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The development of *Gymnophallus australis* Szidat, 1962 (Digenea: Gymnophallidae) from the Patagonian coast (Argentina) from metacercaria to adult, with an amended diagnosis of *Gymnophallus* Odhner, 1905

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Abstract *Parvatrema australis* (Szidat, 1962) Szidat, 1965 was described based on larval stages found in specimens of the mussel *Mytilus edulis* from the Buenos Aires Province, Argentina. Although Szidat later examined hundreds of mussels, this parasite has never been found again until now. In the present study, larval stages, including germinal sacs, found in mytilids from the Patagonian coast were identified as *P. australis*. Metacercariae were incubated *in vitro* at 39°C in physiological solution for 18–20 hours, by which time 80% of the specimens had eggs. *P. australis* is redescribed, on the basis of infective metacercariae and adults obtained in the laboratory, and is reassigned to *Gymnophallus* Odhner, 1900, the genus in which it was originally described. The generic diagnosis of *Gymnophallus* is here amended to include as diagnostic characters the presence or absence of the lateral lips, the form and position of the vitellarium (compact or follicular) and the presence of a pars prostatica (i.e. prostatic cells open into proximal part of the ejaculatory duct). The validity of some characters (i.e. the presence of lateral lips of the oral sucker, the form of the vitellarium and excretory vesicle, the extent of the uterus) as diagnostic at the generic level within the family Gymnophallidae is discussed. It is proposed that the least unambiguous characters that can be used to distinguish gymnophallid genera include the position of the ovary, the presence of a ventral pit and a pars prostatica, and caecal diverticula.

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Introduction

The family Gymnophallidae Odhner, 1905 is a small and homogeneous entity of marine digeneans (Scholz, 2002). Most members use bivalves as first intermediate hosts and, with rare exceptions, charadriiform and anseriform birds as definitive hosts (Bartoli, 1974). The metacercariae never encyst and are usually parasitic in bivalves, but has also been found parasitising gastropods, brachiopods and polychaetes (Bartoli, 1974; Ching, 1995).

In South America, records of gymnophallids are very scarce. There is only one species (*Bartolius pierrei* Cremonte, 2001), whose adult and life-cycle is known (Cremonte, 2001, 2004) and two larval stages have been described from fixed specimens: *Lacunovermis* sp. (see Martorelli & Morriconi, 1998) and *Parvatrema australis* (Szidat, 1962) (see Szidat, 1962, 1965). In addition, Franjola & Gallardo (1991) reported the larval stages of an unidentified gymnophallid in *Kingiella chilenica* (Bivalvia: Cyamiidae).

Parvatrema australis was described as *Gymnophallus australis* by Szidat (1962) based on larval stages found parasitising the mussel *Mytilus edulis* on the coast of Buenos Aires Province, Argentina. Szidat (1962) described a total of 19 fixed larvae, including germinal sacs, which he called 'rediae' and 'parthenogenetic metacercariae', and assigned them to *Gymnophallus* Odhner, 1900. Following the paper by James (1964), describing *Parvatrema homoeotecnum* James, 1964, Szidat (1965) changed the generic assignation of *G. australis* to *Parvatrema* Cable, 1953 due to the similarity of the life-history stage which he then called a 'germinal sac' (Szidat, 1965). It is clear that Szidat's specimens came from an accidental infection, since the infected mussel was collected at a depth of 30–60 m, a habitat unavailable to birds acting as definitive hosts. Moreover, despite the hundreds of mussels later examined by Szidat (1965) and Cremonte (1999) from the type-locality, *P. australis* has never been found again.

The germinal sac is a life-history stage unique among digeneans, consisting of a metacercaria able to reproduce asexually by means of germinal balls that develop firstly into cercariae and then into new metacercariae. It seems that metacercariae formed in this way are distributed within the molluscan host by breaking through the germinal sac wall. Germinal sacs were first reported by James (1960) and later found by Szidat (1962), Ching (1982), Galaktionov (1996) and Galaktionov et al. (2006); all of the records are from gastropods except for that of Szidat.

