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Bc. Thesis



**IS THERE A SOLDIER CAST IN FRESHWATER ECHINOSTOME
TREMATODES?**

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ANNOTATION

Novel data on the morphology and size of the daughter rediae of the echinostomatid trematode, *Echinostoma spiniferum*, is provided and their distributions within the first intermediate snail host, *Planorbarius corneus*, is analysed to test some of the hypotheses of Hechinger et al. (2010) for the existence of social organisation in trematodes.

DECLARATION

Prohlašuji, že svoji bakalářskou práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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1. INTRODUCTION

This study targeted the intramolluscan redial larval stages of a species of *Echinostoma* (Digenea: Echinostomatidae). Rediae are characteristic for trematodes of families considered "more primitive" (e.g. Galaktionov & Dobrovolskij, 2003): Fasciolidae, Echinostomatidae, Paramphistomidae, Psilostomidae, Philophthalmidae and Lepocreadiidae). Current knowledge on the development, morphology, abundance and function of these larval stages in the first intermediate mollusc hosts is mainly based on experimental studies on species of veterinary importance (*Fasciola* spp., and *Paramphistomum* spp.; reviewed in Rondelaud et al., 2009) but also on experiments with some *Echinostoma* spp. and field observations on other echinostomatid species (reviewed in Galaktionov & Dobrovolskij, 2003).

These studies have shown that the development of these trematodes within the snail hosts is characterized by the sequential production of larval stages, including mother sporocysts and two-three successive generations of rediae, finally resulting in the production of cercariae, the second dispersive stage in the trematode life-cycle. Rediae are the developmental stages of trematodes found in highest abundance within the intermediate snail host and responsible for the cercarial production and subsequent dispersal of the parasites. They possess a mouth, pharynx and a blind intestine and are able to ingest host tissue directly, by tearing and swallowing. This histhiopathy is considered as the most traumatic for the snail host feeding mechanism (Galaktionov & Dobrovolskij, 2003). These authors also suggested that young rediae have especially powerful muscular system and are capable of locomotion; they also show marked preference for histhiopathy and cannibalism. Intensive studies on *Fasciola* spp. have shown that the three redial generations characteristic for these species could be differentiated from each other by the redial size, the shape of the pharynx and the intrapharyngeal lumen (e.g. Abrous et al., 2000; Dar et al., 2002; Vignoles et al., 2002).

Recently Hechinger et al. (2010) studied an unidentified species, named *Himasthla* sp. B and presumed to be an echinostomatid, and suggested a drastically different view for the morphology and function of the intramolluscan redial stages *i.e.* that they form cooperative colonies in their mollusc hosts represented by two distinct castes: some individuals are responsible for reproduction ("reproductive caste") while others defend the colony ("soldier caste"). These authors observed two morphologically distinct forms. The "primary morphs" or "reproductives" only had embryos in their body cavities, were large and immobile. The "secondary morphs" or "soldiers" were c. 2% of the body size of the "reproductives", did not reproduce, had large pharynxes in relation to body sizes, exhibited greater mobility, were more abundant in areas of the host where miracidial invasion occurs, and were frequently

observed attacking rediae of other species or colonies. Hechinger et al. (2010) suggested that their finding provides a new avenue by which to study the evolution of social behaviour.

2. AIM AND OBJECTIVES

The study **aims** to provide data on the morphology and size of the rediae of a model freshwater echinostomatid species (*Echinostoma spiniferum*) and their distributions within the first intermediate snail hosts that would help to test the hypothesis of Hechinger et al. (2010) for the existence of social organisation in trematodes.

OBJECTIVES

- (i) To review the literature on the morphology of the redial intramolluscan stages of trematodes, their site selection and temporal variations in abundance.
- (ii) To perform total redial counts from three separate regions of infected snails (foot and mantle; basal visceral mass and hepatopancreas/gonad).
- (iii) To search for the presence of germinal material in the small-sized rediae using chromosomal stain (iron acetocarmine).
- (iv) To evaluate statistically the abundance and size distributions of the rediae in the three regions of infected snails.

3. REVIEW OF THE LITERATURE

3.1. LIFE-CYCLE OF ECHINOSTOMATID TREMATODES

Trematodes of the family Echinostomatidae Looss, 1899 represent a heterogeneous group of cosmopolitan, hermaphroditic digeneans that parasitize, as adults, numerous vertebrate hosts (predominantly birds and mammals and occasionally reptiles and fishes) (Kostadinova & Gibson, 2005). Most species of the family possess a three-host life-cycle involving:

- (i) a vertebrate definitive host;
- (ii) a gastropod mollusc first intermediate host; and
- (iii) an invertebrate or vertebrate second intermediate host.

The following phases of the life-cycle typical for echinostomatids are characteristic for the species of the genus *Echinostoma* (Fig. 3.1.).

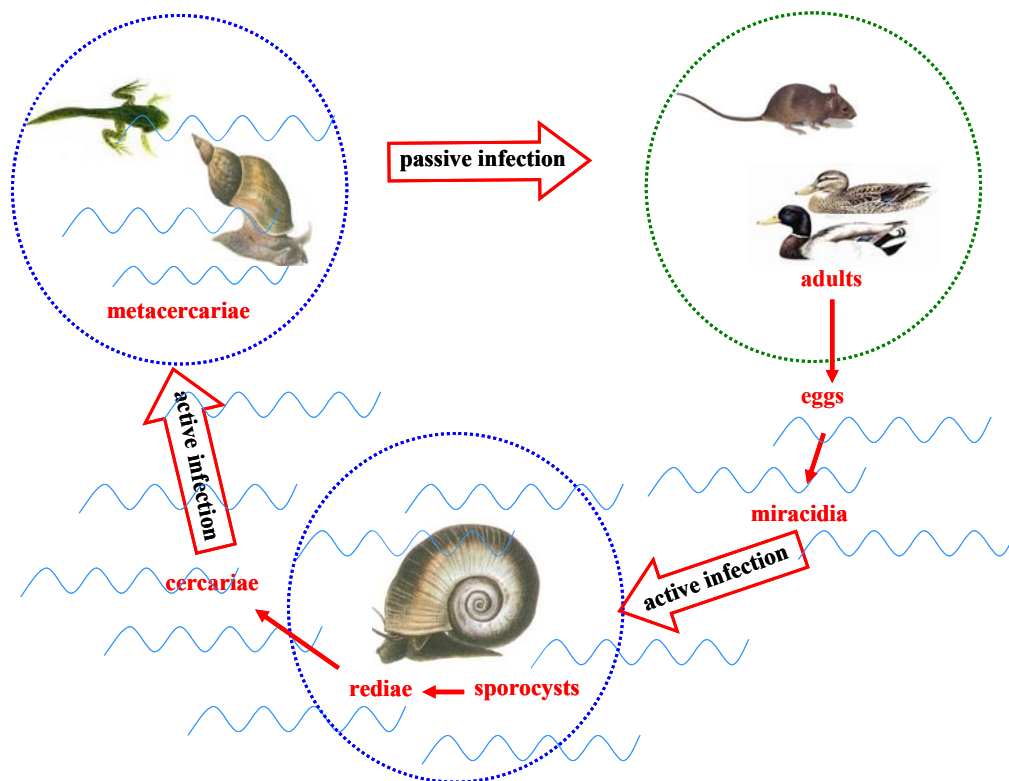


Fig. 3.1. Schematic illustration of the life-cycle of *Echinostoma* spp.

Sexual reproduction occurs in the intestine of the definitive vertebrate host (an aquatic bird or a mammal). Unembryonated eggs are released by mature worms and passed via faeces of the definitive host in the aquatic environment. Eggs hatch after some time and release free-swimming miracidia which search and infect the intermediate snail host, in which asexual reproduction occurs. They actively swim in the water and penetrate the molluscan host. Normally, actively infecting miracidia penetrate through the mantle, foot and tentacles of the snail. Once in the snail, the miracidium transforms into a mother sporocyst.

Mother sporocyst of *Echinostoma* spp. is sac-like, has no digestive system and contains the embryos of the mother rediae. Young mother sporocyst is mobile and migrates along the haemocoel lacunae to the heart of the snail, the site of its final location. After several weeks it begins to produce the first redial generation *i.e.* mother rediae. These initially reside within the ventricle and aorta of the snail, although when these sites became filled, the mother rediae migrate to the digestive gland (hepatopancreas) and gonad of the snail and begin producing individuals of the next generations *i.e.* daughter rediae.

Daughter rediae start their reproduction, giving birth to several rediae and then begin to produce cercariae. The rediae feed on the tissues of the snail, and can castrate it by devouring the gonads. About 40-50 days after the initial infection, daughter rediae begin producing cercariae, and continue so for the life of the snail. The cercariae exit the snail and swim vigorously around until they encounter a suitable second intermediate host (another snail). They penetrate the body surface or enter through an orifice of the second intermediate host and encyst as metacercariae. If an infected second intermediate host is ingested by the definitive host, the metacercariae excyst and develop into adults.

A modification of this pattern was observed in *Echinostoma paraensei* by Sapp et al. (1998). These authors reported the development of a so-called precocious mother redia that remains for 31 days or more with the sporocyst, attached to the ventricle. Its presence reduced the success rate of establishment of subsequent *E. paraensei* sporocysts in the ventricle of the snail host.

3.2. MORPHOLOGY OF THE REDIAL INTRAMOLLUSCAN STAGES

The redia has an elongate cylinder-shaped or more rounded body with conical locomotory projections that enhance its locomotor activity. In echinostomatids the anterior locomotory extension is transformed into a circular folding of the tegumentary-muscular sac (collar). Immediately posterior to the collar is located a small opening (birth pore) through which the individuals of the next generations leave the redia.

Young rediae possess the most powerful muscular apparatus and this is associated with their greater locomotor activity. However, the locomotory appendages may disappear during redial growth; with age such rediae obtain a sac-like shape and their mobility is only manifested as contractility (Galaktionov & Dobrovolskij, 2003). These authors suggested that the structure and functions of the locomotory apparatus of the rediae are closely correlated with the structure and function of the feeding apparatus.

Rediae have reduced digestive system comprising an anterior terminal mouth opening, followed by a cavity with tegumental folds (homologous to the prepharynx in adults), a rounded muscular pharynx, short oesophagus and sac-like intestine with walls lined by flattened epithelial cells. According to Galaktionov & Dobrovolskij (2003) the development of pharynx in redial ontogeny stops already in very young individuals, which continue to grow for quite a long time after that and this leads to an imbalance between the greatly increased body mass and the muscular system.

A large body of experimental studies on snail-*Fasciola* spp. relationship has provided valuable data on the development of the redial generations (reviewed in Rondelaud et al., 2009). Studies carried out on serial sections of experimentally infected snails and on live rediae using an image-processor have shown similar life-cycles of *Fasciola hepatica* and *F. gigantica* with the development of up to four succeeding generations of rediae within the intermediate host. To our knowledge these studies provide the only experimental evidence for the development, morphology and function of the redial generations.

The main criteria used to recognise the redial generations in these studies on *Fasciola* spp. were: (i) the age of infection; (ii) the degree of development of the intraredial developmental stages; and (iii) the general shape of the pharynx (*e.g.* Dar et al., 2002). Ambrous et al. (2000) distinguished four intraredial types of embryos in *F. hepatica*: (i) round morulae; (ii) ovoid procercarial embryos often with a tail rudiment; (iii) procercariae with short differentiating tail; and (iv) fully formed cercariae with well-differentiated tail.

Summarised data from these studies in Table 3.1 indicate a substantial decrease in redial body size as well as changes in pharynx size and shape in the subsequent redial generations. Furthermore, Dar et al. (2002) have observed that the rediae of the second generation had only morulae and embryos in *F. gigantica* whereas Augot et al. (1997) have observed rediae of the next generation in these rediae in *F. hepatica*.

