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Social organization in trematode parasitic flatworms

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Ecology, Evolution and Marine Biology

by

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March 2018

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Guo H, **Garcia-Vedrenne AE**, Isserlin R, Lugowski A, Morada A, et al. (2015). Phosphoproteomic network analysis in the sea urchin *Strongylocentrotus purpuratus* reveals new candidates in egg activation. Proteomics. 15: 4080-4095. (Highlighted with commentary by Wessel, G. M. 2015. Proteomics, 15: 3925-3927)

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Studies in oarfish parasites, prey and potential predators with Armand M. Kuris

Abstract

Social organization in trematode parasitic flatworms

by

Ana Elisa Garcia Vedrenne

As in the most complex animal societies, trematodes (parasitic flatworms) live in colonies characterized by a high degree of cooperative organization. My research started with the unexpected observation that several trematode species have a reproductive division of labor with morphologically and behaviorally distinct reproductive and non-reproductive castes. The non-reproductive individuals are smaller but have relatively large mouthparts. They are more active than their reproductive counterparts and disproportionately common in areas of the host where invasions by other trematodes occur. Finally, only non-reproductive individuals readily attack enemies with their mouths. Thus, it is clear that one major role of these individuals is to defend the colony from enemy trematode invaders; they are soldiers.

The initial discovery was followed by reports revealing the existence of soldiers in five additional trematode species and by research examining the adaptive significance of soldiers. However, descriptions of colony structure were restricted to a single trematode superfamily (Echinostomatoidea), and knowledge of colony demography and caste function was limited to snapshots of the condition of mature colonies. My doctoral dissertation has laid the foundation to explore the

evolution of this remarkable social organization in trematodes, as well as the mechanisms regulating it.

Here I quantify morphology, distribution, and behavior of parasites from both establishing and fully developed colonies of sixteen species of trematodes that infect the California horn snail. While showing that eight additional species have a soldier caste, including four species from a new superfamily, I expand the phylogenetic range for which trematode sociality has been examined. I identify patterns underlying colony structure for trematode species that lack a soldier caste and establish discrete criteria to recognize colonies with and without soldiers. Finally, I develop an *in vitro* system for cultivation of marine trematodes that includes co-culture with *Biomphalaria glabrata* embryonic (Bge) cell line and media with Bge-released factors. The results presented here highlight the promise of these methods to address questions regarding trematode sociality, interspecific interactions, development and caste differentiation.

Trematode colonies are readily replicated, can be maintained in large numbers, and are amenable to *in vitro* studies. Hence, they provide many advantages as model systems to pursue experimental and comparative research probing general principles underlying the ecology and evolution of sociality. Furthermore, there are more than 20,000 species of trematodes worldwide. They cover a wide range of environmental and life history diversity and are both ecologically and medically important. Thus, understanding the mechanisms that shape trematode communities can have substantial public health, veterinary and wildlife disease applications.

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Chapter 1

Trematode Sociality: An overview

Introduction

Most people associate the term “complex sociality” with the classic social insects (bees, ants, termites) – animal groups characterized by a reproductive division of labor, where individuals of helper castes forego reproduction to serve the needs of the colony. Continuing with this word association, when thinking of trematodes (parasitic flatworms), one does not immediately think of colonies or castes. My research started with the unexpected observation that several trematode species have a reproductive division of labor with morphologically and behaviorally distinct reproductive and non-reproductive castes (Hechinger et al. 2011). My thesis work extends these observations and investigates the role that complex sociality plays in trematode biology and ecology.

Rationale and Significance

Trematodes form colonies within their first intermediate molluscan host. The colony is initiated by a single founder larva (miracidium) that infects the host, metamorphoses, and clonally produces large numbers of daughter parthenitae (Figure 1). Some trematode species have rediae: parthenitae that possess a muscular pharynx and a gut. Other species have sporocysts, which lack a pharynx and gut. Both kinds of parthenitae produce more parthenitae and then dispersive offspring (cercariae), which leave the colony to infect the next host in the life cycle.

Once established inside the molluscan host, the colony blocks host reproduction and takes control of the host’s energy allocation to serve the needs of the colony,

primarily diverting energy to parasite reproduction (Kuris, 1974; Baudoin, 1975; Hechinger et al., 2009; Lafferty and Kuris, 2009). The colony commonly occupies the gonad and/or digestive gland of the host, taking up a large portion of the host's soft tissue (10-50%; Fig 2A) (e.g., Hurst, 1927; Bernot and Lamberti, 2008; Hechinger et al., 2009). Given their extensive use of host resources, now directed to parasite reproduction, it is not surprising that such conditions could lead to intense competition should a different trematode clone occupy the same snail host.

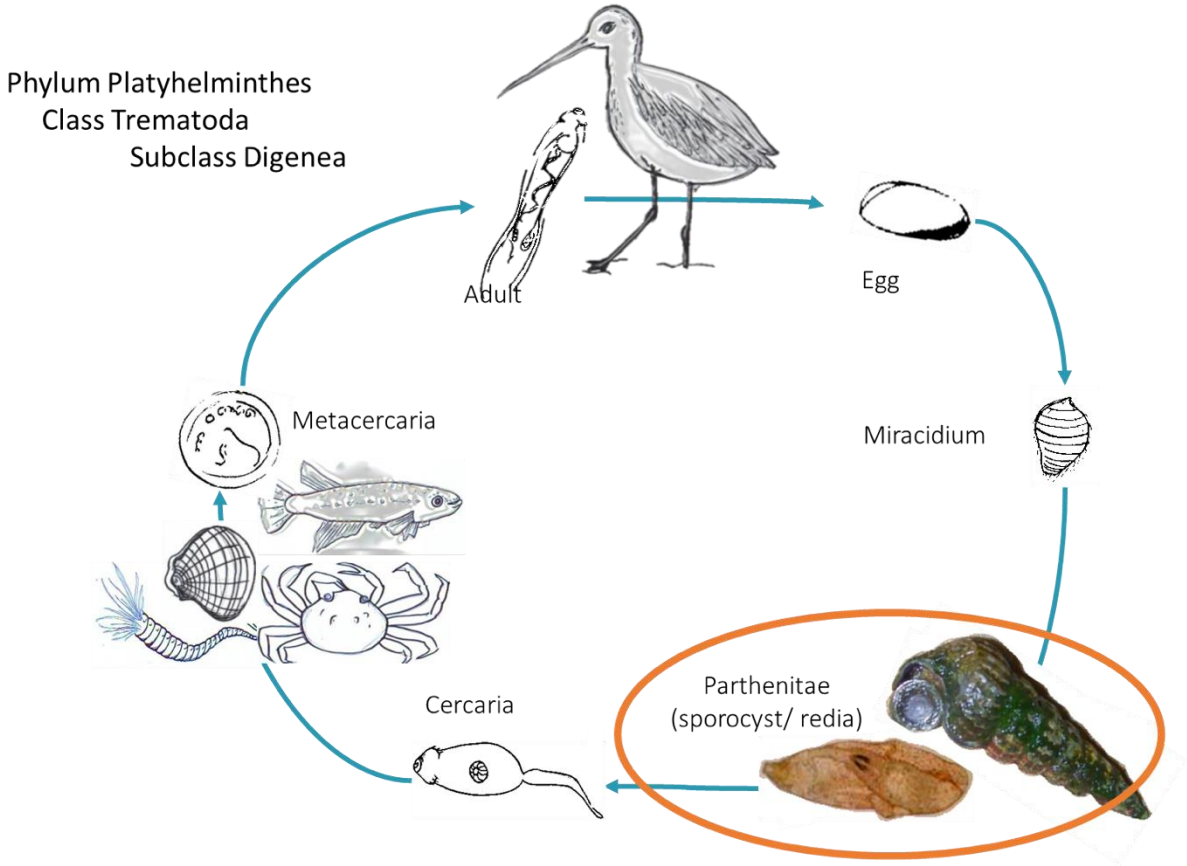


Figure 1. Generalized trematode life cycle.

It has long been known that larval trematodes kill unrelated trematodes when a snail is simultaneously infected with two species. These antagonistic interactions are

hierarchical. Dominant species fend off invasions or displace established colonies of subordinate species. Until recently, this antagonism had been considered to occur via the actions of “totipotent” rediae, those that both reproduce and defend the colony (Lim and Heyneman, 1972; Lie, 1973; Kuris, 1990; Sousa, 1993; but see Lie, 1969). It is now known that some species have a non-reproducing soldier caste (Hechinger et al., 2011), where soldiers are specialized to defend the colony from enemy trematode invaders. In agreement with Hechinger et al (2011), my own investigations have shown that soldiers do not reproduce and are smaller than reproductives but have relatively large mouthparts (Fig. 2B). Only soldiers readily attack enemies with their mouths (Fig. 2C). In addition, soldiers are more active and disproportionately common in areas of the host where invasions occur.

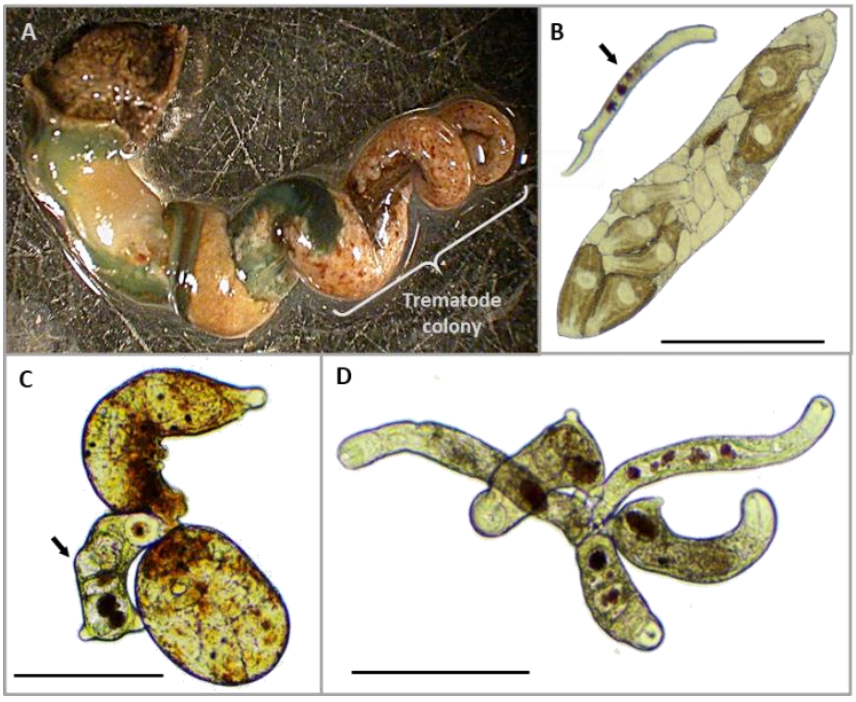
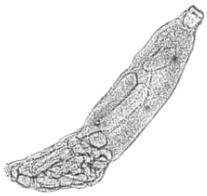


Figure 2. *Cloacitrema michiganensis* (CLOA) social organization. (**A**) De-shelled host California horn snail revealing appearance of an intact colony. (**B**) Examples of CLOA soldier (left) and reproductive (right) (**C**) CLOA soldier attacking a heterospecific (*Euhaplorchis californiensis*). Soldier indicated by black arrow. (**D**) CLOA soldiers adhering to each other at their posterior ends. Scale bar=300 µm.

The initial discovery (Hechinger et al. 2011) was followed by reports revealing the existence of soldiers in five additional trematode species (Leung & Poulin, 2011; Miura, 2012; Nielsen et al., 2014) and by research examining the adaptive significance of soldiers (e.g., Lloyd & Poulin 2012, Kamiya & Poulin 2013). However, descriptions of colony structure were restricted to a single trematode superfamily (Echinostomatoidea), and knowledge of colony demography and caste function was limited to snapshots of the condition of mature colonies.

Table 1. Taxonomy of trematodes that infect the California horn snail

Order	Family	Species (Abbreviation)	
Strigeida	Schistosomatidae	*Austrobilharzia* sp.(AUST)	
	Cyathocotylidae	*Mesostephanus appendiculatus* (MESO)	
		Small cyathocotylid (SMCY)	
Plagiorchiida	Microphallidae	*Probolocoryphe uca* (PROB)	
		Small microphallid (SMMI)	
	Renicolidae	*Renicola* sp. "*polychaetophila*" (REPO)	
		Renicola sp. "*martin*i?" (REMA)	Sporocyst
		Renicola buchanani (RENB)	
		Renicola cerithidicola (RENC)	
	Heterophyidae	*Pygidiopsoides spindalis* (PYGI)	
		Phocitremoides ovale (PHOC)	
		Stictodora hancocki (STIC)	Redia
		Euhaplorchis californiensis (EUHA)	
Echinostomida	Philophthalmidae	*Parorchis acanthus* (PARO)	
		Cloacitrema michiganensis (CLOA)	
	Echinostomatidae	*Himasthla* sp. B (HIMB)	
		Himasthla rhigedana (HIMA)	
		Acanthoparyphium spinulosum (ACAN)	
	Notocotylidae	*Catatropsis johnstoni* (CATA)	

Here I address these gaps by studying sixteen trematode species that form colonies in the California horn snail, *Cerithideopsis californica* (= *Cerithidea californica*). The California horn snail is host for a diverse trematode guild (Table 1) that is characterized by a fairly well-resolved interspecific dominance hierarchy (Kuris, 1990; Sousa, 1993; Hechinger, 2010). This marine snail that can be found in salt marshes and tidal mudflats from northern California, USA, to Baja California, Mexico

A summary of the major findings of this research are as follows:

Chapter 2: This chapter examines colony structure for four trematode species that infect the California horn snail; two species belong to the Family Echinostomatidae (*Himasthla rhigedana* and *Acanthoparyphium spinulosum*) and two others to the Philophthalmidae (*Parorchis acanthus* and *Cloacitrema michiganensis*). In addition, we examine one echinostomatid (*Echinostoma liei*) isolated from the freshwater snail, *Biomphalaria alexandrina*, and maintained in *Biomphalaria glabrata*. We present redia morphology (pharynx and body size), and the distribution of individuals of different castes throughout the snail body. When morphological, developmental, and frequency-distribution evidence indicated the presence of a permanent soldier caste, we assessed behavior by measuring attack rates of the different morphs toward heterospecific trematodes. Our findings permit us to expand the list of trematodes known to have a non-reproductive soldier caste to include the four species examined from the California horn snail, and to document

colony structure for a species (*E. liei*) that explicitly lacks a permanent soldier caste. The contrasting colony structure for species with and without a soldier caste emphasizes the diverse nature of trematode sociality and the promise of the group to permit comparative investigations of the evolution and ecology of sociality.

Chapter 3: This study examines colony structure for four trematode species that belong to the Family Heterophyidae (*Euhaplorchis californiensis, Phocitremoides ovale, Pygidiopsoides spindalis* and *Stictodora hancocki*). The heterophyids we study here have an intermediate position in the dominance hierarchy (they are subordinate to the echinostomatids and philophthalmids described in Chapter 2, but can eliminate or prevent infections of other, more subordinate, species in the guild). We also compare colony structure of a few recent, developing heterophyid colonies to fully developed colonies to shed light on the nature of colony development. We discuss the implications of our results, including alternative interpretations concerning the nature of trematode sociality. Our analysis of morphology, distribution, behavior, and colony development of these four heterophyid species indicates that they also have a soldier caste, and that trematode caste structure takes time to develop, becoming more pronounced with colony age.

Chapter 4: We examine seven species that, having sporocyst parthenitae, cannot have soldiers. We also document patterns for a redia species that lacks a soldier caste. We present data on morphology, reproductive status, and distribution of the parthenitae. By describing such patterns for eight species belonging to five

additional digenean families, we expand the taxonomic range for which colony structure has been examined in the Trematoda. Our findings indicate that trematode colony structure and allometric growth patterns when a soldier caste is absent are in stark contrast to those of species with soldiers: (1) colonies are characterized by unimodal size-frequency distributions with few small and large individuals. (2) Even the smallest colony members contain developing embryos or late-stage cercariae. (3) Individuals of all sizes have similar morphologies and lack structures that are specialized for antagonism. (4) Distribution of parthenitae is restricted to the main infection locus. Thus, we identify easily measurable characteristics that can be used as "markers" to determine whether a soldier caste is present in trematode colonies.

Chapter 5: The development of suitable culture systems for trematodes infecting marine snails would allow us to ask questions concerning trematode sociality such as the fundamentals of interspecific interactions and the mechanics underlying caste dynamics and regulation in mature colonies. However, because most trematodes of medical and veterinary importance infect pulmonate snails, efforts to develop *in vitro* systems have focused on freshwater systems rather than marine ones. Here we report primary *in vitro* culture of rediae of *Euhaplorchis californiensis* (Heterophyidae), *Himasthla rhigedana* and *Himasthla* sp. B (Echinostomatidae) infecting the California horn snail. Our results indicate that these trematodes can be cultured *in vitro* in the commercially available Leibovitz L-15 media that had been previously used for other marine trematodes. However, rediae survival and performance can be improved by using the *Biomphalaria glabrata* embryonic (Bge) cell line, either by co-culturing with

the Bge cells or in media that contains Bge released factors. Rediae do not appear to consume Bge cells but are able to eat rediae and cercariae of heterospecific species. Even though cultured rediae (both reproductive and soldier castes) were mobile and healthy looking, we rarely observed progeny rediae or cercariae being released. This is the first reported cultivation of marine trematodes that includes co-culture with Bge cells and media with Bge factors. Our results highlight the promise of using these methods for cultivation of marine trematodes.

Trematode colonies are readily replicated, can be maintained in large numbers, and are amenable to *in vitro* studies. Hence, they provide many advantages as model systems to introduce students to science and to pursue experimental and comparative research probing general principles underlying the ecology and evolution of sociality. Furthermore, there are over 20,000 species of trematodes worldwide; they cover a wide range of environmental and life history diversity and are both ecologically and medically important. Thus, understanding the mechanisms that shape trematode communities can have substantial public health, veterinary and wildlife disease applications.

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Chapter 2

Social organization in parasitic flatworms- four additional echinostomoid trematodes have a soldier caste and one does not

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Social organization in parasitic flatworms- four additional echinostomoid trematodes have a soldier caste and one does not

Abstract

Complex societies where individuals exhibit division of labor with physical polymorphism, behavioral specialization, and caste formation, have evolved several times throughout the animal kingdom. Recently, such complex sociality has been recognized in digenean trematodes; evidence is limited to 6 marine species. Hence, the extent to which a soldier caste is present throughout the Trematoda is sparsely documented, and there are no studies detailing the structure of a species lacking such a social structure. Here we examine colony structure for an additional 5 echinostomoid species, 4 of which infect the marine snail *Cerithidea californica* and 1 (*Echinostoma liei*) that infects the freshwater snail *Biomphalaria glabrata*. For all species, we present redia morphology (pharynx and body size), and the distribution of individuals of different castes throughout the snail body. When morphological evidence indicated the presence of a soldier caste, we assessed behavior by measuring attack rates of the different morphs toward heterospecific trematodes. Our findings indicate that each of the 4 species from *C. californica* have a permanent soldier caste, while *E. liei* does not. The observed intra- and inter-specific variation of caste structure for those species with soldiers, and the documentation of colony structure for a species explicitly lacking permanent soldiers, emphasizes the diverse nature of trematode sociality and the promise of the group to permit comparative investigations of the evolution and ecology of sociality.

Introduction

In the most complex animal societies, individuals live in colonies characterized by a high degree of cooperative organization and the formation of morphologically and behaviorally distinct reproductive and non-reproductive castes. Non-reproducing individuals specialize on tasks that include gathering food, building nests, or defending the colony from invaders. Such organized and cooperative societies characterize several types of insects (e.g., Wilson, 1971; Aoki, 1977; Crespi, 1992; Kent and Simpson, 1992; review in Myles, 1999), snapping shrimp (Duffy, 1996), and naked mole-rats, (Jarvis et al., 1981). Recent findings (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014) have expanded the existence of complex sociality to a new phylum, the Platyhelminthes (Class Trematoda), by documenting the existence of a reproductive division of labor in rediae involving a non-reproductive soldier caste occurring alongside a reproductive caste. Here, we present experimental and observational evidence for 4 additional echinostomoid species that exhibit such caste formation, and we document the colony structure for an echinostomoid lacking a permanent soldier caste.

Although not traditionally recognized as such, trematode infections in molluscan first intermediate hosts comprise colonies (Hechinger et al., 2011). A single founder larva (a miracidium) infects a mollusk host (usually a snail). After infection, the miracidium metamorphoses, initiates clonal reproduction, and produces large numbers of parthenitae (sporocysts or rediae). Initially, parthenitae produce more parthenitae, but ultimately switch to long-term production of

dispersive offspring (cercariae), which leave the colony to infect the next host in the life cycle. Some trematode species have parthenitae that possess a muscular pharynx and a gut; these parthenitae are called rediae. Other species have parthenitae called sporocysts, which lack those structures. The mass of parthenitae commonly occupies the gonad and/or digestive gland of the snail, and, in mature infections, can constitute a large percentage (up to 50%) of the tissue mass of an infected host (Bernot and Lamberti, 2008; Hechinger et al., 2009). The parthenitae collectively block host reproduction and divert host physiology, generally for the life span of the host, often years (Sousa, 1983; Sorensen and Minchella, 2001), to serve the reproductive needs of the trematode clones (Lafferty and Kuris, 2009; Hechinger, 2010). Thus, the parthenitae work together as a colony, and the host body is an important, limited resource for the trematode colony (Kuris and Lafferty, 1994; Hechinger et al., 2011).

Consistent with the host body representing a limited and critical resource, when 2 trematode species simultaneously infect a snail host, one generally kills the other (Lim and Heyneman, 1972; Lie, 1973; Kuris, 1990; Sousa, 1993; Kuris and Lafferty 1994). This may occur frequently when overall prevalence of trematodes is high (Kuris and Lafferty, 1994). Thus, there can be strong selective pressure for established trematode colonies to protect and defend against new infections. Trematode antagonism has typically been considered, and sometimes clearly documented, as operating via “totipotent” rediae, which not only reproduce, but attack enemies (Lim and Heyneman, 1972; Lie, 1973; Kuris, 1990; Sousa, 1993). However, for some species, certain individual rediae are specialized for

antagonism. Sapp et al. (1998) documented a specialized reproductive morph of *Echinostoma paraensei* that matured early and remained at a prime site to attack invading heterospecific species. Further, Hechinger et al. (2011) reported the discovery of a caste of non-reproductive soldier rediae. In *Himasthla* sp. B, reproductive rediae produce additional parthenitae and dispersive cercariae, while soldiers lack actively developing embryos, and are specialized for inter-trematode antagonism. These non-reproductive soldiers are small, have relatively large pharynxes, and are more common at peripheral sites in the snail where new invasions are likely to be encountered (Hechinger et al., 2011). Soldiers are also more active, and readily attack enemies with their mouth.

The initial documentation of the soldier caste stimulated a search for soldiers in other trematode species. Five additional species have now been reported to exhibit a soldier caste (Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014). All 6 species with soldiers belong to 2 families, the Echinostomatidae and Philophthalmidae, both of which lie within the super-family Echinostomoidea (Olson et al., 2003). This clade includes the most competitively dominant species in most trematode assemblages (Lim and Heyneman, 1972; Lie, 1973; Kuris, 1990). However, the extent to which a soldier caste is present in this clade is still sparsely documented, and there are no studies detailing the structure of trematode colonies that lack a soldier caste. This limits our ability to make empirical generalizations and to carry out comparative tests concerning the nature of sociality.

Here we examine colony structure for an additional 5 echinostomoid species: 2 echinostomatids and 2 philophthalmids that infect the marine California horn snail

(*Cerithidea californica*) and 1 echinostomatid (*Echinostoma liei*) isolated from the freshwater snail, *Biomphalaria alexandrina,* and maintained in *Biomphalaria glabrata*. We present redia morphology (pharynx and body size), and the distribution of individuals of different castes throughout the snail body. When morphological, developmental, and frequency-distribution evidence indicated the presence of a permanent soldier caste, we assessed behavior by measuring attack rates of the different morphs toward heterospecific trematodes. Our findings permit us (1) to expand the list of trematodes known to have a non-reproductive soldier caste to include the four species examined from the California horn snail, and (2) to document colony structure for a species (*E. liei*) that explicitly lacks a permanent soldier caste. The contrasting colony structure for echinostomoids with and without a soldier caste emphasizes the diverse nature of trematode sociality and the promise of the group to permit comparative investigations of the evolution and ecology of sociality.

Materials and Methods

Study systems and sample collection

Species from the California horn snail, *Cerithidea californica* (= *Cerithideopsis californica*) (Potamididae): We collected California horn snails at Carpinteria Salt Marsh, Santa Barbara County, California between July 2013 and May 2014. The snails were maintained in the laboratory in running seawater for up to 6 wk before processing. We identified trematode species following Martin (1972)

and additional unpublished observations. We studied 3 echinostomoids listed by Hechinger et al. (2011) as likely having soldiers: *Himasthla rhigedana* Dietz, 1909 (Echinostomatidae), *Parorchis acanthus* Nicoll, 1907 *(*Philophthalmidae), and *Cloacitrema michiganensis* McIntosh, 1938 (Philophthalmidae), and added a fourth, *Acanthoparyphium spinulosum* Johnston, 1917 (Echinostomatidae). For simplicity and clarity, we will hereafter refer to each species with abbreviations formed by the first four letters of the genus (HIMA, PARO, CLOA and ACAN, respectively).