Life-history stages, including germinal sacs, closely resembling those described by Szidat (1962, 1965), were found in about 80% of mytilids from the intertidal of the region of Comodoro Rivadavia, on the Patagonian coast of Argentina, and were studied by Cremonte (1999).

The aim of this paper is to describe the infective metacercaria of *P. australis* from mytilids on the intertidal region at Comodoro Rivadavia and its development, under experimental conditions, to the sexual adult stage. *P. australis* is re-assigned to *Gymnophallus* and the diagnosis of this genus is amended. Furthermore, the validity of some diagnostic characters used for generic identification within the family Gymnophallidae are discussed.

Materials and methods

During February and May, 1997 and December, 2005, specimens of *Perumytilus purpuratus* (Lamarck), *Mytilus edulis* (d'Orbingy) and *Aulacomya atra* (Molina) (Bivalvia: Mytilidae) were collected at Comodoro Rivadavia (45°52′S; 67°29′W), Chubut Province, Argentina. The study area is a rocky littoral; the epibenthic community in the intertidal zone is dominated by the little mussel *P. purpuratus*; while the blue mussel *M. edulis* and the ribbed mussel *A. atra* are less common components. Mytilids were collected during low tides and maintained in aquaria with aerated seawater. Metacercariae (obtained mainly from *P. purpuratus*) were studied live, stained with neutral red and Nile blue, using a light microscope with DIC optics. They were incubated *in vitro* at 39°C in a small Petri dish with physiological solution and observed at different time intervals in order to study their development to the sexual adult stage. Metacercariae and all incubated specimens were put on a glass-slide, killed with a drop of hot physiological solution and immediately covered with a coverslip. Then the specimens were fixed in AFA, stored in 70% alcohol, stained with Semichon's acetocarmine, cleared in methylsalicylate and mounted in Canada balsam. Illustrations were made with the aid of a drawing attachment. Dimensions, measured on mounted specimens, are given in micrometres with the mean value followed by the range in parentheses. The sucker-ratio was calculated as: oral sucker length/ventral sucker length. The forebody was measured from the anterior extremity to the anterior margin of the ventral sucker.

To calculate the prevalence (P) and the mean intensity (Im) of infection, 122 specimens of *P. purpuratus* [mean shell length 12 (range 2–23) mm], 15 specimens of *M. edulis* [mean shell length 33 (3–54) mm] and 15 specimens of *A. atra* [mean shell length 55 (9–81) mm] were fixed during February and May, 1997 and the parasites counted. Metacercariae and germinal sacs were counted together, because it was not possible to differentiate them under a stereomicroscope. The nomenclature of the habitat of the metacercariae follows that of Bartoli (1974). Some metacercariae were cultivated for about 3 hours, to enable them to escape from their

jelly envelope, and were then fixed in 4% glutaraldehyde in sodium cacodylate buffer (0.1M, pH 7.4), dehydrated, critical-point dried, gold coated and observed under a JEOL JSM 6360 LV scanning electron microscope.

For comparative purposes, syntypes of *Gymnophallus australis* housed in the National Collection of Parasitology, Museo Argentino de Ciencias Naturales, Buenos Aires (Argentina) (MACN-Pa 27 1/7, ex MACN 27801) were studied.

