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The life cycle of *Zygocotyle lunata* (Trematoda, Paramphistomoidea) in the subtropical region of South America

El ciclo de vida de *Zygocotyle lunata* (Trematoda, Paramphistomoidea) en la región subtropical de América del Sur

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Abstract. The life cycle of the trematode *Zygocotyle lunata* (Diesing, 1836) is studied, beginning with cercariae from naturally infected *Biomphalaria peregrina* (d'Orbigny, 1835) and *B. tenagophila* (d'Orbigny, 1835) from Buenos Aires Province and Salta Province (Argentina), respectively. Adults were recovered from *Gallus domesticus* (Linnaeus, 1758) and *Mus musculus* (Linnaeus, 1758). Egg development lasted for 15-23 days at 23-24 °C, and 10-12 days at 28-30 °C; it was delayed after successive passages through mice, and ceased thereafter. In the snail, sporocysts, located beneath the mantle border, occurred up to 37 days post exposure (PE). Rediae invaded the entire snail body; developing cercarial bodies within rediae were recognized by the presence of a short tail, excretory canals, and eye-spot pigment. Cercariae emerged from snails as early as 20-26 days PE, encysting immediately on any substrate. These can be easily differentiated from those of *Paramphistomum* Fischoeder, 1901 spp. and *Cotylophoron* Stiles and Goldberger, 1910 spp., which have lateral excretory tubes forming a ring at the midlevel of the body and usually lack pharyngeal appendages. Metacercariae were infective for at least 6 months. Mature adults were observed 27-32 days PE in mice and 35 days PE in chicks. *Biomphalaria tenagophila* represents a new intermediate natural host record and Salta Province a new geographic area; *Dendrocygna viduata* (Linnaeus, 1766) and *D. bicolor* (Vieillot, 1816) from Venezuela are new host records for *Z. lunata*.

Key words: Digenea, Zygocotylidae, Experimental cycle, *Biomphalaria* spp., larval stages, Argentina.

Resumen. El ciclo de vida del trematode *Zygocotyle lunata* (Diesing, 1836) (Paramphistomoidea: Zygocotylidae) fue estudiado experimentalmente, comenzando con cercarias emergidas de *Biomphalaria peregrina* (d'Orbigny, 1835) y *B. tenagophila* (d'Orbigny, 1835) infectadas naturalmente, provenientes de las provincias de Buenos Aires y Salta (Argentina), respectivamente. Los adultos fueron obtenidos de *Gallus domesticus* (Linnaeus, 1758) y *Mus musculus* (Linnaeus, 1758). El desarrollo del huevo demora 15-23 días a 23-24 °C y 10-12 días a 28-30 °C; demora aun más después de sucesivos pasajes por ratones, no desarrollándose posteriormente. Los esporoquistes se localizan debajo del borde del manto del molusco, y se observaron hasta 37 días después de la infección (PE). Las redias invaden todo el cuerpo del molusco; los cuerpos de las cercarias en desarrollo se distinguen por la presencia de una cola corta, canales excretores y pigmento de manchas oculares. Las cercarias emergen como mínimo después de 20-26 días PE y se enquistan inmediatamente sobre cualquier superficie. Pueden ser fácilmente diferenciadas de las cercarias de *Paramphistomum* Fischoeder, 1901 spp. y *Cotylophoron* Stiles and Goldberger, 1910 spp., las cuales poseen canales excretores laterales formando un anillo a nivel medio del cuerpo, y generalmente no poseen apéndices faringiales. Las metacercarias son infectivas al menos hasta 6 meses. Adultos maduros fueron observados a partir de 27-32 días PE en los ratones y 35 días PE en los pollos. *Biomphalaria tenagophila* constituye un nuevo hospedador intermediario natural y la Provincia de Salta un área geográfica nueva; *Dendrocygna viduata* (Linnaeus, 1766) y *D. bicolor* (Vieillot, 1816) de Venezuela constituyen nuevos hospedadores definitivos de *Z. lunata*.

Palabras clave: Digenea, Zygocotylidae, ciclo experimental, *Biomphalaria* spp., estados larvales, Argentina.