Echinostoma liei in *Biomphalaria glabrata* (Planorbidae): *Echinostoma liei* Jeyarasasingam et al. 1972 (= *E. caproni* in part) (Echinostomatidae) was isolated in Ethiopia c1970 and has been maintained in NIH albino *B*. *glabrata* in our laboratory for approximately 40 yr. General maintenance procedures follow Kuris (1980). *Biomphalaria glabrata* serves as both the first and second intermediate host to *E. liei*. Metacercariae obtained from snail pericardia were administered to Swiss Webster mice (25-30 metacercariae per mouse) via oral gavage. Four months later, we euthanized the mice, extracted adult trematodes from the intestines, and harvested trematode eggs. Following incubation at 26 C for 12 days, miracidia were hatched. Naive snails (5-15 mm diameter range) were exposed to 3-5 miracidia on October 2013 and maintained in aerated tanks at room temperature (22-26 C).

Redia morphology and distribution

To dissect California horn snails, we carefully cracked the shell with a hammer and removed the shell. Following Hechinger et al. (2011), we divided the body into 3 regions: mantle, basal visceral mass (middle), and the gonad/digestive gland. Five snails harboring colonies of each of the 4 horn snail trematode species

were used to quantify redia morphology and distribution. All colonies examined were mature and producing cercariae.

Six snails harboring *E. liei* colonies were examined for redia morphology and distribution. Three of these were examined 6 wk after exposure, and 3 more 12 wk post-exposure (colonies mature 2-4 wk after exposure). To dissect *B. glabrata*, we gently cracked the shells with a glass vial and carefully removed the shell fragments. Because of smaller size and less discrete tissue boundaries, we divided these snails into 2 instead of 3 regions: the snails were bisected just posterior to the heart, into anterior (head, foot and mantle) and posterior (visceral mass and ovotestis) regions for all colonies but one (which was analyzed whole). For 2 of the earlier infections, we collected all present individuals for quantitative analysis, as colony size was small enough for comprehensive redia collection. For the remaining 4 infected *B. glabrata,* we followed the procedures described below.

Using a pipette, a grid, and random numbers, we sampled approximately 30 rediae from each body region, irrespective of redia type. If there were less than 30 rediae in the body region, we sampled all rediae present. Accurate counting of rediae was difficult because cercariae were sometimes densely intermingled with the rediae, so we frequently retrieved and measured more than 30 rediae. Consequently, the number of rediae measured per region ranged from 1-118. Sampled rediae were killed by immersion in hot deionized water, fixed in 70% EtOH, and mounted in glycerin; preliminary trials confirmed this process was suitable for our morphological analyses. Digital pictures of each redia were taken using a compound microscope with a Lumenera Infinity 3 camera at 4x, 10x or 20x

magnification to permit morphological measurements. We measured body and pharynx length and width to the nearest micron using the image analysis software FIJI (Fiji Is Just ImageJ). We calculated total body and pharynx volumes by approximation to a cylinder.

For each body region, we calculated the proportion of each redia morph type observed in the randomly sampled rediae. We assigned each redia to a morph category based on the presence or absence of embryonic or developing offspring (cercariae or rediae). Soldiers lacked free germ balls or later-stage embryos. Reproductives contained at least some late-stage embryos of cercariae or parthenitae. Occasionally, we observed individuals that only had early stage embryos. These individuals were scored as immature reproductives. For statistical analyses we have included these immature rediae in the reproductive category, unless otherwise stated.

Attack Trials

When morphology, reproduction, and size-frequency distributions indicated the existence of a permanent soldier caste, we ran experimental trials to evaluate attack rates to compare behavioral specialization of soldier and reproductive castes. This involved all species from the California horn snail. Rediae from each species were presented with rediae of the heterospecific *Euhaplorchis californiensis* (Heterophyidae). We selected *E. californiensis* (EUHA) as the heterospecific enemy in these trials, as did Hechinger et al. (2011), because it is one of the most common trematodes at Carpinteria Salt Marsh. Hence, in addition to being readily available, it is likely regularly encountered by other species.

Experiments took place in May 2014. We used 96-well plates, with concave bottoms to increase encounter rates. Rediae from 3 colonies of each tested species were presented with reproductive rediae from 2 EUHA colonies (also referred to as “heterospecific rediae”). For each replicate, we placed 10 heterospecific rediae into a single well with sea water, followed by approximately 10 soldiers or 10 reproductives of the focal species. For each combination, we used 5 replicate wells. The wells were held for 90-120 min at ambient room temperature (21-23 C). We then observed each individual well with a stereomicroscope for 20 sec. An attack was recorded whenever a redia was attached to another with its mouth, following Hechinger et al. (2011), or when a redia had already ingested an entire heterospecific redia (this consistently occurred for HIMA soldiers). These were easily distinguishable because the rediae of EUHA are yellow and their developing cercariae have eyespots.

Statistical analysis

All statistical analyses were performed using R 3.1.3 (ran with RStudio 0.98.1103), or JMP Pro 11.0.0. For morphological analyses, we used a mixed-effect general linear model (GLM) on natural log-transformed data and setting colony ID as a random effect to model the relationship between soldier and reproductive total volume, pharynx volume, and pharynx/volume ratios. Interaction terms between colony and redia type were included in the model. We examined residual plots and normal quantile plots to ensure meeting assumptions regarding data normality and homoscedasticity. Cochran-Mantel-Haenszel chi-square tests were used to examine the relationship between rediae type and snail region, while controlling for

colony ID. For the attack rates, we used a binomial regression with a logit link function and tested for the effects of colony ID, redia type and heterospecific colony ID. Colony ID and heterospecific ID were incorporated as fixed effects because we were interested in distinguishing effects of specific colonies.

Results

We processed a total of 2,616 rediae from the 5 trematode species examined. We examined 5 colonies for each of the trematode species infecting the California horn snail, and 6 colonies for the species (*Echinostoma liei)* infecting *B. glabrata*. General patterns were consistent among colonies, and our analyses included data from all colonies. However, for illustration purposes, we provide figures in the main text for 1 representative colony of each species. Additional figures and detailed data for all colonies can be found in the Supplementary material (Figures S1-S5, Table S1).

Morphology

Examination of rediae of the 4 trematode species from the California horn snail revealed distinct soldier and reproductive castes. Reproductive rediae were consistently longer and wider, with no overlap in body volume (Figs. 1, 2A). The size frequency distributions were clearly bimodal (Fig. 1B), and the volume of a reproductive redia was 141 times that of a soldier for CLOA (t_{632}=85.0, p<0.0001), 216x for PARO (t_{480}=26.1, p<0.0001), 21x for HIMA (t_{441}=20.5, p<0.0001), and 39x for ACAN (t_{573}=14.6, p<0.0001) (on average, pooling all rediae per species; results

for each individual colony can be found in Table S1). In absolute dimensions, pharynx size of a reproductive redia was 3.2 times that of a soldier for CLOA (t_{620}=9.7, p<0.0001), 1.9x for PARO (t_{470}=2.2, p=0.0285), 1.8x for HIMA (t_{438}=6.4, p<0.0001), and 1.5x for ACAN (t_{567}=4.3, p<0.0001) (Figure 2b). However, relative to their body size, soldiers had substantially larger pharynxes than did reproductive rediae; it was 40x larger for CLOA (t_{620}=9.6, p<0.0001), 114x for PARO (t_{470}=20.9, p<0.0001), 12x for HIMA (t_{438}=19.2, p<0.0001), and 26x for ACAN (t_{567}=16.8, p<0.0001) (Fig. 2C).

In addition to the difference in size, soldiers had a distinctive body shape (Fig. 1, Supplemental video). They possessed pronounced collars and had larger appendages compared to reproductive rediae. For ACAN, there is also strong dimorphism in color, with reproductive rediae being orange and soldiers being cream-colored. In 1 ACAN colony, however, we observed 1 individual that was shaped like a soldier, but it contained a daughter redia. Following our operational categorization, this individual was recorded as a reproductive redia (see ACAN scatterplot and histogram in Fig. 1).

From the 6 *E. liei* colonies, no evidence of a soldier morph was detected. The size frequency distribution of the colonies had a single mode, and small and large rediae had similar morphology. Additionally, all of the rediae examined had germ balls or developing embryos (Fig. 1C). Individuals from the 12-wk-old colonies were 2.6 times larger than those from 6-wk-old colonies (t_{468}=12.45, p<0.0001; Fig. S5, Table S1).

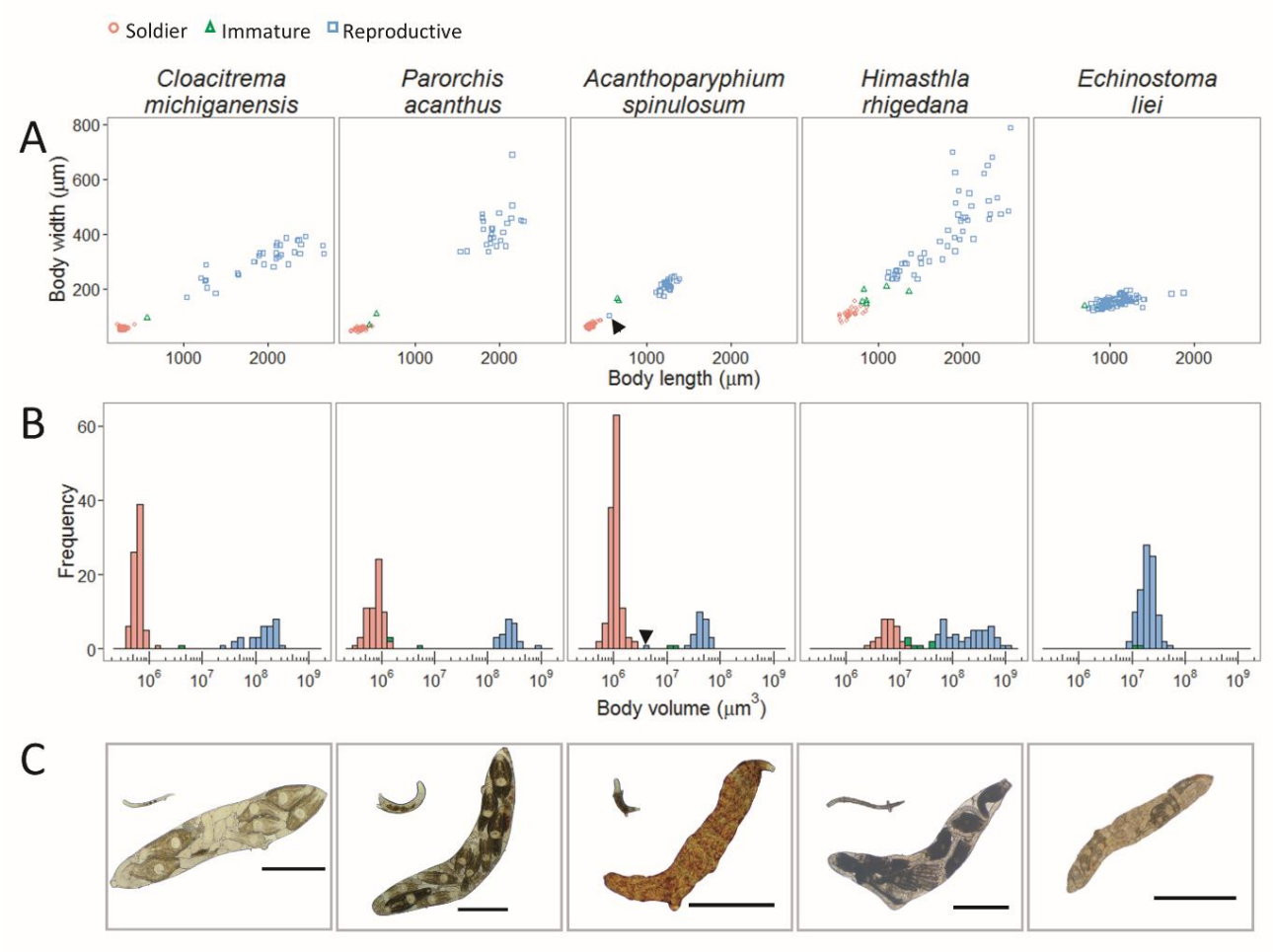

Figure 1. Body size and shape of rediae for the 5 examined species of echinostomoid trematodes. (**A**) Body width to body length relationships. Each point represents a randomly sampled redia from a single trematode colony. (**B**) Frequency distributions of body volume for randomly sampled rediae. X-axis is $\log_{10}$ scale. A and B depict data from a single representative colony of each species (see Supplemental material for all colonies). The arrows on the third panel of B indicate the individual that was shaped as a soldier but contained a daughter redia. (**C**) Photographs of representative redia morphs for each species. Scale bar= 500µm.

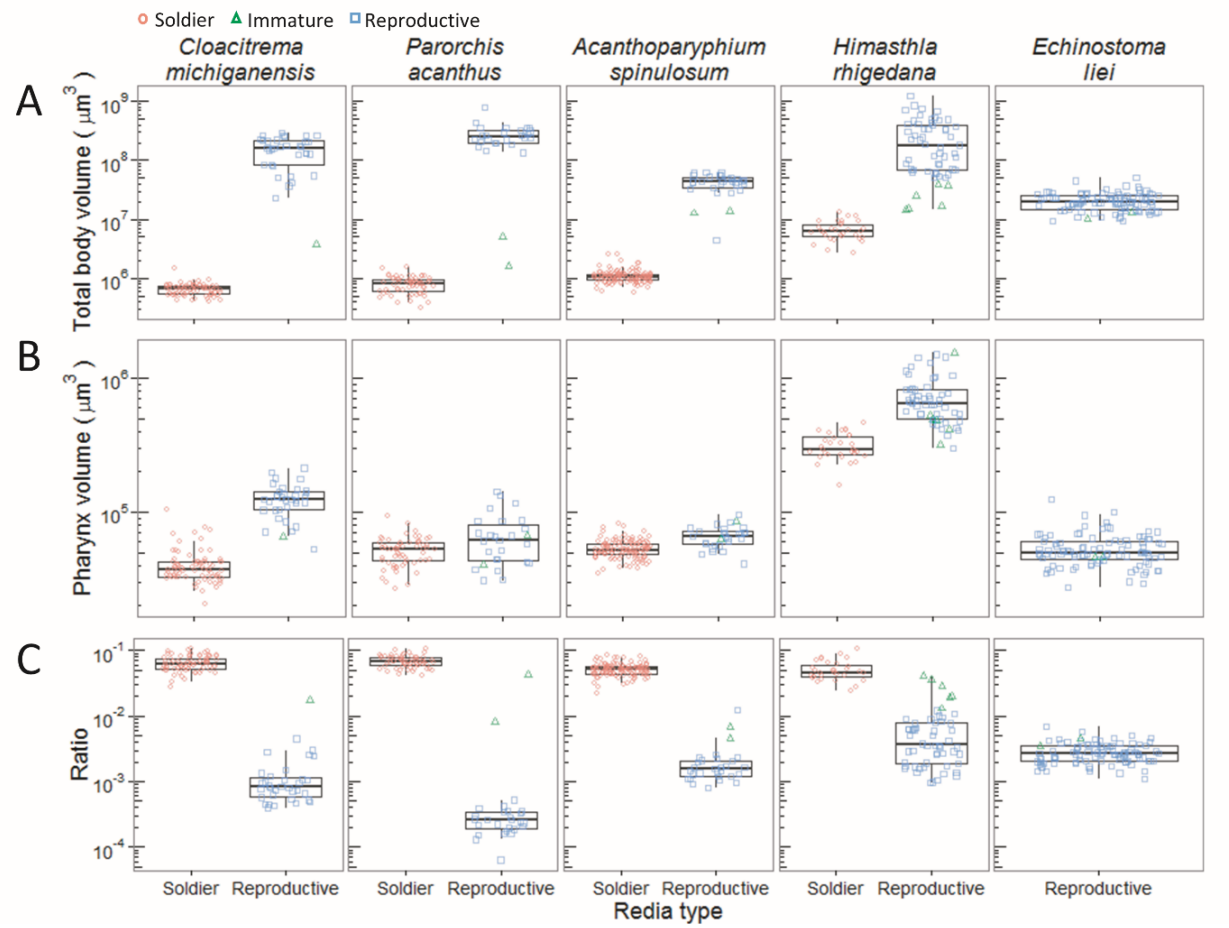

Figure 2. Body and pharynx volumes for rediae of the 5 species examined. For each species, points represent randomly sampled, individual rediae from a single representative colony (see Supplemental material for all colonies). Boxplots indicate median (line), interquartile range (box) and range of data (whiskers) for the following metrics: (**A**) total body volume of individual rediae, (**B**) pharynx size, and (**C**) pharynx volume relative to body volume. Log_{10} scale for Y-axes.

Table I. Percentage of rediae that are soldiers, by host body region, for 4 trematode species infecting the California horn snail.

	Average (and range) percentage of soldiers among colonies			
	CLOA	PARO	HIMA	ACAN
Mantle	85% (45 - 100%)	96% (78 - 100%)	85% (75 - 100%)	100%
Basal Visceral Mass	63% (28 - 80%)	54% (16 - 96%)	57% (44 - 90%)	95% (92 - 98%)
Gonad	23% (11 - 42%)	51% (25 - 74%)	17% (4 - 41%)	55% (38 - 75%)

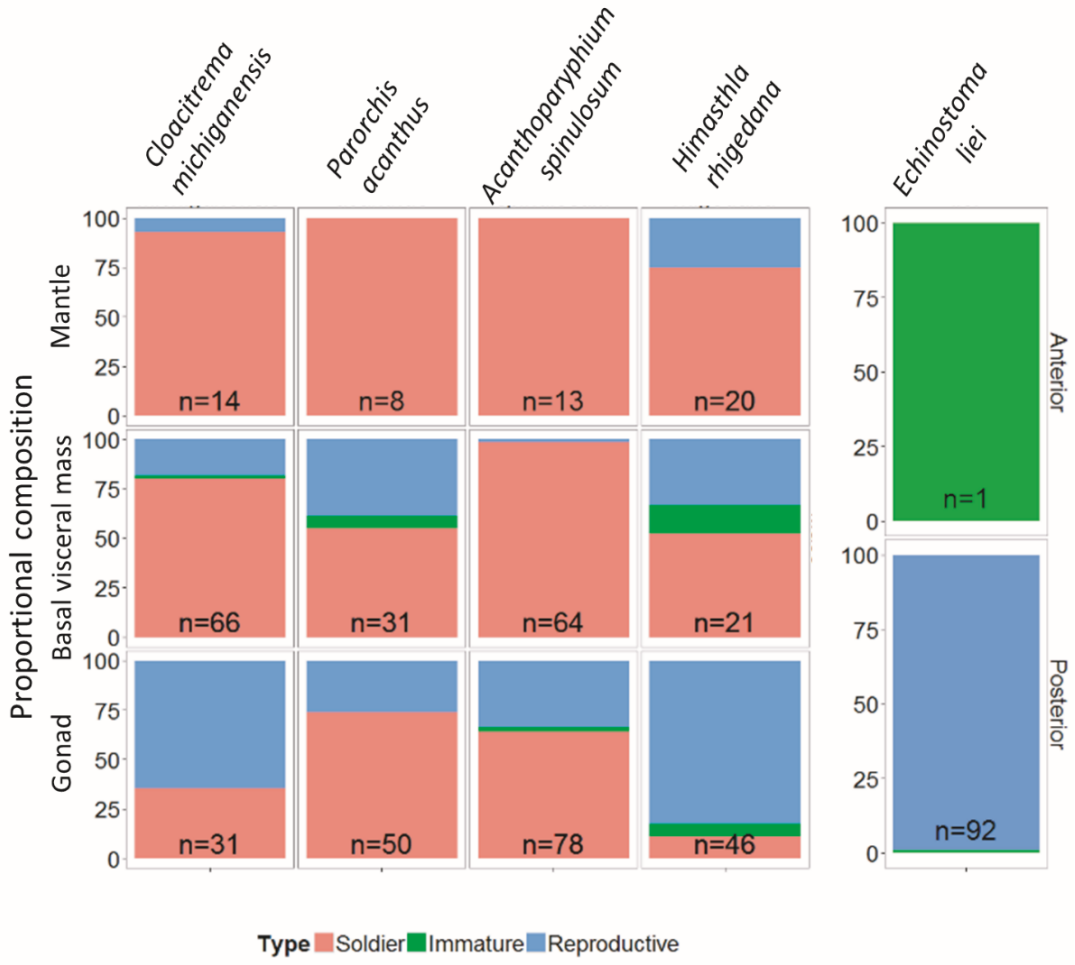

Figure 3. Proportion of reproductive, immature and solider rediae found in each region of the snail. The California horn snail was divided into 3 regions: mantle, basal visceral mass and gonad (and digestive gland); *Biomphalaria glabrata* was divided into 2: anterior and posterior. Numbers in boxes indicate total number of individuals sampled from that given region.

Caste ratios and distribution

For the 4 species examined from the California horn snail, most rediae were found in the gonadal area, but some were also found in the basal visceral mass and the mantle regions. The relative numbers of soldiers and reproductive rediae (caste ratios) varied between regions for each species (Table I, Figs. 3, S1-S4; CLOA: $\chi^2_{1,636}=62.68$, $p<0.0001$; PARO: $\chi^2_{1,485}=1.87$, $p=0.1710$; HIMA: $\chi^2_{1,447}=86.27$,

$p<0.0001$; and ACAN: $\chi^2_{1,578} = 100.27$, $p<0.0001$). However, in all cases, soldiers were relatively much more common in the snail anterior (basal visceral mass and mantle) compared to the gonad region.

For *E. liei*, very few individuals were retrieved from the anterior part of the snail; of the ones retrieved, all were reproductive or immature rediae (Figs. 3, S5).

Immature reproductives: Morphology and frequencies

We occasionally observed some individuals that only had early stage embryos. These individuals were intermediate in size, ranging from the size of soldiers to that of the smallest reproductive rediae with developing embryos (Figs. 1, S1-S5). They were shaped similarly to reproductive rediae, with reduced collars and appendages. These individuals were scored as immature reproductives.

Immature rediae were uncommon in colonies of all species (Figs. 1, S1-S5). Of the total rediae from the 4 species infecting the California horn snail, 77 out of 2,146 rediae were immature. Among colonies within species, the average (and range) proportion of immatures among all rediae was 3.6% (0.9 - 10.2%) for CLOA, 3.8% (0 - 9.3%) for PARO, 6.7% (1.7 - 12.8%) for HIMA, and 0.94% (0 - 2.1%) for ACAN. The average (and range) proportion of immatures considering only reproductive rediae was 9.9% (3 - 30.2%) for CLOA, 10.4% (0 - 30%) for PARO, 16.2% (4.3 - 42.4%) for HIMA, and 3.5% (0 - 7.4%) for ACAN. For *E.* liei, we obtained a total of 470 reproductive rediae, 57 of which were immature. On average 17% of the rediae per colony were immature, However, this percentage was higher in the 6-wk-old colonies (range 21 - 23%), than in the 12-wk-old colonies (2-17%) (Fig. S5, Table S1).

In general, colonies of *E. liei* had a higher percentage of immature rediae (17%) compared to the species infecting the California horn snail (ranging from 1% in ACAN to 7% in HIMA) (logistic regression controlling for trematode species; $\chi^2_{4,26} = 17.77$, $p = 0.0014$). However, when excluding soldiers from the calculation, the proportions of reproductive rediae that were immature were more comparable ($\chi^2_{4,26} = 3.52$, $p = 0.4750$), with HIMA having effectively the same proportion of reproductive rediae that were immature (16%) as did *E. liei*.

Activity and attack rates

In each of the 4 species from the California horn snail, soldiers were qualitatively more active than reproductive rediae, consistent with the previous quantitative data for *Himasthla* sp. B (Hechinger et al., 2011). Reproductive rediae rarely moved when exposed to heterospecific rediae. In contrast, soldiers increased activity in the presence of heterospecific rediae, stretching and contracting their bodies and probing heterospecific rediae with their mouths. In some cases, soldiers attached their mouths to heterospecific rediae (Fig. 4A). We note that soldiers of all species often formed clusters of 2 to 7 individuals, adhering at the posterior ends of their bodies (Fig. 4B). Clustered soldiers were frequently observed attacking heterospecific rediae. This behavior was never observed in reproductive rediae of any of the species examined.

In quantitative attack experiments (Fig. 5), reproductive rediae rarely attacked heterospecific rediae for any of the 4 species from the California horn snail. No attacks were observed for reproductive rediae of CLOA and PARO. For ACAN, 6/323 (1.9%) reproductive rediae attacked, while in HIMA 1/274 (0.4%)

attacked a heterospecific redia. Soldiers, on the other hand, were much more aggressive. For CLOA, 85/280 (31%) soldiers attacked heterospecific rediae, for PARO, 32/278 (12%), and for ACAN, 94/270 (35%). Soldiers of HIMA attacked at a particularly high rate; 223/274 (81%) soldiers attacked heterospecific rediae, and frequently entirely ingested them during the 90-min trial (Fig. 4C). These substantial differences in attack rates between soldier and reproductive rediae were statistically significant for each species (logistic regression for CLOA: $\chi^2_{1,60}=145.4$, $p<0.0001$; PARO: $\chi^2_{1,60}=48.8$, $p<0.0001$; ACAN: $\chi^2_{1,60}=134.7$, $p<0.0001$; and HIMA: $\chi^2_{1,60}=485.6$, $p<0.0001$). Colony of origin of both the focal species and the heterospecific rediae were also significant predictors for attack rates (Fig. 5, Table S2).