Gymnophallus australis Szidat, 1962

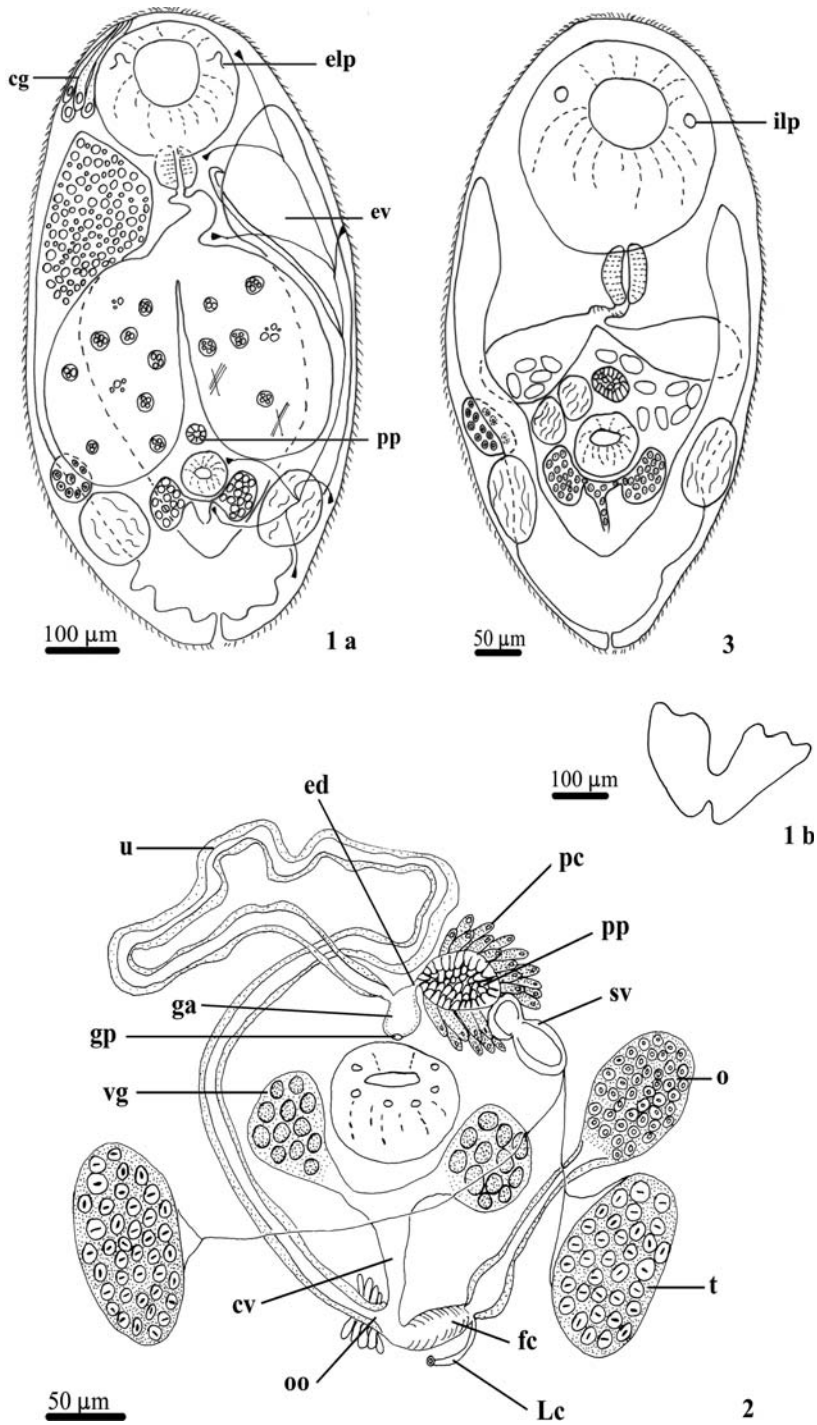
Syn. *Parvatrema australis* (Szidat, 1962) Szidat, 1965

Locality: Comodoro Rivadavia (45°52′S, 67°29′W), Chubut Province, Argentina.
Second intermediate hosts: *Perumytilus purpuratus*, *Mytilus edulis* and *Aulacomya atra* (Bivalvia: Mytilidae).
Site of infection: Metacercariae occur between the valve and mantle, in the central extrapallial space, mainly in its dorsal region, above the level of the digestive gland.
Prevalence and mean intensity: 80% and 17 (*P. purpuratus*), 93% and 61 (*M. edulis*), 47% and 15 (*A. atra*), respectively.
Specimens deposited: National Parasitological Collection, Museo Argentino de Ciencias Naturales (stained whole-mounts of infective metacercariae MACN-Pa 438/1–4 and experimentally obtained adults MACN-Pa 437/1–4), and Natural History Museum, London (stained whole-mounts of infective metacercariae BMNH 2007.2.21.18 and experimentally obtained adults BMNH 2007.2.21.17).