Introduction

Zygocotyle lunata (Diesing, 1836) is a widely distributed species present in North and Central America, Brazil, Asia (Russia, India), and Africa parasitizing wild birds belonging to Anatidae, Recurvirostridae, and Podicipedidae, and some mammals belonging to Cervidae (Sey, 1991). In addition, it can be easily obtained experimentally from laboratory-reared animals (ducks, chicks, rats, and mice). The life cycle and larval stages of *Z. lunata* have been investigated mainly in the United States, where species of the genus *Helisoma* Swainson, 1840 act as first intermediate hosts (Huffman et al., 1991; Etges, 1992; Shostak et al., 1993; Nollen, 1994; Reddy and Fried, 1997; Fried et al., 2009). Among these, only Willey (1941) described in detail the morphology of the larval stages in *Helisoma antrosum* (Conrad).

In Argentina, *Z. lunata* has been found naturally parasitizing *Anas sibilatrix* Poepping, 1829 and *Cygnus melancorypha* (Molina, 1782) (Sutton and Lunaschi, 1987; Digiani, 1997). *Biomphalaria peregrina* (d'Orbigny, 1835) collected from artificial ponds in parks and in the Zoological Garden of Buenos Aires was reported as a natural first intermediate host; aquatic birds of Anseriformes, Ciconiiformes, Pelecaniformes, and Gruiformes, and mammals (*Myocastor coypus* Molina, 1782) occurred in these freshwater environments. Experimental infection was achieved in *B. peregrina*, *B. straminea* (Dunker, 1848), *B. orbignyi* (Paraense, 1975), *B. tenagophila* (d'Orbigny, 1835), and *B. oligoza* (Paraense, 1974), but not in *B. glabrata* (Say, 1818) (see Ostrowski de Núñez et al., 2003).

The aim of this paper is to present unpublished data from an earlier study (Ostrowski de Núñez et al. 2003), and new results about the larval stages of *Z. lunata* in *Biomphalaria* spp. The detailed description will allow their differentiation from those of other amphistomes, especially species of *Paramphistomum* (Fischöder, 1901) and *Cotylophoron* (Stiles et Goldberger, 1910), whose prevalence is apparently increasing in cattle in Argentina and Brazil (Raccioppi et al., 1994; Costa et al. 1999; Miranda and Costa, 1999; Rodríguez Armesto et al., 2002). Additionally, a new natural intermediate host species from Argentina, and 2 new bird hosts from Venezuela, are reported herein.

Materials and methods

The study of the present life cycle was based on 2 different experiments (N: number of specimens revised):

Experiment 1: *Biomphalaria peregrina* was collected (1992–1999; N= 1 424) at a pond in the Zoological Garden in Buenos Aires, Argentina (34°36'S; 58°27'W). Snails were maintained individually in vials with 5 ml of tap water and a piece of lettuce, and checked daily for emergence of cercariae. Cercariae identified as *Z. lunata* emerged from 8 snails between January 1996 and October 1999, and encysted on vial walls, and plants. Adults were recovered after infecting newborn, unfed chicks and mice with these metacercariae. Eggs recovered from the feces of the mice were incubated at temperatures between 23–24 C or 28–30 C and exposed to laboratory-raised *Biomphalaria* spp. (*B. orbignyi*, *B. tenagophila*, *B. oligoza*, *B. peregrina*, *B. straminea*, and *B. glabrata*); finally, uninfected mice were fed metacercariae from the experimentally obtained cercariae. The life cycle was maintained in this way in the laboratory until egg development ceased after successive passages through mice and infections of snails was no longer possible.

Experiment 2: *Biomphalaria tenagophila* was collected (2005–2010; N= 4 448) at a small pond at the confluence of the rivers San Lorenzo and Arias (24°47'S; 65°28'W), in the Lerma Valley located in the suburbs of Salta, Argentina, and maintained as above. Cercariae of *Z. lunata* emerged from 10 snails between June 2005 and April 2010. Mice and unfed chicks were exposed to the cysts from emerging cercariae, and eggs recovered from the feces of mice were incubated at room temperature, 18–27 C. Eggs containing almost fully developed miracidia were exposed to groups of laboratory raised *B. straminea*, for 8–25 days. Some of these snails were dissected up to 37 days post exposure (PE) for the detection of sporocysts and young rediae, whereas the remaining snails were maintained alive until emergence of cercariae and then dissected to study mature rediae.