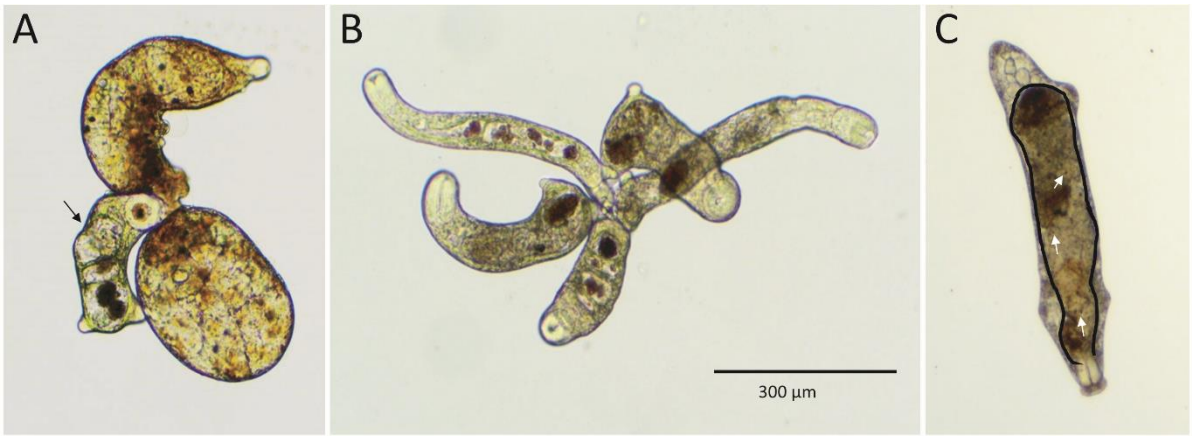

Figure 4. Examples of trematode soldier attack and activity: (**A**) *Cloacitrema michiganensis* (CLOA) soldier attacking a heterospecific redia (*Euhaplorchis californiensis*, EUHA). Soldier indicated by black arrow. (**B**) CLOA soldiers adhering to each other at their posterior ends. (**C**) *Himasthla rhigedana* (HIMA) soldier with a consumed heterospecific (EUHA) redia filling its gut (outlined in black, white arrows indicate eyespots of EUHA cercariae that were developing inside the ingested EUHA redia).

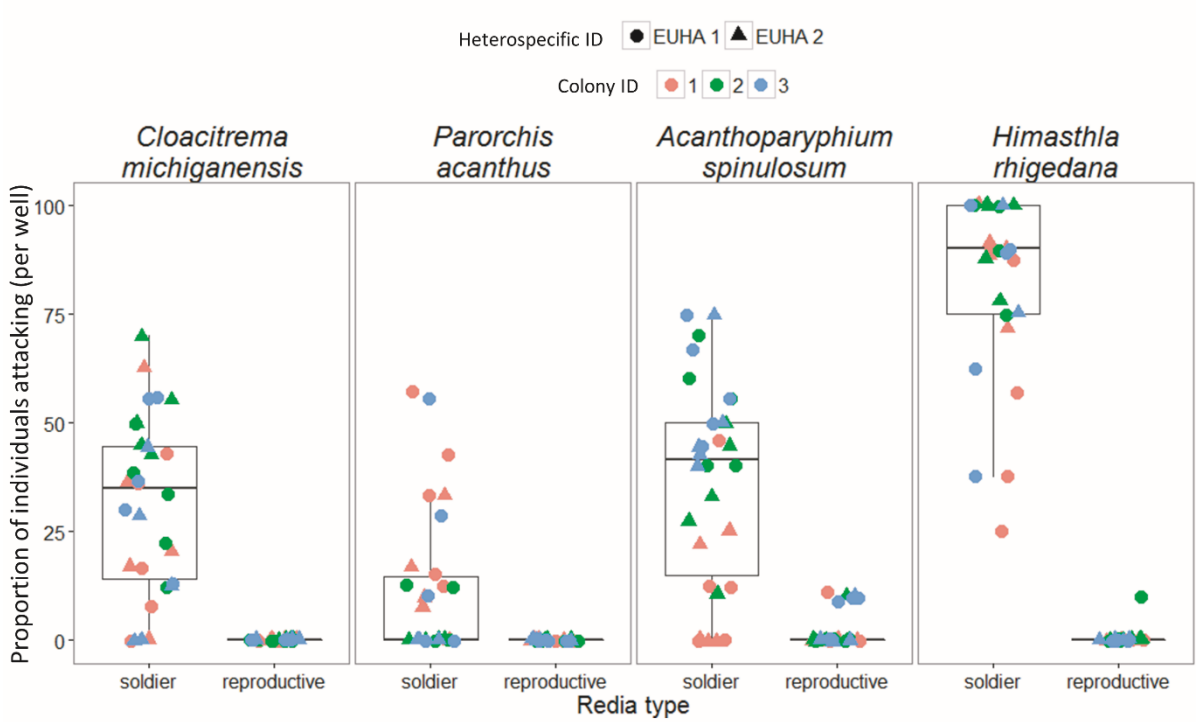

Figure 5. Attack rates of soldiers and reproductives, of the 4 species from the California horn snail on rediae of the heterospecific *Euhaplorchis californiensis* (EUHA). Three colonies of each focal species were used (represented by the different colors), and each was exposed to rediae from 3 colonies of the heterospecific EUHA (represented by different shapes). Points indicate proportion of individuals observed attacking for each replicate well (5 replicates for each of 48 combinations), boxplots indicate median (line), interquartile range (box) and range of data (whiskers). The arrows on the right indicate the average attack rates previously reported for soldiers of other species: *Himasthla* sp. B (figure 3a in Hechinger et al., 2010), *Himasthla elongata* (figure 5 in Nielsen et al., 2014), Philophthalmid sp. I, Philophthalmid sp. II, and *Acanthoparyphium* sp. I (figure 5 in Miura, 2012). We used WebPlotDigitizer (http://arohatgi.info/WebPlotDigitizer/app/) to extract data on attack rates from original published graphs.

Discussion

Patterns of colony structure in trematode species with a soldier caste

Our analyses of morphological, distributional, and behavioral patterns indicate that the 4 species from the California horn snail have a soldier caste. Across those species, reproductive rediae were 21x to 216x larger than soldiers,

and lacked the prominent appendages and collars of the soldiers. Further, soldier and reproductive rediae of the 4 species were unevenly distributed throughout host tissues, with soldiers being disproportionately abundant in the anterior part of the snail, where invasions by other trematodes are initiated. Finally, very few reproductive rediae (0 - 1.9%) attacked heterospecific rediae, while the percentage of soldiers that attacked was substantially higher (12 - 81%). We note that, in addition to being more aggressive, soldiers frequently anchored their posterior ends onto the substrate, or each other, sometimes forming clusters (Fig. 4C). Similar observations have previously been reported *in vitro* for *Philophthalmus* sp. (Lloyd and Poulin, 2012) and, importantly, *in vivo* for *Philophthalmus gralli* (West, 1961), where the small (likely soldier) rediae attached to the walls of the blood passages of the snail host. Attaching to substrate may enhance attack rates, perhaps providing leverage and enabling movement in all directions, thus increasing the ability for soldiers to encounter and attack enemy trematodes.

Among species with a soldier caste, there was variation in the degree of morphological dimorphism between soldiers and reproductive rediae (Figs. 1, S1-S4). The average differences in size between soldier and reproductive morphs was large for CLOA and PARO (141x and 216x, respectively), intermediate for ACAN (39x) and smallest for HIMA (21x). For the first three, there was no overlap in body size between soldier and reproductive rediae, and the size-frequency distributions are distinctly bimodal (Figs. 1, S1-S4). In ACAN, there is also strong dimorphism in color. HIMA colonies, on the other hand, exhibited less morphological divergence. They had the highest proportion of individuals bridging the size-frequency gap

between the modes of soldier and reproductive rediae distributions (Figs. 1, S4). Nonetheless, HIMA colonies were still characterized by bimodal size-frequency distributions and their division of labor is evident given the particularly aggressive behavior of their soldiers (see below).

Aggressiveness of soldier rediae

Although the soldier caste might have some other roles (e.g. Lloyd and Poulin, 2012), it is clear that one major role is defense. During the *in vitro* attack trials, soldiers of each of the 4 species from the California horn snail consistently attacked heterospecific rediae at much greater rates than did reproductive individuals (Figs. 5, Table S2). Despite some variation in methodological details, these values are generally consistent with values reported for other species with soldier and reproductive castes (Fig. 5; Hechinger et al., 2010; Miura, 2012; Nielsen et al., 2014), with the exception of the high 81% attack rate for HIMA.

HIMA soldiers were particularly aggressive. Not only did they have the highest attack rates, which appear to be at least 2 times greater than the other species' attack rates (Fig. 5); HIMA soldiers often completely ingested heterospecific rediae during the observation periods (Fig. 4C). Therefore, although morphological differences were less extreme for HIMA than for the other trematodes from the California horn snail, the attack trials suggest that HIMA has the most aggressive soldiers yet described.

Intraspecific variation in behavior and colony structure (caste ratios)

During the attack trials, there were intraspecific differences in the attack rates of soldiers originating from different colonies. For example, soldiers of ACAN colonies 2 and 3 were more aggressive than those from ACAN 1 (Fig. 5, Table S2). In addition, the antagonistic behavior varied depending on the colony of origin of the heterospecific rediae that soldiers were exposed to (e.g., ACAN soldiers consistently attacked EUHA 1 more often than EUHA 2; Fig. 5, Table S2).

In addition to the differences in antagonistic behavior, the examined colonies presented considerable intraspecific variation in redia morphometrics and colony structure (caste ratios). For example, reproductive rediae ranged from being 65 to 423 times bigger than soldiers among CLOA colonies, and 86 to 673 times bigger for PARO (Table S1). This represents a 6x and 8x difference in reproductive to soldier body-size ratios, respectively. We also note that, although the percentage of soldiers consistently increased in the anterior parts of the snail, the range of values obtained for each region was wide, especially so for CLOA, PARO and HIMA (Table I).

The intraspecific variation in behavior and colony structure could be due to multiple factors, including colony condition, age, or history of threats of attack. For instance, the colonies for this study were collected over 11 mo from different sites at Carpinteria Salt Marsh. Because the risk of interspecific invasion varies by season (Martin, 1955; Yoshino, 1975), and at a fine spatial scale (Lafferty et al., 1994), it is possible that investment in soldiers could be adaptive, being higher in seasons or areas with higher risks of invasion. Variability in colony structure has also been

observed for colonies of *Philophthalmus* sp., (Lloyd and Poulin, 2014) and *H. elongata* (Nielsen et al., 2014) from different geographic locations. Although attempts have been made to explain the variation observed (Kamiya et al., 2013; Lloyd and Poulin, 2013), the mechanisms controlling caste ratio remain unclear.

We also hypothesize that the bimodality of the size-frequency distributions will increase with colony development. Early infections are likely to be dominated by rapidly growing reproductive rediae, and the size-frequency distributions will become more bimodal as the colony matures with the development of more soldiers and fewer developing reproductives. Hence, reproductive to soldier body-size ratios would also increase in older colonies. The horn snails examined here had been naturally infected. Although we only examined developed colonies, we were unable to estimate their exact ages. However, the size and distributional patterns analyzed for *E. liei*, where colony age was known, suggest that some of these factors change with colony age. Specifically, the proportion of immature rediae appeared lower, and the size of reproductive rediae larger in the older *E. liei* colonies (Fig. S5). If soldiers were present, this would also drive an increase in reproductive to soldier size ratios. Further supporting this scenario of colony development, in June 2015, we were able to examine 1 early HIMA colony. The morphological and distributional patterns of this developing colony (unimodal, with many immature reproductives; Fig. S6), are consistent with our prediction of increasing bimodality with colony age.

Finally, molecular genetic evidence indicates that ACAN (Nguyen, 2012; Nguyen et al., 2015) and PARO (Huspeni, 2000) are species complexes. Hence, it is important to consider that some of the intraspecific variation may actually reflect

interspecific variation caused by the 2 or 3 cryptic species that cannot currently be morphologically differentiated.

Not all echinostomoids have a non-reproducing soldier caste

For *Echinostoma liei*, small and large rediae had similar morphology, and all contained developing embryos. There was no evidence for within-colony redia size dimorphism, as body-size distributions were unimodal (Figs. 1, S5). The patterns observed for *E. liei* should be understood the first explicit depiction of colony structure in trematodes lacking soldiers. However, because this strain has been maintained in a laboratory setting without enemies for 40 yrs, examination of wild populations is required to understand whether *E. liei* lacks soldiers in the wild.

Interestingly, in her undergraduate thesis, Zikmundová (2011) also reported a unimodal size distribution for rediae of another echinostomoid, *E. nasincovae* (= *E. spiniferum*) that naturally infected the freshwater snail *Planorbarius corneus*.

The patterns observed for these 2 trematodes are in contrast to those of species with soldiers and provide insight into colony structure and function when a permanent soldier caste is lacking. *Echinostoma liei* is a strong competitor (Heyneman et al., 1972). The fact that it lacks soldiers suggests that soldiers are not required for a species to be dominant in trematode dominance hierarchies and the potential existence of “totipotent” reproductive rediae that can also attack heterospecific rediae. Future examination of the distribution of defensive behavior among reproductive individuals might provide insight into the evolution of a reproductive division of labor in the Trematoda. For instance, we predict that defense in trematodes lacking soldiers will be mostly performed by younger, small

reproductive rediae (these individuals can more easily move through the snail body causing less damage to the host), suggesting that evolution of a permanent soldier caste operated via selection on these young reproductive stages.

Role and origin of immature rediae in trematode colonies

Consistent with previous research (Hechinger et al., 2011; Miura, 2012), for the species with soldiers, immature rediae were relatively rare. Immature rediae represent developing reproductives, which provide 2 functions for trematode colonies: colony growth and replacement of dying reproductives.

New reproductives are added to trematode colonies as colonies increase in size with host growth and age (e.g., Lim and Lie, 1969; Smith, 1984; Hechinger et al., 2011). Colonies that are growing quickly should have a higher proportion of developing reproductives. Supporting this, younger colonies (6-wk-old) in *B. glabrata* appeared to have a higher proportion of immature rediae than did the 12-wk-old ones (Fig. S5). Because we examined developed colonies from the California horn snail, colony expansion would be slow, limited to concordant growth with the infected snail.

Despite us studying developed colonies, there were differences among species in the proportion of immatures. The difference in proportion of immature rediae of *E. liei* to the other 4 trematode species may simply represent the faster development of *E. liei* colonies permitted by their rapidly growing snail host. Because HIMA shares the same host snail as do the 3 other species with soldiers from *C. californica,* horn snails infected with HIMA must have a faster growth rate and/or a greater rate of turnover of reproductive rediae.

Hechinger et al. (2011) did not observe dead or dying rediae in *Himasthla* sp. B. However, in dissections, we have now observed some reproductives with disintegrating teguments that were filled with active, developed cercariae (A. E. Garcia-Vedrenne, pers. obs.). Because these rediae are apparently dying while filled with living offspring, they could have gone unrecognized in Hechinger et al. (2011). The existence of these dying reproductives suggests that some immature rediae are produced to replace dead reproductive rediae, in addition to increasing the total number of reproductives as the host and the colony grows.

It remains an open question the extent to which any interspecific differences in the amount of immatures are caused by variation in turnover of reproductives or faster colony (and host) growth rates. It is also unknown whether immature reproductives originate from a few soldiers that switch roles and transition to become reproductive rediae, or from newly formed daughter rediae that never specialize in defense and that grow directly to reproductive rediae.

Importance of ecology in evolution of sociality

The intra- and inter- specific variation in colonial structure highlights the dynamic nature of social organization among trematodes, even within the single superfamily Echinostomoidea. Hechinger et al. (2011) predicted that trematode soldier castes would most likely evolve in taxa that are typically dominant in interspecific hierarchies, in situations of invasion risk, and among trematodes that infect longer-lived hosts. Although there are still few studies examining the existence of a trematode soldier caste, and all have involved echinostomoid trematodes (Table II), the available information is consistent with those predictions.

First, echinostomoids tend to be high in trematode dominance hierarchies (Lim and Heyneman, 1972; Kuris, 1990), so soldiers would be relatively common in this group. Second, species that use hosts infected by relatively more trematode species (see Table II) might have stronger caste formation. This would be expected because higher trematode diversity generally corresponds to higher overall prevalence (Poulin and Mouritsen, 2003), implying higher invasion probabilities. Hence, the apparently higher aggressiveness of ACAN soldiers compared to the congener infecting *Batillaria attramentaria* (Fig. 5) is consistent with the prediction that invasion probabilities can drive the evolution of a soldier caste. Similarly, Nielsen et al. (2014) suggest that *Himasthla* sp. B has stronger caste formation than *H. elongata*. However, we note several methodological differences between the studies characterizing *Himasthla* sp. B and *H. elongata* (e.g Nielsen et al. (2014) pooled colonies, which would obscure caste body-size differences due to considerable inter-colony variation in caste sizes; other differences included size of arenas in behavioral trials, type of target species of heterospecific enemies, and the method of counting attacks in the trials). Hence, we cannot draw conclusions about the strength of caste formation in those two *Himasthla* species at this point. Finally, the 2 echinostomoid species known to lack soldiers both infect relatively short-lived freshwater snails, whereas those with soldiers infect longer-lived marine snails (Table II). Although life span and habitat variables are presently confounded, the lack of soldiers in the two *Echinostoma* species is consistent with Hechinger et al.'s (2011) prediction that shorter lifespans may result in less selection for soldiers given a lower lifetime risk of invasion. Testing these hypotheses in a comparative

framework will require broader sampling throughout the trematode phylogeny in a way that encompasses substantial life-history and ecological variation.

Table II. Species of trematodes that have been explicitly examined for the occurrence of a division of labor involving a reproductive and a soldier caste of rediae.

Family	Species	Snail host	Snail life span*	Trematodes in guild †	Soldier caste
Echinostomatidae	*Himasthla* sp. B	*Cerithidea californica*	8-10	19	Yes ‡
	Himasthla rhigedana	*Cerithidea californica*	8-10	19	Yes §
	Himasthla elongata	*Littorina littorea*	6-10	6	Yes II
	Acanthoparyphium spinulosum	*Cerithidea californica*	8-10	19	Yes §
	Acanthoparyphium sp. I	*Batillaria attramentaria*	6-10	9	Yes #
	Echinostoma liei	*Biomphalaria glabrata*	1-1.5	8/13††	No §
	Echinostoma nasincovae	*Planorbarius corneus*	3	15	No ¶
Philophthalmidae	Philophthalmid sp. I	*Batillaria attramentaria*	6-10	9	Yes #
	Philophthalmid sp. II	*Batillaria attramentaria*	6-10	9	Yes #
	Philophthalmus sp.	*Zeacumantus subcarinatus*	5-6	9	Yes **
	Cloacitrema michiganensis	*C. californica*	8-10	19	Yes §
	Parorchis acanthus	*C. californica*	8-10	19	Yes §

* Race, 1981; Eveland and Haseeb, 2011; Moore, 1937; Yamada, 1982; Berrie, 1963; Fredensborg et al., 2005
† Sousa, 1993; Esteban, 2011; Blakeslee, 2008; Hechinger, 2007; Brown, 2011; Leung, et al., 2009
‡ Hechinger et al., 2011.
§ Present study,
II Nielsen et al., 2014.
Miura, 2012.
¶ Zikmundová, 2011.
** Leung and Poulin, 2011.
†† In nature / Experimentally.

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Chapter 3

Trematodes with a reproductive division of labor: Heterophyids also have a soldier caste and early infections reveal how colonies become structured

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Trematodes with a reproductive division of labor: Heterophyids also have a soldier caste and early infections reveal how colonies become structured

Abstract

Recent findings have extended the documentation of complex sociality to the Platyhelminthes, describing the existence of a reproductive division of labor involving a soldier caste among the parthenitae of trematode parasites. However, all species examined so far occupy high positions in trematode interspecific dominance hierarchies, and belong to two closely related families, the Echinostomatidae and the Philophthalmidae (Superfamily Echinostomatoidea). Further, the two species documented as lacking soldiers also belong to the Echinostomatidae. Here, we examine four species of intermediate dominance, all belonging to the family Heterophyidae (Superfamily Opisthorchioidea): *Euhaplorchis californiensis, Phocitremoides ovale*, *Pygidiopsoides spindalis and Stictodora hancocki*, all of which infect the California horn snail, *Cerithideopsis californica* (=*Cerithidea californica*). We quantify morphology, distribution, and behavior of rediae from fully developed colonies. We also provide information on colony structure for three developing heterophyid colonies to better understand colony development. We discuss the implications of our findings, particularly with respect to how they suggest alternatives to the conclusions of other researchers concerning the nature of trematode sociality. Our analyses of morphological, distributional, and behavioral

patterns of developed colonies indicate that these heterophyid trematodes have a non-reproductive caste whose function is defense of the colony from invading trematodes. Hence, a soldier caste occurs for species lower in dominance hierarchies than previously known and is present in at least two superfamilies of digenean trematodes, suggesting that selection for a soldier caste may be much more common among the Trematoda than previously recognized.

Keywords

Sociality, colony, soldier caste, defense, rediae, Digenea, Trematoda

Introduction

A complex social system has recently been documented in digenean trematodes (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014, Garcia-Vedrenne et al., 2016). As in the most complex animal societies [e.g. several types of insects (e.g., Wilson, 1971; Aoki, 1977; Crespi, 1992; Kent and Simpson, 1992; review in Myles, 1999), snapping shrimp (Duffy, 1996), and naked mole-rats, (Jarvis et al., 1981)], the parthenita stages of some trematode species live in colonies with morphologically and behaviorally distinct reproductive and non-reproductive castes (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014, Garcia-Vedrenne et al., 2016). Although the non-reproducing caste might have some other roles (e.g. Lloyd and Poulin, 2012; Galaktionov et al., 2015), it is clear that a major role is defense of colonies

(Hechinger et al., 2011; Miura, 2012; Mouritsen and Halvorsen, 2015; Garcia-Vedrenne et al., 2016).

Digenean trematode colonies are formed in the first intermediate host, usually a mollusk. The colony is initiated by a single founder larva (miracidium) that infects the host, metamorphoses, and clonally produces large numbers of daughter parthenitae. Some trematode species have rediae: parthenitae that possess a muscular pharynx and a gut. Other species have sporocysts, which lack a pharynx and gut. Both kinds of parthenitae produce more parthenitae and then dispersive offspring (cercariae), which leave the colony to infect the next host in the life cycle. Once established, the colony blocks host reproduction and takes control of the host's energy allocation to serve the needs of the colony, primarily diverting energy to parasite reproduction (Rothschild & Clay, 1952; Kuris, 1974; Baudoin, 1975; Hechinger et al., 2009; Lafferty and Kuris, 2009). The colony commonly occupies the gonad and/or digestive gland of the host, taking up a large portion of the host's soft tissue (10-50%) (e.g., Hurst, 1927; Bernot and Lamberti, 2008; Hechinger et al., 2009). Given their extensive use and control of host resources, such conditions would lead to intense competition should another trematode invade the same molluscan host.

Typically, when two trematode species infect the same host, one kills the other. These antagonistic interactions are hierarchical. Dominant species fend off invasions or displace established colonies of subordinate species. This displacement may be via chemical mechanisms (known for some species with sporocysts (Basch et al., 1969; Walker, 1979)), but it most commonly occurs via

predation by rediae (Lim and Heyneman, 1972; Lie, 1973; Combes, 1982; Kuris, 1990; Sousa, 1993). Until recently, this antagonism had been considered to occur via the actions of “totipotent” rediae, those that both reproduce and defend the colony (Lim and Heyneman, 1972; Lie, 1973; Kuris, 1990; Sousa, 1993; but see Lie, 1969).

However, recent studies have shown that several trematode species have a division of labor involving a caste of non-reproducing soldiers that are specialized for defense (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014; Garcia-Vedrenne et al., 2016). Despite being smaller than reproductives, soldiers have relatively large pharynxes to attack and kill invaders. Only soldiers readily attack heterospecific and even conspecific enemies. The small size of these soldiers likely facilitates dispersion throughout the host body, supported by the fact that soldiers are more active and disproportionately common in areas of the host where invasions occur. Small rediae have been long observed in trematode infections (e.g. Stunkard, 1930; Kuntz and Chandler, 1956). However, small rediae are classically considered solely as being immature reproductives that are generated early in colony development, to periodically replace dying reproductives, and to permit colony growth as the host body increases in size. The discovery that these small rediae are not solely immatures, and sometimes represent a soldier caste, has expanded our perspective on the nature of trematode infections in first intermediate hosts and opened up new research avenues examining the ecology and evolution of complex sociality.

Hechinger et al. (2011) predicted that soldier castes would most likely evolve in trematode taxa that are typically dominant in interspecific hierarchies. To adequately test this and other hypotheses concerning the evolution of sociality among trematode species, it is necessary to quantify social structure for trematode species encompassing a range of dominance positions and that are spread throughout the trematode phylogenetic tree. Despite there being over 150 families of Trematoda (Cribb and Bray, 2011), all trematodes so far examined for social structure belong to two closely related digenean families: Echinostomatidae and Philophthalmidae (Superfamily Echinostomatoidea) (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014; Garcia-Vedrenne et al., 2016). Echinostomoids tend to occupy high positions in trematode dominance hierarchies (Lim & Heyneman, 1972; Lie, 1973; Kuris, 1990). This restricted taxonomic sampling also includes the two species explicitly shown to lack soldiers (Garcia-Vedrenne et al. 2016). Colony social structure has not been examined for species in any of the other trematode superfamilies.

Here, we examine four trematode species that belong to the Family Heterophyidae of the Superfamily Opisthorchioidea (*Euhaplorchis californiensis, Phocitremoides ovale*, *Pygidiopsoides spindalis and Stictodora hancocki*). These species form colonies in the California horn snail, *Cerithideopsis californica* (= *Cerithidea californica*). The California horn snail is host for a diverse trematode guild that is characterized by a fairly well-resolved interspecific dominance hierarchy (Kuris, 1990; Sousa, 1993; Hechinger, 2010). Five of the most dominant species in this hierarchy (all in the Superfamily Echinostomatoidea) have been documented to

have soldiers. The heterophyids we study here have an intermediate position in the dominance hierarchy (they are subordinate to the echinostomatids and philophthalmids, but can eliminate or prevent infections of other, more subordinate, species in the guild (Kuris, 1990)). We also compare colony structure of a few recent, developing heterophyid colonies to fully developed colonies to shed light on the nature of colony development. We discuss the implications of our results, including alternative interpretations concerning the nature of trematode sociality. Our analysis of morphology, distribution, behavior, and colony development of these four heterophyid species indicates that they also have a soldier caste, and that trematode caste structure takes time to develop, becoming more pronounced with colony age.