The publication of the book "The biology and evolution of the trematodes" by Galaktionov & Dobrovolskij (2003) summarising a large body of data, has substantially advanced our knowledge on the reproduction of the intramolluscan larval stages in trematodes. According to these authors the reproduction starts at very early stages of the formation of the larvae, before the formation of schizocoel by a process of specialisation of some undifferentiated cells of the central embryonic mass of cells into primary germinal cells that start cleavage forming germinal mass.

In some echinostomatids the germinal mass remains sunk into the parenchyma at the posterior end of the redial body during the entire period of its functioning. But normally in the rediae of echinostomatids the germinal mass is displaced into the body cavity, tightly attached

to its posterior wall although in some species it is almost completely separated from the wall of the body cavity connected by only a thin stem. The undifferentiated cells capable of proliferation are concentrated at the base of the rediae body and forming a proliferation zone. This is followed by a zone of maturation of germinal cells and a cleavage zone. The germinal balls are concentrated at the anterior end of the germinal mass, which is closer to the schizocoel. When the embryonic membrane has been formed, embryos detach from the germinal mass and are delivered to the schizocoel (Galaktionov & Dobrovolskij, 2003).

Table 3.1. Summary for the redial generations and cohorts during the normal intramolluscan development of *Fasciola* spp. at different periods after infection (data for *Fasciola hepatica* in d.p.i., days post-infection in days; Rondelaud et al., 2009) with an indication of the sizes and pharynx morphology. Highlighted cells indicate redial stages that cannot be distinguished morphologically.

	Sporocyst		d.p.i.
Redial generations			
First generation rediae (mother rediae)	First mother redia (R1a) (cohort 1) <i>F. gigantica</i> : BL=1,400 µm <i>F. hepatica</i> : BL=1,000-1,200 µm Pharynx: large, oval; up to 105 µm (<i>F. hepatica</i>)		after 7
		Other mother rediae (R1b) (cohort 2) <i>F. gigantica</i> : BL=1,000 µm Pharynx: oval, smaller than in R1a; up to 70 µm (<i>F. hepatica</i>)	after 14
Second generation rediae (daughter rediae)	R2a (cohort 1) <i>F. gigantica</i> : BL=710 µm Pharynx: oval; up to 65 µm (<i>F. hepatica</i>)		after 14
		R2b (cohort 2) <i>F. gigantica</i> : BL=490 µm <i>F. hepatica</i> : BL=300-400 µm Pharynx: spherical, small, thick, with narrow, long lumen; up to 30 µm (<i>F. hepatica</i>)	after 35
Third generation rediae (granddaughter rediae)	R3a (cohort 1) <i>F. gigantica</i> : BL=490 µm <i>F. hepatica</i> : BL=300-400 µm Pharynx: spherical, small, thick, with narrow, long lumen; up to 30 µm (<i>F. hepatica</i>)		after 35
		R3b (cohort 2) Pharynx up to 30 µm (<i>F. hepatica</i>)	after 63
Fourth generation rediae (granddaughter rediae)	R4a Pharynx up to 30 µm (<i>F. hepatica</i>)		after 63

3.3. SITE SELECTION WITHIN THE SNAIL HOST BY THE REDIAE

The rediae retain mobility during their entire life span. Miracidia and juvenile mother sporocysts are normally mobile and can undergo considerable migrations in the body of the molluscan host. According to the observations of Lie (1964), Hsu et al. (1968) and Ataev et al. (1997), echinostomatids migrate especially along the haemocoel lacunae to the heart which is the site of their final settlement of the sporocyst. From there, young rediae of the first generation actively migrate along the haemocoel lacunae or through host tissues to their final location, hepatopancreas and gonad. Galaktionov & Dobrovolskij (2003) suggested that young rediae cause minimal traumatic influence upon the host tissues during their migration due to their minute size whereas the reduced mobility of the considerably larger mature rediae serves to decrease significantly the pathogenic effect of their presence.

These authors also provided examples for two types of migration (tropism) of the miracidia and mother sporocysts: (i) viscerotropism (migration towards the internal organs and tissues; species with actively penetrating miracidia including *Echinostoma*); (ii) dermatotropism (migration towards the epidermal tissues; species developing in sporocysts only and infecting the snail hosts *via* egg ingestion); and (iii) lack of pronounced tropism (*e.g.* *Schistosoma* species whose actively penetrating miracidia and mother sporocysts do not leave the head and „neck“ regions of penetration, reaching only as far as the oesophagus).

3.4. TEMPORAL VARIATIONS IN THE ABUNDANCE OF REDIAL GENERATIONS

The total abundance of the rediae and the physiological state of the snail host determines the structure of microhemipopulations (infrapopulations *sensu* Bush et al. (1997) *i.e.* all intramolluscan individuals in an individual snail, see Galaktionov & Dobrovolskij, 2003). These authors recognised two main types of microhemipopulations in the snail hosts: (i) daughter parthenitae are capable of re-determination of offspring type *i.e.* can switch from producing cercariae to rediae/sporocysts (observed in "primitive" trematodes including family Echinostomatidae, and the orders Schistosomatida and Strigeidida developing in sporocysts only); and (ii) the number of generations is limited to two (mother sporocyst and a single generation of daughter sporocyst which only produces cercariae).

Galaktionov & Dobrovolskij (2003) also emphasized that the stability of the mollusc-trematode systems depends on the capacity of the host to support larval populations that varies among individual hosts and is limited. Thus, host size has been shown as a direct determinant of the redial and sporocyst abundance (*e.g.* Smith, 1959; Dinnik & Dinnik, 1960;

Tihomirov, 1980). Théron et al. (1992) and Gérard et al. (1993) established that the abundance of the daughter sporocysts and the volume of these larvae in the mollusc hepatopancreas depend not only on the number of the penetrated miracidia but also on the initial size of the molluscan host and its growth rate. They suggested the existence of certain regulatory mechanisms in the host-parasite system that confine the use of the host resources to the maximal possible level which does not result in the host death.

On the other hand, as shown by Tihomirov (1980) in *Philophthalmus rhionica*, the structure of redial populations (*i.e.* the relative proportions of different types of rediae (young individuals which have not started the reproduction; rediae producing rediae; rediae producing cercariae; and old degenerating individuals) remains constant at different snail sizes.

Galaktionov & Dobrovolskij (2003) also stressed that a number of conditions should be satisfied in order to separate the seasonal dynamics of the structure of larval infrapopulations from purely age changes. The most important is that both the hosts and the trematodes should have a life span of at least two years. In these cases seasonal changes have been observed in a number of host-parasite systems with regeneration of the composition of the infrapopulations and massive cercarial release in the warm season as opposed to ceasing cercarial production and death of the old individuals during overwintering. On the other hand, in spring the structure of the infrapopulations is restored by larvae first producing their like (*i.e.* sporocysts producing sporocysts and rediae producing rediae, to compensate for the losses due to overwintering) and then resuming cercarial production.

However, perhaps due to the lack of expertise, the number of empirical studies on the seasonal dynamics of intramolluscan larval population structure is still small (Rusanov & Galaktionov, 1984; Galaktionov & Dobrovolskij, 2003). Of particular interest for the present study is the data provided by these authors on the echinostomatid species *Himasthla elongata*, parasitising *Littorina* spp. at the White Sea. In this species the production of rediae by rediae is not timed for a certain season but takes place constantly when the group is functionally active and rediae which have young rediae of the next generation as well as cercariae, can be found in all seasons. Fig. 3.2. [redrawn after Galaktionov & Dobrovolskij (2003) illustrates the structure of larval infrapopulations]. In winter (November) redial populations represent individuals of different age characterised by the total lack of formed cercariae: (i) very young rediae with germinal balls and cercarial embryos (red bars); (ii) larger and more mature (light blue bars); and (iii) old degenerating rediae along with very young ones (dark blue bars). This structure gradually changes in the warm season. Thus, in June the first group *i.e.* very young rediae with germinal balls and cercarial embryos (red bars) predominates over the third winter group (dark blue bars). In July-August the structure is very similar and comprises two groups:

- (i) rediae of different ages, some containing fully developed cercariae (light blue bars); and
- (ii) old degenerating rediae along with very young ones (dark blue bars).

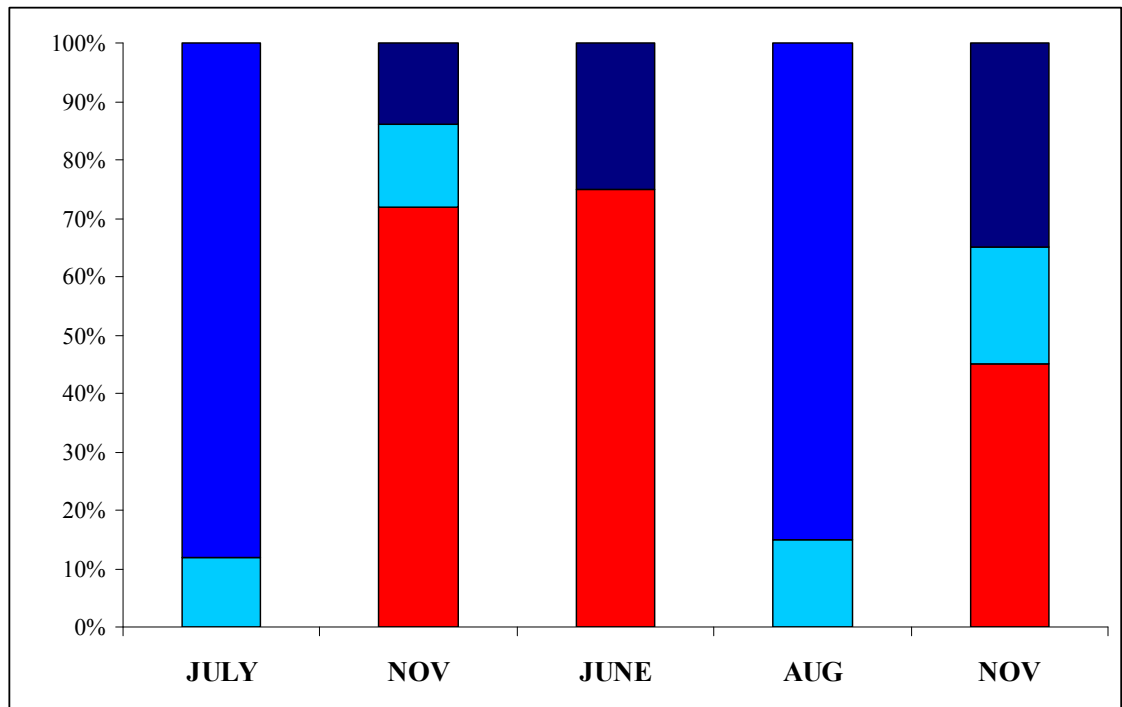


Fig. 3.2. Structure of redial populations of *H. elongata* in *Littorina saxatilis* (data by Galaktionov & Dobrovolskij, 2003). See text for details.

3.5. OBSERVATIONS AND HYPOTHESES OF HECHINGER ET AL. (2010)

Hechinger et al. (2010) suggested that their data on the morphology, reproduction, activity, attack rates, censuses and spatial distribution congruently support the hypothesis of a sharply defined division of labour within *Himasthla* sp. B colonies (*i.e.* infrapopulations) in *Cerithidea californica*. These studies are briefly reviewed below under the authors' original conclusive statements.

3.5.1. "Secondary and primary morphs differ in body size, body shape and relative mouthpart size"

In summer 2006, the authors measured 14-35 individuals of each redia morph (173 small or "secondary" and 143 large or "primary") of *Himasthla* sp. B. from three snail regions (mantle, basal visceral mass and gonad/hepatopancreas) of seven snails. Rediae were fixed in the 10% hot formalin, transferred to 70% alcohol and their length and width were measured on temporary glycerine mounts. Volumes of the redial bodies and pharynxes were calculated and used in all comparisons. Hechinger et al. (2010) found that the secondary morphs are much

smaller and thinner than primary morphs. On average, they represented 0.52-3.8% of the volume, 26-54% of the length and 18-29% of the width of the primary morphs. The two morphs also differed in their body shapes, the secondary morphs possessing distinguishable collar and distinct posterior appendages and the primary morphs lacking these structures. The two morphs exhibited different length-width relationships and relative (to body) pharynx volumes. Pharynxes of the secondary morphs reached 4% of their body volume vs only 0.2% for primary morphs. These observations have led the authors to hypothesise that the two morphs comprise functionally specialized castes.