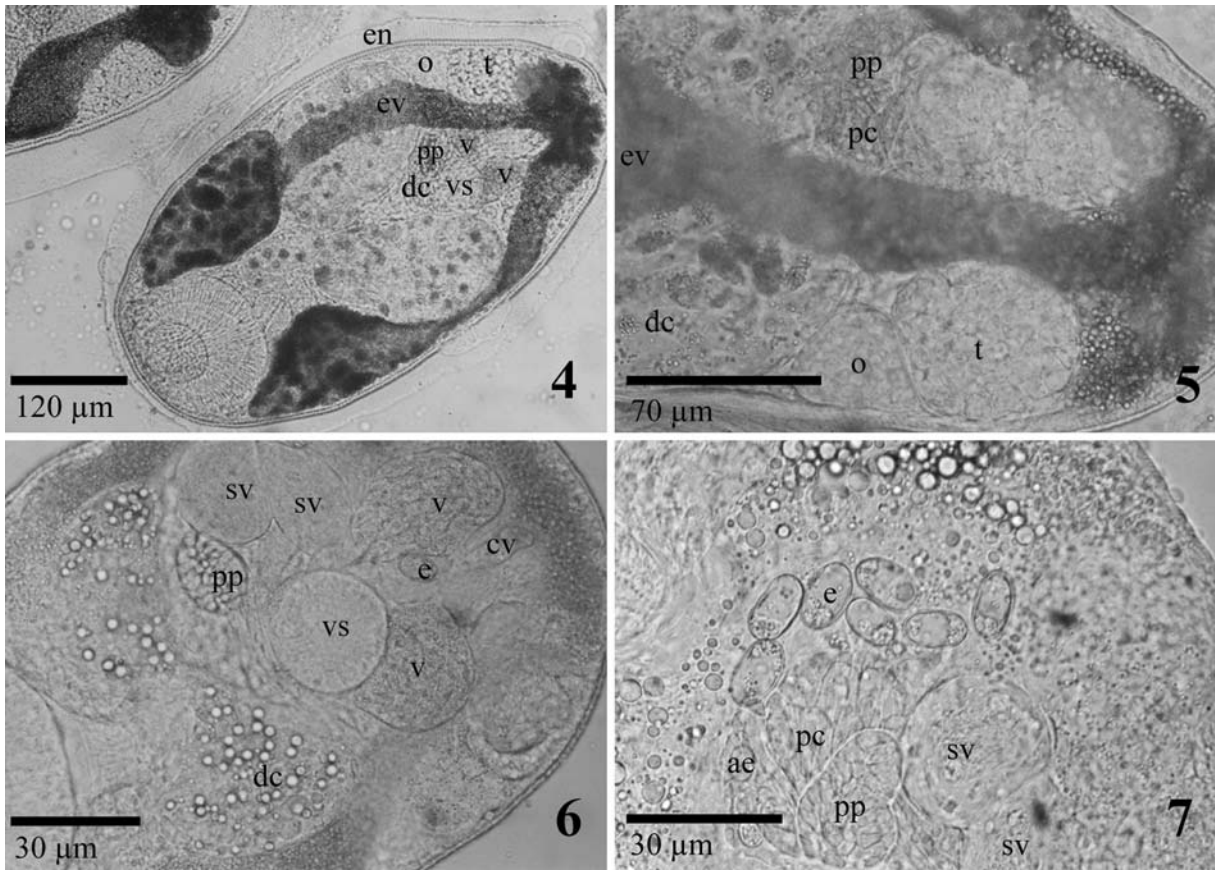
Redescription (Figs. 1–11)