Materials and methods

Study system and sample collection

California horn snails, *Cerithideopsis californica* (=*Cerithidea californica*), (Potamididae) were collected from Carpinteria Salt Marsh, Santa Barbara County, California between July 2013 and May 2016. Snails were maintained in the lab for up to 7 weeks in mesh bags on running sea water tables until processing. Some infections were identified by inducing cercaria emergence, and then dissected. In other cases, the snails were dissected and, if infected with the appropriate species, immediately processed. We identified trematode species following Martin (1972) and additional unpublished observations.

We examined the four species that belong to the family Heterophyidae: *Euhaplorchis californiensis* (EUHA), *Phocitremoides ovale* (PHOC), *Pygidiopsoides spindalis* (PYGI), and *Stictodora hancocki* (STIC). For simplicity and clarity, we will refer to each species by the codes formed by the first 4 letters of their genus, as above.

Redia morphology and distribution

Snails were collected between July 2013 and October 2015. We targeted 3-5 colonies (snails) for each of the study species. All the trematode colonies examined were producing cercariae. We followed the methods described in Garcia-Vedrenne et al. (2016). Briefly, we dissected snails by carefully cracking the shell with a hammer and divided the body into 3 different regions: mantle, basal visceral mass (middle), and the gonad/digestive gland. To ensure unbiased sampling of individuals to depict size-frequency distributions, we used a grid and random numbers to randomly sample approximately 30 parthenitae from each snail region. Sampled parthenitae were killed by immersion in hot water, fixed in 70% EtOH and mounted in glycerin. Digital pictures were taken with a Lumenera Infinity 3 camera mounted on an Olympus BX60 compound microscope. We measured body length and width to the nearest micron; we also measured pharynx width and length. We calculated total body and pharynx volumes by approximation to a cylinder.

We assigned each individual to a morph category based on the presence or absence of developing offspring, regardless of size. Parthenitae that lacked free germ-balls or later-stage embryos were classified as soldiers, while individuals that contained at least some late-stage embryos of cercariae or parthenitae were

identified as mature reproductives. In some cases, we found individuals that had early stage embryos; these were scored as immature reproductives. For statistical analyses, we have included these immature morphs in the reproductive category, unless otherwise stated.

Colony development

In November 2015, California horn snails were collected from Carpinteria Salt Marsh and checked for infection status. Uninfected snails were sprayed with paint and returned to the salt marsh in December 2015. In May 2016, the marked snails were collected and dissected. Three snails harboring newly established heterophyid colonies were found (such infections are readily identifiable because the mother sporocyst is still present in the basal visceral mass region, and the rediae have not yet filled up the gonadal space and are generally creating more rediae compared to cercariae (pers. observations)). Identification to species was not possible due the absence of developed cercariae. Although exact age of the infection cannot be determined, they are likely less than 5 months old. We have organized the relative ages of the colonies from youngest (1) to oldest (3) as determined by the size, number, and distribution of parthenitae, as well as the stage of development of the cercariae within them. Individual rediae were randomly sampled from the whole snail (we did not divide these into the three different regions). Morphology and classification was determined as described in the previous section.

Attack trials

We performed attack trials to compare behavioral specialization of soldier and reproductive castes. Experiments took place between June and November 2015, and during May 2016.

Snails harboring heterophyid colonies were dissected and bisected just anterior to the gonad. Reproductives were isolated from the gonad by teasing apart the tissues and pipetting out the individuals into a separate petri dish with filtered sea water. To isolate soldiers, we first teased the tissues from the anterior part of the snail (mantle and basal visceral mass), and filtered the parasites and tissues using a 75 µm filter. This concentrated the soldiers and helped remove most of the snail tissue and free-swimming cercariae. Soldiers were further isolated and concentrated by pipetting them out into a smaller petri dish before setting up the attack trials.

For the attack trials, 96-well plates with concave bottoms were used to increase encounter rates. Parthenitae from one to three colonies (as available) of each tested species were presented with reproductive rediae from heterospecific colonies. Colonies of PHOC, PYGI and STIC were exposed to EUHA reproductives, following Hechinger et al. (2011) and Garcia-Vedrenne et al, (2016), because EUHA is one of the most common trematodes at the collection locality. To evaluate the attack rates of EUHA, reproductive individuals of the heterospecific STIC were used. For each replicate, we placed approximately 10 heterospecific rediae into a single well with sea water, followed by approximately 10 soldiers or 10 reproductives of the focal species. For each combination, we used 2 to 4 replicate

wells. The wells were held for 90 min at ambient room temperature (21-23 C). We then observed each individual well with a stereomicroscope for 20 sec. An attack was recorded whenever a redia was attached to another with its mouth, following Hechinger et al. (2011).

Statistical analysis

All statistical analyses were performed using R 3.1.3 (ran with RStudio 0.98.1103), or JMP Pro 12.0.0. For morphological analyses, we used a mixed-effects general linear model (GLM) on natural log-transformed response data and set colony ID as a random effect to model the relationship between soldier and reproductive total volume, pharynx volume, and pharynx/volume ratios. Interaction terms between colony and morph type were included in the model as needed. We examined residual plots and normal quantile plots to ensure meeting assumptions regarding data normality and homoscedasticity. Cochran-Mantel-Haenszel Chi-square tests were used to examine the relationship between rediae type and snail region, while controlling for colony ID. For the attack rates, we used a binomial regression with a logit link function and tested for the effects of redia type. Colony ID was set as random effect.

Results

Morphology

We processed a total of 2,067 parthenitae from the 4 species. We examined four colonies of EUHA, three of PHOC, three of PYGI, and five of STIC. General

patterns were consistent among colonies, and our statistical analyses include data from all colonies. However, for illustrative purposes, we provide figures in the text for one representative colony of each species. Additional figures and detailed data are in the Supplementary material (Supplementary Figs. S1-S4, Supplementary Table S1).

There was strong dimorphism among colony members for each of the four species. Soldiers were clear and transparent, while the tegument of reproductives had a light yellowish-orange pigmentation. Reproductives were consistently longer and wider (Figure 1B), with no overlap in body volume (Figure 1C). The size-frequency distributions were clearly bimodal (Figure 1C) and reproductives had, on average, a volume 28 times larger than soldiers for EUHA (t_{504}=80.0, p<0.0001), 69x for PHOC (t_{618}=141.0, p<0.0001), 32x for PYGI (t_{483}=98.7, p<0.0001), and 19x for STIC (t_{445}=16.6, p<0.0001 (pooling all rediae per species; Supplementary Table S1 shows results for individual colonies).

For three species, absolute pharynx sizes of reproductive morphs were not significantly different from those of soldiers, being 0.98 times that of a soldier for EUHA (t_{449}=-0.14, p=0.8893), 0.94x for PHOC (t_{554}=-0.72, p=0.4702), and 0.92x for STIC (t_{367}=-1.15, p=0.249) (Figure 1C). However, for PYGI the size of the pharynx of the large reproductive was actually smaller than that of the soldiers (0.87x, t_{401}=-2.74, p=0.0065) (Figure 1D).

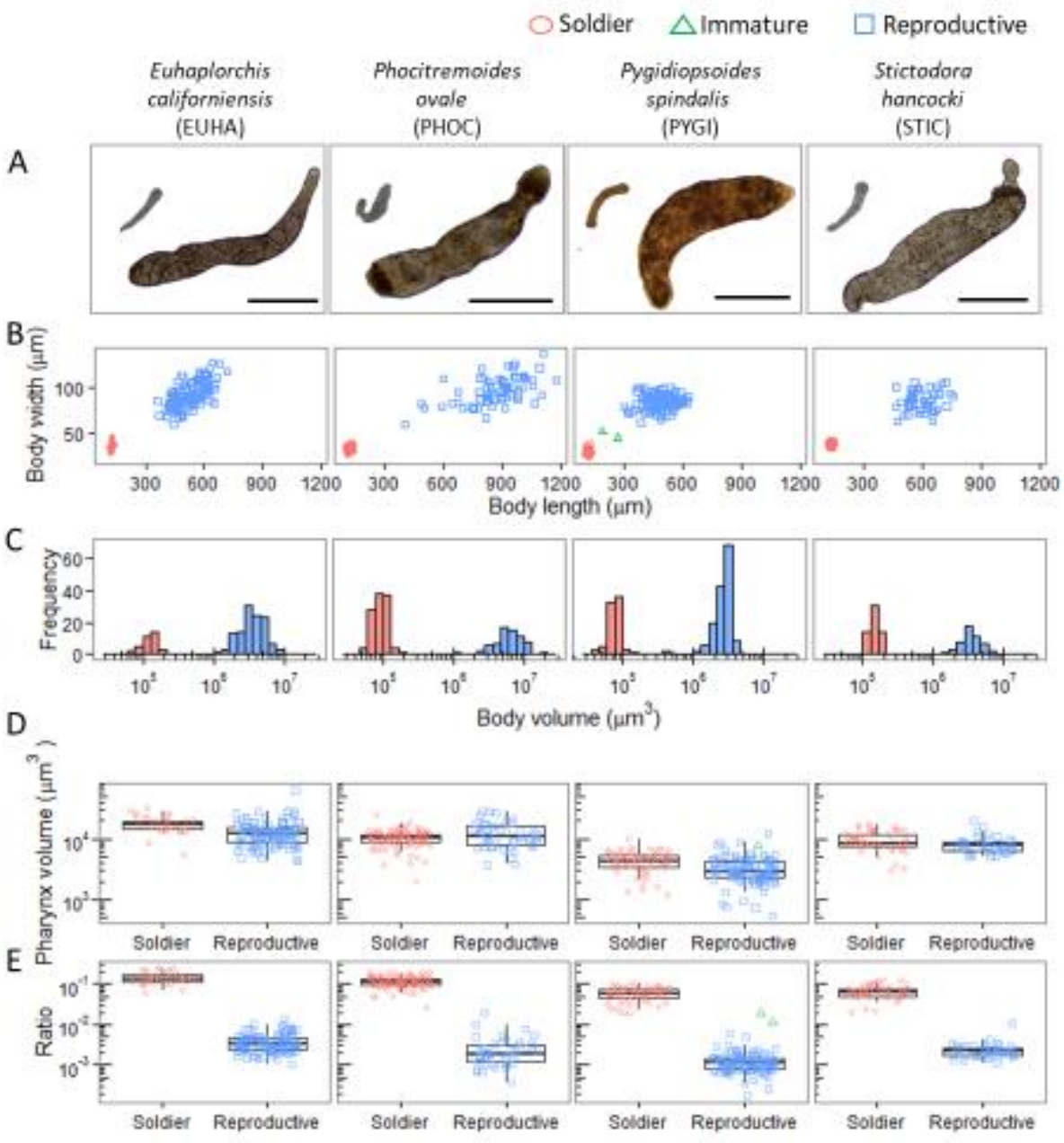

Figure 1. Morphological attributes of redia morphs for each of the 4 species of examined heterophyid trematodes. Data for figures are of a single, representative colony, but statistical analyses used data from all colonies and Supplemental Material includes figures for each colony examined. (A) Photographs of representative redia morphs: soldiers on the left, reproductives on the right. Scale bar= 200µm. (B) Body width to body length relationships. (C) Frequency distributions of body volume for randomly sampled rediae. Note the $\log_{10}$ scale of X-axes. (D-E) Boxplots indicating median (line), interquartile range (box) and values that are within 1.5 * interquantile range (whiskers) of data for (D) absolute pharynx volume and (E) pharynx volume relative to body volume. Note the $\log_{10}$ scale for Y-axes.

Both reproductive and soldier parthenitae in these four Heterophyidae lacked collars and locomotory appendages (lappets), consistent with the long-known general morphology of this group.

Caste ratios and distribution

For the 4 species examined, most parthenitae were located in the gonadal region, but some were also found in the basal visceral mass and the mantle. The relative numbers of soldiers and reproductive parthenitae (caste ratios) varied between regions for each species (Table 1, Figure 2, Supplementary Figs. S1-S4; EUHA: $\chi^2_{1,508}=20.5$, $p<0.0001$; PHOC: $\chi^2_{1,622}=32.5$, $p<0.0001$; PYGI: $\chi^2_{1,487}=26.9$, $p<0.0001$; and STIC: $\chi^2_{1,450}=26.2$, $p<0.0001$). However, in all cases, soldiers were relatively more common in the anterior portion of the snail (basal visceral mass and mantle) compared to the gonad region.

Table 1. Percentage of parthenitae that are soldiers, by host body region. Actual counts can be found in Table S1 and Figures S1-S4.

	Average (and range) percentage of soldiers among colonies			
	EUHA	PHOC	PYGI	STIC
Mantle	79 (50 - 100)	92 (89 - 94)	72 (57 - 88)	98 (89 - 100)
Basal Visceral Mass	47 (6 - 69)	80 (71 - 95)	55 (25 - 71)	79 (42 - 97)
Gonad	8 (3 - 17)	14 (8 - 24)	9 (4 - 16)	9 (0 - 17)

Immature individuals were uncommon in mature colonies of all species (Figures 2, Supplementary Fig. S1-S4, and Supplementary Table S1). Of the total parthenitae examined from the 4 species infecting the California horn snail, 23 out of 2,067 were immature. Among colonies within species, the average (and range)

proportion of immatures among all parthenitae examined was 0.8% (0 – 1.7%) for EUHA, 0.2% (0 – 0.5%) for PHOC, 1% (0 – 1.6%) for PYGI, and 2.9% (0 – 7.2%) for STIC. The average (and range) proportion of immatures considering only reproductive morphs was 1.1% (0 – 2.8%) for EUHA, 0.4% (0 – 0.8%) for PHOC, 1.6% (0 – 2.8%) for PYGI, and 4.9% (0 – 9.5%) for STIC.

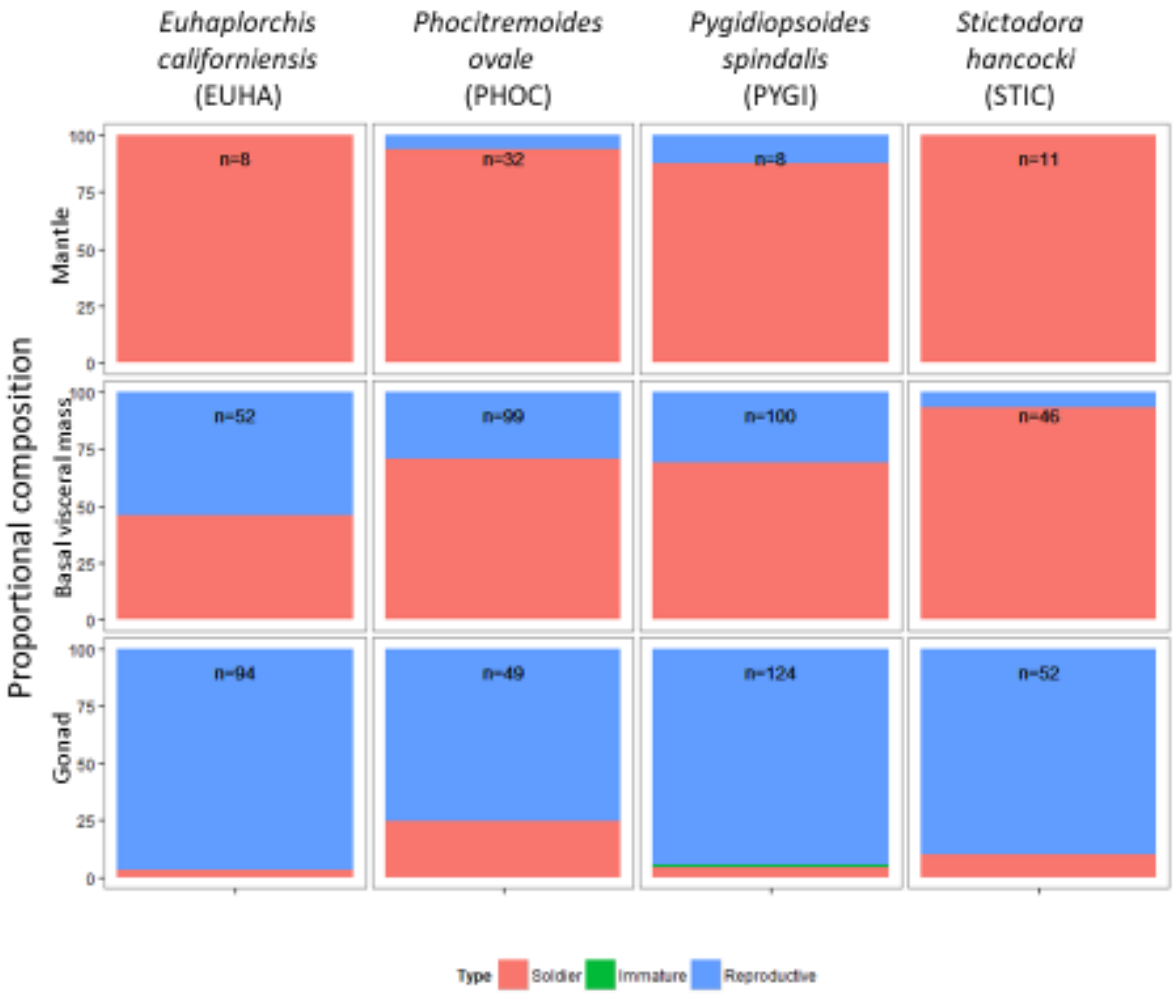

Figure 2. Proportion of different morphs found in the three snail body regions: the mantle, basal visceral mass and gonad (and digestive gland). Data for figures are of a single, representative colony, but analyses used data from all colonies and Supplemental Material includes figures for all colonies. Numbers in boxes indicate total number of parthenitae sampled from the given region.

Table 2. Percentage of parthenitae of each morph type in three newly established heterophyid colonies. 1= youngest colony; 3= oldest colony.

	1	2	3
Reproductive	6%	47%	60%
Immature	77%	29%	21%
Soldier	17%	24%	19%
N sampled	83	143	226

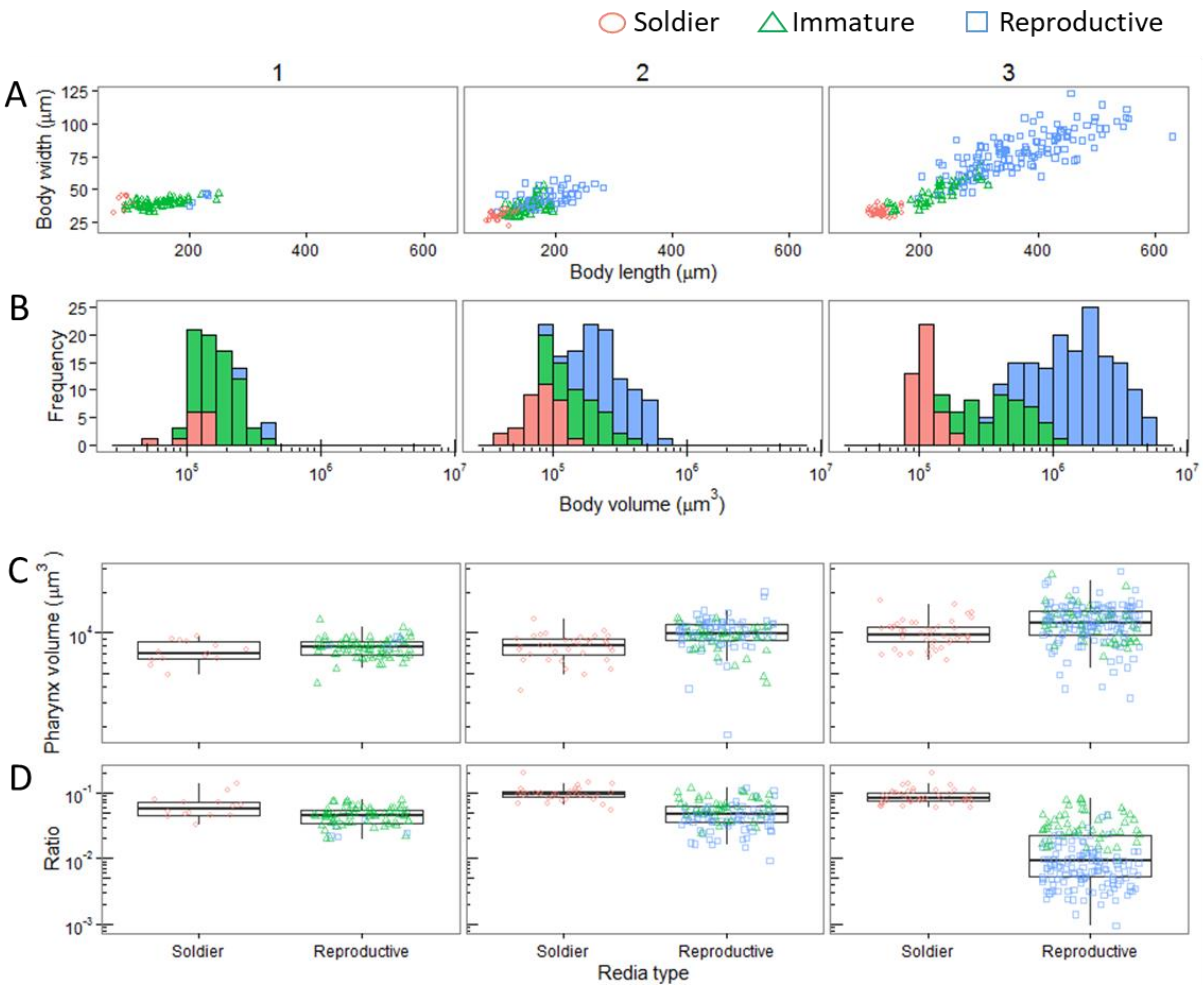

Figure 3. Morphological attributes of developing heterophyid colonies. Colony 1 is inferred as being the youngest, Colony 3 as the oldest. (A) Body width to body length relationships. (B) Frequency distributions of body volume for randomly sampled rediae. Note the $\log_{10}$ scale of X-axes. (C-D) Boxplots indicating median (line), interquartile range (box) and values that are within 1.5 * interquantile range (whiskers) of data for (C) absolute pharynx volume and (D) pharynx volume relative to body volume. Note the $\log_{10}$ scale for Y-axes.

Colony development

We processed an additional 453 parthenitae from snails with newly established heterophyid colonies. Among these, the proportion of immature rediae was significantly different (χ^2_4=102.7, p<0.0001), ranging from 21% immatures in the oldest colony to 77% in the youngest colony (Table 2; age was determined by number and size of parthenitae, as well as the stage of development of cercariae inside of them). The degree of bimodality of the size-frequency distributions increased as the colony matures (Figure 3).

Activity and attack rates

For all species, soldiers qualitatively appeared more active than were reproductive morphs. Reproductive individuals rarely moved when exposed to heterospecific parthenitae. In contrast, soldiers increased activity, stretching and contracting their bodies and probing heterospecific parthenitae with their mouths. In some cases, soldiers attached their mouths to heterospecific parthenitae (Fig. 4), sometimes pulling the heterospecific's tegument into the pharyngeal lumen. Similar to what has been reported for echinostomoid soldiers (West, 1961; Lloyd and Poulin, 2012; Garcia- Vedrenne et al. 2016).), soldiers of each species often formed clusters of 2 to 7 individuals, adhering at the posterior ends of their bodies (Fig. 4B). This clustering behavior was rarely observed among the reproductive morphs.

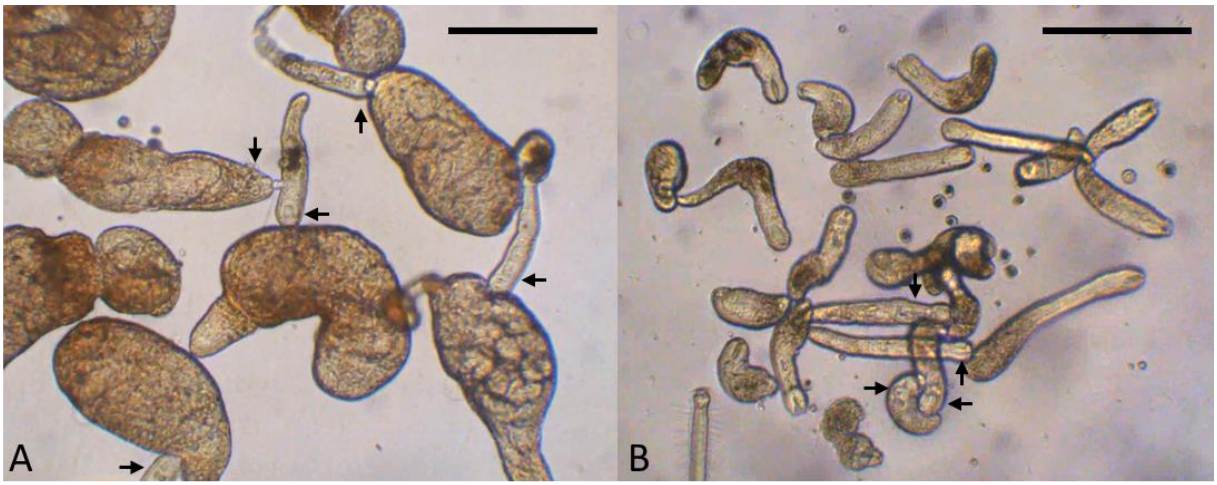

Figure 4. Examples of trematode soldier attack and activity. Individuals attacking are indicated by black arrows. (A) Attacks between *Stictodora hancocki* (STIC) soldiers and *Euhaplorchis californiensis* (EUHA) reproductives. (B) STIC and EUHA soldiers attacking each other. Note also soldiers adhering to each other at their posterior ends. Scale bar= 200µm.

