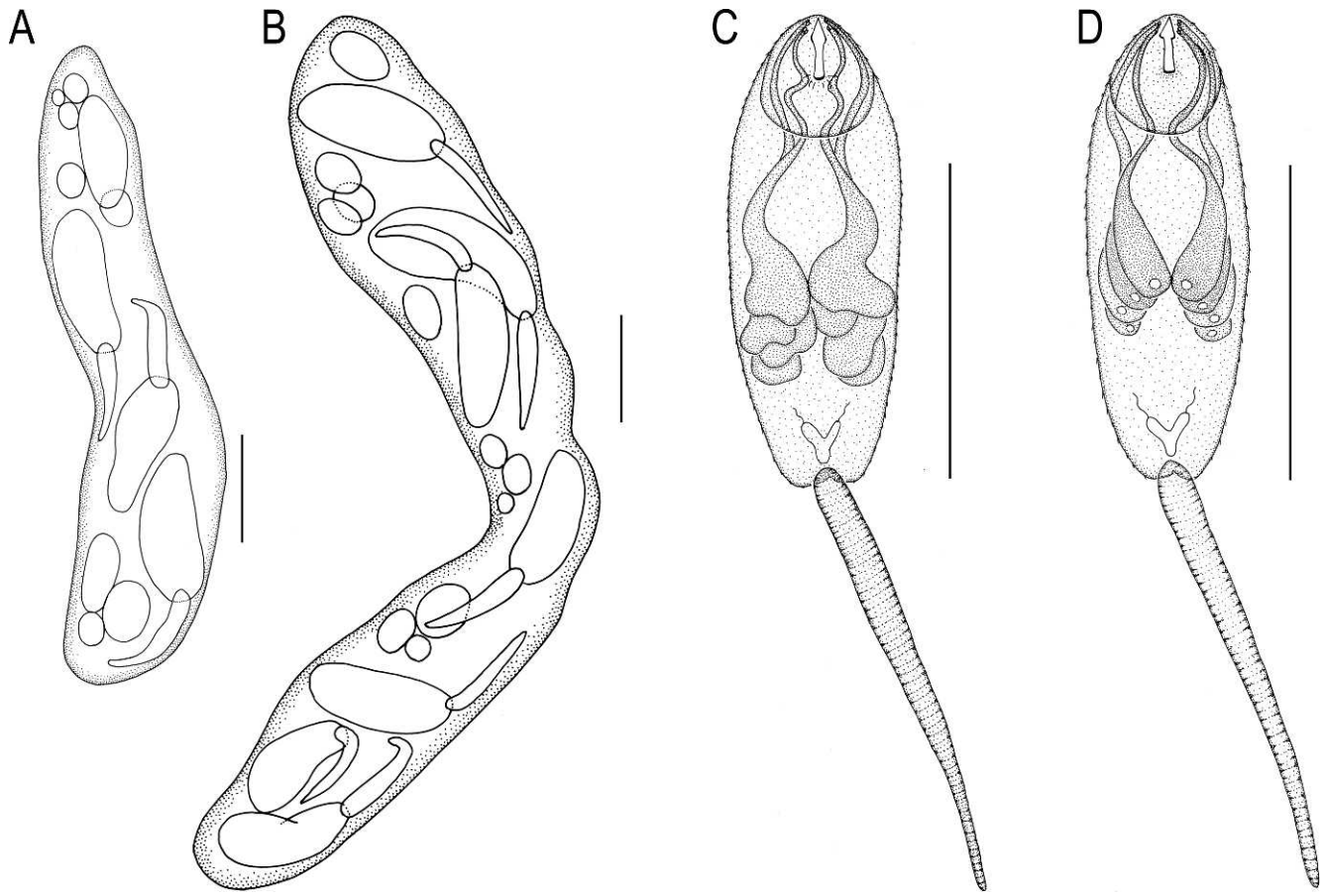

FIGURE 1. Line drawings. Sporocysts of (**A**) *Maritrema orensense* and (**B**) *Maritrema bonaerense* parasite of *Heleobia australis*; cercaria of (**C**) *Maritrema orensense* and (**D**) *Maritrema bonaerense* shed from *Heleobia australis*. Scale bars: 100 μm.

extent just over 2/3 way from anterior to posterior body margin, with rounded margins, filled with fine granular inclusions, with visible nuclei; ventral pairs shorter and stain lighter with neutral red than dorsal pairs; dorsal and ventral cephalic gland ducts undulate and separate just posterior to oral sucker into 2 bundles of 2 ducts each, open near anterior of stylet with ventral gland ducts opening posteriorly to dorsal gland ducts. Excretory bladder V-shaped, opening at posterior body extremity. Flame cell formula $2([2+2]+[2+2])=16$.

Metacercariae round with 2 hyaline layers, freely encysted, usually in ventral side of primary filament of crab's gill (Table I; Figs. 4A, B, D, 5B, D). Also found in hepatopancreas, coelom, muscle, and gonad of crabs; these are larger. Body folded in half inside cyst with typical brown vitellarium arranged in a ring. Morphology of excysted metacercariae closely resembles adults except for absence of eggs. Excysted body broadly oval to pyriform, somewhat thinner at ovary level, with slightly pointed anterior end. Tegument covered with spines widely spaced from each other (generally >1 spine width), distributed regularly over body, most dense on ventral surface; spines ~0.7 μm long, hand-shaped, deeply inserted in tegument, with 9–10 fingers projected posteriorly (fingers only visible with SEM). Oral sucker round and subterminal. Digestive system formed by prepharynx almost as long as esophagus, muscular pharynx, long esophagus, and 2 intestinal ceca terminating before anterior of testes. Ventral sucker small and round. Cirrus sac long, curved. Ovary oval and middle-dextral. Testes oval, symmetrical, posterior to ovary, enclosed between lateral and anterior branch of vitellarium. Brown vitellarium with oval and round follicles arranged in ring, sometimes incomplete posteriorly, that encloses testes. Excretory vesicle V-shaped; flame bulb formula $2([2+2]+[2+2])=16$.