3.5.2. "Secondary morphs are more active than primary morphs"

Hechinger et al. (2003) carried out *in vitro* experiments in filtered sea water during spring and summer 2008, to quantify the body movement for nearly 200 rediae of each morph from 11 snails. They found that secondary morphs were more active, proportionally they moved five times more than primary morphs, extending, bending and contracting their bodies whereas the primary morphs made a minimal movement. These authors also performed trials to detect whether the secondary morphs change their activity in the presence of heterospecific species (*Euhaplorchis californiensis*) but did not record any changes in the activity.

3.5.3. "Secondary morphs readily attack heterospecifics and conspecifics from other colonies"

Hechinger et al. (2010) carried out another set of *in vitro* experiments in filtered sea water in autumn 2009 to quantify the frequency of attacks by primary secondary morphs against heterospecific non-kin and kin-conspecific rediae. They observed each well with rediae for 30 seconds under a stereomicroscope and scored attack when a redia used its mouth to latch onto another redia. They found that in all trials secondary morphs attacked rediae of the heterospecific *E. californiensis* and conspecific secondary morphs of *Himasthla* sp. B from different snails but never secondary morphs from the same snail. They either eat the heterospecific or lacerated their body wall. On the other hand, attacks by primary morphs occurred at much lower rates. The authors concluded that these observations are consistent with the hypothesis that secondary morphs are specialized to recognise and kill invading rediae.

3.5.4. "Secondary morphs do not reproduce"

Hechinger et al. (2010) failed to observe germinal balls in 173 randomly selected secondary morphs and concluded that their small size precludes them from producing the dispersive offspring (*i.e.* cercariae) that primary morphs produce.

3.5.5. "Secondary morphs do not appear to generally transition to become primary morphs"

Although Hechinger et al. (2010) observed intermediate morphs but did not assess this quantitatively and only indicated that these were "rare" and of "small number". They examined 42 rediae of this type and found that these forms are of intermediate size and shape and some of them (26) had germinal balls and two had procercariae. They hypothesised that "secondary morphs do not appear generally to initially specialise on defence and then transition to become reproductives".

3.5.6. "Secondary morphs are disproportionately common at invasion fronts"

Hechinger et al. (2010) performed redial counts in each of the three body regions (*i.e.* mantle, basal visceral mass and gonad/hepatopancreas) of 51 infected snails collected between February 2006 and January 2007. They counted the rediae under stereomicroscope by squashing tissue between two glass plates and found that "secondary morphs comprised almost 100 per cent of the individuals in the mantle" whereas the primary morphs were located in the visceral mass and gonadal region. The authors suggested that the spatial positioning of the secondary morphs at invasion fronts is consistent with the hypothesis of the defensive role of these morphs.

Based on the discrete morphologies, rarity of intermediate stages, and lack of primary morph turnover, Hechinger et al. (2010) concluded that secondary and primary morphs form two relatively permanent and physically distinct castes in *Himasthla* sp. B: a non-reproductive soldier caste and a reproductive caste. They predicted that trematode soldier castes may evolve in taxa that are dominant in interspecific hierarchies and in systems where trematodes typically infect a high proportion of hosts.

4. MATERIALS AND METHODS

4.1. MATERIALS

A different to Hechinger et al. (2010) snail-trematode system was used for this pilot study. Examinations were conducted on a locally common echinostomatid trematode species, *Echinostoma spiniferum*, which infects the great ramshorn snail, *Planorbarius corneus*. A total of nine snails was examined: two were used for morphological and morphometric examination of the rediae and the remaining seven were used for redial counts for determination of redial abundance in three different parts of the snail body. Snails were collected at different times from three ponds (Table 4.1). They have been kept in individual bakets (0.5l) with dechlorinated tap water at constant temperature (17°C) and fed on lettuce until dissection. Snails spent in laboratory 6-17 months. Since all infections with *E. spiniferum* were patent at the time of collection, the minimum age of infection was estimated as the time spent in the laboratory plus 2 months (Table 4.1).

Table 4.1. Snail codes, shell size, date and locality of collection, and estimated age of infection.

	Snail code	Snail width	Snail height	Date of collection	Pond	Infection age (months)
Snails used for redial size measurements	1V1PC63ES	27.88	12.61	9 June 2010	Vlkovský	11
	3B1PC1ES	24.66	11.94	5 September 2010	Bohdanečský	8
Snails used for redial counts	ES2N	32.71	15.43	10 May 2010	Korenský	12
	4PV67ES	26.6	12.4	12 October 2009	Vlkovský	19
	4PV154ES	30.3	13.6	12 October 2009	Vlkovský	19
	1B2PC37ES	35.3	14.13	5 June 2010	Bohdanečský	11
	2B1PC45ES	32.95	13.33	5 August 2010	Bohdanečský	9
	4PV63ES	23.1	11.6	12 October 2009	Vlkovský	19
	1V1PC40ES	29.97	12.75	9 June 2010	Vlkovský	11

4.2. SNAIL DISSECTION AND REDIAL COUNTS

Snail shell was carefully cracked and removed. Snail body was divided using a scalpel into three regions, corresponding to the subdivision generally used by Hechinger et al. (2010): (i) foot, head and mantle (further referred to as foot and mantle); (ii) basal visceral mass; and (iii) hepatopancreas and gonad (Fig. 4.1). The only difference was that we examined, in addition

to mantle, the head-foot region of the snail, the main target for miracidial penetration. The three body regions were placed in Petri dishes with tap water. All rediae located in the foot and mantle were dissected out by tearing apart the muscle fibres with fine forceps and needles; rediae were then placed in 4% formaldehyde solution.

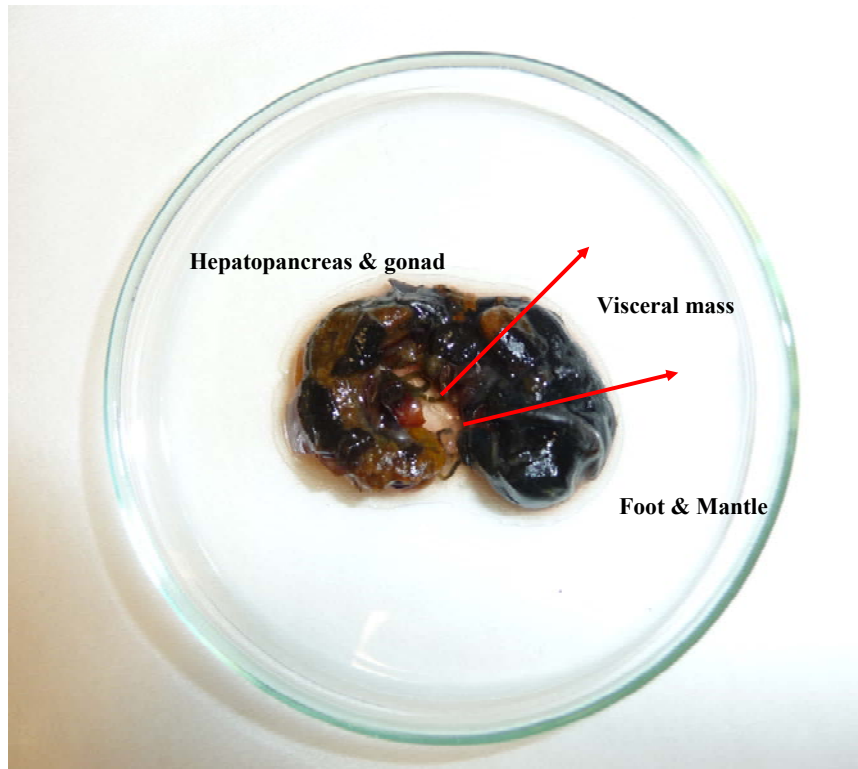


Fig. 4.1. Division of the body of *Planorbarius corneus* into three regions (red arrows).

Rediae from the basal visceral mass and hepatopancreas/gonad were dissected out in a similar manner. However, due to the extremely large number of rediae, counts from these body parts were performed on aliquots. The homogenised pieces (c. 1-2 mm in size) of the visceral mass and hepatopancreas/gonad and the extracted rediae in the dissection Petri dishes were transferred to 200 ml H₂O and 300 ml H₂O, respectively. Then five subsamples of 1 ml were taken from each beaker while stirring vigorously the mixture. These were pipetted into plastic vessels and immediately fixed in 4% formaldehyde solution. Rediae were counted in each subsample per body part and their total numbers estimated from these aliquots.

Rediae were initially assigned to two morphs as recognised by Hechinger et al. (2010):

- (i) "large morph" corresponding to their "primary morph" or "reproductive caste"; and
- (ii) "small morph" corresponding to "secondary morph" or "soldier caste".

Redial counts of these two categories were performed under a stereomicroscope for each body part.

4.3. PREPARATION FOR MORPHOLOGICAL AND MORPHOMETRIC STUDY

All rediae were stained with iron acetocarmine (Georgiev et al., 1986), dehydrated through ethanol series (70-80-90-96-100%), cleared in dimethyl phthalate and examined as temporary mounts in a drop of dimethyl phthalate. Photographs of the rediae were taken with DP70 Digital Camera System and computer program QuickPHOTO MICRO 2. 1. on an optical research microscope Olympus BX51. The presence of germinal mass and the numbers of germinal balls, cercarial embryos and cercariae and cercariae were recorded for each redia. Measurements for four variables (Fig. 4.2) were taken from photos using QuickPHOTO MICRO 2. 1. All measurements are in micrometres.

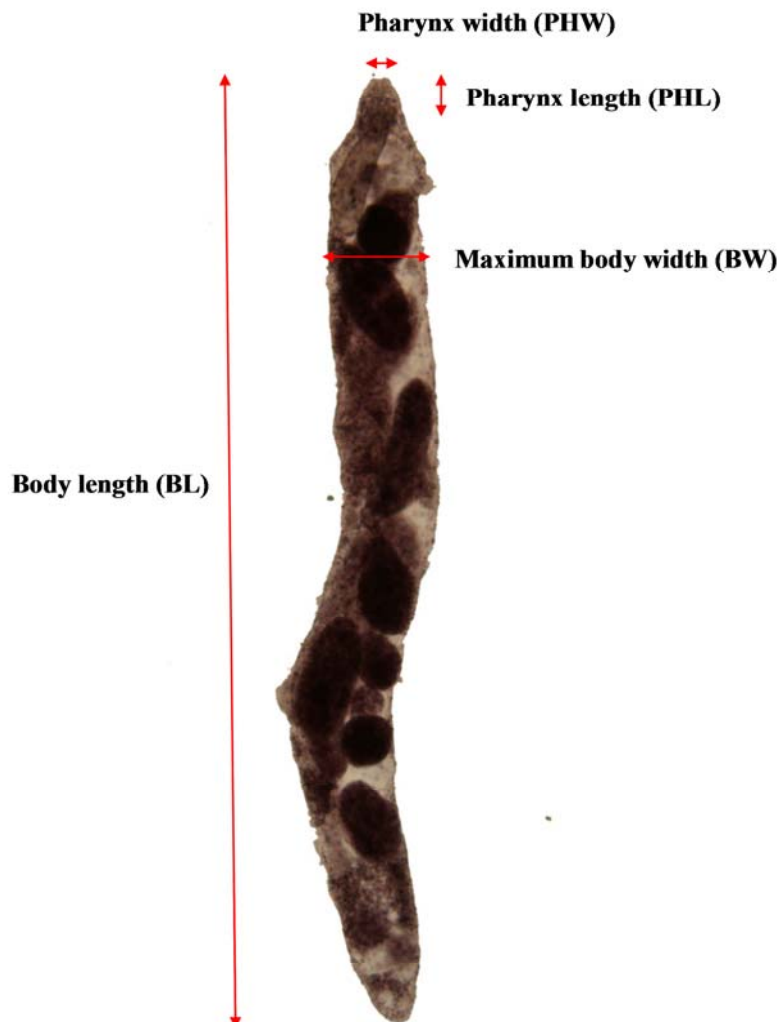


Fig. 4.2. Variables measured for each redia.