In quantitative attack experiments (Figure 5), reproductive rediae rarely attacked heterospecific rediae. Only 1/324 reproductive rediae engaged in antagonism (0.3%). No attacks by reproductive rediae were observed for EUHA (0/105), PHOC (0/86) or PYGI (0/32) in the experimental trials. Only 1/101 (1%) STIC reproductive redia was recorded attacking a EUHA reproductive. We note that one EUHA reproductive was seen attacking a STIC soldier from a dissected snail, outside of the experimental trials (Figure 4A).

In contrast, for two of the heterophyid species, soldiers more readily engaged in antagonism (Figure 5). A total of 5/63 (7.9%) EUHA soldiers attacked STIC reproductives ($\chi^2_{1,14}$=10.7, p<0.0001), and 28/91 (30.8%) STIC soldiers attacked EUHA reproductives ($\chi^2_{1,60}$=485.6, p<0.0001). However, only 1/62 (1.6%) of PHOC

soldiers attacked EUHA reproductive ($\chi^2_{1,12}$=1.7, p=0.19), and no PYGI soldiers were seen engaging in antagonistic behavior (0/22) ($\chi^2_{1,4}$=0, p=1).

We also set up attack trials versus fellow colony members. In one of the dissected colonies, we observed 1 STIC soldier out of 90 attach its mouth to a member of its own colony.

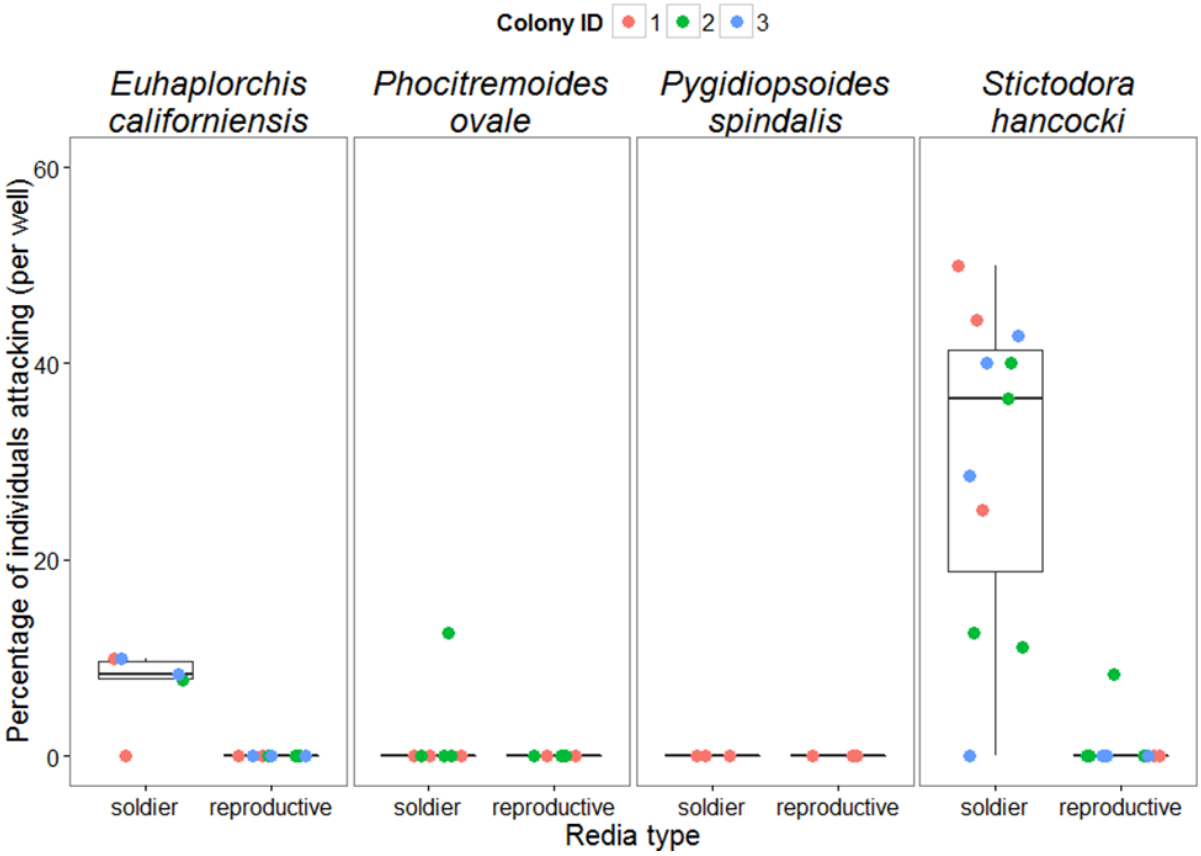

Figure 5. Attack rates of soldiers and reproductives on heterospecific reproductive rediae for each of the 4 heterophyid species from the California horn snail. One to three colonies (as available) of each focal species were used (represented by the different colors). For EUHA, the heterospecific species used was STIC, while PHOC, PYGI, and STIC were exposed to colonies of EUHA. Points indicate proportion of individuals observed attacking for each replicate well (2-4 replicates for each combination), boxplots indicate median (line), interquartile range (box) and values that are within 1.5 * interquantile range (whiskers) of data.

Discussion

The morphological aspects of the rediae in the four examined heterophyid species are generally consistent with the patterns previously observed for the echinostomoid trematodes having a soldier caste (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014; Garcia-Vedrenne et al., 2016). Across all species, reproductive rediae were 18x to 32x larger than soldiers. Despite the fact that soldiers are much smaller than reproductive rediae, pharynx size of both kinds of rediae greatly overlapped in size. The heterophyid soldiers examined here lack the collars and locomotory extensions that have been relatively pronounced in soldier rediae in the Echinostomatidae and Philophthalmidae (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014; Garcia-Vedrenne et al., 2016), clarifying that these features are not required for functioning as a soldier.

Garcia-Vedrenne et al. (2016) hypothesized that, for any given species with soldiers, developing infections would be dominated by rapidly growing reproductive rediae and characterized by unimodal size-frequency distributions. The size-frequency distributions would become more bimodal as the colony matured. Garcia-Vedrenne et al. (2016) present data for one developing infection and five established infections of the echinostomatid, *Himasthla rhigedana*, that are consistent with that hypothesis.

Our observations on established and developing colonies of heterophyid trematodes further support this characterization of colony development. The size-frequency distributions for the fully developed heterophyid colonies were strongly

bimodal, with very few immature rediae, if any, bridging the gap between the two modes. In fact, these heterophyids appear to have the lowest proportion of immatures of any of the species yet examined (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014; Garcia-Vedrenne et al., 2016). Despite this, one colony did stand out as having many immatures (see Supplementary Fig. S4); however, STIC 4 appears to have been a colony that was still becoming established, based on the small amount of mature cercariae observed at dissection, and the large number of reproductive rediae containing only early-stage cercariae.

We also examined three heterophyid colonies that were just becoming established and provide evidence for colony development. Although species identification and exact age were unknown, we know they belong to the Heterophyidae and that colonies were likely less than six months old. The relative ages of the colonies were inferred based on size, number, and distribution of parthenitae, as well as the stage of development of the cercariae within them. In the youngest colony, the distribution was unimodal, and many immature rediae were present (Figure 3, Table 2). The bimodality of the size-frequency distributions was more pronounced in the (putatively) older colonies. The number of immature rediae decreased, more reproductive rediae contained late-stage cercariae, and the number of soldiers increased. Hence, these observations lend further support to the hypothesized characterization of colony development given by Garcia-Vedrenne et al. (2016) and highlight the need for research on experimentally initiated colonies to carefully quantify the details of trematode colony development.

Galaktionov et al. (2016) argue that digenean trematodes do not have a soldier caste. They posit that the overall bimodality reported for redia size-frequency distributions is partly determined by a constraint of redia growth, whereby young rediae initially undergo cellular proliferation, resulting in very little growth for a period of time, followed by a rapid increase in size via cellular extension. This scenario is not consistent with the clear documentation of unimodal size-frequency distributions for several trematode colonies. First, several species with soldiers and strong bimodal size-frequency distributions in developed colonies actually have unimodal size-frequency distributions in early colony development. This is the case for the developing heterophyid colonies reported here, and for the developing colony of *Himasthla rhigedana* described in Garcia-Vedrenne et al. (2016). In these developing colonies, the size-frequency distributions are dominated by immature reproductives with no evidence of arrested growth causing bimodality. Second, examination of established colonies from two species of echinostomatid trematodes that lack soldiers (*Echinostoma liei* in Garcia-Vedrenne et al. (2016), and *E. nasincovae* (= *E. spiniferum*) in (Zikmundová, J. 2011. Is there a soldier cast in freshwater echinostome trematodes? B.S. Thesis. Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic, 36 p.)) consistently showed unimodal size-frequency distributions in established colonies. Garcia-Vedrenne et al. (2016) examined *E. liei* colonies of different ages. They found that, although the proportion of immatures decreases with infection age, the size-frequency distributions are always unimodal. Further, non-reproductive individuals are never seen, consistent with their not having a soldier caste (Garcia-Vedrenne et al., 2016).

Finally, we have observed similar unimodal size-frequency distributions in developed colonies for several species with sporocysts, and another redial species lacking soldiers (Garcia-Vedrenne, unpublished data for species infecting the California horn snail). The presence of these unimodal size-frequency distributions, including those in developing colonies of species with soldiers, confirms that a universal constraint for redia growth does not explain the bimodality characterizing developed colonies of trematodes with a soldier caste.

Galaktionov et al. (2016) also argue that the bimodality of the size-frequency distributions is explained by young rediae experiencing a developmental arrest, driven by density-dependent suppression of their growth by developed rediae. This does not conflict with the hypothesis that small rediae are soldiers. However, Galaktionov et al. (2016) do not attribute a defensive function for those small, developmentally arrested rediae. They evaluate these rediae solely as being a reserve of immatures that will ultimately grow to become reproductives.

As Hechinger et al. (2010) point out, soldiers may have the physiological ability to mature, but the available data suggest the typical soldier does not transition to a reproductive. However, we still lack conclusive evidence for whether digenean trematode soldiers represent a permanent caste or a temporal caste. Intramolluscan development has not been well studied for heterophyids, and a careful investigation of colony development and of the progeny of reproductive rediae would shed the most light on the permanency of the soldier caste. However, similar to Hechinger et al.'s evaluation of the data for *Himasthla* sp. B, the large numbers of heterophyid soldiers, coupled with the rarity of immatures, strongly

supports the hypothesis that the transition from non-reproductive to reproductive is an infrequent occurrence.

Although the permanency of members of the soldier caste remains an open question, the notion that they lack a defensive function is contradicted by several other lines of evidence, including their morphology, distribution within the host, and, particularly, their attack behavior *in vivo* and *in vitro*.

The distribution of the heterophyid rediae is consistent with the patterns characterizing echinostomoid trematodes with soldiers (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Nielsen et al., 2014; Garcia-Vedrenne et al., 2016). Reproductives were mostly located in the visceral mass, particularly in the gonad region (Figures 2, Supplementary Fig. S1-S4). On the other hand, soldiers comprised the majority of the individuals in the basal visceral mass, and particularly in the mantle (72-98%). Hence, soldier and reproductive rediae of the 4 species were unevenly distributed throughout host tissues, with soldiers being more common at invasion fronts. This is consistent with the hypothesis that they are a defensive caste.

Also consistent with the caste hypothesis, heterophyid soldiers tended to attack enemies at greater rates than did reproductives. Although the reproductive rediae have the ability to attack with their mouthparts, they do so at much lower rates than their non-reproductive counterparts. Very few reproductive rediae (0-1.6%) attacked heterospecific rediae, while the percentage of soldiers attacking was substantially higher: 8% of EUHA soldiers and 31% of STIC soldiers were observed engaging in aggressive interactions. Colonies of PHOC and PYGI were rarely

encountered in our sampling, and hence fewer replicates of the attack trials were possible. The lack of attacks by the soldiers in these species might be related to smaller sample size, or that they are indeed less aggressive. Although no attacks were observed for these two heterophyid species, the other lines of evidence (morphology, distribution, etc.) are congruent with them having soldiers. This, along with the fact that attacks were observed for EUHA and STIC, is also consistent with the heterophyids having a non-reproductive soldier caste.

In addition to being more aggressive, the soldiers frequently attached to each other at their posterior ends, forming clusters (Figure 4b). This behavior has previously been reported in soldiers in the Echinostomatidae and Philophthalmidae (West, 1961; Lloyd and Poulin, 2012; Garcia-Vedrenne et al. 2016). Garcia-Vedrenne et al. (2016) hypothesized this behavior could enhance attack rates in snail blood sinuses, providing leverage and enabling movement in all directions.

Also consistent with previous work, soldiers and reproductives of these four species were not generally observed to attack fellow colony members. In the attack trials, no reproductive rediae attacked fellow colony members, and only one STIC soldier was seen attaching to a colony member. This may have been a behavioral artifact of the dissection.

Hence, these distributional and behavioral results indicate that there is a defensive function for the small, non-reproductive rediae. Galaktionov et al. (2016) recognize that “small” and “large” rediae have distinct behaviors and are not randomly distributed throughout the host, suggesting that this is due solely to age-related feeding preferences, conceiving this as a form of “niche segregation”

unlikely to be associated with the ‘colony’ defense against invaders. However, it is well known that these trematode colonies can incur relatively high rates of invasion by enemies in the field (Kuris, 1990; Sousa, 1993; Kuris et al., 1994; Lafferty et al., 1994). Given that soldiers disproportionately attack these enemies *in vitro*, it is parsimonious to attribute defensive function *in vivo*. Further, we have repeatedly observed soldiers aggregating around and attacking invading trematodes in dissections of mixed-species infections (Garcia-Vedrenne, Hechinger, pers. observations; first noted in Hechinger et al. (2010)). Attacking and killing invading trematodes would increase the survivorship and reproductive output of trematode colonies.

Hechinger et al. (2011) predicted that trematode soldier castes would most likely evolve in situations of higher invasion risk, among species that infect longer-lived hosts, and in taxa that are typically dominant in interspecific hierarchies. The California horn snail is long-lived (>8 years (Race, 1981)), and it hosts a species-rich guild of trematodes with a fairly well-resolved interspecific dominance hierarchy (Kuris, 1990; Sousa, 1993; Hechinger, 2010). Five of the most dominant species in this hierarchy (all echinostomoids) have soldiers (Hechinger et al., 2011; Garcia-Vedrenne et al., 2016). However, the four heterophyid species examined here occupy a middle position in the hierarchy, being subordinate to the echinostomoids, but able to kill other, more subordinate species in the guild (Kuris, 1990). The morphological, distributional and behavioral evidence presented here indicates that these heterophyids also have soldiers. Hence, a soldier caste is more broadly distributed throughout the trematode phylogenetic tree, and among species lower in

dominance hierarchies than was previously known. The identification of soldiers in the Family Opisthorchioidea represents a doubling of the taxonomic range for which soldiers have been documented. This suggests an independent evolution of soldiers (or its loss) multiple times within the Trematoda and indicates that selection for a soldier caste may be much more common among digenean trematodes than previously recognized.

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Chapter 4

Degree of sociality varies across trematodes– finding measurable markers that underlie caste function

Degree of sociality varies across trematodes– finding measurable markers that underlie caste function

Abstract

Trematode flatworms form colonies in their first intermediate molluscan hosts, and these colonies can vary in degree of sociality. It is now evident that for some trematode species, social organization can include the formation of a non-reproducing soldier caste. However, studies on species that lack a soldier caste are limited. By examining colonies from eight soldier-less species that infect the California horn snail, we found that, in contrast to species with soldiers, (1) colonies are characterized by unimodal size-frequency distributions with few small and large individuals. (2) Even the smallest colony members contain developing embryos or late-stage cercariae. (3) Individuals of all sizes have similar morphologies and lack structures that are specialized for antagonism. (4) Distribution of parthenitae is restricted to the main infection locus. The documentation of consistent, easy to measure aspects of colonies that reflect functional caste differences indicates that we may use those attributes as “markers” for the existence of a soldier caste. The relatively rapid identification of a soldier caste will facilitate future research on the evolution and strength of social organization among trematodes.

Introduction

"We have defined a caste intuitively as a set of individuals, smaller than the society itself, that is specialized to perform one or more roles. Because this is a purely functional definition based on the behavior of sets of individuals, it is difficult to express in quantitative terms. If we can find other, more easily measurable characteristics that correlate well with the behavioral roles- that is, if we can use "markers"- then the task of empirically determining caste characteristics will be greatly facilitated."

Oster & Wilson, 1978

Trematode flatworms form colonies in their first intermediate molluscan hosts, and these colonies can vary in degree of social organization (Hechinger et al. 2011; Miura 2012; Nielsen et al. 2014; Garcia-Vedrenne et al. 2016; Garcia-Vedrenne et al. 2017). Trematode colonies are initiated when a single founder larva (miracidium) infects a host, metamorphoses, and clonally produces large numbers of daughter parthenitae. These daughter parthenitae produce more parthenitae, and ultimately, most switch to produce dispersive offspring (cercariae) that will leave the colony to infect the next host in the life cycle. Larval trematodes are parasitic castrators, subsuming host resources in a physiologically sophisticated manner (Lafferty & Kuris, 2009; Hechinger et al, 2009). The mass of parthenitae in the snail cooperatively live together, often for years, to reproduce and operate the host phenotype (Kuris and Lafferty 2005; Hechinger et al. 2009). Full utilization of a limited resource that is directly related to colony reproductive output for an extended time presents a strong selective pressure to secure such resources and vigorously

defend them. It is now evident that for some trematode species, sociality can be developed so far as to include the formation of a morphologically and behaviorally distinct non-reproducing soldier caste that defends the colony form other invading trematodes (Hechinger et al 2011). Although documentation of a soldier caste is currently limited to three trematode families (Garcia-Vedrenne et al. 2017), there is growing evidence that such caste systems are widespread and have evolved multiple times (Leung and Poulin 2011; Miura 2012; Nielsen et al. 2014; Garcia-Vedrenne et al. 2016; Garcia-Vedrenne et al. 2017). There are more than 20,000 species of trematodes worldwide (Cribb & Bray 2011); they cover a wide range of environmental and life history diversity and are both ecologically and medically important. Thus, understanding the mechanisms that shape trematode communities can have substantial public health, veterinary and wildlife disease applications. A comparison of the well-described colonies with soldiers to colonies of species that lack a caste-based social structure will facilitate future research on the evolution and strength of sociality among trematodes.

Trematode colonies with soldiers share several traits: (1) Such colonies are characterized by bimodal size-frequency distributions with many small soldiers and large reproductives, but few individuals of intermediate sizes (Fig 1). (2) The smaller colony members are not actively reproducing (although they may contain germ cells and germ balls). (3) Despite their size, smaller individuals have a mouthpart that is larger relative to their body size. (4) Small individuals are disproportionately common in areas of the host body where invading infections often start. (5) Finally, the small colony members are more active and aggressive than are the large,

sluggish reproductive individuals that are filled with offspring. Hence, these smaller, non-reproducing members are “soldiers”, having distinctive physical and behavioral features underlying their defensive caste function. Colony structure and individual morphology has now been characterized for 14 trematode species that have a soldier caste (Hechinger et al. 2011; Leung and Poulin 2011; Miura 2012; Nielsen et al., 2014; Garcia-Vedrenne et al. 2016; Garcia-Vedrenne et al., 2017).

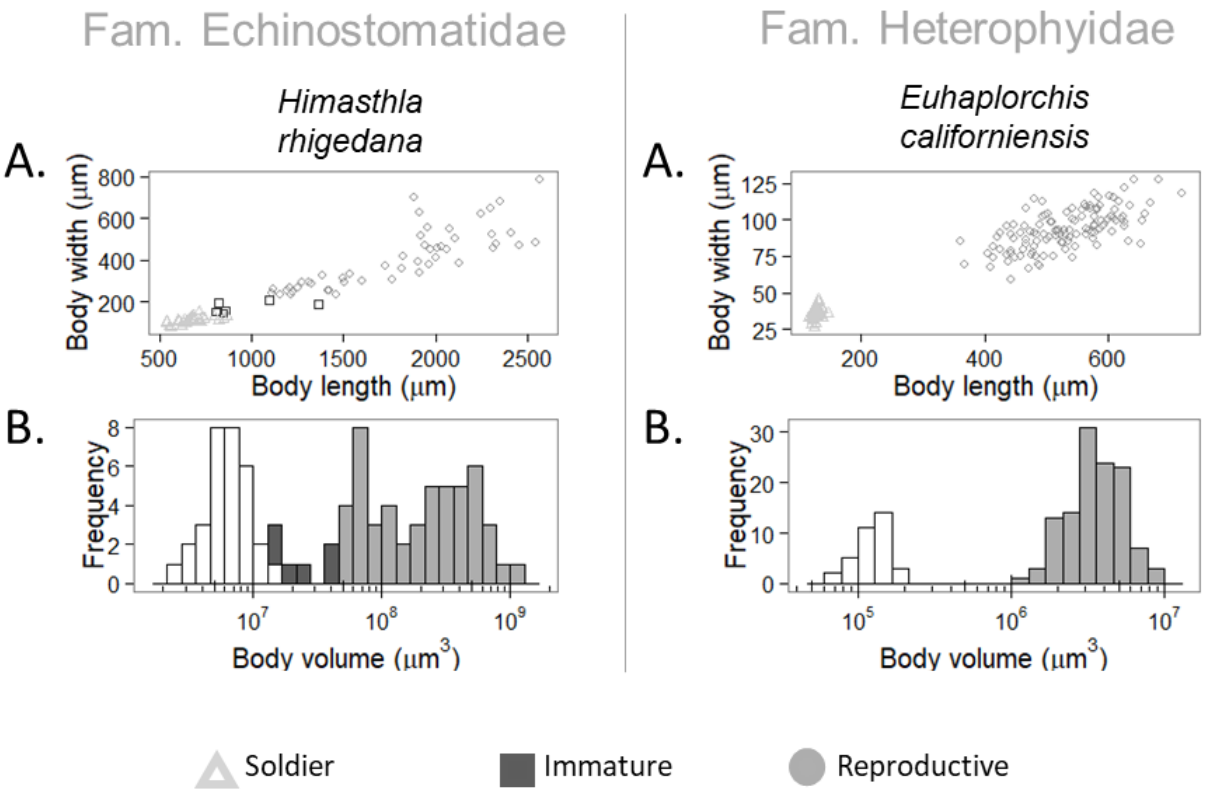

Figure 1. Morphological attributes of redia morphs of *Himasthla rhigedana* (HIMA) and *Euhaplorchis californiensis* (EUHA). Figures modified with permission from Garcia-Vedrenne et al. (2016 and 2017). Data for figures are of a single, representative colony. Each point represents a randomly sampled redia from the trematode colony. (A) Body length to body width relationships. (B) Frequency distributions of body volume for randomly sampled rediae. Note the log10 scale of X-axes.

Conversely, few studies have characterized colony structure and individual morphology for trematode species that lack a soldier caste. The only clear depiction is from a species that had been maintained in a laboratory setting without enemies for 40 years (Garcia-Vedrenne et al. 2016). No wild populations had been explicitly examined. Here we characterize colony structure for eight species lacking soldiers.

Trematode species have one of two general types of daughter parthenitae, which bears on the colony's capacity to have soldiers. Redia species have parthenitae that possess a mouth, muscular pharynx and gut; they can use these structures to actively prey on snail tissue or heterospecific parasites. Thus, redia species may have soldiers. Sporocyst species, on the other hand, have parthenitae that lack a mouth and digestive system; they are generally immotile and absorb nutrients through their body surface. Phylogenetic analyses indicate that sporocysts are a derived trait that has evolved multiple times from ancestors with redia (Cribb et al. 2003). Once the mouth is lost, soldiering, as currently understood, is no longer possible. Hence, sporocyst species can be used to confirm the patterns characterizing colony structure for species that lack soldiers.

Here we examine seven species that, having sporocyst parthenitae, cannot have soldiers. We also document patterns for a redia species that lacks a soldier caste. We present data on morphology, reproductive status, and distribution of the parthenitae. By describing such patterns for eight species belonging to five additional digenean families, we expand the taxonomic range for which colony structure has been examined in the Trematoda. Our findings indicate that trematode colony structure and allometric growth patterns when a soldier caste is absent are in

stark contrast to those of species with soldiers. Thus, we identify easily measurable characteristics that can be used as "markers" to determine whether a soldier caste is present in trematode colonies.

Materials and methods

We examine colony structure for trematode species that infect the California horn snail, *Cerithideopsis californica* (=*Cerithidea californica)* (Potamididae). The California horn snail has a diverse trematode guild that is characterized by an interspecific dominance hierarchy (Kuris, 1990; Sousa, 1993; Hechinger, 2010). Nine species in three families of this guild have been found to have soldiers (Hechinger et al. 2011; Garcia-Vedrenne et al. 2016; Garcia-Vedrenne et al. 2017). Here, we examine colony structure for eight additional species from five different families (Table 1). For simplicity and clarity, we will refer to each species with the abbreviations given in Table 1.

Table 1. Trematode species that infect the California horn snail and are the focus of this paper

Order	Superfamily	Family	Parthenitae	Species	Abbreviation
Plagiorchiida	Pronocephaloidea	Notocotylidae	Redia	*Catatropis johnstoni*	CATA
	Microphalloidea	Microphallidae	Sporocyst	*Probolocoryphe uca*	PROB
			Sporocyst	Small microphallid	SMMI
		Renicolidae	Sporocyst	*Renicola buchanani*	REBU
			Sporocyst	*Renicola* sp. "polychaetophila"	REPO
Strigeida	Diplostomoidea	Cyathocotylidae	Sporocyst	*Mesostephanus appendiculatus*	MESO
			Sporocyst	Small cyathocotylid	SMCY
	Schistosomatoidea	Schistosomatidae	Sporocyst	*Austrobilharzia* sp.	AUST*

* In double infection with *Himasthla rhigedana* (HIMA), for which colony structure was described by Garcia-Vedrenne et al. (2016)

California horn snails were collected from Carpinteria Salt Marsh, Santa Barbara County, California between July 2013 and October 2015. Some infections were identified by inducing cercaria emergence and held up to seven weeks prior to dissection. Other snails were dissected up to one week after collection, and, if infected, trematode species were identified. All colonies examined were mature, producing cercariae. We identified trematode species following Martin (1972) and additional unpublished observations.