Adults widely oblong distomes covered with spines (Table II; Fig. 6B). Oral sucker round and subterminal. Digestive system formed by long prepharynx, small muscular pharynx, long esophagus, and 2 thin and long intestinal ceca terminating before anterior end of testes; posterior portion of intestinal ceca usually enlarged. Cirrus sac nonmuscular, transverse, long, curved, thin-walled, located between intestinal ceca and ventral sucker. Seminal vesicle occupying 2/3 of cirrus sac. Ductus ejaculatorius short and slightly curved. Prostatic cells located in lateral margins of cirrus sac and 10–15 in number. Pars prostatica vesicular. Cirrus unspined and folded when invaginated, sometimes observed evaginated. Ovary oval, sometimes lobed, and middle-dextral. Ootype and Mehlis's gland posterior to ovary. Laurer's canal and seminal receptacle not seen. Testes oval to rounded, slightly irregular, symmetrical, posterior to ovary, enclosed between lateral and anterior branch of vitellarium. Brown vitellarium with oval and round follicles arranged in ring, sometimes incomplete posteriorly, that encloses testes. Metraterm curved and thick-walled; uterus extends from anterior of testes to posterior vitellarium. Genital pore sinistral to ventral sucker. Eggs operculated, light yellow to brown. Excretory vesicle V-shaped; flame bulb formula $2([2+2]+[2+2])=16$.

Taxonomic summary

Natural definitive host: Larus dominicanus, *Larus maculipennis* Lichtenstein 1823, and *Larus atlanticus* (see Etchegoin and Martorelli, 1997; Cremonte et al., 1999; La Sala et al., 2009).

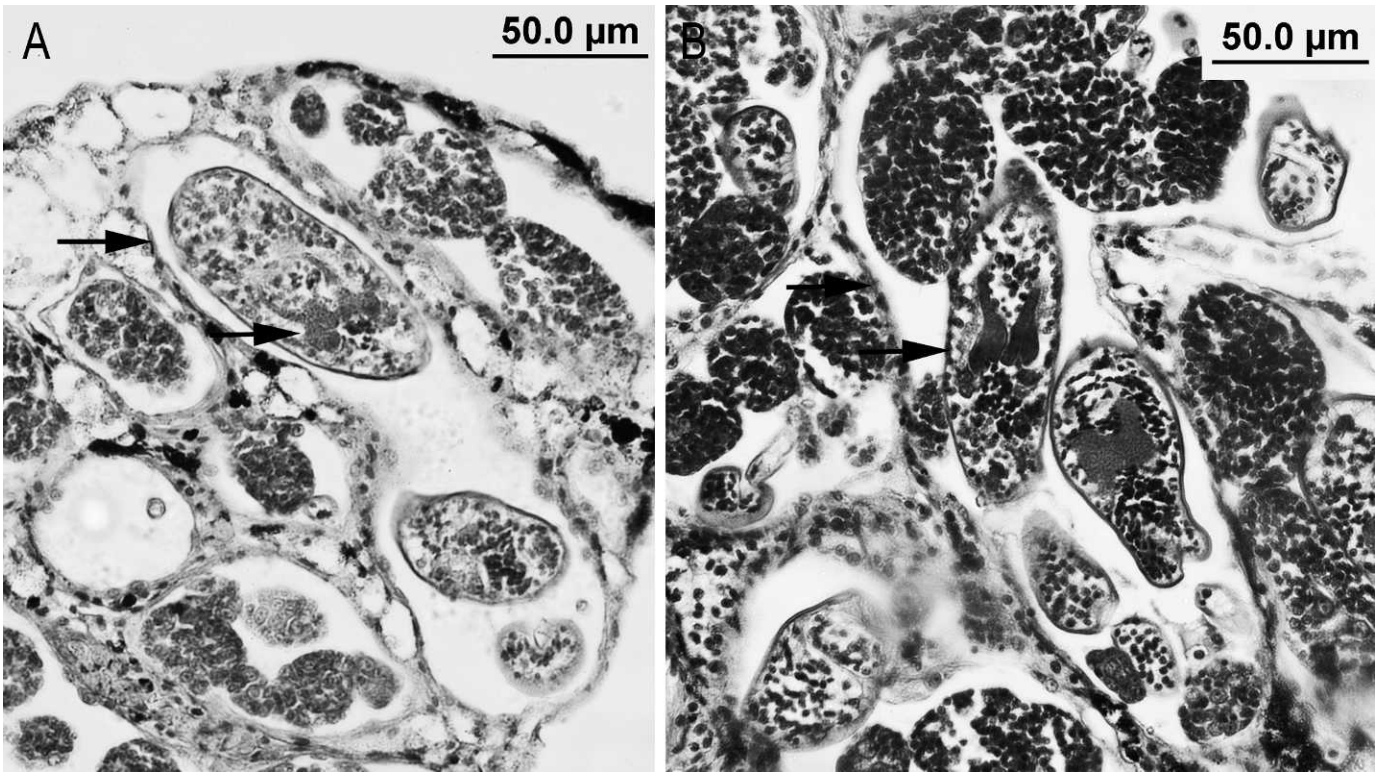

FIGURE 2. Cross sections (H&E) of snail's tissue parasitized with (**A**) *Maritrema orensense* and (**B**) *Maritrema bonaerense* showing both types of cephalic glands of cercaria and sporocyst's wall (rows).

Experimental definitive host: Gallus gallus domesticus.

Site of infection: Intestine.

First intermediate host: Heleobia australis.

Second intermediate host: Cyrtograpsus angulatus and Neohelice granulata.

Locality: Bahía Blanca Estuary, Argentina.

Specimens deposited: Sporocysts and cercariae from *Heleobia australis* (no. 6525), metacercariae from *Cyrtograpsus angulatus* (no. 6526), and adults experimentally obtained in chicks (no. 6527) deposited in the MPHC.

Remarks

This section provides a comparison of the 2 species based on the life stage morphometrics presented in Tables I and II.

Daughter sporocysts of *M. orensense* were on average 25% shorter than those of *M. bonaerense* ($t_{(20)} = -3.022$, $P = 0.007$), despite substantial overlap of body length range, but were equal in width ($t_{(20)} = 0.099$, $P = 0.992$). Hence, *M. orensense* sporocysts tend to be stockier than *M. bonaerense* sporocysts (*t*-test on body length/body width [BL/BW]: $t_{(20)} = -2.471$, $P = 0.022$). *Maritrema orensense* also had about 1/3 to 1/4 the number of developed cercariae per individual than did *M. bonaerense* ($t_{(20)} = -5.752$, $P < 0.0001$).