Infective metacercariae (10 specimens measured unless otherwise indicated) (Figs. 1, 2, 4, 5, 7, 8–11). Body oval to elongate, 364 (235–416) × 239 (210–263) in maximum width. Spines arranged transversely covering entire body surface, except in region of ventral sucker. Forebody 252 (212–292) in length. Oral sucker 119 (90–140) × 126 (106–148), with circle of 10 papillae surrounding mouth (Fig. 9) and 2 lateral eversible lips. Ventral sucker 46 (40–50) × 50 (45–54), with outer circle of 6 conspicuous papillae (Fig. 10). Sucker-ratio 1:2.6 (2–3). Cephalic glands numerous. Pharynx ovoid, 44 (40–48) × 43 (39–50). Oesophagus 37 (35–40) (n = 3) in length. Caeca sacciform, 97 (73–123) × 60 (38–82) (n = 8), reach to level of ventral sucker, filled with brownish material and some excretory granules. Testes ovoid, located at level of ventral sucker or slightly posterolaterally; testis posterior to ovary may be slightly diagonal with respect to other testis; left testis 71 (50–93) × 57 (45–67) (n = 8); right testis 67 (49–82) × 57 (40–66) (n = 9). Vasa efferentia arise from middle or anterior region of testes, unite at base of bipartite seminal vesicle. Pars prostatica 21 (17–25) × 17 (15–20), surrounded by numerous prostatic cells. Remainder of ejaculatory duct short. Genital atrium oval. Genital pore inconspicuous, located very close to anterior margin of ventral sucker. Ovary rounded, 57 (45–72) × 48 (35–62), located anterior to right or left testis, sometimes displacing testis slightly posteriorly. Oviduct short, originating dorsal to ovary. Laurer's channel present. Fertilisation chamber ciliated, located in widened part of oviduct; common vitelline duct opens at distal end of oviduct, just before oötype. Canalicular seminal receptacle absent. Uterus ascends towards genital atrium, forming long loop anterior to ventral sucker. Vitelline masses paired, compact, oval, close to ventral sucker, 45 (38–61) × 30 (23–43). Flame-cell formula 2 [(2 + 2) + (2 + 2)] = 16. Excretory vesicle Y-shaped, with very short stem and 2 long dorsal arms extending to oral sucker and expanding towards mid-line and 2 short arms directed ventrally, distended and filled with spherical excretory granules. Infective metacercariae always enclosed by radially striated and translucent or melanised jelly-like envelope (Fig. 4).

Sexual adult (10 specimens measured unless otherwise indicated) (Figs. 3, 6, 7). Body oval, 343 (300–390) × 258 (226–303) in maximum width, slightly pointed at posterior end. Forebody 217 (169–270) in length. Oral sucker 143 (125–155) × 156 (140–175), with circle of 10 papillae surrounding mouth and 2 lateral eversible lips. Ventral sucker 50 (45–57) × 54 (50–60), with outer circle of 6 conspicuous papillae. Sucker-ratio 1:2.9 (2.6–3.1). Pharynx ovoid, 40 (30–50) × 41 (32−50). Oesophagus 28 (16–50) (n = 5) in length. Caeca short, 78 (48–110) × 54 (27–90) (n = 9) in maximum width, never reaching to ventral sucker, empty. Testes ovoid, located at level of ventral sucker or slightly posterior it, in lateral fields; left testis 55 (41–70) × 45 (23–57); right testis 60 (40–85) × 41 (29–50).



Figs. 1–3 *Gymnophallus australis.* 1a. Infective metacercaria, ventral view, flame-cells on the right side omitted and cephalic glands of the left side omitted, excretory granules represented only in the distal dextral part of the excretory vesicle. 1b. Ventral branches of the excretory vesicle. 2. Genital system of infective metacercaria, dorsal view. 3. Adult experimentally obtained ventral view. *Abbreviations*: cg, cephalic glands; cv, common vitelline duct; ed, ejaculatory duct; ev, excretory vesicle; fc, fertilisation chamber; ga, genital atrium; gp, genital pore; ilp, invaginated lateral lips; Lc, Laureŕs canal; elp, evaginated lateral lips; o, ovary; oo, oötype; pc, prostatic cells; pp, pars prostatica; sv, seminal vesicle, testis; u, uterus; vg, vitelline gland



Figs. 4–7 Photomicrograph of live *Gymnophallus australis*. 4. Infective metacercaria, ventral view. 5. Infective metacercaria, detail of genitalia, dorsal view. 6. Adult obtained after 20 hours of cultivation, posterior half of body, dorsal view. 7. Adult obtained after 20 hours of cultivation, detail of the pars prostatica, seminal vesicle and eggs. *Abbreviations*: ae, abortive egg; cv, common vitelline duct; dc, digestive caecum; e, egg; en, envelope; ev, excretory vesicle; o, ovary; pc, prostatic cells; pp, pars prostatica; sv, seminal vesicle; t, testis; v, vitelline gland; vs, ventral sucker