4.4. STATISTICS

First, exploratory data analysis was carried out. Metrical data and redial counts were summarised with the aid of bar charts, histograms, scatterplots, box-and-whisker plots and the standard descriptive statistics (range, mean, standard deviation, median) were computed. Comparisons of medians and measures of variation in the two groups of rediae were visualized in box-and-whisker plots. The box shows the location of the middle half of the data, median is indicated by a horizontal line and one quarter of data are located along each whisker. Scatterplot was a useful summary for the set of bivariate data (redial body length and body width) which were examined in order to visualise the type of the relationship between the two variables before calculating a correlation coefficient or fitting a regression line.

The relation between variables was measured by Spearman's rank correlation coefficient (r_s) in cases when sample sizes were small (*e.g.* redial abundance and snail size). Its formula uses the squared difference (d^2) between ranked values for each pair of data (ranked for each variable separately) and sample size as follows:

$$r_s = 1 - \frac{6\sum d^2}{n(n^2-1)}$$

Correlation coefficients range from -1.00 (a perfect negative correlation) to +1.00 (a perfect positive correlation). A value of 0.00 represents a lack of correlation.

Regression analysis was carried out on ln-transformed data to describe the average change in redial width for a unit change of redial length. In general, the goal of linear regression is to find the line that best predicts Y from X. This is reached by finding the line that minimizes the sum of the squares of the vertical distances of the points from the line.

Since count data did not have normal distribution the differences between independent groups (samples) were assessed by the nonparametric alternatives for (i) the t-test for independent samples (Mann-Whitney U test for two samples; abbreviated in the text as M-W test), and (ii) for the analysis of variance (Kruskal-Wallis nonparametric ANOVA for multiple samples; abbreviated in the text as K-W test).

5. RESULTS

5.1. REDIAL MORPHOLOGY: GENERAL OBSERVATIONS

We examined in detail the morphology of a total of 232 rediae dissected out from two infected snails with an estimated minimum age of infection 8 and 11 months (see Table 5.1 for a breakdown by site in the snail body). In this sample young rediae (33) predominated in the foot and mantle whereas mature rediae predominated in the hepatopancreas and gonad.

Table 5.1. Numbers of the young and mature rediae measured broken down by site of location. F & M, foot & mantle; V; basal visceral mass; HG & G, hepatopancreas & gonad.

Snail code	Young rediae			Mature rediae			Total
	F & M	V	HG & G	F & M	V	HG & G	
1V1PC63ES	14	3	4	11	2	103	137
3B1PC1ES	19	5	3	4	11	53	95
Total	33	8	7	15	13	156	232

Detailed examination of the redial morphology revealed a more complex picture than that observed by Hechinger et al. (2010). First, there were different types of small rediae possessing large pharynxes (in relation to body size) and distinct collars and locomotory appendages. After staining we found that some of the small rediae contained germinal balls in their body cavity (*e.g.* Fig. 5.1.1. A,C), some with a poorly detectable germinal mass (*e.g.* Fig. 5.1.1.B) and some with many well-developed germinal balls and embryos (7-9 in number, *e.g.* Fig. 5.1.2.C). Secondly, there were at least two types of large rediae that all had developed collars and locomotory appendages (Fig. 5.1.2. A,B). The first type was characterised by elongate, narrow body and contained many germinal balls, cercarial embryos and fully mature cercariae (Fig. 5.1.2. A). Rediae of the second type had shorter and much wider bodies and contained a large number of germinal balls and cercariae at different stages of development (Fig. 5.1.2. B).

Thus, a general division between the small rediae with relatively large pharynxes (corresponding to "secondary morphs" or "soldiers" of Hechinger et al., 2010) and the large, actively reproducing rediae with relatively small pharynxes (corresponding to "primary morphs" or "reproductives" of Hechinger et al., 2010) was possible. However, considering the different degrees of development of the intraredial developmental stages observed, we preferred to assign the rediae of *E. spiniferum* into categories associated with their development: young and maturing/mature (referred to as "mature" for simplicity) (see details of morphology in Figs 5.1.1. and 5.1.2.).

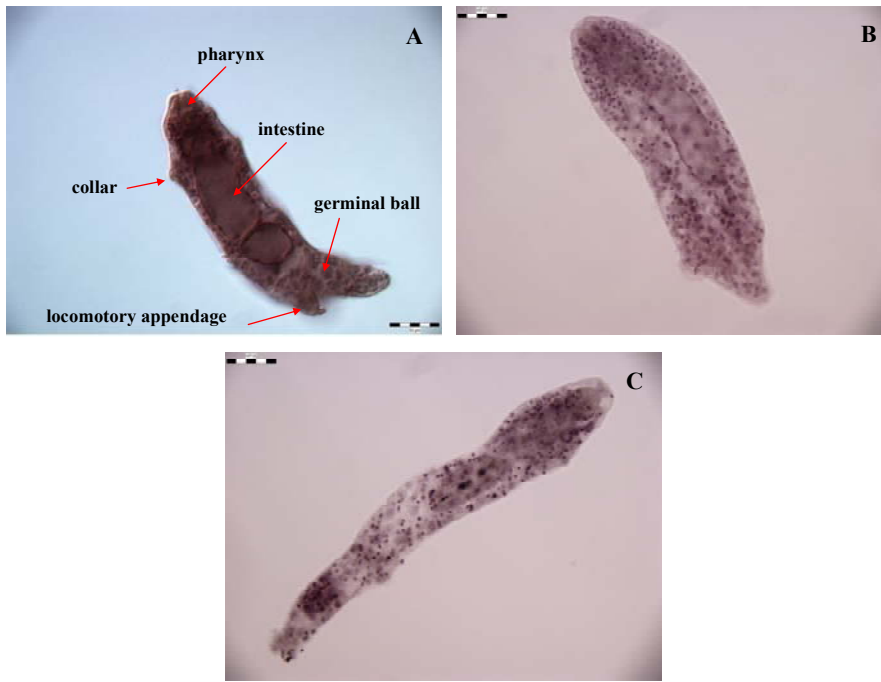


Fig. 5.1.1. Microphotographs of young rediae stained with iron acetocarmine and mounted in dimethyl phthalate. *Scale-bars:* 50 μm .

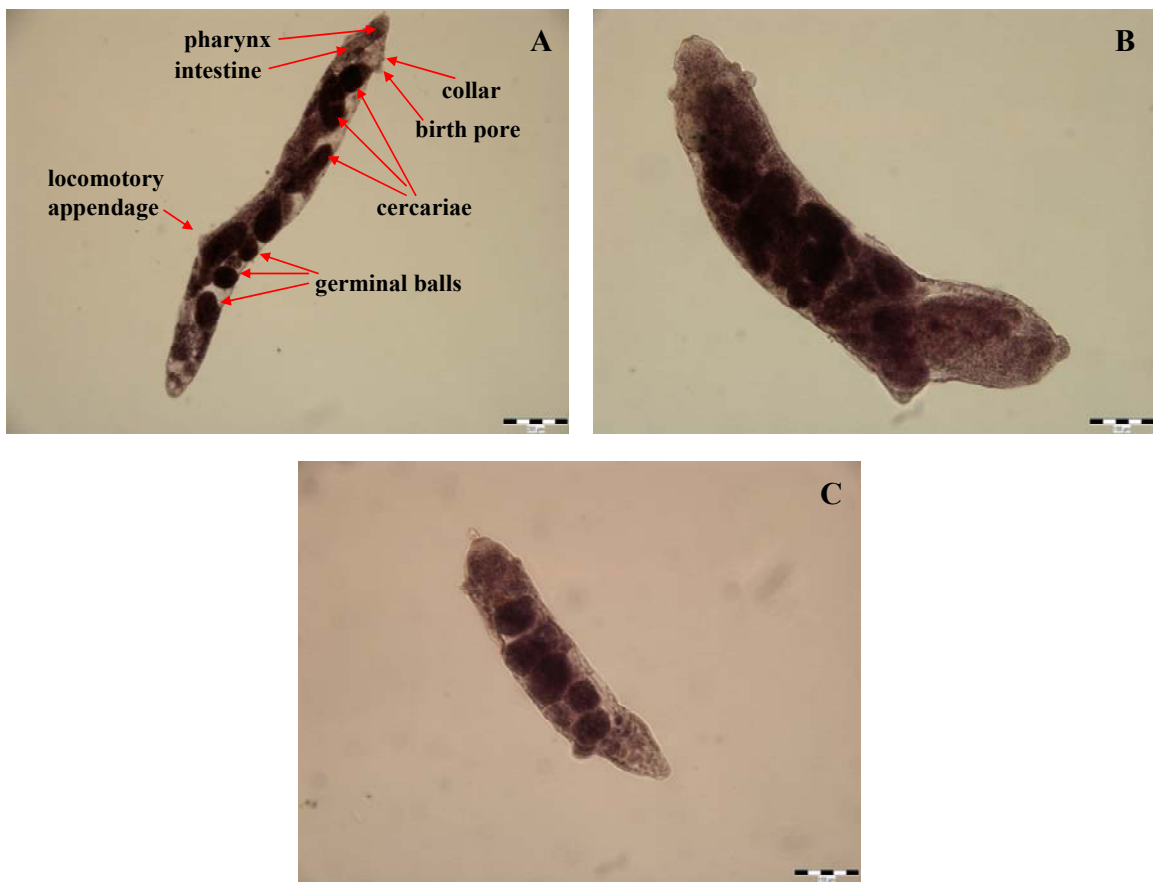


Fig. 5.1.2. Microphotographs of mature rediae stained with iron acetocarmine and mounted in dimethyl phthalate. *Scale-bars:* A, 200 μm ; B, C, 100 μm .

5.2. REDIAL MEASUREMENTS

The histogram in Fig.5.2.1 shows a frequency distribution of redial lengths. It was unimodal and agreed with a normal distribution (Kolmogorov-Smirnov test $d = 0.08119$, $p < 0.15$). The largest proportion of the rediae had an intermediate length (within the range of 400-800 μm) and only few had a length $> 1,000 \mu\text{m}$. The graph therefore, indicates a gradual increase in redial length and no detectable group separation between the young and mature rediae in relation to their body length in the sample of 232 rediae of *E. spiniferum* examined.