Cercariae of *M. orensense* had on average 10% shorter ($t_{(40)} = -2.577$, $P = 0.014$) but similarly wide ($t_{(40)} = 1.396$, $P = 0.170$) bodies, 11% shorter ($t_{(40)} = -4.04$, $P < 0.001$) and 12% wider ($t_{(40)} = 3.642$, $P < 0.001$) oral suckers, 13% shorter ($t_{(40)} = -2.473$, $P = 0.017$) and 12% narrower ($t_{(40)} = -2.572$, $P = 0.014$) tails, and 10% shorter ($t_{(36)} = -3.335$, $P = 0.002$) but similarly wide ($t_{(35)} = -1.449$, $P = 0.156$) oral stylets. Morphometric ratios (BL/BW) showed that *M. bonaerense* cercaria was significantly more elongate ($t_{(40)} = -2.291$, $P = 0.027$). Despite these differences on average, the range of measurements overlapped for all the above characters. Notwithstanding the overlap of univariate characters, discriminant analysis, which uses all the characters simultaneously, provided 88% correct classification of cercariae to species (Fig. 7A). The separation along the discriminant eigenvector appeared to be almost total. The canonical correlation coefficient from discriminant function 1 was 0.804—i.e., the proportion of variance resulting from the difference between groups (the coefficient^2 × 100) was 64.6%. The score values for *M. orensense* (centroid at –1.353 on the discriminant eigenvector) ranged from –3.652 to 1.203, but for *M. bonaerense* (centroid at 1.282) they ranged from –0.727 to 2.469. Based on a 0.30 cutoff discriminant loading criterion, the structure matrix revealed 4 morphometric variables that significantly loaded on the discriminant function, i.e., oral sucker length (0.488), oral sucker width (–0.483), stylet length (0.443), and tail width (0.399).

Encysted metacercariae of *M. orensense* found in gill's crabs showed on average 20% narrower cysts ($t_{(16)} = -3.895$, $P < 0.001$) and 38% ($t_{(16)} = -3.619$, $P = 0.002$) and 22% ($t_{(16)} = -2.279$, $P = 0.037$) thinner outermost and innermost walls, respectively, compared to *M. bonaerense*. We found no significant differences in cyst length ($t_{(16)} = -1.920$, $P = 0.073$). Length to width ratios (CL/CW) showed differences in cyst shape ($t_{(16)} = 3.100$, $P = 0.007$); *M. orensense* cyst was oval, while *M. bonaerense* cyst was spherical. Discriminant analysis yielded 81% correct classification (Fig. 7B). The canonic correlation coefficient from discriminant function 1 was 0.784, i.e., the proportion of variance resulting from the difference between groups (the coefficient^2 × 100) was 61.5%. The score values for *M. orensense* (centroid at –1.065 on the discriminant eigenvector) ranged from –3.111 to 0.638, but for *M. bonaerense* (centroid at 1.331) they ranged from 0.253 to 3.050. The structure matrix revealed 3 morphometric variables that significantly loaded on the discriminant function, i.e., cyst width (0.771), outermost wall thickness (0.692), and innermost wall thickness (0.451).

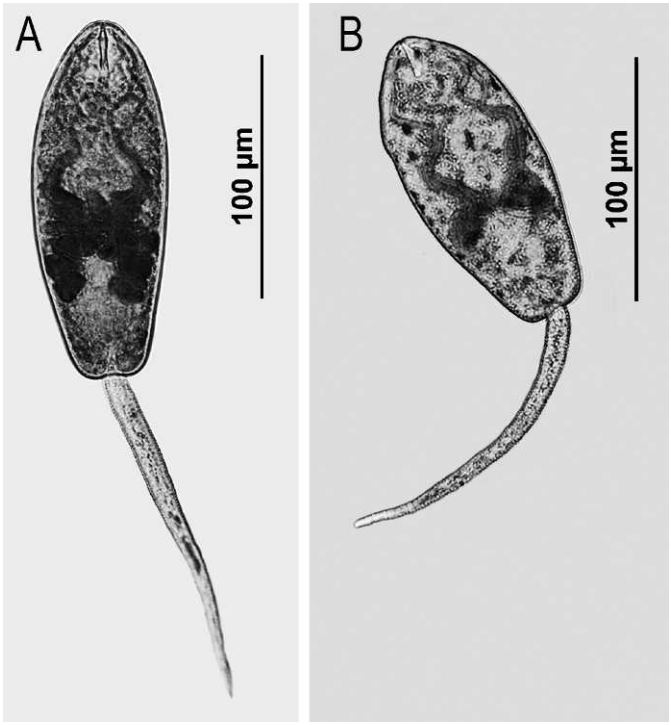

FIGURE 3. Neutral red–stained cercaria of (**A**) *Maritrema orensense* and (**B**) *Maritrema bonaerense* shed from *Heleobia australis*.