Cercariae were studied alive under coverslip pressure immediately after emergence because they encysted rapidly on vial walls, snail shells, and plants. For histological studies, 2 snails were fixed, embedded in paraffin wax, cut at 7 µm, stained with hematoxylin and eosin, and mounted in Canada balsam. Adults recovered from infected mice and chicks at different times PE were fixed in hot 4% formalin, stained with alcoholic hydrochloric carmine, and mounted in Canada balsam. Measurements of eggs, sporocysts, metacercariae, and adults were taken from living and/or stained material, and measurements of cercariae and rediae were taken from unstained material fixed in hot 4% formalin, unless otherwise stated. The body and tail of cercariae were measured without coverslip pressure, but suckers could only be visualized after a slight pressure due to the heavily pigmented body. Measurements are given in micrometers, with the range followed by the mean in

parentheses. *Dendrocygna viduata* (Linnaeus, 1766) and *D. bicolor* (Vieillot, 1816) were captured near Caracas, Venezuela, in August 1978. Specimens were deposited at the Helminthological Collection of the Museo Argentino de Ciencias Naturales (Buenos Aires), MACN-Pa 497.

Results

Egg (Fig 1-3): freshly laid eggs 129-142 long by 91-98 wide. Egg development takes 15-23 days at 23-24 C, with hatching starting on day 17; or 10-12 days at 28-30 C, with hatching starting on day 10. Development is delayed after successive passage through mice, and ceases thereafter.

Sporocyst (Figs. 4, 5, 9): located beneath the mantle border near the kidney in *B. straminea*, appears as a rounded cyst-like body 440-500 long by 188-377 wide, 12-20 days PE. Living sporocysts freed from the mantle tissue have irregular elongated bodies, 525-690 long by 200-216 wide, with irregular borders, containing numerous undeveloped germinal masses and young rediae, 160-200 long by 70-90 wide; pharynx 25 in diameter. Two stained, slightly flattened specimens 880-912 long by 272 wide (Fig. 9).

Redia (Figs. 6, 10, 11): freshly released, living redia from *B. straminea* (N= 10) 202-483 (326) long by 84-283 (139) wide, with peculiar tapered posterior end; pharynx 22-55 (32) long by 28-55 (36) wide. Cecum 133-267 (208) occupies half or more of body length (Fig. 6). Formalin-fixed rediae, approximately 38-50 days PE, from *B. straminea*, (N= 10) 491-825 (629) long by 101-145 (124) wide, small pharynx 26-52 (28) long by 32-42 (37) wide, short cecum 54-96 (77) long, representing 8-19 (13) % of body length. Redia from naturally infected *B. peregrina* variable in length, (N= 10) 608-912 (786) long by 160-240 (195) wide; small pharynx 29-45 (34) long by 35-51 (40) wide; short cecum 96-176 (118) long, representing 10.5-19 (15.2) % of body length; birth pore, when visible, 144-176 from anterior end. Large rediae from hepatopancreas and gonad contain 1-5 distinguishable cercarial bodies and several undifferentiated germ balls. Excretory vesicle with 2 chambers on each side of body: a relatively large distal chamber, and a spherical anterior chamber connected to a single duct receiving collecting ducts of 1 anterior, and 2 posterior protonephridia up to 26.8 long (Fig. 11).