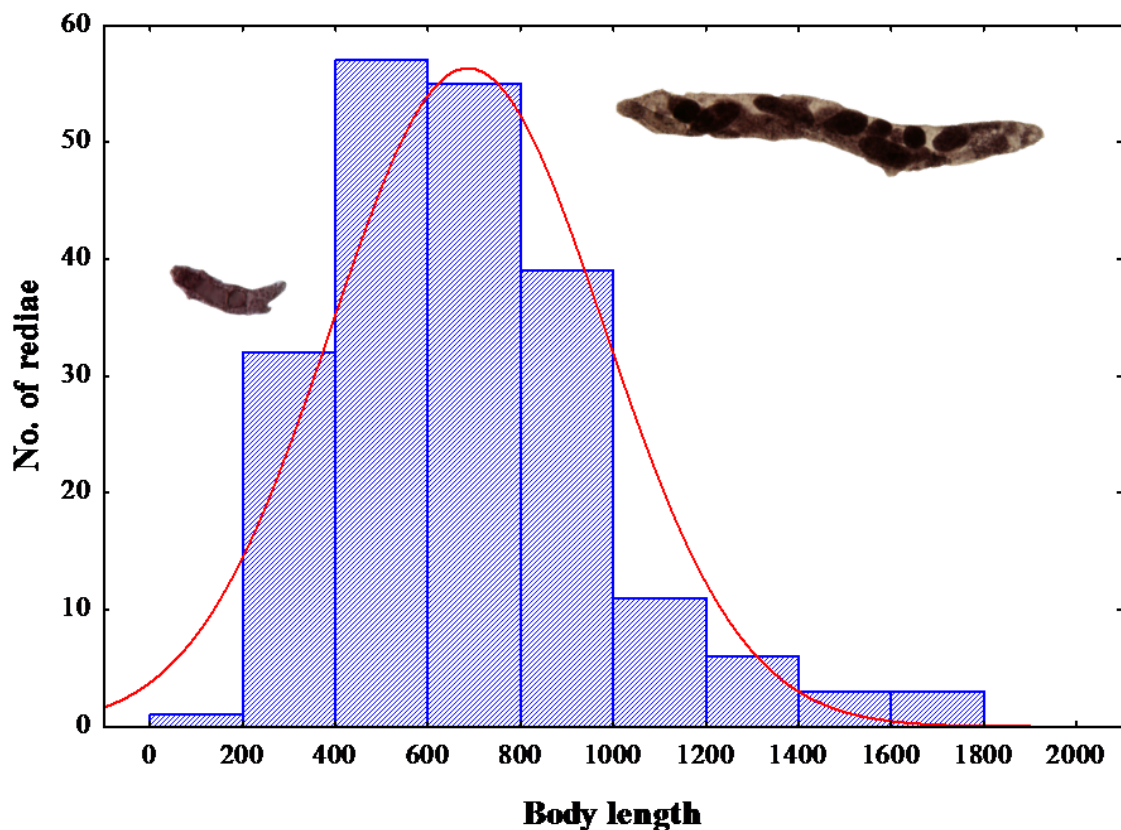


Fig. 5.2.1. Histogram showing the distribution of redial body length (in μm).

A plot of all specimens studied showing the relationship between redial body length and width is presented in Fig. 5.2.2.A. The scatterplot indicates an overlap between young and mature rediae when both measures for body size are considered. Although the young and mature rediae generally formed two groups, there was a significant overlap at the small size-range (c. 200-600 μm in length and c. 60-140 μm in maximum width). This is in contrast with the observations of Hechinger et al. (2010), the most pronounced difference being the rates of increase in redial width with increased length.

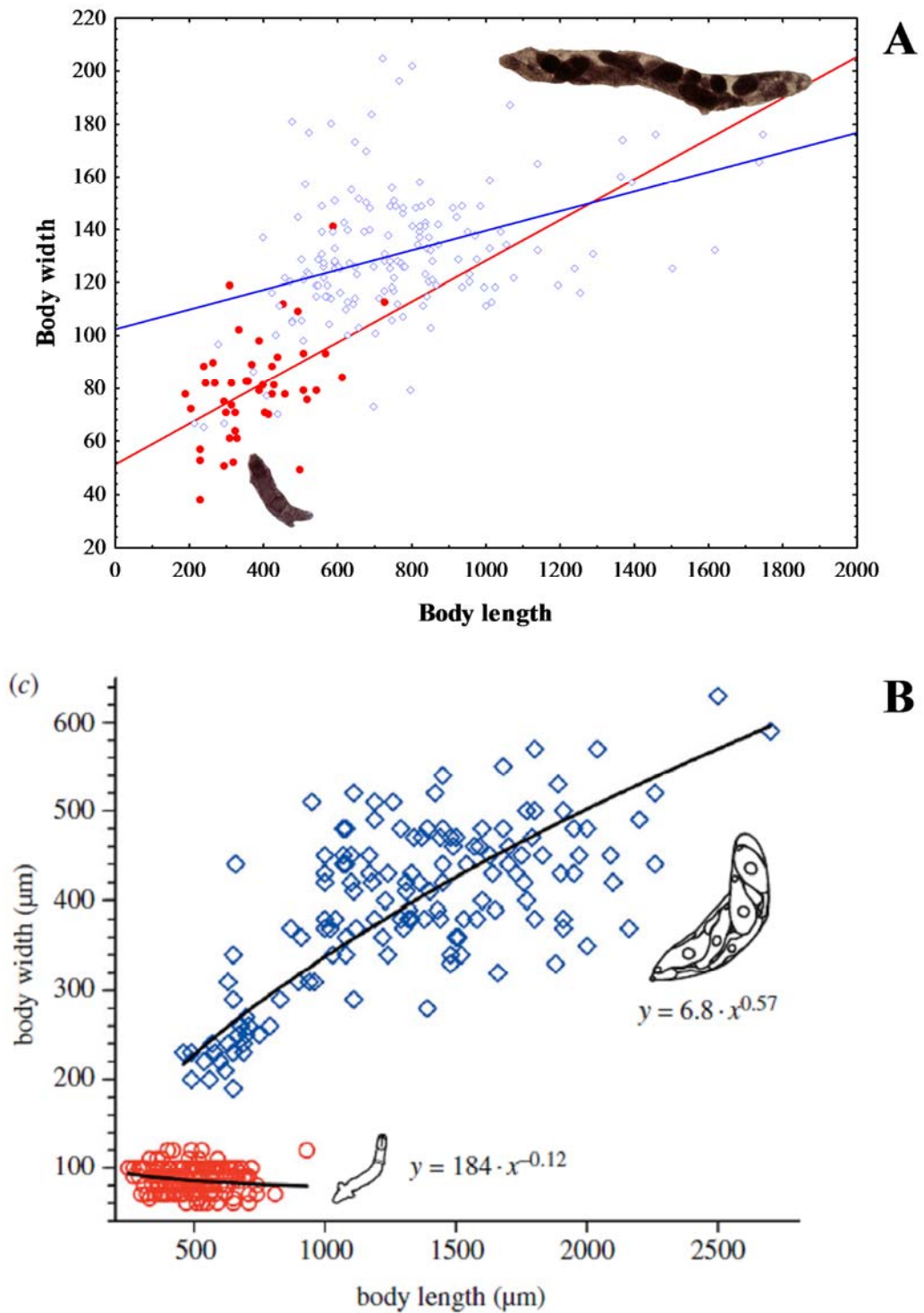


Fig. 5.2.2. Relationship between redial body length and width (μm) for the sample examined in the present study (**A**) compared with that observed by Hechinger et al. (2010) (**B**: Fig. 1c in the original paper). Young rediae (**A**) and "soldier caste" (**B**) in red, mature rediae (**A**) and "reproductive caste" (**B**) in blue.

There was a significant strong linear correlation between body width and length in the entire dataset of rediae of *E. spiniferum* ($R^2=0.50$; $b\pm SE=0.47\pm 0.03$; $F_{(1,205)}=205.37$, $p<0.0001$). In other words, when the body length increases also the body width increases. Separate regressions carried out on the data for young and mature rediae were also significant: young ($R^2=0.20$; $b\pm SE=0.36\pm 0.11$; $F_{(1,43)}=10.86$, $p<0.002$); mature ($R^2=0.24$; $b\pm SE=0.28\pm 0.04$; $F_{(1,160)}=46.66$, $p<0.0001$). The slopes of the regression lines (Fig. 5.2.2.A) indicate a steeper increase in width in the young rediae of *E. spiniferum*. This is in contrast with the non-linear growth patterns in the data for *Himasthla* sp. B (Fig. 5.2.2.B): a negative increase in width with length in the "soldier caste" vs a positive increase in the "reproductive caste". This difference results in a distinct gap between the two morphs with respect to redial width.

Table 5.2. provides a summary of the metrical data for the young and mature rediae of *E. spiniferum* studied; these data are graphically illustrated in Fig. 5.2.3. A-C

Table 5.2. Metrical data (in μm) for the rediae of the two categories studied.

Features/Category	Young				Mature			
	n*	Range	Mean	SD**	n	Range	Mean	SD
BL	45	188 - 725	380	120	162	216 - 1,748	766	271
BW	48	38 - 141	81	19	179	65 - 205	130	25
PHL	46	20 - 45	37	5	175	28 - 66	42	6
PHW	46	20 - 43	34	4	175	27 - 70	40	6
PHL/BL (%)	43	5 - 21	11	3	153	2 - 17	6	2

* n, sample size; ** SD, standard deviation.

In spite of the range overlaps (Table 5.2.) there were statistically significant differences between the young and mature rediae with respect to body length (M-W test, $z=8.90$, $p<0.0001$; $n_y=45$, $n_m=162$), pharynx length (M-W test, $z=5.76$, $p<0.0001$; $n_y=46$, $n_m=175$) and the relative length of pharynx (M-W test, $z=-8.40$, $p<0.0001$; $n_y=43$, $n_m=153$). As illustrated in Fig. 5.2.3.A, half of the young rediae had body length within the 300-500 μm range as opposed to mature rediae (range 600-850 μm). Additionally, the variation of the body length of mature rediae was much higher than that of the young rediae. Mature rediae had significantly longer pharynxes (Fig. 5.2.3.B) but pharynx length in the young rediae was considerably larger when related to body length (Fig. 5.2.3.C) thus showing a pattern similar to that observed in *Himasthla* sp. B (inset in Fig. 5.2.3.C). However, a significant gap in the relative size of pharynx was observed in the latter species while our data exhibited a wide overlap (5-21% of body length in young rediae vs 2-17% in mature rediae).

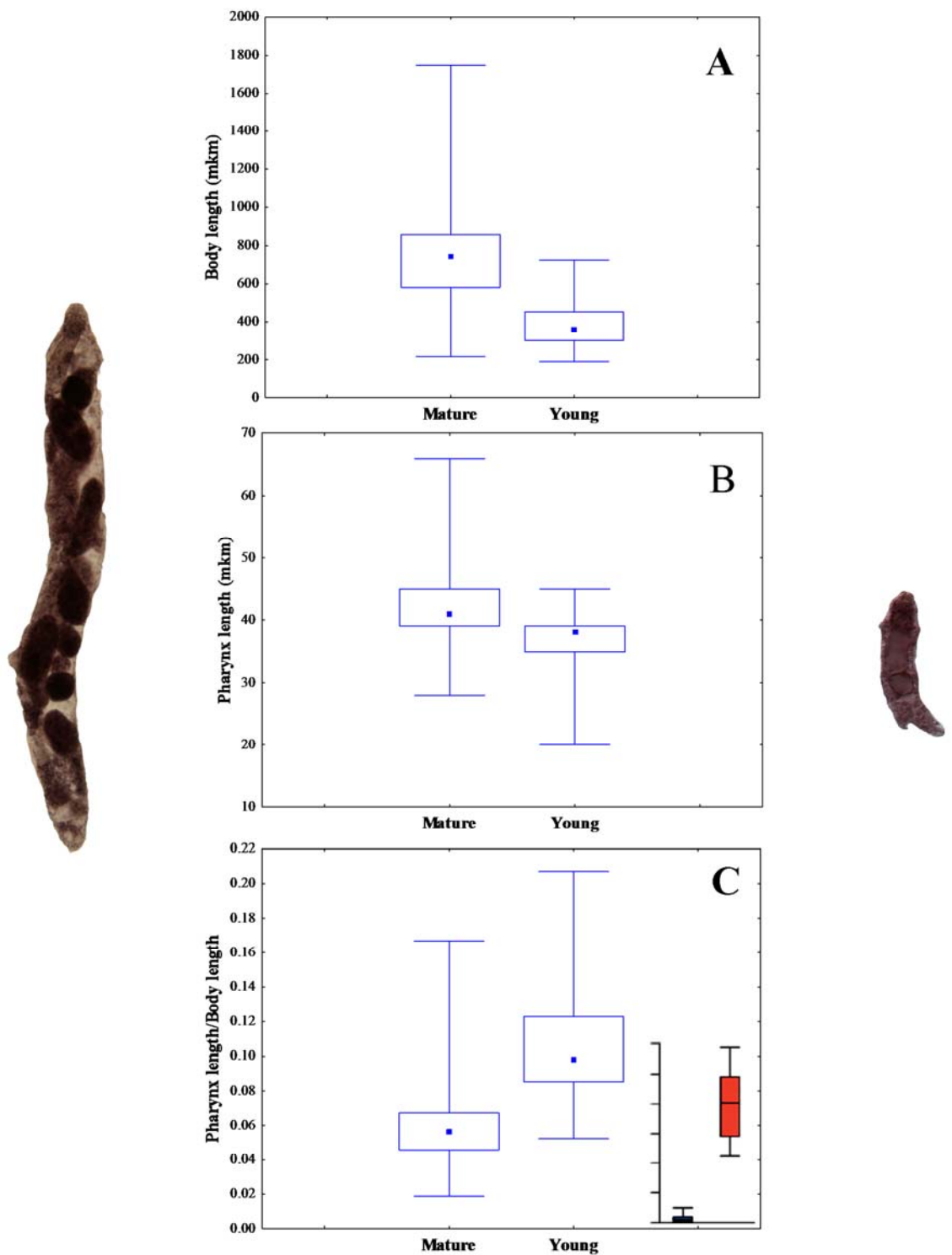


Fig. 5.2.3. Box-and-whisker plots (range, interquartile range and median) for: (A) redial length; (B) pharynx length; and (C) relative size of pharynx for the mature and young rediae studied. Inset in C: Relative size of pharynx for primary (left box) and secondary (right box) rediae of *Himasthla* sp. B (Fig. 1e of Hechinger et al., 2010).