Excysted metacercariae of *M. orensense* had on average 48% larger ($t_{(18)} = -10.029$, $P < 0.001$) and 49% wider ($t_{(18)} = -15.892$, $P < 0.001$) bodies, 35% larger ($t_{(17)} = -9.369$, $P < 0.001$) and 47% wider ($t_{(17)} = -9.819$, $P < 0.001$) oral sucker, 21% larger ($t_{(18)} = -4.002$, $P < 0.001$) and 39% wider ($t_{(18)} = -8.139$, $P < 0.001$) pharynx, 73% larger esophagus ($t_{(18)} = -9.806$, $P < 0.001$), 71% larger intestinal ceca ($t_{(17)} = -22.499$, $P < 0.001$), 61% larger ($t_{(17)} = -25.037$, $P < 0.001$) and 59% wider ($t_{(15)} = -19.060$, $P < 0.001$) ventral sucker, 52% larger ($t_{(16)} = -10.964$, $P < 0.001$) and 34% wider ($t_{(16)} = -3.692$, $P = 0.002$) cirrus sac, and 69% larger ($t_{(14)} = -10.321$, $P < 0.001$) and 63% wider ($t_{(14)} = -5.889$, $P < 0.001$) ovary. We found no significant differences in morphometric ratios (BL/BW) ($t_{(18)} = 1.769$, $P = 0.094$), prepharynx length ($t_{(17)} = 1.137$, $P = 0.271$), and intestinal ceca width ($t_{(18)} = -1.987$, $P = 0.062$). Variables such as testes length and width and cirrus length and width were not included in the analysis, since only a few, or no, measurements were obtained from individuals of *M. orensense*. Consistent with the lack of overlap in 10 of the single variables, discriminant analysis yielded a 100% correct classification (Fig. 7C). The canonic correlation coefficient from discriminant function 1 was 0.996, i.e., the proportion of variance resulting from the difference between groups (the coefficient$^2 \times 100$) was 99.2%. The score values for the *M. orensense* (centroid at –10.151 on the discriminant eigenvector) ranged from –11.907 to –8.650, but for *M. bonaerense* (centroid at 10.151) they ranged from 8.142 to 11.752. The classification based on a discriminant function strongly separated species mainly on the basis of intestinal ceca and body size. From all variables entered into discriminant analysis, the structure matrix revealed only 3 morphometric variables that significantly loaded on the discriminant function, i.e., intestinal ceca length (0.662), body length (0.369), and body width (0.354).

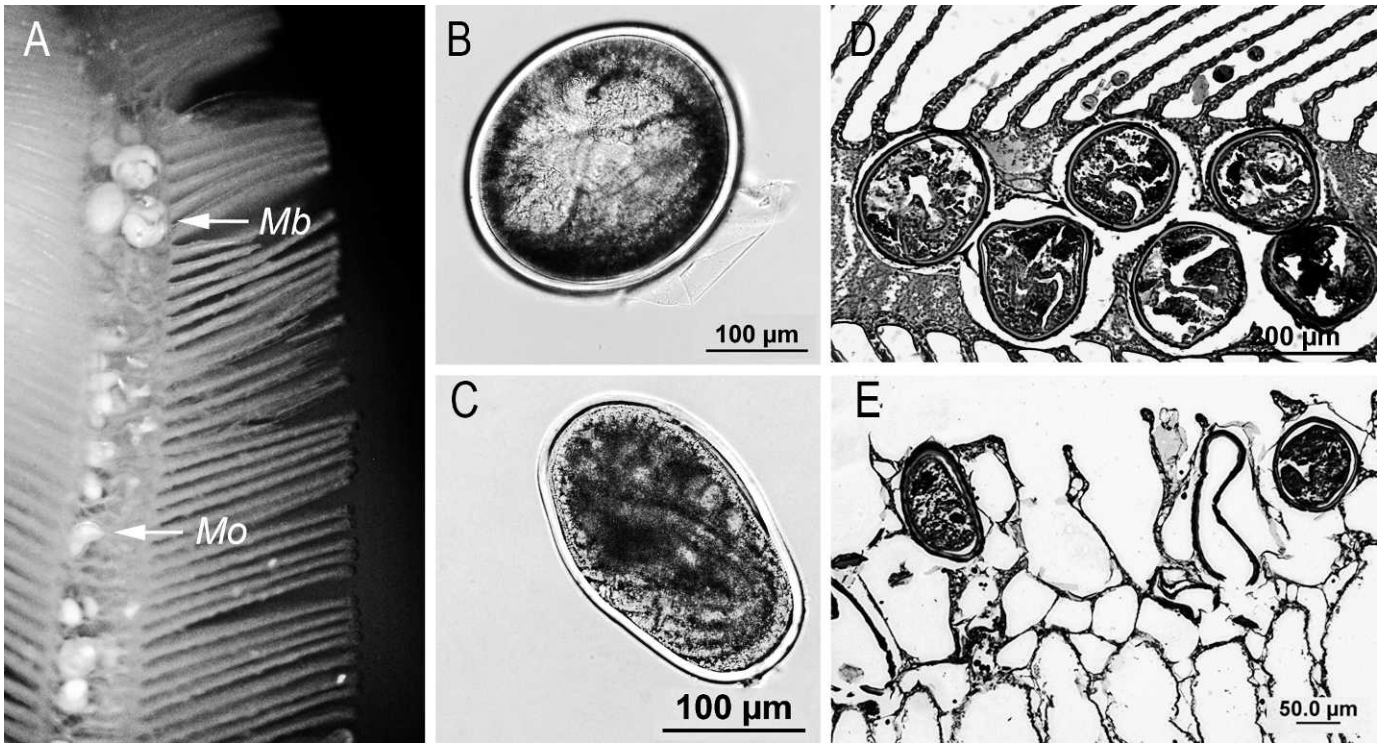

FIGURE 4. (**A**) Gill's crab with cysts of metacercaria of *Maritrema bonaerense* (Mb) and *Maritrema orensense* (Mo); cyst of metacercaria of (**B**) *Maritrema bonaerense* and (**C**) *Maritrema orensense*; cross sections (H&E) of gill's crabs parasitized with (**D**) *Maritrema bonaerense* and (**E**) *Maritrema orensense*.