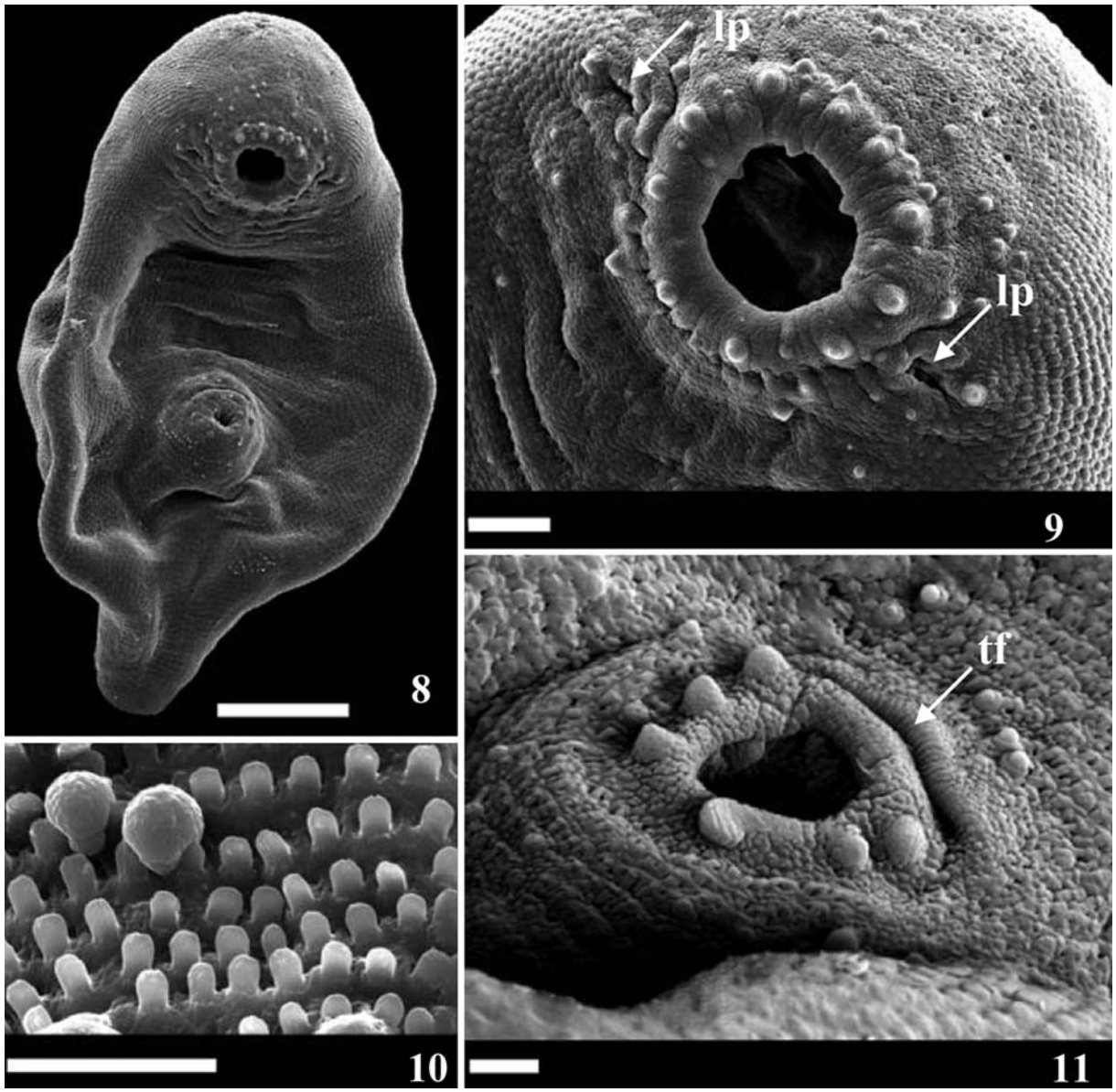
Vasa efferentia arise from middle or anterior region of testes, unite at base of seminal vesicle. Seminal vesicle bipartite, 44 (30–61) × 37 (27–54). Pars prostatica 31 (24–45) × 32 (25–38), surrounded by numerous prostatic cells. Remainder of ejaculatory duct short. Genital atrium oval. Genital pore inconspicuous, located very close to anterior margin of ventral sucker in mid-ventral line. Ovary ovoid, 60 (43–87) × 49 (32–61), located anterior to either right or left testis, sometimes slightly displacing testis posteriorly. Oviduct short; common vitelline duct opens into its distal end. Seminal receptacle absent. First eggs in forebody; egg size 21 (17–23) × 11 (10–13). Vitelline glands paired, compact, close to ventral sucker, 52 (41–62) × 38 (28–46). Flame-cell formula 2 [(2 + 2) + (2 + 2)] = 16. Excretory vesicle smaller and with fewer excretory granules than in metacercaria.