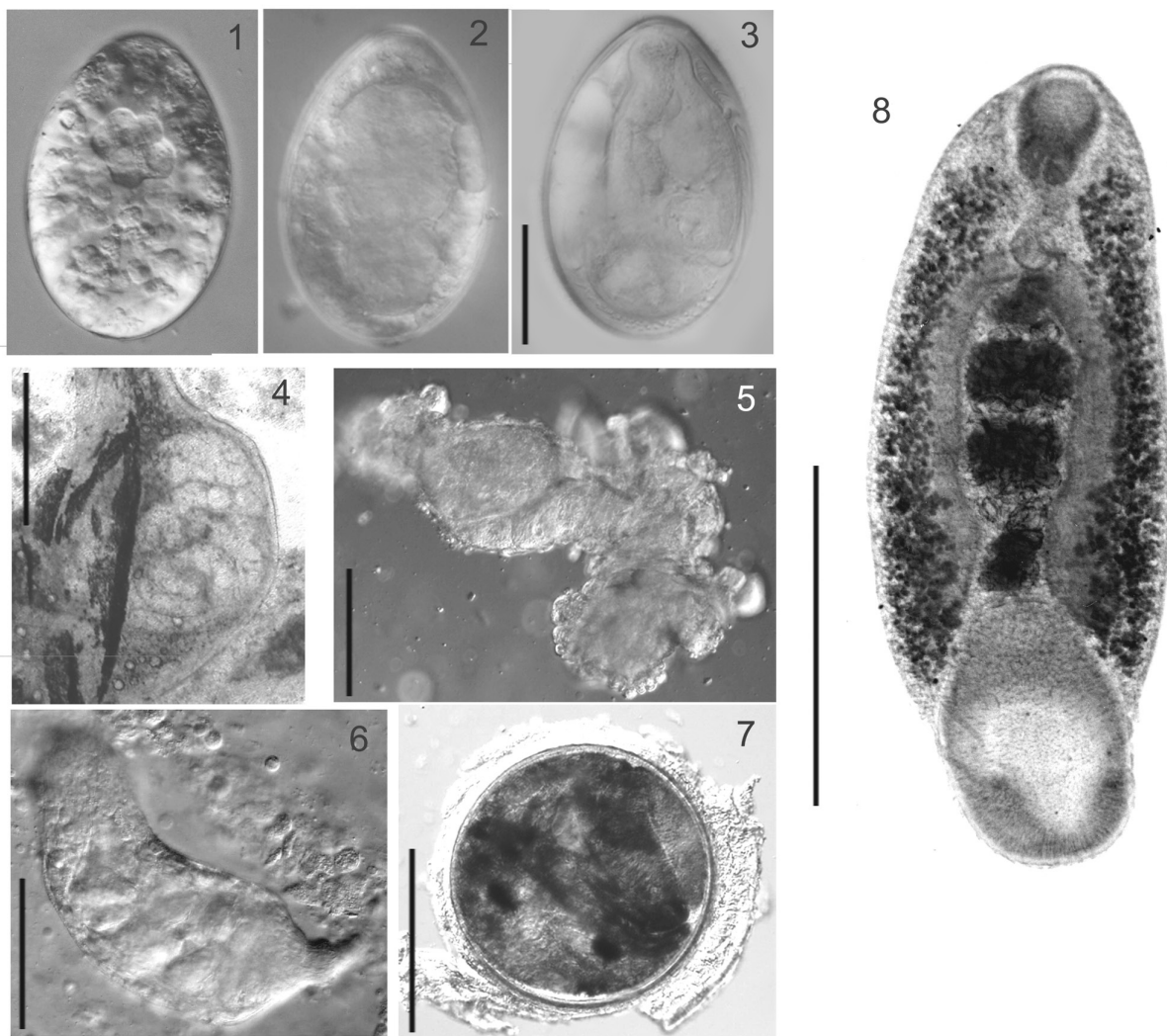
Cercaria (Figs. 12, 13): measurements of cercariae (N= 20) that emerged naturally from *B. peregrina*: heavily pigmented body 264-333 (298) long by 239-276 (261) wide, tail 640-880 (739) long by 64 wide at proximal end, with several papillae with short setae on lateral borders; oral opening followed by pharynx 63-72 (67) long by

63-79 (69) wide, with pharyngeal appendages; ventral sucker 95-117 (103) long by 117-148 (135) wide, with typical well-developed lip at distal border; pigmented eye-spots 28-41 (34) long by 25-32 (30) wide, situated 63-95 (85) from anterior end. Main collecting ducts with lateral diverticula, join shortly anterior to ventral sucker, enter posterior margin of excretory vesicle (Fig. 12).