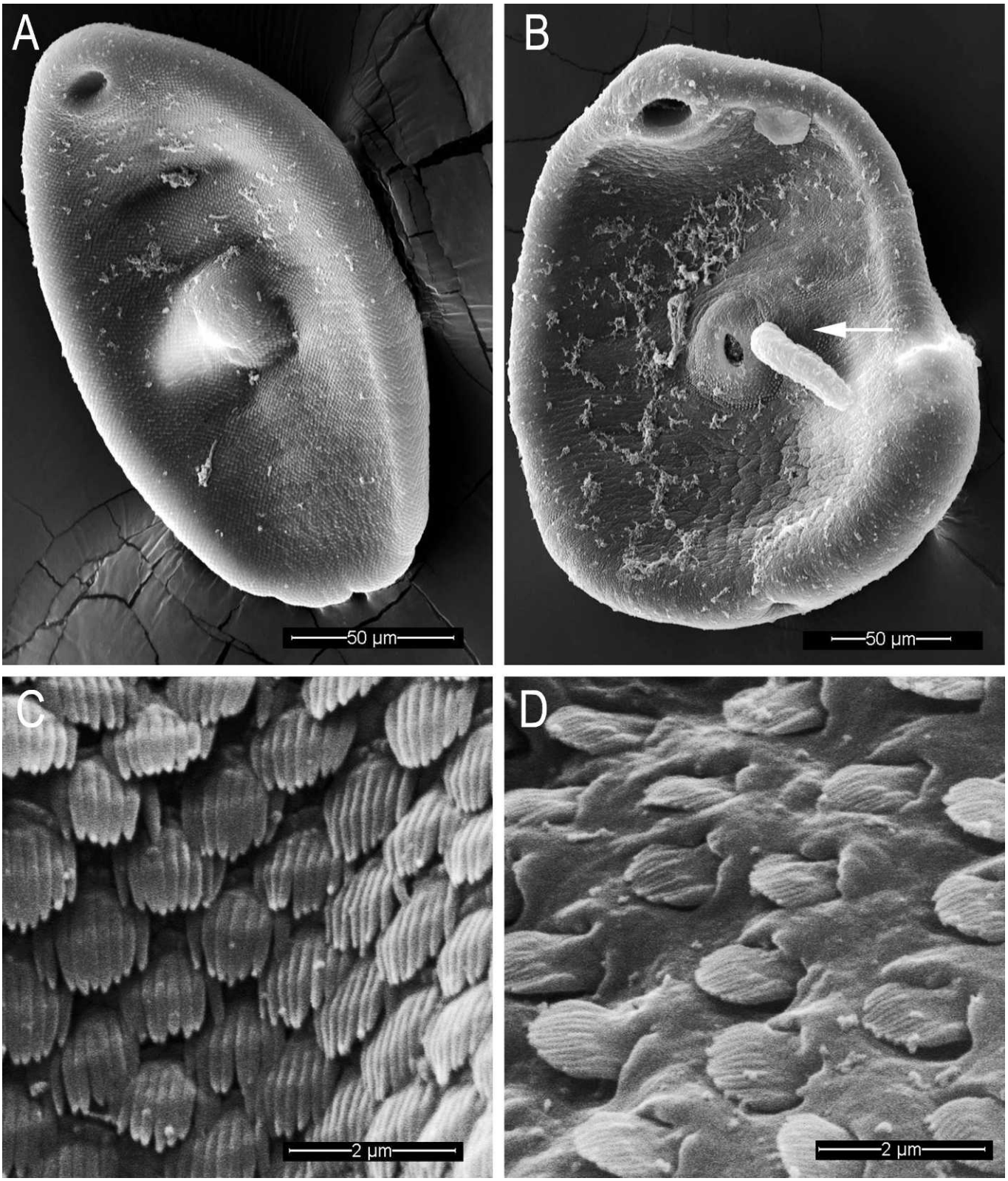

FIGURE 5. Excysted metacercaria of (**A**) *Maritrema orensense* and (**B**) *Maritrema bonaerense* showing cirrus evaginated (row), SEM. Tegument near ventral sucker of metacercaria of (**C**) *Maritrema orensense* and (**D**) *Maritrema bonaerense*.

TABLE II. Descriptive statistics for adult stage of *Maritrema orensense* and *Maritrema bonaerense* obtained from experimental infections in chicks. SD: standard deviation; CV: coefficient of variation; N: number of specimens observed; BL: body length; BW: body width; OSL: oral sucker length; OSW: oral sucker width; PpL: prepharynx length; PL: pharynx length; PW: pharynx width; EL: esophagus length; IL-l: left intestinal cecum length; IW-l: left intestinal cecum width; IL-r: right intestinal cecum length; IW-r: right intestinal cecum width; VSL: ventral sucker length; VSW: ventral sucker width; CiSL: cirrus sac length; CiSW: cirrus sac width; OvL: ovary length; OvW: ovary width; TeL-l: left testis length; TeW-l: left testis width; TeL-r: right testis length; TeW-r: right testis width; CiL: cirrus length; CiW: cirrus width; SemVL: seminal vesicle length; SemVW: seminal vesicle width; PCD: prostatic cells diameter; EggL: egg length; EggW: egg width; ND: no data.

	Maritrema orensense				*Maritrema bonaerense*	
	Mean ± SD	Range	CV	N	Mean ± SD	N
BL	0.344 ± 0.040	[0.286–0.395]	0.12	12	0.567	1
BW	0.159 ± 0.017	[0.136–0.181]	0.11	12	0.273	1
OSL	0.039 ± 0.004	[0.034–0.046]	0.10	12	0.050	1
OSW	0.041 ± 0.004	[0.034–0.047]	0.10	12	0.057	1
PpL	0.020 ± 0.005	[0.014–0.029]	0.25	11	0.020	1
PL	0.026 ± 0.005	[0.019–0.031]	0.19	11	0.020	1
PW	0.021 ± 0.004	[0.017–0.027]	0.23	11	0.023	1
EL	0.029 ± 0.005	[0.022–0.034]	0.17	11	0.073	1
IL-l	0.086 ± 0.008	[0.075–0.100]	0.09	12	0.253	1
IW-l	0.014 ± 0.002	[0.012–0.017]	0.14	12	0.013	1
IL-r	0.085 ± 0.008	[0.073–0.098]	0.09	12	0.267	1
IW-r	0.014 ± 0.002	[0.010–0.017]	0.14	12	0.010	1
VSL	0.028 ± 0.003	[0.024–0.032]	0.11	11	0.050	1
VSW	0.029 ± 0.004	[0.024–0.036]	0.14	11	0.060	1
CiSL	0.122 ± 0.009	[0.108–0.136]	0.07	12	0.193	1
CiSW	0.028 ± 0.005	[0.022–0.037]	0.18	12	0.033	1
OvL	0.041 ± 0.017	[0.022–0.078]	0.41	12	0.057	1
OvW	0.047 ± 0.013	[0.034–0.069]	0.28	12	0.097	1
TeL-l	0.044 ± 0.012	[0.029–0.059]	0.27	12	0.070	1
TeW-l	0.045 ± 0.006	[0.037–0.056]	0.13	11	0.073	1
TeL-r	0.042 ± 0.011	[0.031–0.061]	0.26	12	0.077	1
TeW-r	0.043 ± 0.008	[0.031–0.053]	0.19	12	0.083	1
CiL	0.038 ± 0.019	[0.017–0.054]	0.50	7	0.113	1
CiW	0.020 ± 0.010	[0.014–0.032]	0.50	7	0.023	1
SemVL	0.026 ± 0.002	[0.024–0.029]	0.08	9	0.067	1
SemVW	0.014 ± 0.003	[0.010–0.019]	0.21	9	0.030	1
PCD	0.005 ± 0.001	[0.003–0.007]	0.20	9	ND	1
EggL	0.014 ± 0.004	[0.008–0.016]	0.29	8	0.020	1
EggW	0.010 ± 0.003	[0.007–0.015]	0.30	8	0.010	1