5.3. REDIAL COUNTS

Figures 5.3.1. and 5.3.2. show the variations in the distributions of young and mature rediae by site of location in the seven snail hosts examined. Most of the young rediae were located in hepatopancreas and gonad and the visceral mass. However, there was a notable variation and extreme cases: in one snail (No. 2) we found a huge number of young rediae in the visceral mass whereas in another (No. 5) the young rediae in the hepatopancreas and gonad exhibited similar high abundance. Mature rediae had distinctly high abundance in the hepatopancreas and gonad (Fig 5.3.2). However, mature rediae were found in the visceral mass in six snails (range 40-1680 individuals) and in the foot and mantle in all seven snails studied (range 1-334 individuals).

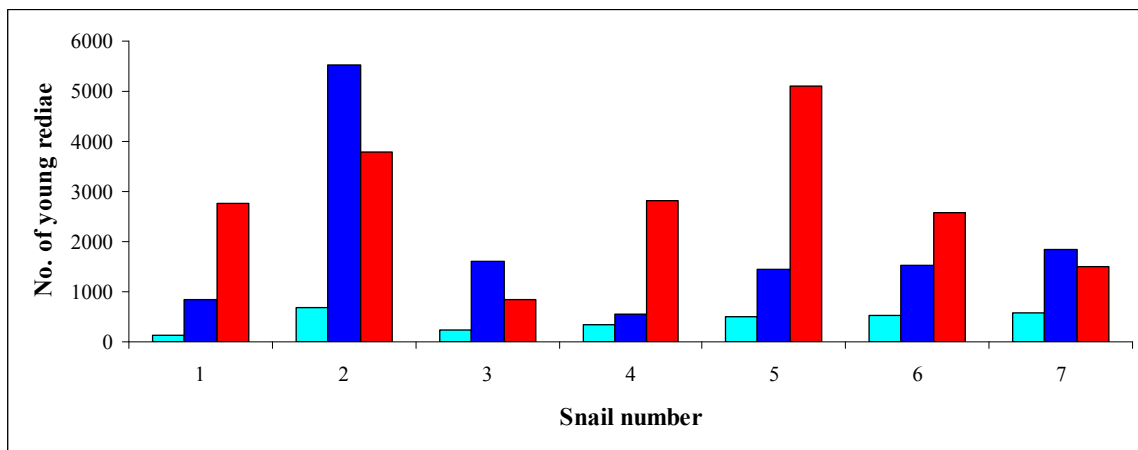


Fig. 5.3.1. Distribution of the young rediae within the three sections of snail body: (i) foot & mantle (light blue bars); (ii) basal visceral mass (dark blue bars); and (iii) hepatopancreas & gonad (red bars).

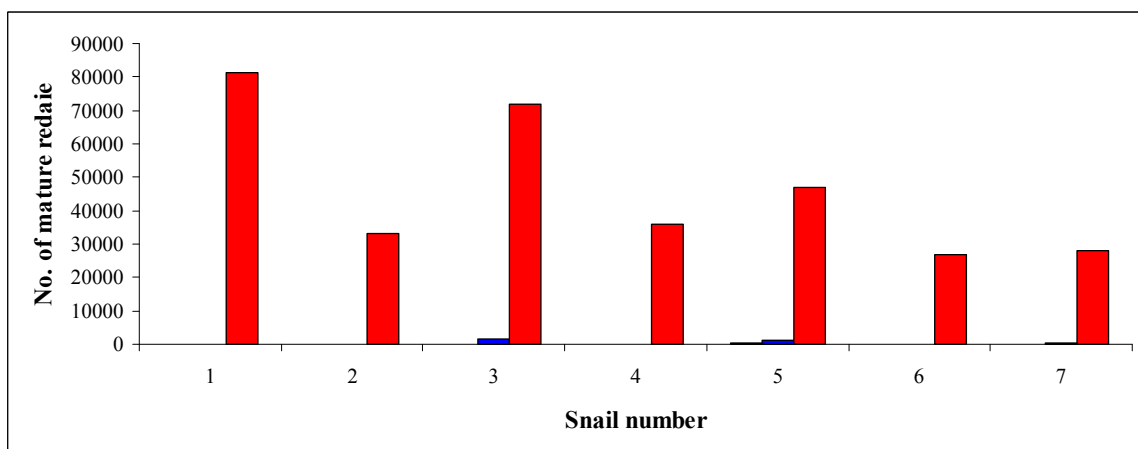


Fig. 5.3.2. Distribution of the mature rediae within the three sections of snail body: (i) foot & mantle (light blue bars); (ii) basal visceral mass (dark blue bars); and (iii) hepatopancreas & gonad (red bars).

There was a positive correlation between snail width and the number of mature rediae ($r_s=0.64$, Fig. 5.3.3.A) following a pattern similar to that observed by Hechinger et al. (2010) (Fig. 5.3.3.B) and a negative correlation ($r_s= - 0.43$) between snail width and the abundance of young rediae. However, due to the small sample size examined, these associations had no statistical significance (all $p>0.05$).

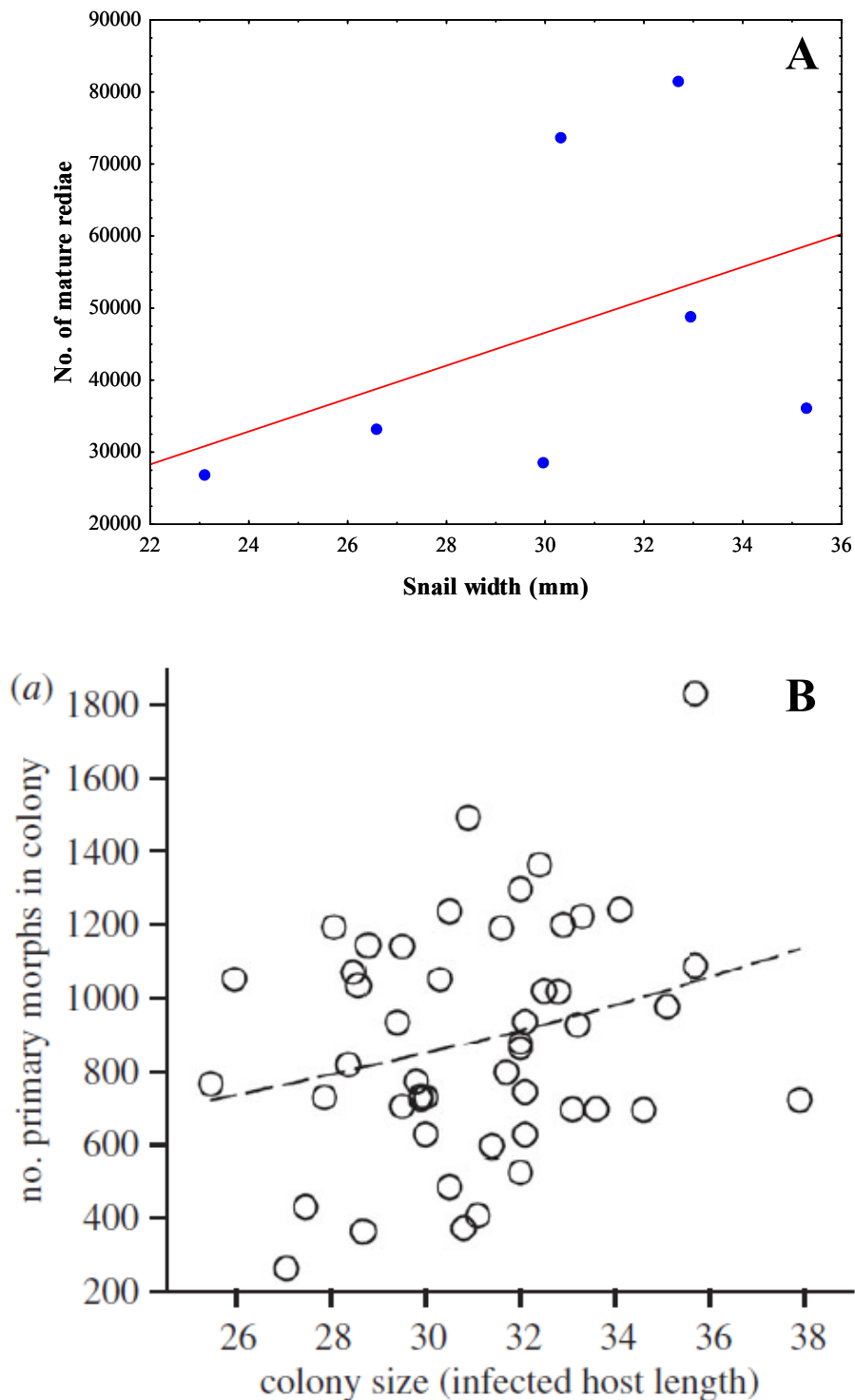


Fig. 5.3.3. Relationship between the abundance of young mature rediae and snail size in: (A) present study; (B) *Himasthla* sp. B (Fig. 5a of Hechinger et al., 2010).

Perhaps associated with the size of the snail, there was a negative but non-significant correlation between the numbers of young and mature rediae ($r_s = -0.46$, $p > 0.05$, see Fig. 5.3.4.A), a trend opposite to the one observed in *Himasthla* sp. B (Fig. 5.3.4B), *i.e.* disproportionately increased numbers of the "soldier caste" with increased numbers of the "reproductive caste".