Cercariae (N= 20) emerging naturally from *B. tenagophila* slightly larger: body 304-393 (330) long by 157-334 (255) wide; tail 530-923 (748) long by 59-88 (72) wide; ventral sucker 88-115 (106) long by 96-115 (106) wide; pigmented eye-spots 22-47 (31) long by 20-32 (24) wide, situated 42-98 (66) from anterior end.

The source for sporocysts was *B. straminea* experimentally exposed in groups to the eggs containing almost fully developed miracidia for 8-25 days; therefore, their exact infection date is unknown. In snails dissected up to 37 days PE, rediae had already invaded the gonad whereas 1-5 sporocysts were located beneath the mantle border. Young rediae are densely packed within the developing germinal masses, so that they could be clearly distinguished only after being released by manipulating the coverslip. The young rediae seem to leave the sporocyst by rupture of the body wall. In mature rediae, it is impossible to distinguish between daughter rediae and developing cercarial bodies, which can only be identified when the excretory canals, a short tail and eye-spot pigment are being developed. In older infections the rediae invade the whole body of snails, and are easily detected through the shell at low magnification. The size of the pharynx is similar in freshly released and older rediae, whereas the length of the cecum appears larger in the former, which may be due to the different development of the body cavity containing the germinal masses. Immature cercariae emerge from rediae and continue their development in the snail tissues.

Some measurements of cercariae were taken on living material for comparison with the data by Willey (1936, 1941; measurements of Willey in parentheses): body length 1 000 (980-1 200), body width 500 (310-450), pharynx length 107 (106) without pharyngeal appendages and 186 (165) with them; ventral sucker length 214, width 271 (210 in diameter). Tail 1 071 long by 143 wide (1 267 by 107 according to fig. 1 in Willey, 1936). Cercariae fixed with hot formalin became contracted, adopting a spherical or oval body, considerably different from that of living material. Internal organs could only be seen in flattened specimens because the body is heavily pigmented. Cercariae emerged from experimentally infected snails maintained at 26 C as early as 20-26 days PE, but at room temperature emergence was delayed for at least 60 days. Cercariae encysted immediately on the vial wall, snail shell, and pieces of lettuce.