Ontogenetic development

After some minutes *in vitro* at 39°C, the metacercariae greatly increased their movements. The oral sucker attaches to the bottom of the Petri dish, while the body starts to alternately stretch and contract. After 3–4 hours, most of the metacercariae had escaped from their envelope through the anterior end.

After 6–7 hours, the metacercariae possess active spermatozoa within the testes, although the seminal vesicle and common vitelline duct were empty. The caeca are reduced in size and the excretory vesicle contained fewer excretion granules than in matured metacercariae.

After 12–13 hours, 17% of the specimens (n = 29) possessed 1–4 (mean 2) eggs in the uterus. The ovary, which is rounded and smaller than the testes in the



Figs. 8–11 SEM photomicrographs of *Gymnophallus australis* metacercaria without jelly-like envelope. 8. Infective metacercaria, ventral view. 9. Oral sucker. 10. Detail of body spines with secretions of unknown origin. 11. Ventral sucker. *Abbreviations*: lp, invaginated lateral lips; tf, transverse fold of the ventral tegument. *Scale-bars*: 8, 50 μm; 9, 10 μm; 10,11, 5 μm

metacercariae, became larger and oval. The common vitelline duct was also distended and filled with vitelline cells. At this stage, spermatozoids were observed inside the uterus, and the caeca showed a great reduction in size.

After 18–20 hours, 80% of the specimens (n = 15) possessed *c*.4–27 (mean 13) eggs in the terminal part of the uterus (Fig. 7). Abortive eggs were often observed in the uterus (Fig. 7). The excretory vesicle had few excretory granules.

Remarks on the type-specimens of *Gymnophallus australis*

Except for the presence of a pars prostatica in the life-history stage referred to by Szidat (1962) as an

'encysted metacercaria', no other details from the type-material can be added to the original description. Szidat had considered this organ to represent a cellular mass forming a genital primordium.

Discussion

Since the internal organs of gymnophallids are difficult to observe, especially in mounted specimens, the systematics of this family is extremely confusing, a fact that has discouraged many authors from its studying this group. The generic allocation of species using the identification keys of James (1964), Ching (1995) and Scholz (2002) is very difficult. As an example, the adult specimens experimentally obtained in the present study do not agree with any of the generic diagnoses included in these keys. In this regard, the generic diagnosis of *Gymnophallus* given by Scholz (2002) is here amended to include the presence or absence of lateral lips, the form and position of the vitellarium (paired or not, compact or follicular) and the presence of a pars prostatica (i.e. prostatic cells opening directly into the proximal region of the ejaculatory duct).

Gymnophallus Odhner, 1900

Amended diagnosis. Gymnophallidae. Body small, oval. Oral sucker with or without lateral lips. Caeca short in adults, without dorsal diverticula. Ventral sucker usually in posterior third of body. Ventral pit absent. Seminal vesicle usually bipartite. Pars prostatica well developed. Ovary pre-testicular. Vitelline glands paired or not, compact or follicular, close to ventral sucker. Seminal receptacle present or absent. Uterus mainly in forebody. Genital pore inconspicuous, very close to anterior margin of ventral sucker. Excretory vesicle Y-shaped, with short stem, reaching to oral sucker. Flame-cell formula: 2 [(2 + 2) + (2 + 2)] = 16 or [(2 + 2 + 2) + (2 + 2 + 2)] = 24. Parasites of gall-bladder, bursa Fabricii or intestine of charadriiform and anseriform birds. Type-species: *G. deliciosus* (Olsson, 1893).