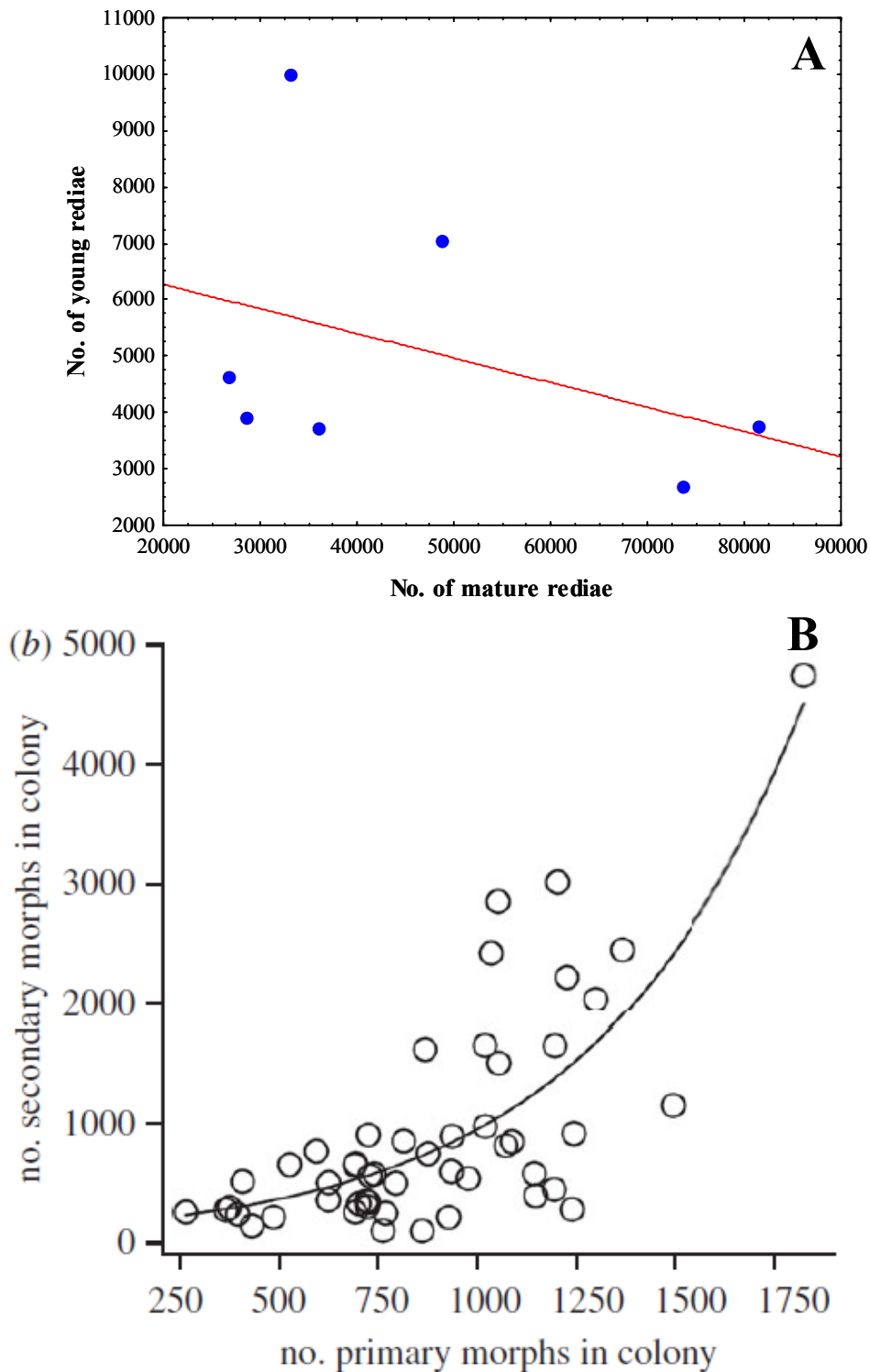


Fig. 5.3.4 Relationship between abundances of young and mature rediae in: (A) present study and (B) *Himasthla* sp. B (Fig. 5a of Hechinger et al., 2010).

The distributional pattern of the total redial counts for *E. spiniferum* shown in Fig. 5.3.5.A revealed that the majority of the rediae are located in the hepatopancreas and gonad (K-W test, $H_{(2,21)}=16.79$, $p=0.0002$). Thus redial abundance of *E. spiniferum* although not comparable with the much lower levels observed in *Himasthla* sp. B, showed a similar trend in relation to site-selection by the rediae (Fig. 5.3.5.B).

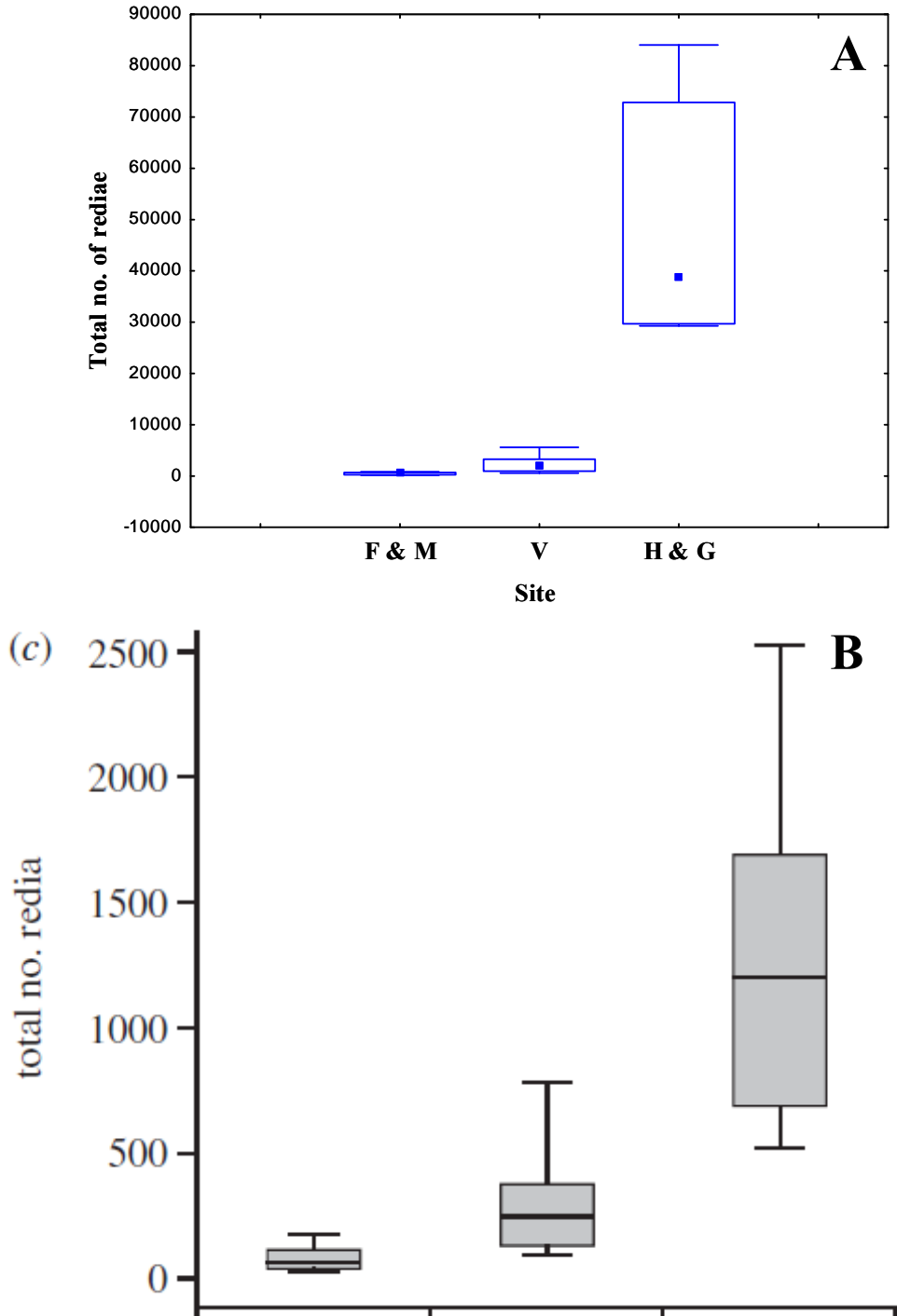


Fig. 5.3.5. Box-and-whisker plots for redial abundance in the three sites of snail body. **A.** Present study. **B.** *Himasthla* sp. B (Fig. 5c of Hechinger et al., 2010).

However, young individuals represented significantly higher proportions of the rediae located in the foot and mantle (range 60-100%, median 99%) and the basal visceral mass (range 49-100%, median 88%) compared to hepatopancreas and gonad (range 1-10%, median 7%) (K-W test, $H_{(2,21)}=14.03$, $p=0.0009$; multiple comparisons $p=0.001$ and 0.018 , respectively; see Fig. 5.3.6.A). This distribution is similar to that of the "soldier caste" of *Himasthla* sp. B (Fig. 5.3.6.B) although the probability to find an individual of this caste in the visceral mass and gonad shows much wider and overlapping ranges. It is worth stressing the "inverse" meaning of the data presented in Fig. 5.3.6 is, that up to 40% of the rediae of *E. spiniferum* located in the foot and mantle, represent mature rediae (up to 20% of the "reproductive caste" in *Himasthla* sp. B). Furthermore, half of the rediae of *E. spiniferum* in the visceral mass were mature whereas the proportion of the rediae of the "reproductive caste" of *Himasthla* sp. B reaches as high as 70%. Finally, the "soldier caste" of *Himasthla* sp. B represents a substantially higher proportion of the rediae in the gonad (range 0-65% vs 1-10% in *E. spiniferum*).

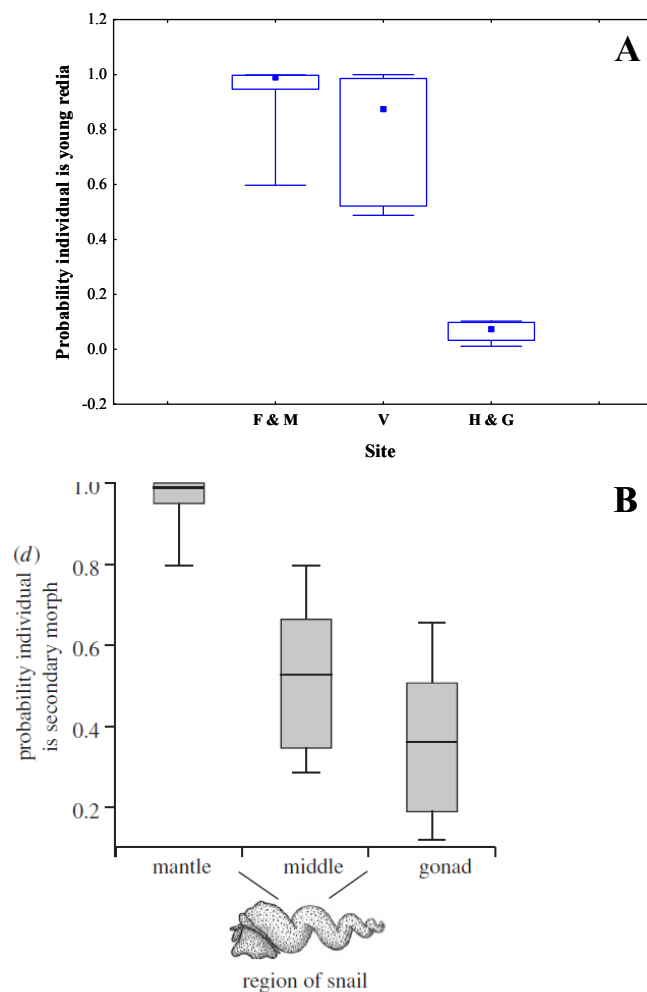


Fig. 5.3.6. Box-and-whisker plots for the probability of a redia to belong to the young category in each of the three sites of the snail body. **A.** Present study. **B.** *Himasthla* sp. B (Fig. 5d of Hechinger et al., 2010).

6. DISCUSSION

Evidences in line with the two main characteristics observed by Hechinger et al. (2010) *i.e.* that (i) rediae of different generations showing differences in body and pharynx sizes exist simultaneously in the same individual snail host (reviewed by Rondelaud et al., 2009 and Galaktionov & Dobrovolskij, 2003); (ii) young rediae possess a powerful muscular apparatus and this is associated with their greater locomotor activity (Galaktionov & Dobrovolskij, 2003); and (iii) rediae of the family Echinostomatidae show predatory behaviour (reviewed by Lie et al., 1968 and Lim & Heyneman, 1972) have long existed. What makes their study unique, is the hypotheses linking these two lines of evidence.

This first pilot study on a freshwater snail-trematode system was restricted, due to time limitations, to tests of the predictions of Hechinger et al. (2010) concerning the morphology and site-selection by rediae. The selection of the model species *E. spiniferum* reflects numerous lines of evidence (reviewed by Lie et al., 1968) suggesting that rediae of the genus *Echinostoma* (Echinostomatidae) are strong competitors that exhibit predatory behaviour. This species is also one of the most prevalent parasites of *P. corneus*. Thus the model system of the study meets two of the assumptions of Hechinger et al. (2010) for the systems, in which "trematode soldier castes will more probably evolve".