We targeted 5 colonies (snails) for each of the study species. However, some species have low prevalence and fewer than the targeted 5 colonies were encountered. For these we show results for all colonies examined. Two of the species that infect the California horn snail, *Renicola* sp. “martini” and *Renicola cerithidicola* were not encountered.

We followed the methods described in Garcia-Vedrenne et al. (2016). Briefly, we carefully dissected the snails, dividing the body into 3 different regions: mantle, basal visceral mass (middle), and the gonad/digestive gland. From each region, we randomly sampled approximately 30 individuals. Sampled parthenitae were killed by immersion in hot water, fixed in 70% EtOH and mounted in glycerin. Digital pictures were taken and used to measure body length and width to the nearest micron. For rediae, pharynx width and length were also measured. We calculated total body and pharynx volumes by approximation to a cylinder.

We assigned each individual to a morph category based on the presence or absence of developing offspring. Parthenitae that had a defined body cavity but lacked free-germ balls and later-stage embryos were classified as non-reproductive

(soldiers), while individuals that contained at least one late-stage embryo of cercariae or parthenitae were identified as reproductives. Individuals with only early stage embryos were scored as immature reproductives. For all statistical analyses, we included immature morphs among the reproductive category, unless otherwise stated.

Results

We processed 1,602 individuals from eight trematode species from the California horn snail, including three colonies of PROB, two of SMMI, six of REBU, two of REPO, five of MESO, two of SMCY, two of AUST, and four of CATA. Patterns were consistent among colonies, and statistical analyses include data from all colonies. However, for illustrative purposes, we provide figures in the main text for one representative colony of each species. Additional figures and detailed data are in the Supplementary material (Figures S1-S8, Table S1).

No morphological dimorphism was observed among colony members of the seven trematode species with sporocyst parthenitae. The size-frequency distributions of the colonies had a single mode (Fig 2, 3), and small and large sporocysts had similar morphologies. Additionally, all sporocysts examined contained either developing embryos or cercariae at different stages of maturation.

Similarly, there is no evidence of a soldier caste in CATA, the only redia species examined here. All the rediae had free germ balls, developing embryos, or cercariae at various stages of development. In contrast to redia species with soldiers, the size-frequency distributions of CATA colonies had a single mode (Fig 3), and small and large rediae had similar morphologies. The average size of the

pharynx across colonies was 70,555 ± 31,780 μm^3, and average pharynx to body ratio was 0.0067± 0.0039 (Fig 3, 4). The pharynx to body ratio of smaller individuals did not differ from that of larger ones. No pharynx to body ratios fell within the range of values reported for soldiers of species with a reproductive division of labor (Fig 4).

Some of the parthenitae had only early stage embryos. These individuals were identified as immature reproductives. Immature parthenitae were uncommon in colonies of all species. Among colonies, the average (and range) proportion of immatures among all parthenitae sampled was 0.86% (0-1.43%) for PROB, 0.61% (0-1.22%) for SMMI, 0.57% (0-2.30%) for REBU, 1.85% (0-7.14%) for REPO, 3.90% (0-7.41%) for MESO, 1.11% (0-1.89%) for SMCY, 1.75% (0-2.94%) for AUST, and 0.45% (0-1.75%) for CATA.

Parthenitae from PROB, SMMI, REPO, SMCY and AUST were recovered from the basal visceral mass and gonad regions. Parthenitae from REBU, MESO and CATA only occurred in the mantle and the basal visceral mass of the infected snails.

AUST was only recovered from snails that were co-infected with HIMA. The results for these two HIMA colonies are provided in the Supplemental Material (Fig. S8, Table S1), and the more detailed analyses of colony structure for this species can be found in Garcia-Vedrenne et al. (2016).

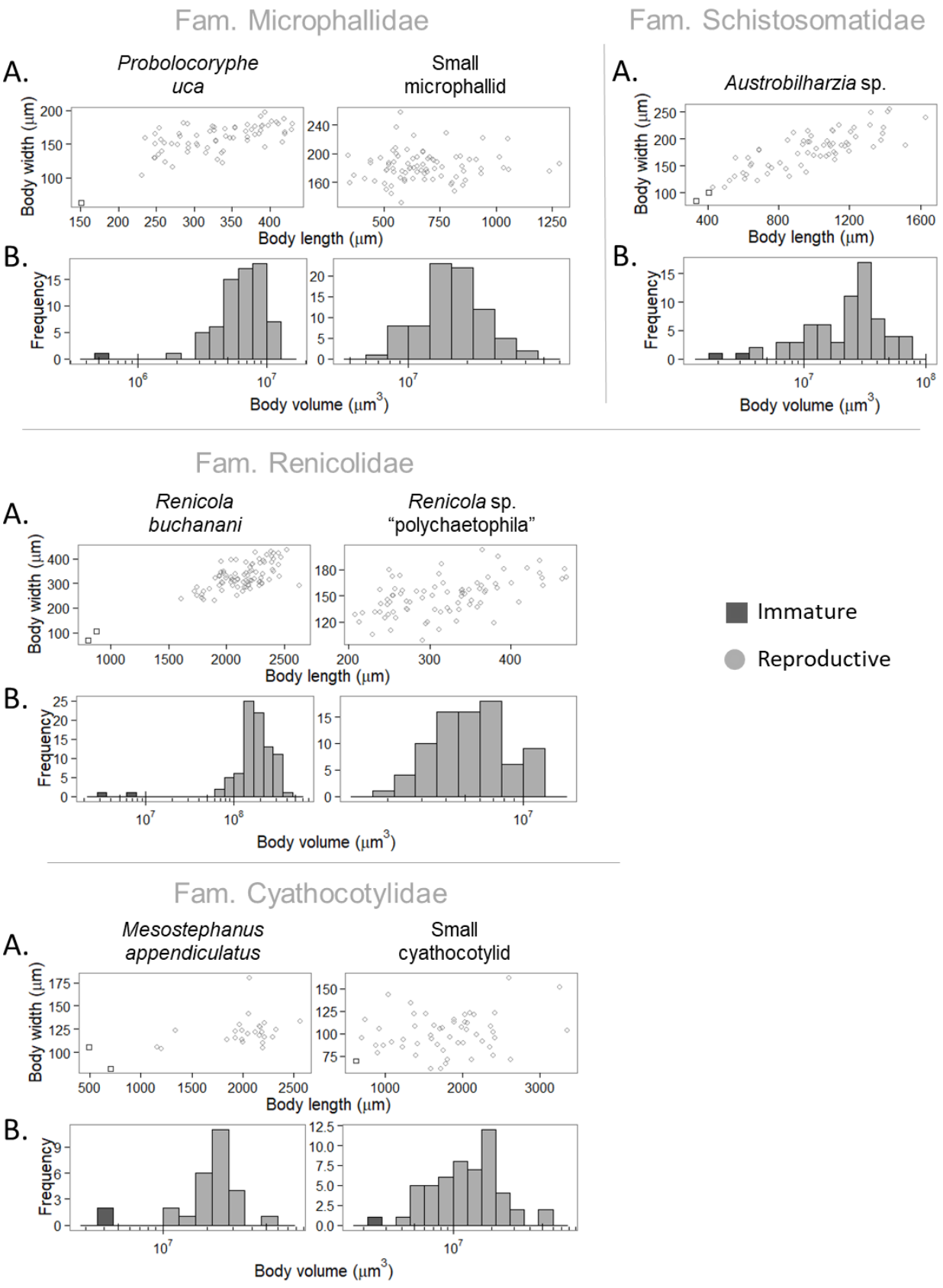
Fam. Microphallidae
Fam. Schistosomatidae
Fam. Renicolidae
Fam. Cyathocotylidae
A.
B.
Probolocoryphe uca
Small microphallid
Austrobilharzia sp.
Renicola buchanani
Renicola sp. "polychaetophila"
Mesostephanus appendiculatus
Small cyathocotylid
Body width (µm)
Body length (µm)
Frequency
Body volume (µm3)
Immature
Reproductive

Figure 2. Morphological attributes of sporocyst species that infect the California horn snail. Data for figures are of a single, representative colony, but statistical analyses used data from all colonies and Supplemental Material includes figures for each colony examined. Each point represents a randomly sampled sporocyst from a single trematode colony. (A) Body length to body width relationships. (B) Frequency distributions of body volume for randomly sampled sporocysts. Note the log10 scale of X-axes.

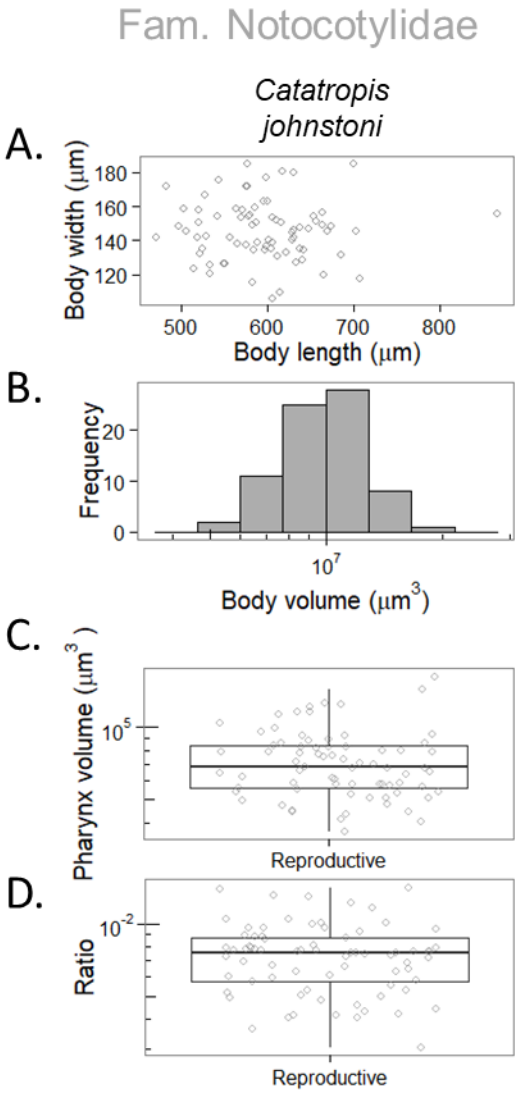

Figure 3. Morphological attributes of redia morphs of *Catatropis johnstoni* (CATA). Data for figures are of a single, representative colony, but statistical analyses used data from all colonies and Supplemental Material includes figures for each colony examined. Each point represents a randomly sampled redia from a single trematode colony. (A) Body length to body width relationships. (B) Frequency distributions of body volume for randomly sampled rediae. Note the $\log_{10}$ scale of X-axes. (C-D) Boxplots indicating median (line), interquartile range (box) and values that are within 1.5 * interquartile range (whiskers) of data for (C) absolute pharynx volume and (D) pharynx volume relative to body volume. Note the $\log_{10}$ scale for Y-axes.

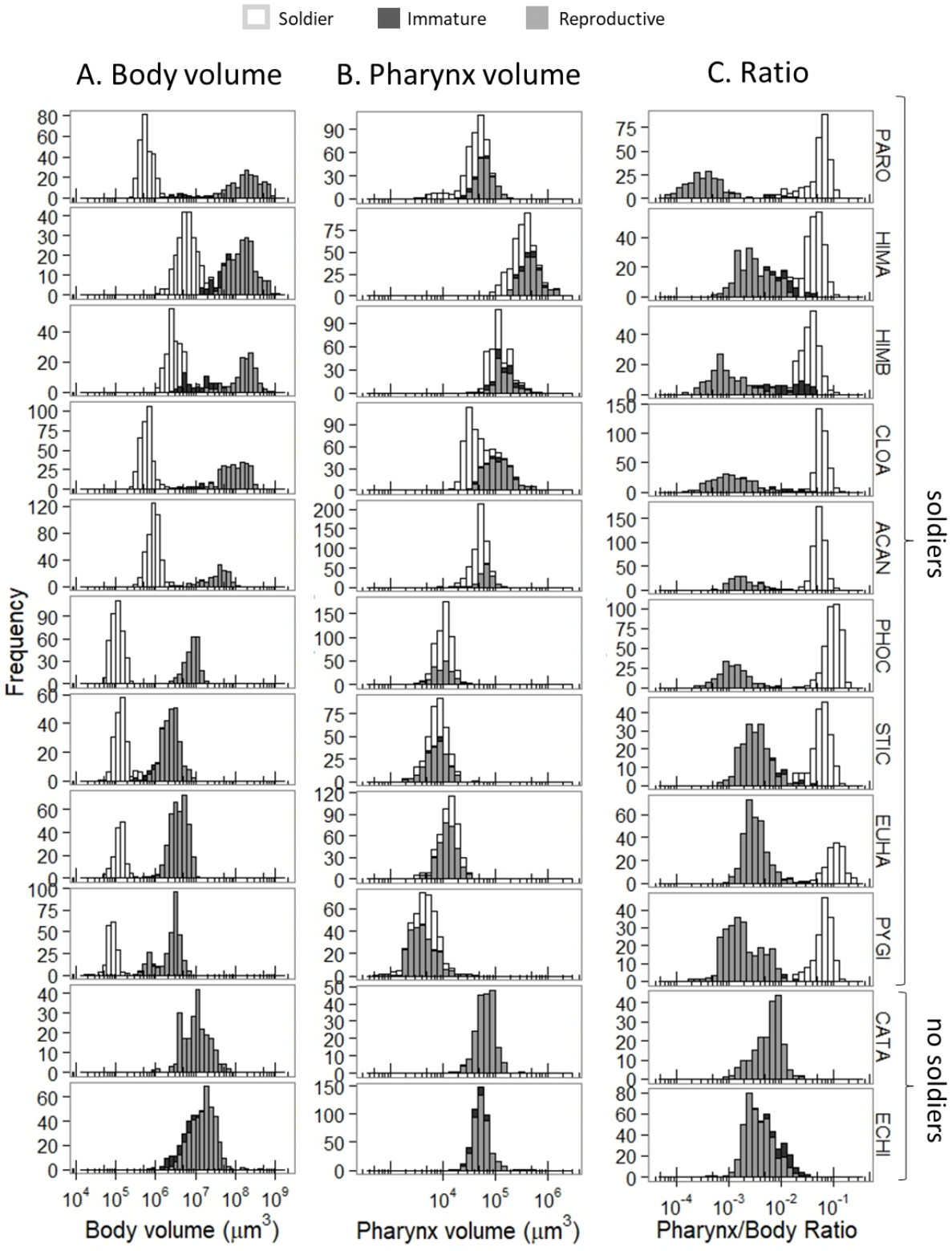

Figure 4. Variation in (A) body volume, (B) pharynx volume, and (C) pharynx/body ratio across redia species with and without soldiers. Figures include the pooled results of all redia colonies examined from the California horn snail and *Echinostoma liei* from

experimental infections in *Biomphalaria glabrata* (data for all but CATA obtained from Hechinger et al (2011) and Garcia-Vedrenne et al. (2016; 2017)). Abbreviations stand for species names- PARO: *Parorchis acanthus;* HIMA: *Himasthla rhigedana;* HIMB: *Himasthla* sp. B; CLOA: *Cloacitrema michiganensis;* ACAN: *Acanthoparyphium spinulosum;* PHOC: *Phocitremoides ovale;* STIC: *Stictodora hancocki;* EUHA: *Euhaplorchis californiensis;* PYGI: *Pygidiopsoides spindalis;* CATA: *Catatropis johnstoni;* ECHI: *Echinostoma liei.*

Discussion

Because they lack feeding structures (mouth, pharynx, gut, etc.) and are often immobile, sporocyst parthenitae cannot use predation to defend the colony. Therefore, by analyzing sporocyst species, we can characterize colony structure for trematode species lacking a soldier caste. Those patterns can then inform our interpretation of colony structure for redia species that lack soldiers. We found that the colony structure characterizing seven sporocyst species largely parallels colony structure for two redia species that lack soldiers. Further, colonies of all the examined soldier-less species share several traits that greatly differ from species with soldiers. First, soldier-less colonies are characterized by unimodal size-frequency distributions, with few small and large individuals. Second, even the smallest colony members are actively reproducing and contain developing embryos or late-stage cercariae. Third, individuals of all sizes have similar morphologies and the redia species lack pharynx-body size dimorphism. Finally, parthenita distribution is restricted to the main infection locus.

Sporocysts species do not have soldiers

In contrast to what has been seen for colonies with soldiers, colony structure for sporocyst species revealed unimodal size-frequency distributions. Close

examination showed that all colony members were actively reproducing: most parthenitae contained at least some late stage embryos, although immature reproductives harboring only early stage embryos were also found. On average, immatures ranged from 0.57–3.90% of the individuals in a colony across sporocyst species.

Sporocysts generally lack defense structures and are subordinate in trematode dominance hierarchies (Lim & Heyneman 1972; Kuris 1990; Fernandez & Esch 1991; Soldanova et al. 2012). This is true for six of the seven such species studied here (PROB, SMMI, REBU, REPO, MESO, and SMCY). Careful search for morphological differences between small and large sporocysts did not detect differences in shape, nor the presence of other structural defenses. A literature search for any documentation of sporocyst defense structures revealed that the schistosome *Trichobilharzia cameroni* has small individuals with spines that are lost as the sporocyst matures (Wu, 1953). The spines may be used in migrating through host tissues (Wu, 1953). Because those small individuals contained developing embryos (see Fig. 15 in Wu, 1953), they are immature sporocysts, not a non-reproductive caste. Hence, most sporocyst species are low in interspecific dominance hierarchies and lack soldiers.

A few sporocyst species are dominant in trematode dominance hierarchies (Walker 1979; Kuris 1990). AUST, for example**,** has sporocysts, but is effectively a dominant species because it is not eliminated by any of the species with large rediae (Kuris 1990). In fact*, Austrobilharzia* species may generally be obligate secondary invaders (Walker 1979; Kuris 1990). Species in this genus may only

become established if the defense mechanisms of the snail have been ‘switched off’ by another species of trematode (Walker 1979). The AUST colonies examined here were in co-infections with HIMA (Fig. S7). Based on the appearance of the HIMA colony, it is likely that AUST was slowly suppressing the development of HIMA rediae, similar to the impact of *A. terrigalensis* on other trematodes (Walker 1979; Appleton 1983). Given that all the sporocysts contained developing embryos, it appears that this trematode is able to suppress competitors by a mechanism other than the use of a specialized caste. Apparently, even sporocyst species at high positions in dominance hierarchies lack a soldier caste.

Small and large individuals of all sporocyst species were restricted to the colony’s main locus. These distributions are contrary to the dispersion documented for trematodes with soldiers, where a substantial number of soldiers are found in areas away from the colony locus.

Redia size-frequency distributions and reproductive status

Redia species may also lack a soldier caste, and the patterns observed for such redia colonies are comparable to those observed for sporocysts. The CATA colonies examined here have unimodal size-frequency distributions and all rediae have developing embryos inside of them; only one of the four colonies examined had immature rediae present. *Echinostoma liei*, in long-term laboratory culture, exhibited a similar pattern (Garcia Vedrenne et al. 2016). Other studies have partially documented comparable patterns for other rediae species. Gonchar & Galaktionov (2017) examined a notocotylid, *Tristriata anatis* with a unimodal size distribution for rediae. Likewise, in her undergraduate thesis, Zikmundová (2011)

reported a unimodal size distribution for rediae of an echinostomoid, *E. nasincovae* (= *E. spiniferum*) that naturally infected the freshwater snail *Planorbarius corneus*. In both cases, “young rediae” contained germinal balls and embryos and were scarce. Hence, these species likely lack a soldier caste.

Concerning species that have a bimodal size-frequency distribution, a purported alternative to the soldier caste hypothesis is that such a pattern could be explained by 1) a constraint on parthenita growth (whereby young parthenitae initially undergo cellular proliferation, resulting in very little growth for a period of time, followed by a rapid increase in size via cellular extension), and 2) small parthenitae experiencing developmental arrest, thus resulting in reduced reproduction (Galaktionov et al. 2015). This hypothesis does not counter the evidence that those small individuals are soldiers, particularly that they attack enemies at much greater rates than reproductives. Moreover, the gradual increase in parthenita size observed for the species that lack a soldier caste, and the lack of small parthenitae undergoing developmental arrest reject the developmental constraint hypothesis. There is no universal constraint on parthenita growth that yields a bimodal size-frequency distribution. Bimodal distributions occur only when a soldier caste is present. Thus, the small individuals evaluated as soldiers by Hechinger et al. (2011), Leung and Poulin (2011), Miura (2012), Nielsen et al. (2014), and Garcia-Vedrenne et al. (2016 and 2017) are not just a “reservoir” of individuals that will eventually produce cercariae. They represent a specialized trematode caste that has foregone reproduction to defend the colony.

Rediae defense structures

Morphological differences among castes frequently underlie caste function (Oster & Wilson 1978). For many social animals, allometric growth differences can lead to individuals of a colony having distinctive body shapes and proportions. Such physical differences allow individuals in the colony to perform their specific roles more efficiently.

For species with rediae, pharynx size is an important correlate with a species' ability to kill heterospecifics (Kuris, 1990). In species with a soldier caste, small individuals have a large pharynx relative to the size of their body (Fig 6), presumably enhancing their ability to perform their defensive role. In CATA colonies, however, the smaller individuals had a relatively small pharynx (Fig 4D, S1D). We note that the rediae of CATA and *E. liei* (Garcia-Vedrenne et al. 2016) lacked pharynx:body volume ratios within the range characteristic of soldier rediae (Fig. 5). Thus, soldier-less species have monophasic growth patterns, whereas species with a soldier caste have diphasic allometric growth or almost complete dimorphism (see Wilson 1953). This variation in allometric growth curves further highlight the importance of pharynx size in antagonism and the usefulness of relative pharynx sizes to identify species with a soldier caste.

Colony locus and soldier distribution

For trematodes infecting the California horn snail, CATA is the only redia species that lacks soldiers and also the only redia species that has a colony locus in the mantle. This may represent an alternative strategy to soldier investment that still allows a species to escape being killed by some invaders. Notably, the confamilial

T. anatis does not develop in the mantle (Gonchar & Galaktionov, 2017), but it appears to also lack a soldier caste. Similarly, colonies of *E. liei* develop in the visceral mass and ovotestis of *Biomphalaria glabrata* (Garcia-Vedrenne et al. 2016). Thus, even if CATA's use of mantle tissue is related to its lack of soldiers, such a strategy is not the only explanation for why some rediae species might lack soldiers.

The distribution patterns of soldier-less rediae colonies show that, despite being mobile, the younger reproductive rediae never disperse. This is contrary to what is seen for species with soldiers, were rediae occupy the gonad as their preferred location (Hechinger et al. 2009), but small soldier rediae also frequently occur in the anterior parts of the snail (Hechinger et al. 2011; Garcia-Vedrenne et al. 2016; Garcia-Vedrenne et al. 2017). Galaktionov et al. (2015) have proposed that such distribution patterns are merely a result of age-related differences in feeding preferences and activity levels that characterize trematode species with rediae. If "soldiers" were simply mobile, young rediae that prefer active predation on tissues while older, sluggish rediae absorb nutrients through tegument, one would expect most rediae species to have similar distribution patterns. However, the small parthenitae of both sporocyst and redia species that lack soldiers were restricted to the colony locus. Therefore, parthenita developmental stage is not the sole factor driving the distribution patterns in colonies with soldiers. Rather, the dispersal of most soldiers from the main colony locus suggests that their distribution underlies their caste function. Peripheral areas are often sites of invasion by other trematodes (Hechinger et al. 2011). Thus, the spatial patterns provide evidence of surveillance against invaders by members of a soldier caste.

Conclusion

Here we have laid out the patterns underlying colony structure for trematode species that lack a soldier caste and contrasted them to those characterizing species with soldiers. Demographic, morphological, and distributional patterns reliably distinguish colonies with and without caste systems. The documentation of easy to measure aspects of colonies that reflect functional caste differences indicates that we may use those attributes as “markers” of the existence of a soldier caste. There are at least 20,000 trematode species that encompass substantial life-history and ecological variation, many of which are ecologically and medically important. The relatively rapid identification of a soldier caste will facilitate future research on the occurrence and strength of social organization among trematodes.

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Chapter 5

In vitro systems for intramolluscan stages of trematodes—will the trick used for freshwater systems also work for marine ones?

In vitro systems for intramolluscan stages of trematodes—will the trick used for freshwater systems also work for marine ones?

Abstract

Is it possible to follow the success of freshwater *in vitro* methods and use snail cell lines for marine trematode culture? Two findings from freshwater systems using the *Biomphalaria glabrata* embryonic (Bge) cell line suggest a possible approach. First, co-cultivation with Bge cells is not necessary for culture success; media that have been only preconditioned with Bge cells has permitted trematode growth and development. Second, the Bge cell line works even for trematodes that use freshwater snails other than *B. glabrata* as first intermediate host. Hence, we asked whether the Bge cell line might also promote the *in vitro* survivorship and development of marine trematode parthenitae. Here we report primary *in vitro* culture of rediae of *Euhaplorchis californiensis* (Heterophyidae), *Himasthla rhigedana* and *Himasthla sp. B* (Echinostomatidae) infecting the California horn snail, *Cerithideopsis californica (=Cerithidea californica),* (Potamididae). Survivorship was evaluated both quantitatively and qualitatively under five experimental treatments i) sterile sea water, ii) the previously described marine L-15 medium, iii) Bge-conditioned marine-adjusted medium, iv) Bge (non-conditioned) marine-adjusted medium and v) Co-cultivation with Bge cells at fresh-water osmolarity. To determine survivorship, parthenitae were counted and classified as alive or dead based on tegument integrity, movement, and shape. Video footage was used in blind trials to qualitatively gauge parthenitae activity *in vitro*. Our results indicate that trematodes infecting the California horn snail can be cultured *in vitro* in

the L-15 medium that had been previously used for other marine trematodes. However, rediae survival and performance can be improved by the presence of Bge cells or Bge released factors. Rediae do not appear to consume Bge cells but are able to eat rediae and cercariae of heterospecific species. Even though cultured rediae (both reproductive and soldier castes) were mobile and healthy looking, we rarely observed progeny rediae or cercariae being released. This is the first reported cultivation of marine trematodes that includes co-culture with Bge cells and media with Bge factors. Our results highlight the promise of using these methods for the cultivation of marine trematodes.