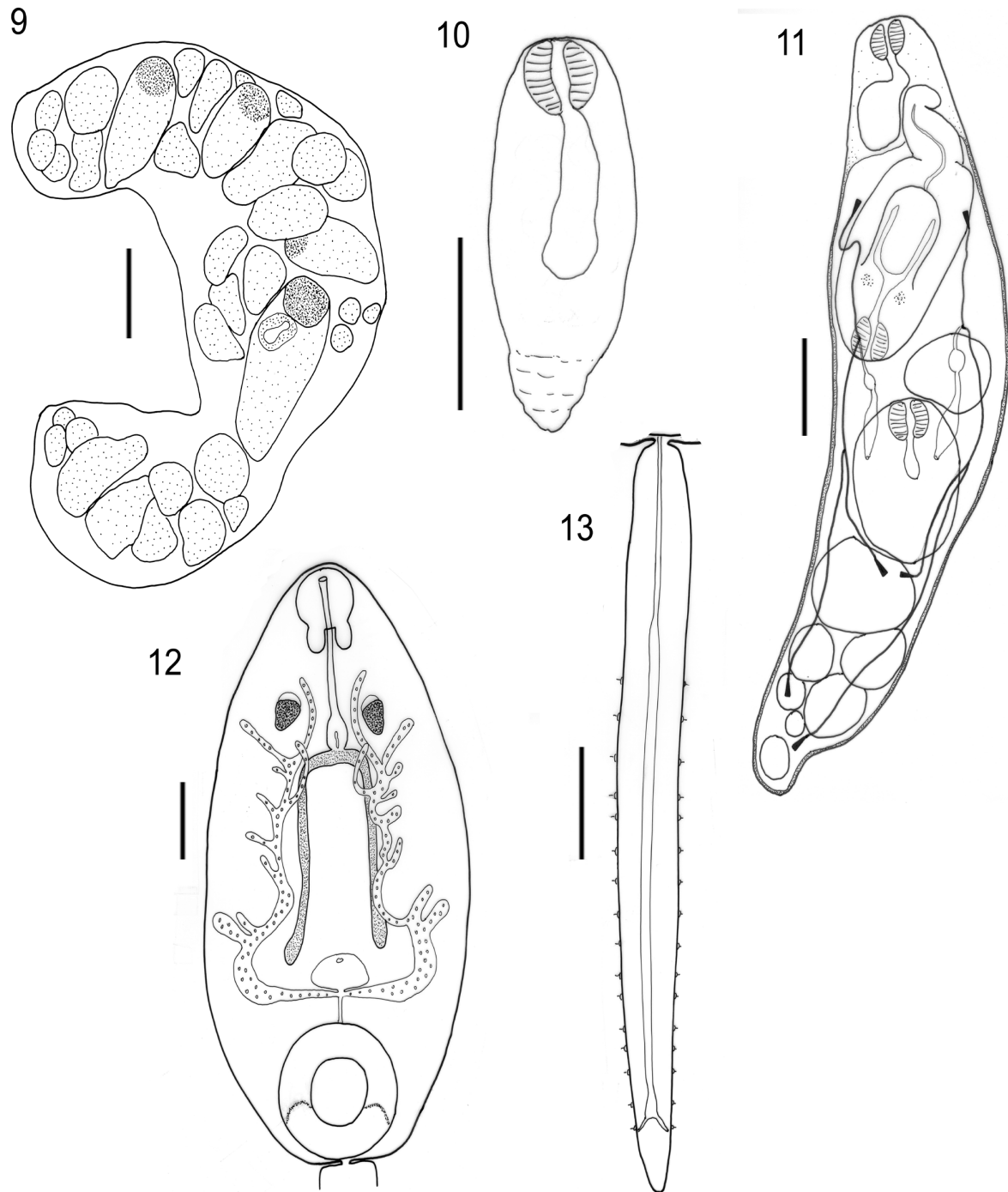


Figures 1-8. 1, freshly laid egg. 2, egg 10 days. 3, egg 17 days (scale bar: 50 μ m). 4, sporocyst, beneath the mantle border (scale bar: 500 μ m). 5, sporocyst freed from tegument (scale bar: 200 μ m). 6, young redia (scale bar 100 μ m). 7, encysted metacercaria (scale bar 200 μ m). Figures 1-7 live specimens. 8, adult, 35 days PE from chick (fixed specimen, scale bar: 1600 μ m).

Metacercaria (Fig. 7): cysts of cercariae emerging from naturally infected *B. peregrina* nearly spherical, 239-264 (250) long by 239-251 (248) wide (N= 20), heavily pigmented, surrounded by gelatinous cyst wall 12-40 thick, with irregular borders. Cysts 150 and 180 days old held at room temperature were still viable and infective, which was experimentally confirmed by successful development of few adults in a mouse and 2 chicks, respectively. Cysts of cercariae emerging from naturally infected *B. tenagophila*: 245-274 (254) long by 247-274 (257) wide (N= 10); gelatinous cyst wall 5-20 (14).

Adult (Fig. 8): measurements of experimentally obtained ovigerous adults from mice and chicks (indistinctly from source of metacercariae) in comparison with that of Sutton and Lunaschi (1987) and Digiani (1997) from natural hosts in Argentina and the specimens obtained in Venezuela are given in Table 1.

Body oval, elongate. Pharynx terminal, with paired small appendages, esophagus short, with well developed bulb. Ceca simple, extending near anterior border of ventral sucker. Ventral sucker ventroterminal, provided with a well developed lip on posterior border. Testes tandem,



Figures 9-13. 9, sporocyst (fixed specimen). 10, young redia. 11, mature redia. 12, cercaria (body, slightly pressed). 13, cercaria (tail). Figures 10-13 from live specimens. Scale bars: Figures 9-11: 200 μ m; Figures 12, 13: 100 μ m.

with regular to lobed borders, intracecal, in middle third of body. Seminal vesicle long and convoluted. Genital pore posterior to intestinal bifurcation. Ovary median, post-testicular, near anterior border of ventral sucker. Mehlis gland and seminal receptacle posterior to ovary. Uterine coils intracecal. Vitelline follicles in lateral bands, from level of pharynx to nearly mid-level of ventral sucker. Eggs numerous.