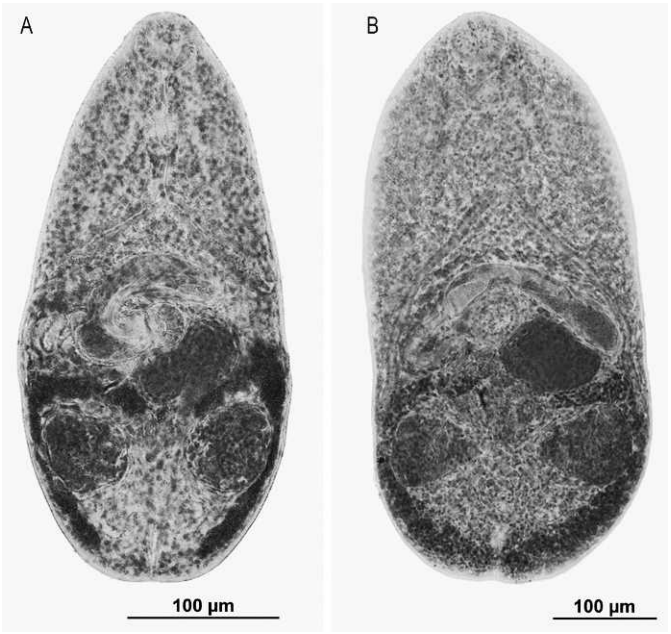

FIGURE 6. Adult of (**A**) *Maritrema orensense* and (**B**) *Maritrema bonaerense* experimentally obtained in chicks.

DISCUSSION

Experimental infections in crabs and chicks allowed us to describe for the first time the life cycle of *M. orensense* and to confirm the life cycle of *M. bonaerense* previously described by Etchegoin and Martorelli (1997). Both species use the cochliopid snail *Heleobia australis* as first intermediate host and the grapsid crabs *Cyrtograpsus angulatus* and *Neohelice granulata* as second intermediate hosts (Fig. 8). Gulls have been reported as the definitive hosts of both species (Etchegoin and Martorelli, 1997; Cremonte and Martorelli, 1998; Cremonte et al., 1999; La Sala et al., 2009).

Morphology and measurements of metacercariae of *M. orensense* obtained from experimental infections agree with those reported by Alda et al. (2011). We found differences in adult specimens of *M. orensense* with respect to those measurements reported by Cremonte and Martorelli (1998). In the present study, adults possessed a narrower body (0.136–0.181 vs. 0.19–0.27), a smaller seminal vesicle (0.024–0.029 × 0.010–0.019 vs. 0.035–0.072 × 0.021–0.037), smaller prostatic cells (0.003–0.007 vs. 0.009–0.011), and a shorter cirrus (0.017–0.054 vs. 0.058–0.074). These differences, however, could likely be due to the young age of our specimens (1 day old). The adults experimentally obtained in chicks in this study showed a subspherical to ovoid ovary and few eggs, whereas Cremonte and Martorelli (1998), who obtained adults from natural infections, described a trilobed ovary. However, our observations of holotypes of adult individuals naturally obtained by Cremonte and Martorelli (1998) lead us to consider that the ovary shape was triangular rather than trilobed. These adults also showed different shape and more eggs than adults experimentally obtained. Therefore, we suspect that the discrepancies in ovary shape are explained by ontogenetic variation—subspherical to ovoid in juveniles and triangular in fully developed adults. This indicates that caution is necessary when using ovary shape to identify and compare juvenile and adult stages of different species.

We observed that the sporocysts of *M. bonaerense* have a wider body compared to that reported by Etchegoin and Martorelli (1997) (0.121–0.221 vs. 0.079–0.093). Furthermore, the authors reported sporocysts in the digestive gland of *H. australis*, while we primarily observed them in the gonad region, but also some in the digestive gland. Except for this difference,