1. Introduction

In vitro systems for parasitic trematodes have long been a valuable tool for parasitology research (reviewed by Coustau and Yoshino, 2000). With them, it is possible to answer questions about the development and differentiation of parasites at the biochemical and physiological level, and gain insight into complex host-parasite interactions such as mechanisms of immunosuppression and pathogenesis. Because most trematodes of medical and veterinary importance infect pulmonate snails (*Biomphalaria, Lymnaea, Bulinus*), efforts to develop *in vitro* systems have focused on freshwater systems rather than marine ones.

However, the development of culture systems for trematodes infecting marine snails is also of interest. Many marine trematodes have relatives infecting pulmonate hosts, and the development of tools for the study of marine snails and their trematodes can provide insight into how properties of snail defense systems

might differ (Yakovleva et al., 2001). Further, the development of appropriate culture techniques could provide valuable tools needed to broaden our understanding of several aspects of trematode complex sociality, currently described for marine species only (i.e. Hechinger et al., 2011; Miura 2012; Garcia-Vedrenne et al. 2016; Garcia-Vedrenne et al. 2017). Suitable *in vitro* systems would allow us to ask questions concerning trematode sociality such as the fundamentals of interspecific interactions and the mechanics underlying caste dynamics and regulation in mature colonies.

To our knowledge, only two published studies have aimed to develop *in vitro* culture methods for marine trematodes. In 2002, Gorbushin & Shaposhnikova designed an axenic system for the maintenance of rediae of *Himasthla elongata*, which infect the marine snail *Littorina littorea*. This technique uses sterile seawater enriched with the commercially available Leibovitz's L-15 medium, adjusting osmolarity as needed for marine invertebrates. This technique supported *H. elongata* rediae survival for up to 163 days *in vitro*. A similar technique was used by Lloyd & Poulin (2011). Five trematode species that infect the marine snails *Zeacumantus subcarinatus, Diloma subrostrata,* and *Cominella glandiformis* survived from 8 to 56 days in this medium. Although the osmotically-adjusted Leibovitz's L-15 medium appears to permit parthenita survival, there is currently a lack of a successful, long-term media for *in vitro* culture of marine trematodes that allow continuous propagation of the colony.

Continuous colony propagation has been achieved for fresh-water snail-trematode systems. Following years of effort, highly successful co-cultivation

methods were finally achieved for freshwater systems. Rediae were co-cultured with cells derived from embryos of an albino strain of *Biomphalaria glabrata* snails, which acts as first intermediate host for *Schistosoma mansoni* (Hansen, 1976). Incorporation of this embryonic cell line ("*Bge* cell line") into culture media was key to permit continuous propagation of freshwater trematodes (reviewed by Coustau & Yoshino, 2000).

Is it possible to follow the success of freshwater *in vitro* methods and use snail cell lines for marine trematode culture? Unfortunately, despite much effort, no marine snail cell lines have been successfully developed (Yoshino et al., 2013). However, two findings from freshwater systems using the *Bge* cell line suggest an alternative approach. First, co-cultivation with *Bge* cells is not necessary for culture success; media that have been only preconditioned with *Bge* cells has permitted parthenitae growth and development (Yoshino & Laursen, 1995). Second, the *Bge* cell line works even for trematodes that use freshwater snails other than *B. glabrata* as first intermediate host (e.g. Coustau et al. 1997; Laursen & Yoshino, 1999). Hence, we asked whether the *Bge* cell line might also promote the *in vitro* survivorship and development of marine trematode parthenitae.

Here we describe cultivation of marine trematodes that includes both co-culture with Bge cells and media containing Bge factors. We report primary *in vitro* culture of rediae of *Euhaplorchis californiensis* (Heterophyidae), *Himasthla rhigedana* and *Himasthla sp. B* (Echinostomatidae) infecting the California horn snail, *Cerithideopsis californica (=Cerithidea californica),* (Potamididae). We cultured both soldier and reproductive castes of these species to address the following questions:

1) How well do trematodes infecting the California horn snail survive *in vitro* using the L-15 medium and conditions previously described for other marine trematodes?

2) Is redia survival and performance improved by using media containing or conditioned with Bge cells?

3) Are there intraspecific and interspecific differences in survival?

4) Under any of these *in vitro* conditions, do rediae actively produce offspring (new rediae or cercariae)? If so, over what time interval?

5) Can Bge cells or predation on rediae and cercariae of the same or different species serve as nutrient sources for rediae cultured *in vitro*?

2. Materials and Methods

2.1 Study System and Sample Collection

California horn snails were collected between March 2010 and May 2016 from Carpinteria Salt Marsh, Santa Barbara County, California, USA. Snails were maintained in the laboratory for up to two weeks in mesh bags on running sea water tables. Snails were screened for infections by *Euhaplorchis californiensis* (EUHA), *Himasthla rhigedana* (HIMA) and *Himasthla* sp. B (HIMB). Snails were removed from running sea water for 2 days. Then, they were individually placed in wells with filtered seawater and exposed to incandescent lamps for 2-3 hours to stimulate release of cercariae. Cercariae were examined and identified to species according to Martin (1972) and additional unpublished observations.

2.2 Rediae isol*a*tion

Prior to snail dissections, the work area and dissection tools were carefully cleaned and wiped down with 70% ethanol. Snail dissections and parasite isolation were performed in the aseptic area created around the flame of a Bunsen burner. Snails' shells were wiped with 70% ethanol then placed into a sterile petri dish. Each snail was dissected by gently cracking the shell with a hammer and dividing the body into two distinct regions: mantle/ basal visceral mass and gonad/digestive gland. We briefly submerged the two body segments for two seconds in 70% ethanol to kill any bacteria present on the outside tissue, and then transferred them to separate sterile petri dishes containing sterile sea water (SSW; see below). The gonads and digestive gland were teased apart to release rediae from the surrounding tissue. The pool of rediae sampled from one host individual was termed "colony". Individual rediae were identified as being a member of either the soldier or reproductive caste (Hechinger et al., 2011; Garcia-Vedrenne et al., 2016), and carefully transferred with a glass pipette into a separate petri dish containing colony members of the same caste. If few soldiers were retrieved from the gonad and digestive gland, the mantle/ basal visceral mass were also examined. Once enough individuals had been isolated, the dishes containing pools of reproductive and soldier rediae were rinsed three times with sterile seawater to remove any snail tissue and cercariae that might have been transferred along with the rediae. A stock solution of 100 mL SSW supplemented with 0.4mL of Streptomycin/ penicillin solution (Fisher Scientific; #BP2959-50) was added to the dishes for approximately 45 min to reduce bacterial contamination. Afterwards, rediae were transferred to 96-

well plates with concave wells for the experiments. Approximately 10-15 rediae were added to each well of a 96 well-plate with the aid of a glass pipette. Any excess SSW that was introduced with the rediae was removed before adding media to the wells. Each media treatment had 2-5 replicate wells.

During our first experiments we noted differences in redia activity and appearance during dissections, and the fact that those differences seemed to relate to early mortality. These observations resulted in a more stringent selection procedure. When selecting rediae to add to wells from the pool of available individuals, we targeted those that looked healthier. Such rediae had smooth teguments and exhibited vigorous bending movements, as well as longitudinal contractions and expansions of body and pharynx (Fig 1A). If the majority of the colony did not meet these criteria, a new snail was dissected.

2.3 Media preparation

Preliminary experiments used media that were similar in composition, pH and osmolality to those used in the culture of tissue of marine organisms (Birmelin, et al., 1999; Tucker, 1970). The medium reported by Gorbushin and Shaposhnikova (2002) for culture of marine trematodes was also tested. Out of the three media formulations, the latter was found to produce the best results (results not shown) and was therefore used in subsequent experiments to compare to the new media formulations suggested in this study.

Six different media were used throughout the experiments. All media were sterilized by filtration through 0.22-micron sterile syringe filters (Millipore) and 0.2 mL of Streptomycin/ penicillin solution were added to 100mL stock solutions.

Details on the preparation of each of these are included below:

1. Sterile sea water (SSW): Prepared with 80% natural sea water and 20% deionized water, pH adjusted to 7.8
2. Leibovitz L-15: As used by Gorbushin & Shaposhnikova (2002). Briefly, we added 0.8 g of L-15 medium (Sigma–Aldrich L-4386) to 100 mL of SSW, and adjusted pH to 7.8.
3. L-15 with FCS: 10% heat inactivated fetal bovine serum (Sigma F4135) was added to the L-15 medium described above.
4. Co-cultivation with Bge cells at fresh-water osmolarity: Bge cells were grown as described by Hansen (1976). Briefly, the Bge medium used to culture cells was prepared by combining 22mL of Schneider's Drosophila Medium (Gibco #172), 0.2 mL of phenol red, 68 mL nanopure water, 0.45 g Lactalbumin hydrolysate (Bacto™) and 0.13g of galactose (Difco™). We adjusted pH to 7.2 before adding heat inactivated fetal calf serum to 10% of total and 0.2 mL of Penincillin/Streptomycin solution. Cells were added to the 96 well-plate 2 days before the start of the experiment to allow cells to attach and to start dividing. No additional Bge cells were added once the cultures were initiated.
5. Bge (unconditioned) marine-adjusted medium: Bge medium as described above that was never in contact Bge cells; 0.936g of Instant Ocean were added to 50mL of Bge medium.

6. Bge-conditioned marine-adjusted medium: Bge medium that had been co-cultured with Bge cells for one week was collected and sterilized by filtration with 0.22-micron sterile syringe filters (Millipore). Once enough Bge-conditioned medium had been gathered, 0.936g of Instant Ocean were added to 50mL of Bge conditioned medium.

Although we took several measures to avoid contamination, both bacterial and fungal contamination were observed in some of the wells (Fig 1H). Live individuals in contaminated wells were censored and 70% ethanol was added in an attempt to stop the spread of the contamination. Replicate wells would often remain free of contamination.

2.4 Culture procedure

In a laminar flow hood, we added approximately 0.2 mL of the corresponding media to each well. Cultures were incubated at 24 C under normal atmospheric condition in an incubator (Isotemp Incubator, Fisher Scientific) and kept in the dark, except for when the condition of rediae was monitored or culture media was replaced. Monitoring of rediae health status was done twice a week using an inverted microscope. Culture media was changed once per week by removing 0.1 mL of media and replacing with approximately 1.5 mL (to account for any evaporative loss). Altogether, fourteen colonies from three different trematode species were monitored.

2.4.1 Survival in L-15 medium

We first sought to determine whether trematodes infecting the California horn snail could survive *in vitro* under the conditions that had been previously used for other marine trematodes. Rediae from two EUHA colonies (EUHA-1 and EUHA-2) and two HIMB colonies (HIMB-1 and HIMB-2) were isolated and prepared for the *in vitro* system, three plates were prepared, all of them containing rediae from the four infected snails. The plates were individually maintained at a) 15°C, b) room temperature for 5 hours a day and 15°C at night, c) room temperature (19°C). L-15 medium was used, except for the wells that contained SSW and were used as control experiments.

2.4.2 Intraspecific and interspecific variation in survival

Because we observed that in both the EUHA and HIMB colonies (especially the latter) one colony performed well and the other one did poorly under the L-15 treatment, we decided to repeat our tests with additional colonies to better examine intra- and inter-specific variation. The snails from the previous experiment were used for this analysis, and two additional EUHA colonies (EUHA-3 and EUHA-4) and two HIMB colonies (HIMB-3 and HIMB-4) were dissected and cultured in L-15 medium.

2.4.3 Co-culture with Bge cells and Bge factors

We wanted to test if rediae survival and health could be enhanced by the presence of Bge cells or Bge released factors, similar to what has been achieved in freshwater systems. One HIMB colony (HIMB-5), one EUHA colony (EUHA-5) and

one HIMA colony (HIMA-1) were used in this experiment and exposed to all six treatments described in section 2.3 (Media preparation). A second experiment was set up so that, in addition to survivorship, activity of individual rediae could be qualitatively and quantitatively assessed. One HIMB colony was used for this experiment and exposed to all treatments except L-15 with FCS. The details of this additional experiment are explained below in section 2.6 (Activity).

2.4.4 Food trial

Given that trematodes have been observed to prey on Bge cells (Loker et al., 1999) and on heterospecifics *in vitro* (Basch & Diconza, 1975; Garcia-Vedrenne et al., 2017), we wanted to test whether such nutrient sources could increase rediae performance *in vitro*. Thus, we tested performance of HIMA soldiers and reproductives under different food regimes. These included a control of no food (rediae were cultured in Bge conditioned medium), Bge cells as food source, or heterospecifics of EUHA as food source. In this case, roughly ten EUHA reproductives were added every time the media was changed. Procedures to obtain and isolate these reproductive followed the sterile techniques described in section 2.2 (Rediae isolation).

2.5 Survivorship

Individual rediae in each well were monitored for survival once or twice a week using a Biostar inverted microscope at 100-200x magnification and classified as alive/healthy, dying, or dead (Fig 1 and Supplementary video). Survival scores were based on three criteria: tegument integrity, body shape, and amount of

movement exhibited. Healthy rediae had smooth teguments and exhibited vigorous bending movements, as well as longitudinal contractions and expansions of body and pharynx. Dead rediae were identified by the opacity of bodies, loss of tegument integrity, and lack of movement. Living, motile cercariae inside of deceased rediae were noted on occasion. However, there was no movement of the pharynx and tegument integrity was compromised in these cases. Dying rediae were identified as such because of their rounded bodies, limited movement and wrinkled tegument. These rediae were scored as dying but ultimately included in alive counts, as they would sometimes recuperate when media were changed.

2.6 Activity

Although rediae had similar survival rates in all experimental treatments (see results), qualitative observations indicated that overall health was lowest in L-15 medium and highest in the media associated with Bge cells (Bge cells co-culture (FW) and Bge conditioned (SW)) (as determined by the integrity of the tegument and motility). The activity analysis was designed to test whether the apparent differences in health status between rediae in L-15 medium those in media associated with Bge cells (Bge cells (FW) and Bge conditioned (SW)) could be reliably quantified.

For the second part of experiment 2.4.3 (Co-culture with Bge cells and Bge factors), ten seconds of video footage was taken of each well once a week by attaching a digital camera (MU500, Amscope) to the inverted microscope. Each video was blindly scored for parthenita activity (Fig 1H) by each of four people (AD,

GH, IC and AG) using the rubric provided as Table 1 and instructions provided in Supplementary video 1.

Table 1. Scoring rubric for redia health ranging from 4 (very active and healthy) to 0 (dead) based on movement, behavior, tegument integrity and shape.

Reproductive rediae						
Score	**State**	**Body**	**Pharynx**	**Opacity**	**Tegument**	**Cercariae**
4	Healthy, thriving	Bending/ contracting	Extending/ contracting	Clear or brightly colored	Smooth	Indicate if moving, but does not affect score
3	Healthy, surviving	Bending/ contracting	Extending/ contracting	Clear or brightly colored	Wrinkled	Indicate if moving, but does not affect score
2	Dying	Some rounding	Extending/ contracting	Either	Wrinkled	Indicate if moving, but does not affect score
1	Near dead	Rounded	Slight movement	Opaque	Wrinkled	Indicate if moving, but does not affect score
0	Dead	Rounded	No movement	Opaque	Crumbling	Be aware that some rediae might appear to move because of wiggling cercariae inside. If the pharynx is not moving, mark as dead.
Soldier rediae						
Score	**State**	**Body**	**Pharynx**	**Opacity**	**Tegument**	**Behavior**
4	Healthy, thriving	Bending, extending, contracting		Clear	Smooth	Interacting with other soldiers
3	Healthy, active	Bending, extending, contracting		Clear	Smooth	Active
2	Less active	Not bending, but extending/contracting		Clear	Smooth	Less active
1	Near dead	Barely active	Slight movement	Opaque	Wrinkled	Barely moving
0	Dead	Inactive	No movement	Opaque	Crumbling	

2.5 Statistical analysis

The R survival package (Therneau 2013) was used to construct Kaplan-Meier curves and conduct statistical analyses. Kaplan Meier survival curves were

generated for each set of treatments. Individuals in wells that became contaminated or that were still alive when the experiment ended were censored. We calculated mean survival time for each species and treatment combination. Note, however, that mean survival time will depend on what value is chosen for the maximum survival time. By default, this assumes that the longest survival time is equal to the longest survival time in the data. Hence, we have included the restricted mean upper limit in all our statistics. A log-rank test was used to determine if there was a difference between two or more survival curves.

3. Results

Generally, soldiers and reproductives derived from the same colony had similar survivorship (though we do note that soldiers exhibited a slightly higher survival rate; results not shown). For simplicity, we have combined reproductives and soldiers for most of our results. The one exception was observed in HIMA colonies in the food trial, where soldiers far outlived reproductives. Results for the food trials report survivorship for soldiers and reproductives separately (see Section 3.4).

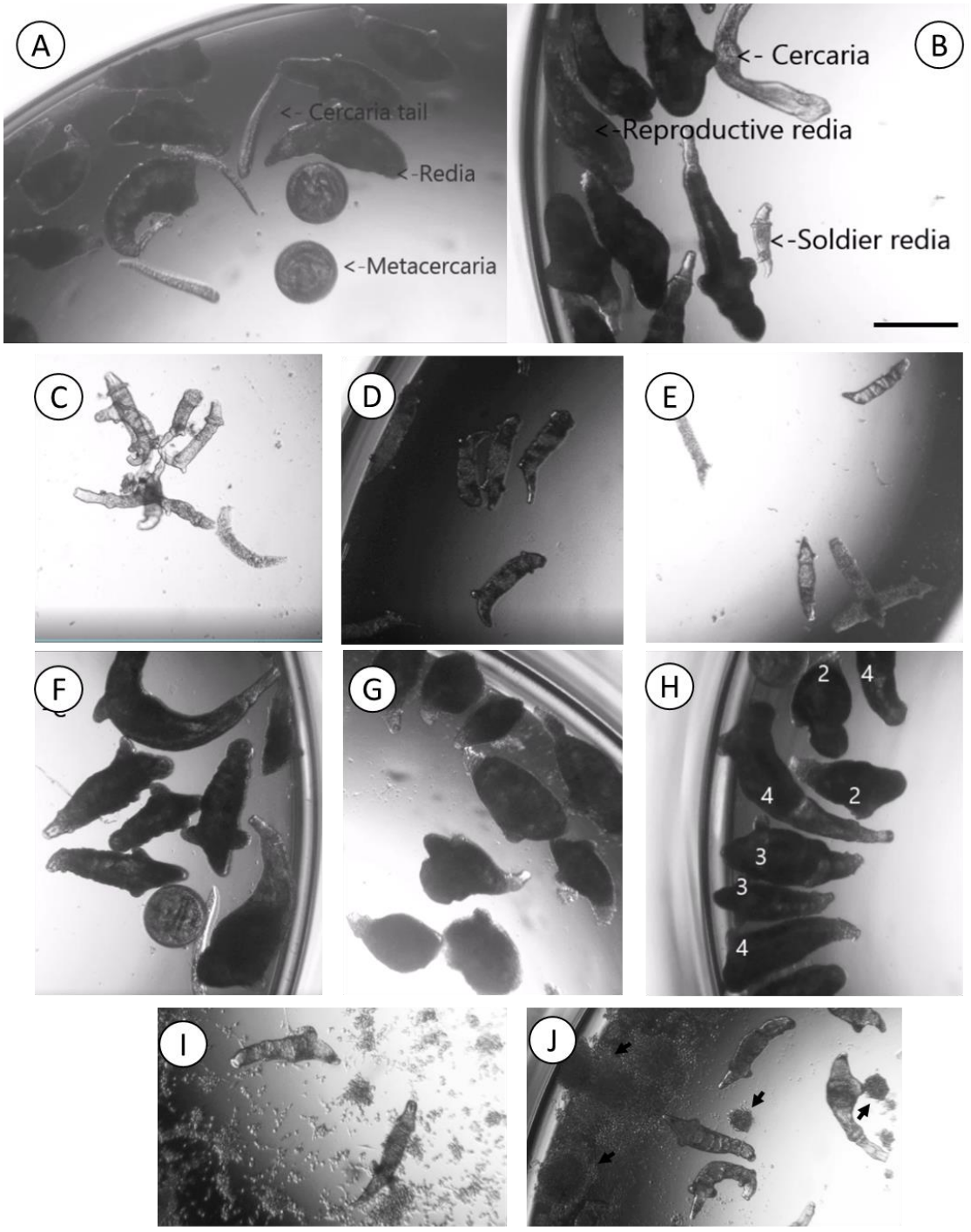

Figure 1. Photographs of *Himasthla* sp. B in culture A) Reproductive rediae and encysted cercariae (metacercariae) with loose tail; B) Reproductive rediae, soldier redia and cercaria body; C) Healthy soldier rediae with smooth teguments, bending movement and longitudinal contractions and expansions of body and pharynx and attached by posterior end; D) Dying soldiers with opaque bodies and limited motility; E) Dead soldiers; F) Healthy reproductive rediae with smooth teguments, bending movement and longitudinal contractions and expansions of body and pharynx; G) Dying rediae with rounded bodies,

limited movement and wrinkled tegument; H) Example of rediae being scored during activity analysis; I-J) Soldiers co-cultured with Bge cells where I) shows Bge cells growing normally and J) arrows indicate clusters of cells post-addition of rediae with some sterile sea water (SSW). Scale bar = 500µm

3.1 Survival in L-15 and intra- and inter-specific variation

Our first experiment was designed to test whether HIMB and EUHA could survive in the media formulation that had worked for other marine trematodes (Gorbushin & Shaposhnikova, 2002; Lloyd & Poulin, 2013). Rediae in sterile sea water (SSW) control cultures were initially motile and releasing cercariae. Within a week, they were much less motile, and their tegument had become opaque and rough instead of clear and smooth. Most individuals were dead within 15-25 days (Figs 2A, 3).

On the other hand, rediae in the Leibovitz L-15 (as used by Gorbushin & Shaposhnikova (2002)) had higher survival rates (Figs 2, 3). Rediae routinely survived for longer than 30 days in culture, up until experiments were terminated. The most robust colony had >80% of rediae surviving after 50 days of cultivation. However, there were differences in survival rate both within species (Figs 2B and 2C; Table 2, 3) and across species (Figs 2, 3, Table 2, 3). For example, mean survival estimates ranged from 8 to 45 days for HIMB colonies and 7 to 26 days for EUHA colonies.

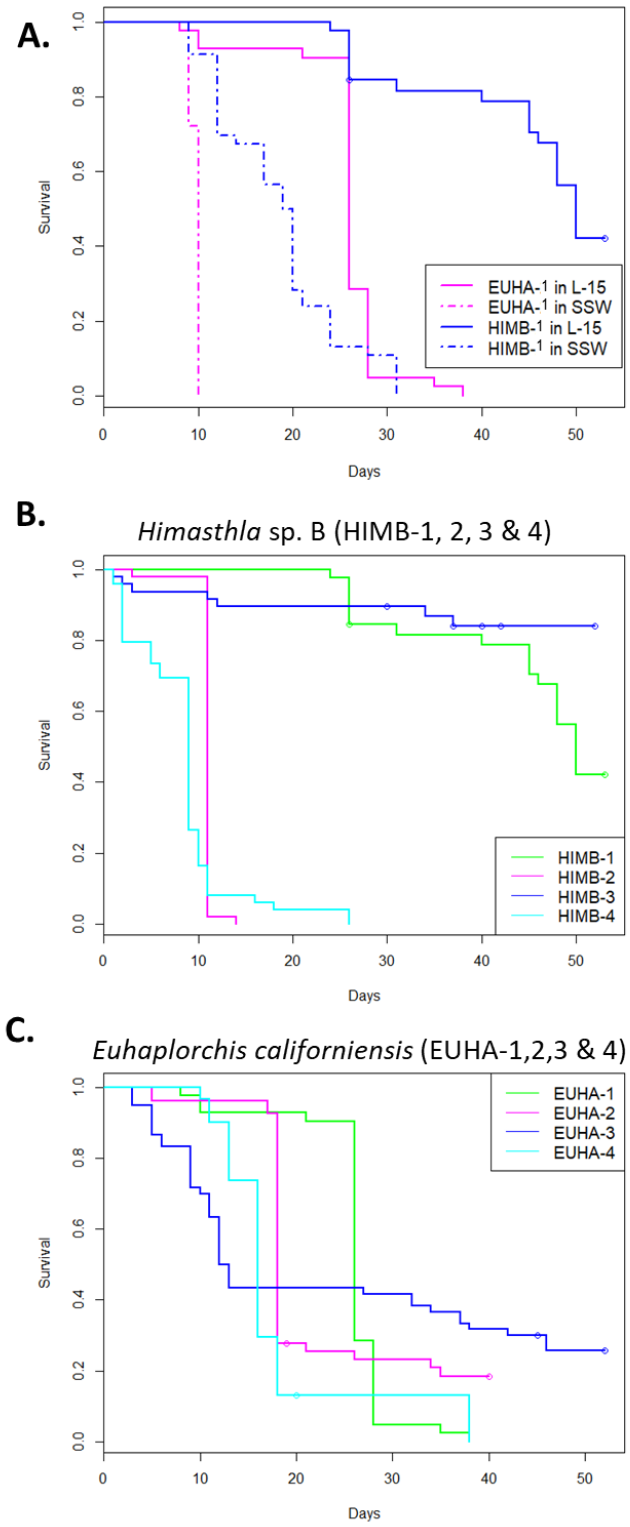

Figure 2. Survival curves for A) representative colonies of *Himasthla* sp. B and *Euhaplorchis californiensis* in the control treatment with sterile sea water (SSW) and Leibovitz L-15 (previously used for marine trematodes); B) four different HIMB colonies and C) four different EUHA colonies all maintained in L-15 medium at 15 C.