All mice (N= 42) and 6 of 8 chicks exposed to metacercariae of *Z. lunata* in experiment 1 and all mice (N= 4) and chicks (N= 2) exposed to metacercariae in experiment 2 resulted positive by presence of eggs in feces or adults in the cecum after dissection. Five mice surviving infection for over a year (up to 447 days) shed eggs throughout this period. Eggs were found in feces as early as on day 27 PE. The uterus of adults from mice appeared full of eggs 32 days PE, and in chicks adults produced eggs from day 35 PE onward. The eggs in the

uterine coils of fixed specimens collapsed, and appeared smaller than the freshly laid eggs. The 2 specimens from *D. viduata* and the single specimen from *D. bicolor* (found in the intestine) had no eggs, and were smaller than adults from mice and chicks 35 days PE.

Discussion

The occurrence of larval stages of *Z. lunata* in *B. tenagophila* collected from water bodies in Lerma Valley, Salta Province, constitutes a new, natural intermediate host record and a new geographic area for this trematode. In addition, this is the first record of *D. viduata* and *D. bicolor* from Venezuela as definitive hosts of *Z. lunata*.

So far, studies on *Z. lunata* have focused on different aspects of adult morphology and development, mating behavior, and effects on the host (Huffman et al., 1991;

Table 1. Measurements (μm) of *Zygocotyle lunata*. Range (mean)

	<i>Mice</i> 35-392 days PE N= 10	<i>Chicks</i> 35 days PE N= 4	<i>A. sibilatrix</i> Sutton and Lunaschi 1987 (N= 2)	<i>C. melancorypha</i> Digiani 1997 (N= 1)	<i>D. viduata</i> N= 2	<i>D. bicolor</i> N= 1
Body L	4064-5872 (4690)	3504-3792 (3632)	9110	6110	2880-3424	2672
Body W	1056-2704 (1666)	1168-1504 (1360)	2930	1500	880-1136	896
pharynx L	448-560 (487)	352-400 (376)	590	560	224-288	256
pharynx W	464-592 (539)	352-400 (376)	640	520	224-304	240
pharyngeal sac L	239-314 (291)	201-251 (221)	260	190-210	144-157	144-157
pharyngeal sac W	138-251 (207)	126-157 (133)	280	240	88-132	82
esophagus	94-251 (245)*	314-333 (319)*	1130	430	448-512	400
esophageal bulb L	157-377 (280)	195-220 (206)		330	170-126	176
esophageal bulb W	157-283 (239)	163-220 (199)	350	200	88-126	151
ventral sucker L	1040-1568 (1236)	1120-1248 (1164)	1700	1590	656-800	736
ventral sucker W	672-1520 (965)	752-912 (856)	1240	1110	480-672	640
ovary L	240-512 (310)	144-176 (160)	250	260		
ovary W	240-800 (402)	192-272 (228)	530	340		
anterior testis L	432-720 (547)	192-320 (256)	520**	380	320-336	188
anterior testis W	480-1280 (744)	336-528 (416)	850**	490	352-512	220
posterior testis L	448-720 (544)	192-320 (280)		380	288-320	176
posterior testis W	480-1088 (698)	400-480 (452)		470	304-480	188
eggs L	113-157 (136)	119-138 (131)	140	131-138	-	-
eggs W	57-94 (69)	57-82 (69)	93	73-82	-	-

*Distance from posterior end of oral sucker to anterior border of esophageal bulb; ** both testes included.

Shostak et al., 1993; Nollen, 1994; Reddy and Fried, 1997), whereas the information on larval stages is scarce and mainly limited to the metacercaria (Fried et al., 2009).