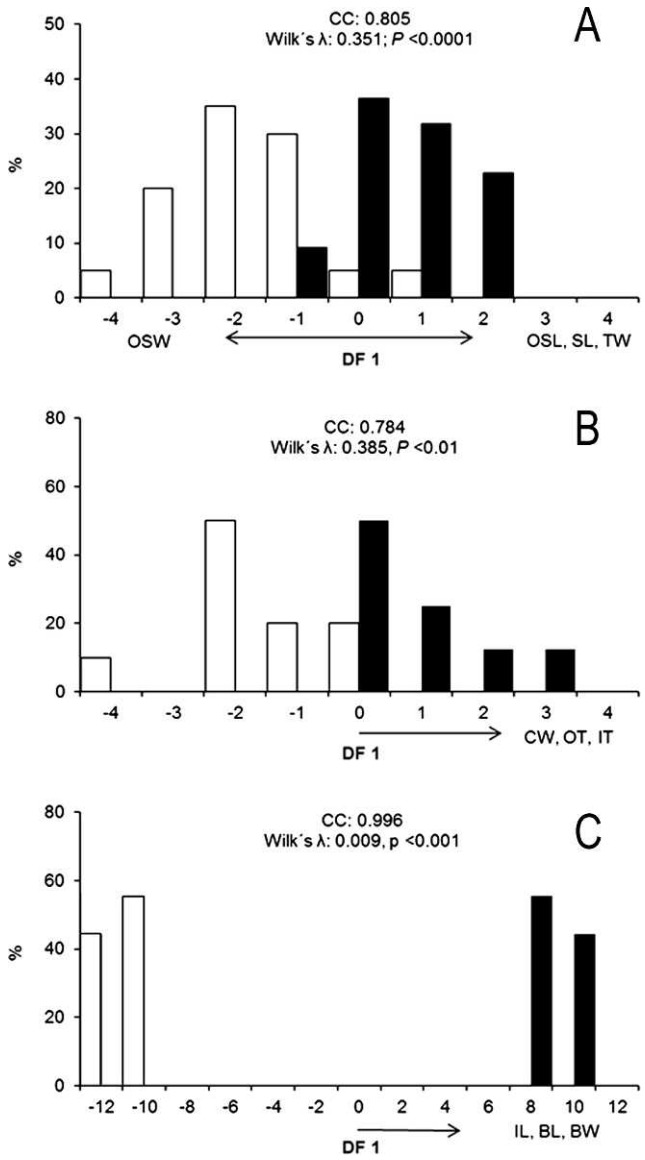

FIGURE 7. Frequency histogram of discriminant function 1 (DF1) corresponding to the discriminant analysis performed for morphological measurements of *Maritrema orensense* (white columns) and *Maritrema bonaerense* (black columns). Wilks's lambda (λ), and canonical correlation (CC) are indicated. (**A**) Variables from cercaria stage of *M. orensense* yielded 90% of correct classification and 73% for *M. bonaerense*; (**B**) variables from encysted metacercariae of *M. orensense* gave 80% of correct classification and 63% for *M. bonaerense*; (**C**) variables from excysted metacercariae of *M. orensense* yielded 100% of correct classification and 100% for *M. bonaerense*. References are indicated in Tables I and II.

the morphology and measurements of sporocysts and cercariae of *M. bonaerense* mentioned in our study were similar to those reported by Etchegoin and Martorelli (1997). We documented similar morphology and measurements of metacercariae obtained from experimental infections to those obtained from naturally infected crabs and to those reported by Etchegoin and Martorelli (1997) and Alda et al. (2011), except that the cyst observed herein has 2 hyaline layers instead of 1 as described by the previous authors. The single adult specimen of *M. bonaerense* found in chicks prevented us from statistically comparing measurements. Although the morphology was similar to that reported by Etchegoin and Martorelli (1997) and most measurements were within the range given by the authors, we estimated a wider cirrus sac (0.033 vs. 0.011–0.017), a wider ovary (0.097 vs. 0.073–0.085), shorter testes (left: 0.070 vs. 0.079–0.2; right: 0.077 vs. 0.083–0.2), a shorter and wider cirrus (0.113×0.023 vs. $0.16–0.21 \times 0.011–0.017$), and a smaller seminal vesicle (0.067×0.030 vs. $0.11–0.17 \times 0.04–0.06$). We suspect that these differences reflect intraspecific variability, as fixation techniques were the same in the 2 studies.

The *t*-tests of most morphometric variables of each stage of *M. orensense* and *M. bonaerense* revealed significant differences between the variables despite substantial overlap in range (Table I). Further, the multivariate classification procedure of discriminant functions analysis allowed us to maximize group differences and determine the morphometric variables that contributed the most to the discrimination between species.

Morphometric ratios showed that sporocysts of *M. bonaerense* were more elongated with a higher number of developed cercariae than *M. orensense*. Both species clearly castrated the snails, as the sporocysts took up the entire gonadal space of the snail. Shed cercariae of *M. bonaerense* had a longer body and stylet, and longer but thinner oral sucker. The cercariae of *M. bonaerense* had smaller, shorter, and rounded cephalic glands, and ventral gland pairs stained lighter with neutral red than dorsal gland pairs. On the contrary, the cercariae of *M. orensense* had longer, larger, and more undulating cephalic glands, and dorsal and ventral glands stained similar with neutral red. Significant differences found in morphometric ratios were consistent with the qualitative morphology descriptions; *M. bonaerense* cercariae had a more elongated shape than *M. orensense*. Discriminant analysis allowed us to mainly differentiate both species stages using the oral sucker length and width of the cercariae.

We found 10 metacercariae of *M. orensense* in 1 crab 3 wk PI and 100 metacercariae of *M. bonaerense* in 1 crab 5 wk PI. This could represent variation in cercarial output, host specificity, or chance, something that can be elucidated with future experiments with adequate design. Similarly, we found 1 adult of *M. bonaerense* in 1 chick 3 days PI and 10 adults of *M. orensense* in 1 chick 1 day PI, but our study was not designed to discern whether this was due to variation in host specificity or longevity of adults, versus simply to obtain stages for morphological description.

The metacercaria stage of both species infects crab's gills, though we found that *M. orensense* metacercariae were more deeply encysted in the primary filament. Also, whereas metacercariae of *M. bonaerense* migrate to other tissues and compartments and encyst in hepatopancreas, coelom, muscle, and gonad (Alda et al., 2011), metacercariae of *M. orensense* infect the crabs' gills only. Cyst lengths of metacercariae of *M. orensense* and *M. bonaerense* encysted in crab's gills were similar, but the shape was different, i.e., more oval for *M. orensense* and more spherical for *M. bonaerense*. The outer and the innermost cyst walls were