The specimens studied here were originally considered by us as belonging to *Parvatrema australis* (Szidat, 1962), because of their close resemblance to Szidat's (1962) specimens (in the relative size and position of the suckers, the form of the vitellarium and the presence of a pars prostatica). This species is here re-assigned to *Gymnophallus*, mainly because of the presence of a pars prostatica, a structure absent in *Parvatrema* (see James 1964).

The character 'presence or absence of pars prostatica' was used by James (1964) to distinguish between two subfamilies within Gymnophallidae: the Gymnophallinae, with a pars prostatica, and the Parvatrematinae, lacking this structure; in the latter, numerous cells surround and open directly into genital atrium (such cells are not strictly 'prostatic'). Despite the fact that this character is readily seen, it was disregarded in the identification key of Scholz (2002). On the other hand, the presence of lateral lips appears to be a variable character among gymnophallid species. Therefore, it is considered to be of little use for distinguishing gymnophallid genera. The extent of the uterus (i.e. in the fore- or hindbody) varies with the age of the specimens [the first eggs appear in the terminal part of the uterus, as observed in the present material and also in *Bartolius pierrei* Cremonte, 2001 (see Cremonte, 2001)]. Consequently, this should not be considered as a useful diagnostic character. Regarding the form of the vitellarium, there is sometimes not a striking difference between the follicular and compact arrangements, the vitellarium being more or less follicular. Moreover, the form of the vitellarium has been reported as varying with the age of the specimens (Cremonte, 2001). Another confusing character is the form of the excretory vesicle (Y- or V-shaped), because the stem of a Y-shaped vesicle may be very short, in some cases almost resembling a V-shape. In conclusion, the main unambiguous characters proposed here as useful for distinguishing gymnophallid genera are the position of the ovary (pre-testicular, post-testicular or inter-testicular) and the presence or absence of a ventral pit, a pars prostatica and caecal diverticula.

Regarding the development of *G. australis* from metacercaria to adult, egg production started under experimental conditions very quickly in most specimens, even when compared with other gymnophallids (12 hours in *G. australis* versus 40 in *Bartolius pierrei* and 48 in *Lacunovermis macomae* Lebour, 1908 (see Cremonte, 2001; Pekkarinen, 1984). An even faster development from metacercaria to adult was observed by Bartoli (1963) in *Parvatrema timondavidi* Bartoli, 1963, in which some individuals

had eggs after only five hours. Because of the particularly advanced degree of development reached by metacercariae in the bivalve host, it seems that merely an increase in temperature is enough to trigger egg production. The variability observed in the time needed for the start of egg production in *G. australis* is possibly due to differences in the development of the metacercariae at the beginning of the incubation experiments. Ovigerous specimens obtained experimentally were, on average, slightly smaller than the metacercariae, possibly due to the reduction in the size of the caeca. While the size of both suckers was larger in the adults, the sucker-ratio remained similar. Furthermore, the process of fixation under a coverslip may cause an artificial increase in dimensions due to the slight pressure resulting from the mounting procedure; nevertheless, this is the only method readily available which makes the internal organs visible (and measurable). Considering that the size of the suckers, changes due to the age of the specimens and the mounting procedure, such measurements must be used with caution as a basis for distinguishing gymnophallid species.

The life-cycle of *G. australis* has still to be elucidated. In this regard, it should be noted that a small erycinid clam, *Lasaea adansoni* (Gmelin), which lives among the byssus threads of mytilids in the study area, was found to host unidentified gymnophallid sporocysts. If these actually belong to *G. australis*, then they may serve as the first intermediate host (Cremonte, 1999). It seems probable that the charadrifom birds *Larus dominicanus* Lichtenstein and *Haematopus palliatus* (Temminck) act as definitive hosts for *G. australis*, since they prey upon mytilids on the study area (pers. obs.).

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