Are there two redial morphs corresponding to "soldier" and "reproductive" castes that differ in body size, body shape and relative pharynx size?

We found that a general division between small rediae with relatively large pharynxes and large, actively reproducing rediae with relatively small pharynxes is possible in the intramolluscan populations of *E. spiniferum*. Thus, the two morphs corresponding to "secondary morphs" or "soldier caste" and to "primary morphs" or "reproductive caste" of Hechinger et al. (2010) were presumably present in our material. However, considering the different degrees of development of the intraredial developmental stages observed, we preferred to assign the rediae of *E. spiniferum* into categories associated with their development: young and maturing/mature. Nevertheless, although the "small" rediae that possessed germinal balls and early stage embryos were classified to the second group, maturing/mature rediae (*i.e.* "reproductives" of Hechinger et al., 2010), there were significant differences between the young and mature rediae with respect to body length, pharynx length and the relative length of pharynx. These observations agree with the observations of Hechinger et al. (2010) on *Himasthla* sp. B.

However, although young and mature rediae generally formed two groups, we observed a substantial overlap in body size (length and width) and this is in contrast with the observations of Hechinger et al. (2010). Furthermore, the rates of increase in redial width with increased length differed, especially due to the steeper increase in width in the young rediae of *E. spiniferum*, and this resulted in the lack of a distinct gap between the two morphs with respect to redial width observed by Hechinger et al. (2010). Our data thus indicate a continuous linear growth in both morphs suggesting a transition of aging larvae rather than occupancy of discrete areas of morphospace supporting the hypothesis that "secondary and primary morphs comprise two functionally specialized castes".

Do rediae of the "soldier" caste possess ability to reproduce and transition to become "reproductives"?

Hechinger et al. (2010) stated that none of the rediae of the "soldier" caste studied under high magnification had germinal balls (early-stage embryos lose in the body cavity). However, these authors did not attempt to visualise the germinal material. Staining with a chromosomal stain revealed the existence of different types of small rediae possessing large pharynxes (in relation to body size) and distinct collars and locomotory appendages (i) rediae with poorly detectable germinal masses; (ii) rediae containing germinal balls; (iii) rediae with many well-developed germinal balls and cercarial embryos. Our data strongly suggest, that young rediae have the potential to reproduce and do reproduce and this is in contrast with the main assumption of the hypothesis of Hechinger et al. (2010) for the division of labour between the "soldier" and "reproductive" caste. Furthermore, the observation of different stages of intraredial development in the maturing/mature rediae indicates a gradual process of maturation of the young rediae and the simultaneous existence of several redial generations of *E. spiniferum* within individual *P. corneus*.

Do rediae of the "soldier" caste transition to "reproductive" caste?

Hechinger et al. (2010) detected a small number of intermediate morphs. After examination they confirmed that these are maturing primary morphs but stressed on their rarity and concluded that the rediae in *Himastha* sp. B "do not appear generally to initially specialize on defence and then transition to become reproductives". In contrast, our morphological observations on the intraredial development of the germinal material, coupled with the high abundance of the intermediate forms and the significant morphometric overlap between young

and mature/maturing rediae in *E. spiniferum* suggest a normal process of gradual renewal of the redial populations in individual snails. This is supported by the opposite trend we observed between the abundance of the two groups of rediae; more dissections need to be performed to confirm or reject this relationship.

Are rediae of the "soldier" caste disproportionately common at invasion fronts?

This appears to be the only hypothesis of Hechinger et al. (2010) supported by our observations. Actively infecting miracidia penetrate through the mantle, foot and tentacles of the snail, so these parts of snail's body can be considered as main "invasion fronts". However, although stressing on "invasion fronts" these authors did not examine the foot and head of the snails. Our data based on careful dissection of the foot and head muscles clearly indicate that the picture of redial proportions would have been different in *Himasthla* sp. B. Thus we found up to 700 young rediae of *E. spiniferum* in the foot and head of *P. corneus*. Nevertheless, the redial abundance of *E. spiniferum* although not comparable with the much lower levels observed in *Himasthla* sp. B, showed a similar trend in relation to redial site-selection.

Two questions arise with respect to the site-selection by rediae. As stressed in the Results section, up to 40% of the rediae of *E. spiniferum* up to 20% of the "reproductive caste" in *Himasthla* sp. B located in the foot and mantle represent mature rediae. For example we found 334 mature rediae in the foot of one snail. Furthermore, half of the rediae of *E. spiniferum* in the visceral mass were mature and the proportion of the rediae of the "reproductive caste" of *Himasthla* sp. B reaches as high as 70%. Finally, the "soldier caste" of *Himasthla* sp. B represents a substantially higher proportion of the rediae in the gonad young (range 0-65% vs 1-10% in *E. spiniferum*).

The two questions, therefore, are: First, if we accept the statement of (Hechinger et al., 2010) that "the spatial positioning at invasion fronts is consistent with the hypothesis of a defensive role for the "soldier caste", how can we explain the presence of mature rediae or "reproductive" caste at the "invasion fronts"? Secondly, if we accept the "division of labour" hypothesis, does it really result from evolutionary mechanisms associated with a selective advantage to defend?

Hechinger et al. (2010) state that "soldiers" defend the stolen host body against invading trematodes. However, studies on snail immunity clearly indicate that the "host body" has much more powerful systems to counteract trematode infections (*e.g.* Adema & Loker, 1997; Coustau et al., 2009). Furthermore, their assumption is based on observations suggesting predatory behaviour of the rediae. However, all existing documented (*i.e.*

photographed) predatory acts illustrate small (or large) rediae "eating" or "attacking" large (*i.e.* mature) rediae or cercariae. Since these stages do not represent invading larvae, this evidence does not reject the hypothesis that rediae are indiscriminate feeders (Esch et al., 2002; Galaktionov & Dobrovolskij, 2003). Rediae consume host tissue by tearing and ingesting it directly and in this process they would ingest not only host tissue but also larval stages of other trematodes they encounter (Esch et al., 2002). Unfortunately, the experimental procedure in the predation experiment of Hechinger et al. (2010) had no control e.g. pieces of host tissue or small invertebrates similar in size and activity to the heterospecific rediae exposed to attacks. Therefore, we consider these results inconclusive.

Hechinger et al. (2010) were the first to publish a photograph of a "soldier" of *Himasthla* sp. B. attacking a heterospecific "soldier" (*Parorchis acanthus*). However, the "war" can be considered lost if there are heterospecific "soldiers" already formed in the snail. To win the "war" would require elimination of the hundreds to thousands of mature rediae of this species that have already occupied large space within the snail. Therefore, even if we accept the reasoning of Hechinger et al. (2010) of the defensive role of the "soldier" caste, experimental evidence is definitely required for "soldier" attacks against the invading stages that occur at the "invasive fronts" *i.e.* migrating miracidia and mother sporocysts.

To conclude, the combined evidence from redial morphology and morphometry of the rediae of *E. spiniferum* and their site-selection in the snail host, *P. corneus*, do not provide support to three of the four hypotheses of Hechinger et al. (2010) tested in the present study. Our data indicate a gradual development of the redial generations with a complex structure of the infrapopulations as described by Galaktionov & Dobrovolskij (2003). We suggest two hypotheses for the contrasts between our system and that studied by Hechinger et al. (2010). First, they may have examined a species with an aberrant structure of the intramolluscan redial populations. However, the taxonomy of the species studied in *C. californica* is far from clarified both at the generic and family levels. Thus, as stated by Hechinger et al. (2010), they have studied the species named by Martin (1955) a "fin-tailed echinostome", and included in his key as "*Echinoparyphium* sp." (Martin, 1972). The species has been reported under the latter name in many studies on trematode communities in *C. californica*. However, none of the known species of *Echinoparyphium* or *Himasthla* possess a fin-fold on the tail and the only echinostomatid genera having cercariae with fin-folds are *Moliniella* and *Neoacanthoparyphium*, both with freshwater representatives only.

Secondly, Hechinger et al. (2010) may have described a temporal "snapshot" of a redial population structure in a *Himasthla* spp. Data by Galaktionov & Dobrovolskij (2003) strongly support this suggestion. Thus, although Hechinger et al. (2010) did not specify the

dates of snail collection for their morphological study (*i.e.* "in summer 2006"), Galaktionov & Dobrovolskij (2003) have found in another species of *Himasthla* (*H. elongata*) that the production of rediae by rediae is not timed for a certain season but takes place constantly leading to the fact that rediae which have young rediae of the next generation as well as cercariae, can be found in all seasons. Of particular interest is the structure found by these authors in July-August that comprised only two groups: (i) rediae of different ages, some containing fully developed cercariae; and (ii) old degenerating rediae along with very young ones (Fig. 3.2.).

Finally, we still have no plausible explanation for the higher abundance of the young rediae in the foot, head and mantle. If an exotic hypothesis is to be suggested we propose the "suicide soldier" hypothesis, *i.e.* that young rediae migrate to invasion fronts in order to activate snail's immune system and thus prevent secondary infections by both conspecific and heterospecific trematodes. Doing so they would also become victims and die (hence "suicide" part of our suggestion) but the life span and individual fate of these developmental stages are of little importance in view of the great reproductive potential of the inrapopulation. Further increase of the sample size for the present system and a comparative approach, *i.e.* examination of another common species of *Echinostoma* in a different snail host (*Echinostoma miyagawai* in *Planorbis planorbis*) would help to assess the validity of the findings of the present study.

7. CONCLUSIONS

The following conclusions can be drawn as a result of the present study:

7.1. The choice of the study system (*i.e.* a species from a group with ***proven dominant position*** in the hierarchy of competitive relationships among trematodes, and exhibiting high levels of infection in its host, *Planorbarius corneus*) and the methodology used (*i.e.* examination of ***all organs*** of the snail; dissecting out of ***all rediae*** from the foot and head regions; and staining with a chromosomal stain) are best suited to provide evidences for testing the hypotheses of Hechinger et al. (2010) for the existence of social organisation in the intramolluscan redial communities of trematodes.

7.2. Two morphs, small and large rediae, corresponding to "secondary morphs" or "soldier caste" and to "primary morphs" or "reproductive caste" of Hechinger et al. (2010) are present in larval redial infracommunities of *Echinostoma spiniferum*, thus partially supporting the observations of Hechinger et al. (2010).

7.3. The two groups, young and mature rediae of *E. spiniferum*, exhibited a substantial overlap in body size with no gaps in the bi-dimensional morphometric space due to the different growth relationships. In contrast with the observations of Hechinger et al. (2010), these data suggest a continuous linear growth in both morphs, therefore indicating a transition of aging larvae rather than occupancy of discrete areas of the morphospace.

7.4. Staining with a chromosomal stain revealed the existence of different types of small rediae in relation to intraredial development of the germinal material and that young rediae have the potential to reproduce. This is in contrast with the main assumption of the hypothesis of Hechinger et al. (2010) for the division of labour between the "soldier" and "reproductive" caste. The developmental patterns in the rediae of *E. spiniferum* within *P. corneus* indicate gradual maturation of the young rediae and simultaneous existence of several redial generations.

7.5. The high abundance of intermediate forms and the significant morphometric overlap between young and mature/maturing rediae in *E. spiniferum* are in disagreement with the hypothesis of Hechinger et al. (2010) that the "soldier" caste does not transition to

"reproductive" caste and suggest a gradual renewal of the redial populations in individual snails.

7.6. The observations of the redial site-selection support the finding of Hechinger et al. (2010) that young rediae are proportionally more abundant in the mantle and visceral mass and provided new data that they are even more abundant in the head and foot region of the snail.

7.7. The combined evidence from redial morphology and morphometry of the rediae of *E. spiniferum* and their site-selection in the snail host, *P. corneus*, do not provide support to three of the four hypotheses of Hechinger et al. (2010) tested in the present study.

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