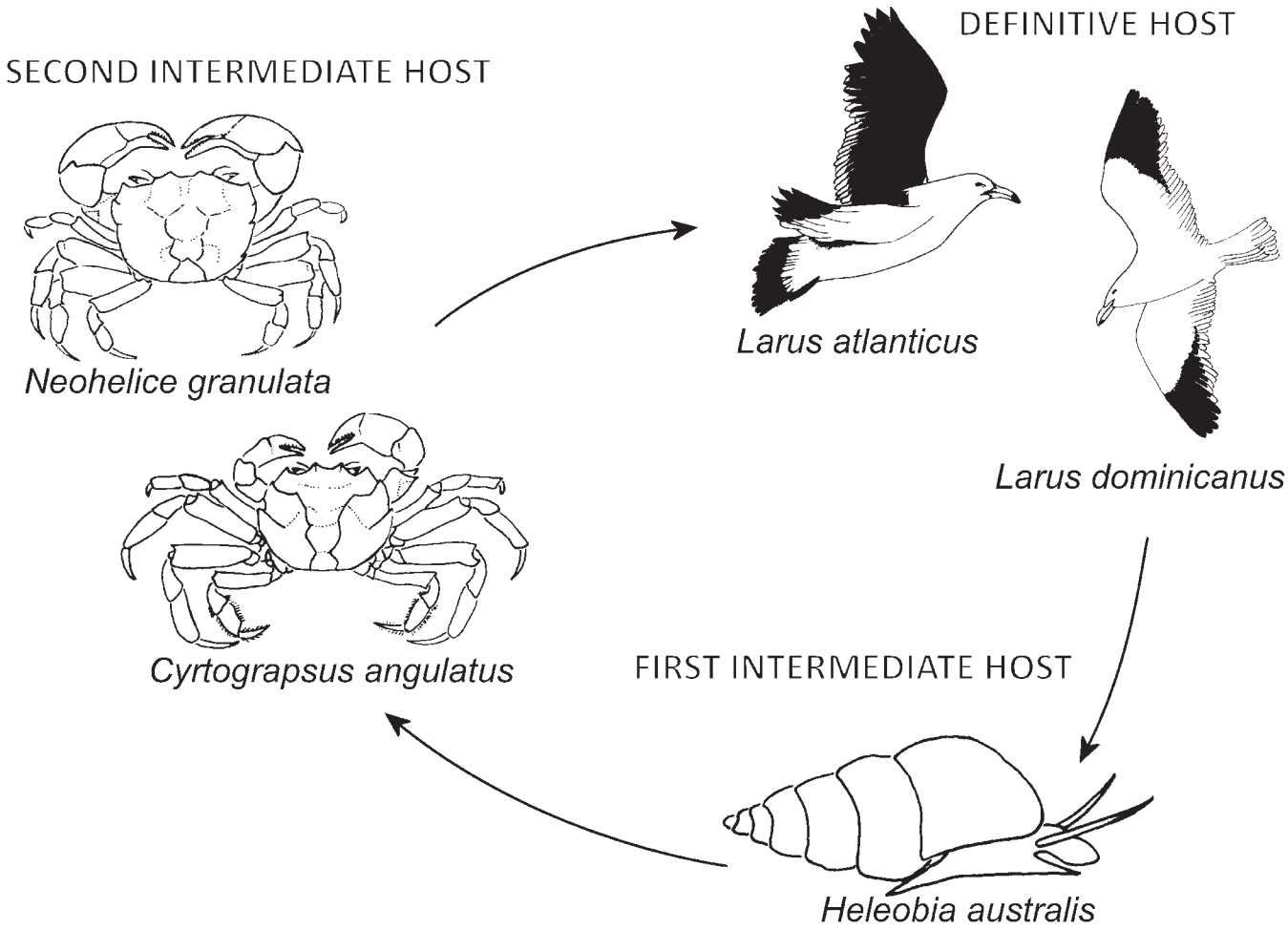

FIGURE 8. Life cycle of *Maritrema orensense* and *Maritrema bonaerense*.

thinner in *M. orensense*. Discriminant analysis showed that the most relevant variables to differentiate both species were cyst width and outermost wall thickness. The shape of the excysted metacercariae also differed, particularly at the anterior body (acutely tapering for *M. orensense* and obtusely tapering for *M. bonaerense*). As in adults, metacercariae of both species were easily differentiated by the intestinal ceca, not reaching the testicular fields in *M. orensense*, and by the shape of the vitellarium—a posteriorly incomplete ring in *M. orensense* and a complete ring in *M. bonaerense*. Also metacercariae of both species differed in measurements of body, oral sucker, pharynx, and esophagus, being always smaller or shorter in *M. orensense*. Regarding morphometric ratios, we found that both species had a similar shape, but different morphometric dimensions. Consistent to the description mention previously, the discriminant analysis showed that the most relevant variable to differentiate both species stages was intestinal ceca length. Although we did not perform a comparative analysis of adult stage of both species, since there were not enough adult individuals for *M. bonaerense*, we observed that they were clearly different in the length of the intestinal ceca, the shape of the vitellarium, and the size of the eggs. These results are consistent with Cremonte and Martorelli's (1998) observations.

Hence, although *M. orensense* and *M. bonaerense* share host species (cochliopid snails, grapsid crabs, and gulls) and certain habitats, the descriptive and comparative analyses of different stages obtained from natural and experimental infections confirm that the species are readily distinguishable from one another at all life stages.

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LITERATURE CITED

Alda, P., L. La Sala, P. Marcotegui, and S. R. Martorelli. 2011. Parasites and epibionts of grapsid crabs in Bahía Blanca Estuary, Argentina. Crustaceana **84:** 559–571.

Cremonte, F., J. A. Etchegoin, and S. R. Martorelli. 1999. Nuevos hospedadores de los digeneos *Maritrema bonaerensis* (Microphallidae) y *Stephanoprora podicipei* (Echinostomatidae) en Argentina. *Stephanoprora manei* Holcman-Spector *y* Olagiie, 1986 como un nomen nudum. Neotrópica **45:** 105–107.

———, and S. R. Martorelli. 1998. Description of a new species of *Maritrema* (Digenea: Microphallidae) from *Larus dominicanus* (Aves: Laridae) in Buenos Aires coast, Argentina. Folia Parasitologica **45:** 230–232.

Etchegoin, J. A., and S. R. Martorelli. 1997. Description of a new species of *Maritrema* (Digenea: Microphallidae) from Mar Chiquita coastal lagoon (Buenos Aires, Argentina) with notes on its life cycle. Journal of Parasitology **83:** 709–713.

Hair, J. F. Jr. R. E. Anderson, R. L. Tatham, and W. C. Black. 1999. Análisis multivariante. Prentice Hall Iberia, Madrid, Spain, 832 p.

La Sala, L. F., S. R. Martorelli, P. Alda, and P. Marcotegui. 2009. Some digeneans from Olrog's gull *Larus atlanticus* Olrog, 1958 (Aves: Laridae) from the Bahía Blanca Estuary, Argentina. Comparative Parasitology **76:** 113–116.