Egg development varies with temperature, e.g., eggs from feces of mice hatched between 30-45 days PE at 20-22 °C (Shostak et al., 1993), eggs from worms in mice and rats hatched between 21-26 days PE, and eggs from worms in hamsters hatched between 40-45 days PE at room temperature (Huffman et al., 1991); eggs from worms in mice hatched between 26-48 days PE at 23-26°C (Etges, 1992). In the experiments carried out in this study at higher temperatures, hatching occurred earlier, between 10-17 days PE onward. Contrary to the life cycle of *Schistosoma mansoni*, which could be maintained nearly indefinitely in the laboratory as eggs are viable after years of passage through mice, the life cycle of *Z. lunata* lasted about 5 years when egg development began to fail, and infection of snails was no longer possible.

Data on the period between infection of snails with *Z. lunata* and emergence of cercariae are scarce: Willey (1941) reported 32-49 days PE, and Etges (1992) 46-50 days PE. In this study, the prepatent period was about 60 days PE at room temperature, decreasing to 20-26 days PE under controlled temperature conditions.

The body length of worms recovered from mice was smaller and the onset of egg production occurred later in this study than in the study of Shostak et al. (1993) (4.1-5.9 mm vs 6.9-8.3 mm and 27-32 vs 17 days PE, respectively). Similar results were obtained for adults recovered from chicks, with worms being smaller and their egg production beginning later in this study than in Fried's study (1970) (3.5-3.7 mm vs 3.5-5.0 mm and 35 vs 12 dpi days PE, respectively). Worms from natural infections (Sutton and Lunaschi 1987, Digiani 1997) were nearly twice as large as those from experimental infections (Table 1). The natural hosts, *A. sibilatrix* and *C. melanocoryphus*, reported by Sutton and Lunaschi (1987) and Digiani (1997), are present in the pond of the Zoological Garden, and may have been the source of infection for *B. peregrina*. *Biomphalaria tenagophila* may have been infected by wild birds visiting the small pond in the Lerma Valley. According to Willey (1941), the sporocyst of *Z. lunata* is a simple saccate structure containing few young rediae and germ balls and its excretory system, retained from the miracidium, consists in a pair of flame cells with collecting ducts opening on the lateral sides of the body. He provided measurements of a sporocyst (297 by 195) found in a snail dissected 47 days PE; this finding was exceptional since all other snails dissected 28 days PE failed to yield sporocysts. In this study, larger sporocysts (up to 690) occurred in snails dissected up to 37 days PE, and their excretory system could not be observed because of the densely packed germ

balls and developing rediae. It remains to be elucidated whether these differences were due to different snail host species (*H. antrosom* vs *B. straminea*).

In this study, rediae containing cercarial bodies were similar in size, pharynx, and extension of cecum to those reported by Willey (1936), but differed in having only 3 pairs of protonephridia instead of 6 pairs; the author reported 3 pairs only for very young rediae. Furthermore, Willey (1936) observed both a single daughter redia and cercariae in all older rediae. This could not be found in the present material, where developing rediae are difficult to distinguish from cercariae, except for more advanced cercarial stages showing a tail, excretory canals, and occasionally, eye-spot pigment.

The cercariae from the natural infection of *B. peregrina* and *B. tenagophila* can be assigned to the group "Cercaria intermedia" proposed by Sey (1991), with pharyngeal appendages, pigmentation and antero- and posterolateral diverticula of the main excretory tubes, and are identified as belonging to *Z. lunata* based on the description of Willey (1936, 1941). These cercariae are easily differentiated from those of *Paramphistomum* spp. and *Cotylophoron* spp., which have lateral excretory tubes that join to form a ring at mid-level of the body and usually lack pharyngeal appendages (Sey, 1991). The presence of a well-developed lip on the posterior border of the ventral sucker is a clear, distinctive characteristic of the cercaria of *Z. lunata*.

The measurements on living cercariae under coverslip pressure obtained here are similar to those reported by Willey (1936, 1941) (see remarks). The cercariae conform to the descriptions of Willey (1936, 1941), and additionally show several papillae with short setae on the lateral borders of the tail. The cysts of metacercariae reported by Willey (1941) are larger than those observed in the present study (277-343 vs 248-250, and 245-274).

Zygocotyle lunata represents an ideal model for life cycle studies, at least for a limited period of time, based on the long survival period of metacercariae (up to 6 months) and of adults in mice (over a year), and the easy availability of its intermediate host snails.

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