Table 2. Survival of rediae in sterile sea water and Leibovitz L-15 medium and statistics on intra- and interspecific variation in survival rates.

Experiment	Species ID	Treatment	N total	N dead	*Restricted mean	*Std Error	*Restricted mean upper limit	Log-rank test
Survival in L-15 medium	EUHA-1	L-15	42	42	25.6	0.8	34.5	χ_3^2= 228 p<0.0001
	EUHA-1	SSW	36	36	9.7	0.1		
	HIMB-1	L-15	45	22	33.0	0.5		
	HIMB-1	SSW	46	46	18.6	0.9		
Intraspecific Variation (all in L-15 medium)	HIMB-1	L-15	45	22	36.7	0.7	39	χ_3^2= 221 p<0.0001
	HIMB-2	L-15	48	48	10.9	0.2		
	HIMB-3	L-15	48	7	35.4	1.5		
	HIMB-4	L-15	49	49	8.5	0.7		
	EUHA-1	L-15	42	42	25.7	0.8	39	χ_3^2= 24.6 p<0.0001
	EUHA-2	L-15	54	43	22.4	1.3		
	EUHA-3	L-15	60	43	21.5	1.9		
	EUHA-4	L-15	61	55	18.2	1.0		

We noted that the starting health of the colony is an important determinant of whether the colony will survive *in vitro* or not. Colonies that died within the first couple of weeks were often already unhealthy looking during the dissection. Rediae were less motile, would become rounded in SSW rinses, their tegument was slightly more opaque, and dead rediae were encountered more frequently. Conversely, colonies that would generally perform well were characterized by motile individuals that were constantly bending, expanding and contracting their bodies and pharynx. Their tegument was smooth, and the colors were very bright and clear. Healthy colonies of HIMB would reliably survive for long periods of time. EUHA colonies, on the other hand, had a lower overall survivorship.

3.2 Techniques involving Bge cells

Ideally, rediae would be cultured along with host snail cells. Because no marine snail cell lines are available (Yoshino et al., 2013), we explored the possibility of using freshwater Bge cells. Two general approaches were used. First, rediae were co-cultured with Bge cells under freshwater conditions (Bge cells (FW)); and second, rediae were cultured in the supernatant collected after culturing Bge cells for a week and adjusting for marine conditions (Bge conditioned (SW)). These treatments were compared to redia survival in SSW, L-15, L-15 supplemented with fetal bovine serum (L-15 + FCS) and in Bge media adjusted for marine conditions but that had never been in contact with Bge cells (Bge unconditioned (SW)).

When rediae were first added to culture wells containing Bge cells (FW), some sea water was inevitably added along with them. This was removed as promptly as possible and replaced with excess Bge medium. However, this was generally enough of a stressor for the cells to detach from the well and clump (Fig 1J, 4B). The cells were able to recover and continued growing normally afterwards (Fig 1I). We did not observe Bge cells growing on ("encapsulating") either live or dead rediae.

Observations of rediae in SSW were consistent with the results described in Section 3.1. Rediae in L-15 medium with FCS deteriorated rapidly. Otherwise, survival across the remaining treatments was similar (Figs 3, 4; Table 3). Mean survival rates in L-15, Bge conditioned (SW), Bge unconditioned (SW) and Bge cells (FW) ranged from 31 to 33 for HIMB, 41 to 47 for HIMA and 6 to 9 for EUHA.

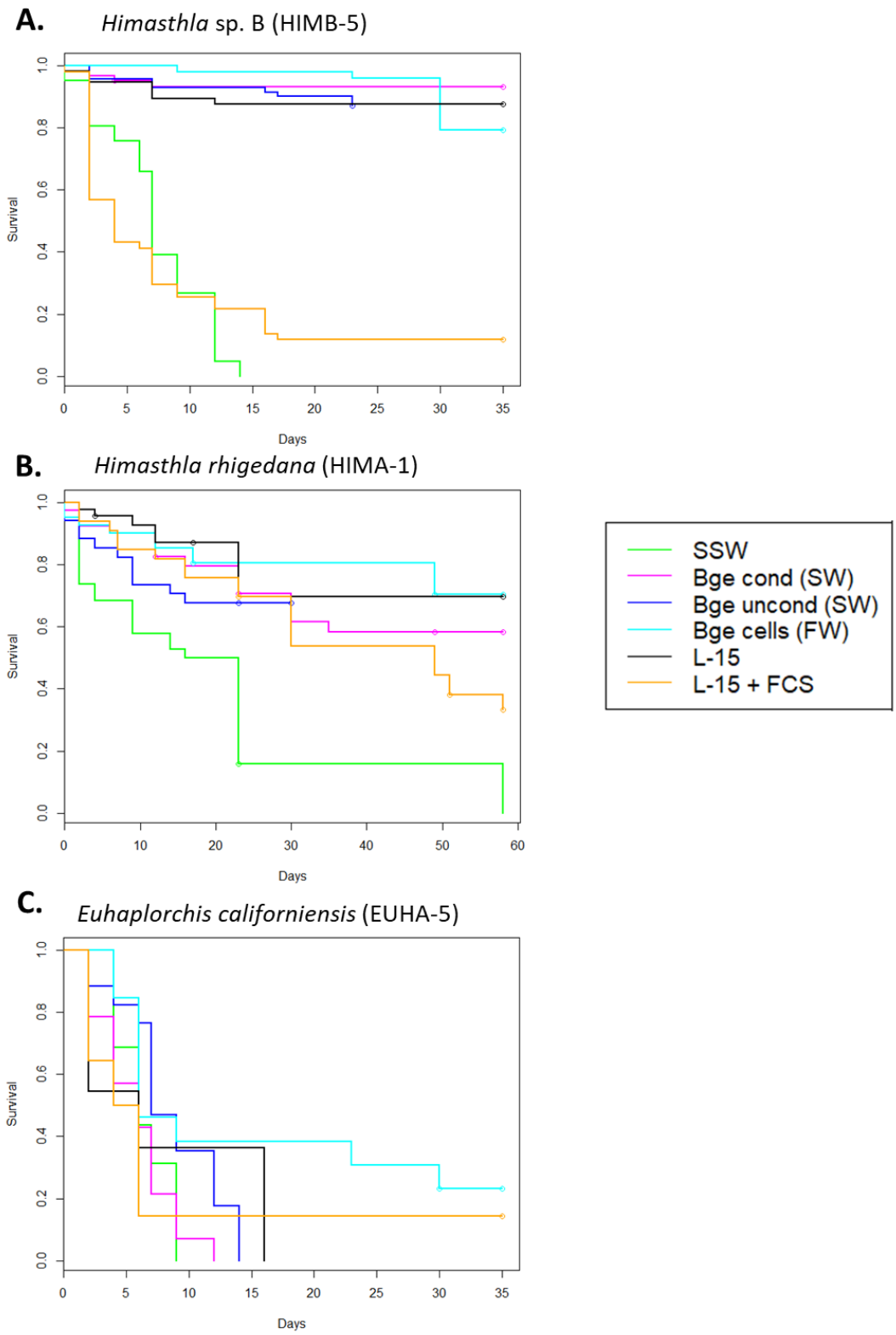

Figure 3. Survival curves for colonies of A) *Himasthla* sp. B; B) a *Himasthla rhigedana*; C) a *Euhaplorchis californiensis* in each of the six experimental treatments. Treatment abbreviations stand for i) Sterile Sea Water prepared with 80% sea water and 20%

deionized water (SSW); ii) Leibovitz L-15 medium as used by Gorbushin & Shaposhnikova (2002) (L-15); iii) 10% heat inactivated fetal bovine serum was added to the L-15 medium (L-15 + FCS); iv) Co-cultivation with Bge cells at fresh-water osmolarity (Bge cells (FW)); v) Bge medium that was never in contact Bge cells adjusted to marine conditions (Bge uncond (SW)) and vi) Bge medium where Bge cells cultured for one week and then filter sterilized to remove cells (Bge cond (SW)). Note: All rediae in the Bge unconditioned (SW) in figures A-C were censored on day 23 because of generalized bacterial contamination.

Table 3. Survival of rediae of *Himasthla* sp. B, *Himasthla rhigedana* and *Euhaplorchis californiensis* in each of the six treatments tested.

Experiment	Species ID	Treatment	N total	N dead	*Restricted mean	*Std Error	*Restricted mean upper limit	Log-rank test
Techniques with Bge cells	HIMB-5	SSW	41	41	7.4	0.6	35	χ^2_5= 283 p<0.0001
	HIMB-5	Bge cond (SW)	61	4	32.9	1.0		
	HIMB-5	Bge uncond (SW)	70	9	31.9	1.0		
	HIMB-5	Bge cells (FW)	48	10	33.4	0.6		
	HIMB-5	L-15	57	7	31.4	1.3		
	HIMB-5	L-15 + FCS	51	45	8.8	1.5		
	HIMA-1	SSW	38	36	19.9	3.0	58	χ^2_5= 63.4 p<0.0001
	HIMA-1	Bge cond (SW)	40	15	41.4	3.5		
	HIMA-1	Bge uncond (SW)	34	11	41.4	4.2		
	HIMA-1	Bge cells (FW)	41	12	47.4	3.1		
	HIMA-1	L-15	45	11	45.5	3.2		
	HIMA-1	L-15 + FCS	33	21	38.1	3.6		
	EUHA-5	SSW	16	16	6.4	0.5	15	χ^2_5= 8.4 p=0.134
	EUHA-5	Bge cond (SW)	14	14	5.8	0.8		
	EUHA-5	Bge uncond (SW)	17	17	8.5	0.9		
	EUHA-5	Bge cells (FW)	13	10	9.4	1.3		
	EUHA-5	L-15	11	11	7.5	1.8		
	EUHA-5	L-15 + FCS	14	12	5.6	1.1		
Activity analysis	HIMB-6	SSW	133	89	14.8	0.5	50	χ^2_4= 240 p<0.0001
	HIMB-6	Bge cond (SW)	134	16	45.6	1.1		
	HIMB-6	Bge uncond (SW)	129	27	41.6	1.3		
	HIMB-6	Bge cells (FW)	137	20	43.9	1.4		
	HIMB-6	L-15	126	35	37.9	1.8		

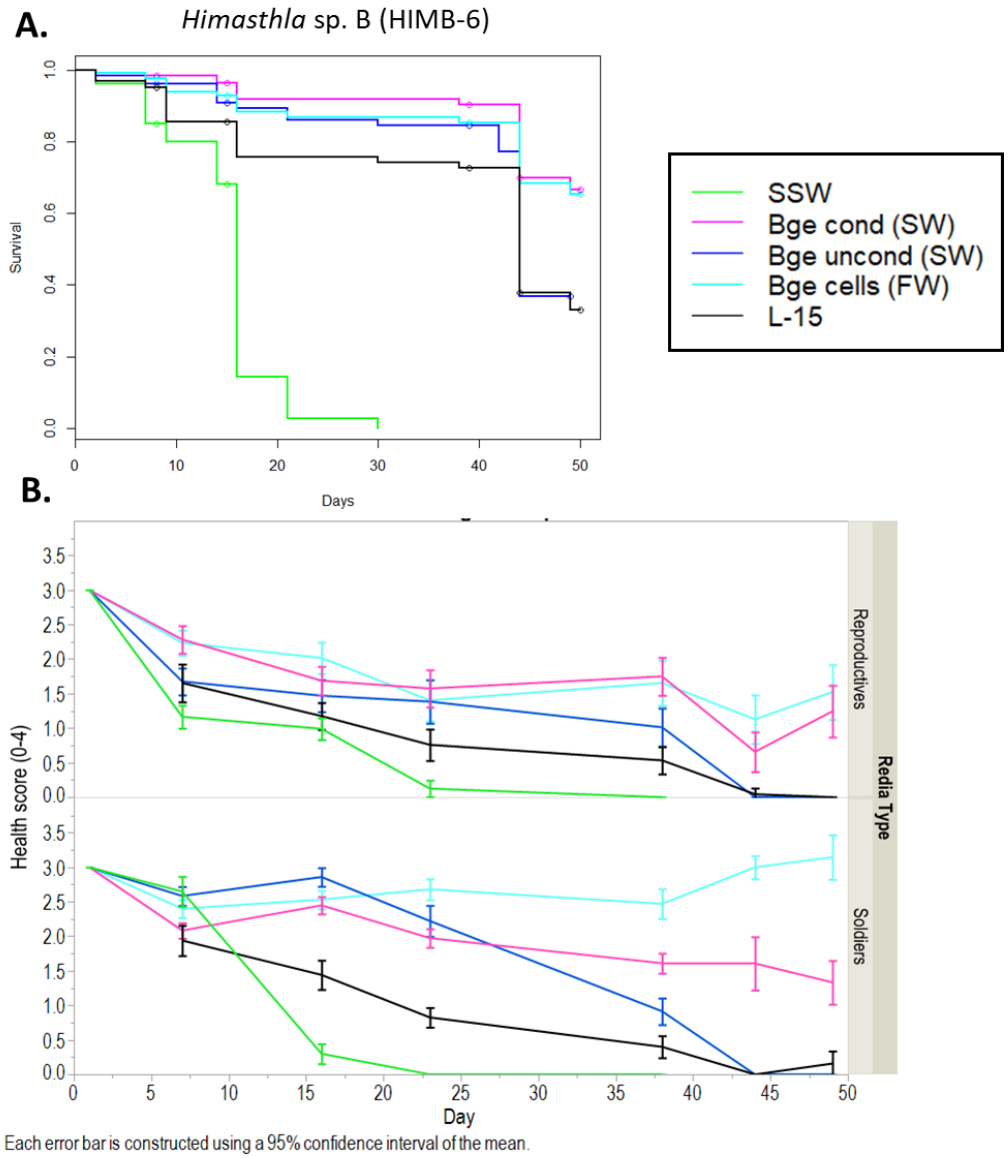

Figure 4. Results of the experiment that examined both survival and overall health and activity; A) Survival curve for one *Himasthla* sp. B colony and B) Results of redia activity and health evaluation. Scores ranged from 4 (healthy, thriving) to 0 (dead).

Although survival was similar across treatments, redia activity and health score was not (Fig 4). After 50 days in culture, reproductive rediae of HIMB were still moving and had an index of ~1.5, while reproductives in L-15 and Bge

unconditioned were very close to dead. Similarly, soldiers in Bge conditioned medium also had an index of ~1.5. However, those that were co-cultured with Bge cells were extremely active, with a health score of ~3. We did not, however, that these individuals appeared swollen, likely from being exposed to hypotonic conditions.

3.3 Rediae development and offspring production

Reproductive rediae often had mature cercariae inside of them that would emerge within the first days of the experiments. Although cercariae emergence was also observed later in the experiment, it was much less frequent. Late cercariae emergence was often associated with the fact that the redia holding the cercariae was about to die. These cercariae were likely pre-developed inside the snail and achieved little, if any development *in vitro*.

Cercariae of HIMB had normal swimming behavior and would often encyst in culture (despite rarely encysting in sea water) (Fig 1A). In contrast, HIMA cercariae often developed knobs on the body wall and rarely encysted in culture (despite easily encysting in sea water). Finally, EUHA cercariae had normal swimming behavior after emerging and never encysted. Moribund cercarial bodies separated from their tails were also noted for all species.

3.4 Food trials

This experiment was designed to test whether nutrient sources such as predation on heterospecifics or on Bge cells could increase redia performance *in*

vitro. Survival across treatments was similar, although soldier survival was much higher than that of reproductive colony members (Fig 5A, Table 4).

Bge cells were not observed sticking to living rediae or cercariae. As noted above clumping of Bge cells did occur when rediae first added (Fig 5B). Often, the space around the rediae would be free of Bge cells, likely because movement of rediae would detach cells and push them away. Rediae did not appear to consume or otherwise directly damage Bge cells.

In no occasion were rediae observed to either attack colony members, or feed on dead neighbors. Similarly, cercariae bodies and tails were never observed to be ingested by rediae of the same species. However, when rediae of HIMA and EUHA were cultured together, HIMA reproductive and soldiers would often eat EUHA reproductive within 3-5 days (Fig. 5C). HIMA rediae continued to prey on EUHA heterospecifics for the first couple of times such individuals were added with media change. After 3-4 weeks, predation rates appeared to decrease significantly Consumption of released cercariae of the heterospecific species was similarly observed.

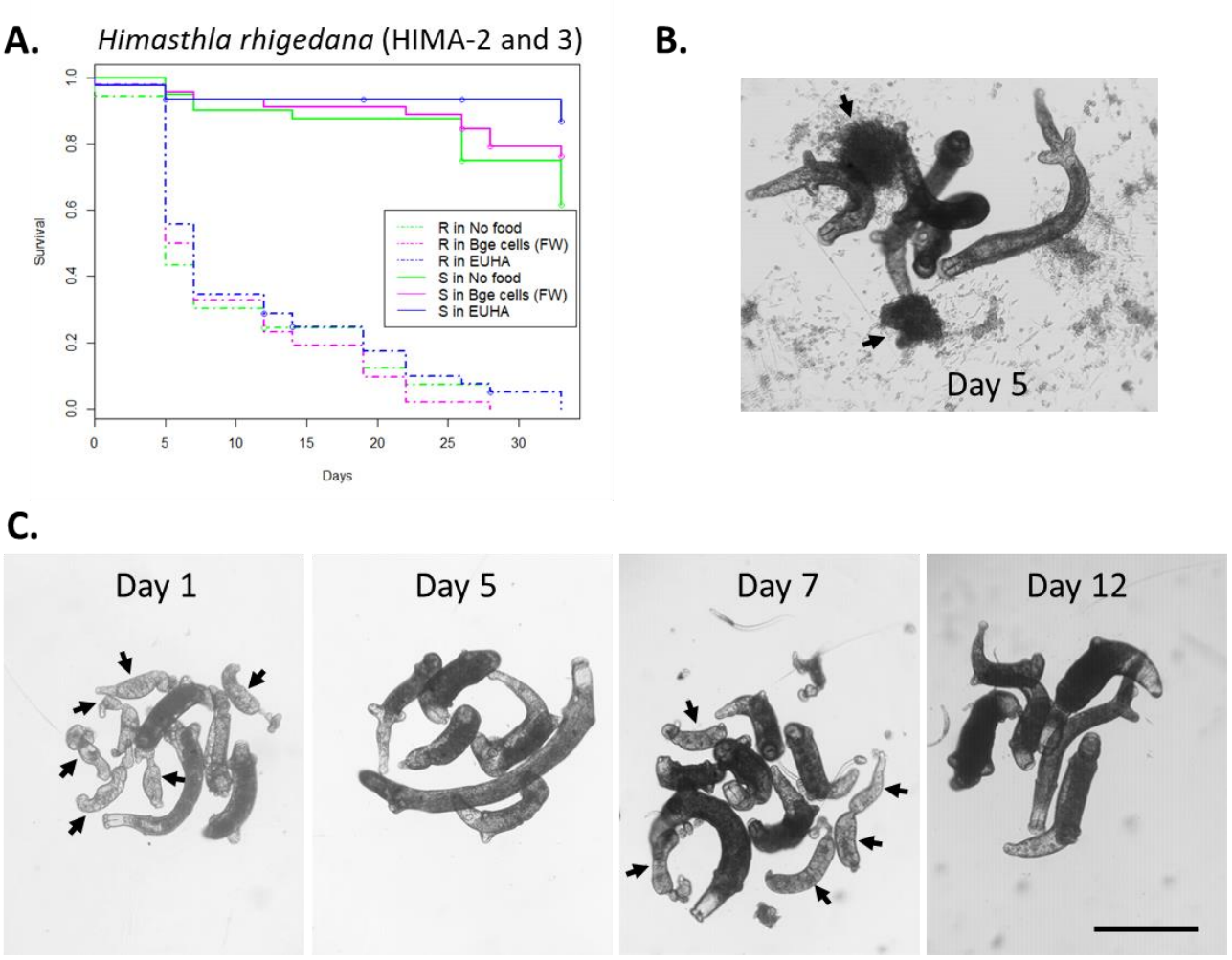

Figure 5. *Himasthla rhigedana* (HIMA) colonies under three different food treatments A) Survival curves for soldiers and reproductives of two HIMA colonies (results from individual colonies pooled) under three different food treatments: control of Bge conditioned medium (no food), Bge cells and heterospecifics of *Euhaplorchis californiensis* (EUHA). B) HIMA soldiers in Bge cells treatment. Arrows point at clusters of cells from when rediae were added. Note normal growth patterns of newer cells around rediae. C) Sequence of photos of HIMA soldiers in EUHA treatment. Note EUHA rediae (black arrows) in Days 1 and 7 when medium was first added/changed. EUHA rediae were completely consumed five days later. Scale bar= 400µm

Table 4. Survival of *Himasthla rhigedana* rediae under three different food regimes.

Experiment	Species ID	Treatment	N total	N dead	*Restricted mean	*Std Error	*Restricted mean upper limit	Log-rank test
Food Experiment	HIMA 2 & 3 repro	No food	53	50	9.9	1.2	33	χ_5^2= 229 p<0.0001
	HIMA 2 & 3 repro	Bge cells (FW)	52	52	9.4	0.9		
	HIMA 2 & 3 repro	EUHA	52	48	11.0	1.2		
	HIMA 2 & 3 soldiers	No food	40	15	29.0	1.3		
	HIMA 2 & 3 soldiers	Bge cells (FW)	45	10	29.8	1.2		
	HIMA 2 & 3 soldiers	EUHA	46	5	31.1	1.1		

4. Discussion

Trematodes infecting the California horn snail survive in L-15 medium

Here we report primary *in vitro* culture of rediae of *Euhaplorchis californiensis* (Heterophyidae), *Himasthla rhigedana* and *Himasthla sp. B* (Echinostomatidae). A total of fourteen colonies were cultured over the course of these experiments. We monitored survival for both soldier and reproductive rediae of these species.

As the first objective, we established that trematodes infecting the California horn snail can survive *in vitro* in the L-15 medium that had been previously used for other marine trematodes (Gorbushin & Shaposhnikova, 2002; Lloyd & Poulin, 2011) for at least 50 days, when experiments were terminated. At this point, several colonies had well over 50% survivorship, suggesting that rediae could potentially survive for longer time periods.

Survival varied greatly within species and across species. Some of the intraspecific variation appeared to be explained by the starting health of the colony. Colonies that died within the first weeks often appeared unhealthy during the initial dissection. Healthy colonies of HIMB and HIMA appeared to consistently survive for long periods of time. EUHA, a heterophyid trematode, on the other hand, had a lower overall survivorship. This suggests that members of families other than the Echinostomatidae might require special formulations to improve performance and survivorship.

Our results are comparable to what has been reported for cultures of other marine trematodes. Gorbushin & Shaposhnikova (2002) achieved the longest culture times with 50% survival at 163 days. However, other colonies they worked with had lower survival rates (see Fig 2 of Gorbushin & Shaposhnikova (2002)). Lloyd & Poulin (2011) also tested the L-15 media but supplemented it with chicken serum. They cultured six species that infect three different marine snail species, showing that this medium formulation can be broadly used. However, they note that survival varies greatly across species, ranging from only 8 days with *Galactosomum* sp. to about 50 days with *Philophthalmus* sp. In fact, they found that *Philophthalmus* sp. survived the longest (56 days) when the L-15 medium was supplemented with medium F (see Supplementary files for Lloyd & Poulin (2011) for recipe). This, and other findings discussed below suggest that medium F might provide essential factors for continuous development and proliferation.

Table 5. Survival, and development of various species of trematodes cocultured with Bge cells (modified from Coustau & Yoshino 2000).

Parasite species	Snail genus	Max survival in culture medium (days)	Max survival with Bge cells (days)	Production of progeny
Schistosoma mansoni	*Biomphalaria*	18	Continuous	+
Schistosoma japonicum	*Oncomelania*	28	210	-
Schistosoma mattheei	*Bulinus*	7	25	+
Schistosoma intercalatum	*Bulinus*	21	63	+
Echinostoma caproni	*Biomphalaria*	14	119	-
Echinostoma magna	*Lymnaea*	12	>90	-
Himasthla elongata	*Littorina*	163	-	-
Philophthalmus sp.	*Zeacumantus*	56	-	-

Rediae survival and performance is enhanced by the presence of Bge cells or factors released from the cells

Our second objective was to determine whether rediae survival and performance could be enhanced by the presence of Bge cells or Bge released factors. These treatments had similar survivorship to what was observed for the L-15 medium. However, overall health of rediae seemed superior in the Bge cell related media (i.e. Bge co-culture and Bge conditioned). Such findings are consistent with what has been reported regarding freshwater trematodes (Table 5). For instance, freshwater trematodes were initially co-cultured with insect cell lines before the Bge cell line was created (DiConza & Hansen, 1973). Once the Bge cell line became available, it was observed that, although co-culture in direct contact with Bge cells was best, schistosome cultures with a Bge cell conditioned medium outperformed those cultures that were only in Bge medium (Yoshino & Laursen, 1995).

Rediae in the SSW control treatment died within the first 2-3 weeks, indicating that starving rediae can use internal energy stores up to a certain point. Rediae maintained in the Bge-conditioned medium did slightly better than those in the L-15, suggesting that some nutritional components were satisfied with the former but not the latter. Despite being cultured in hypotonic conditions, rediae co-cultured with Bge cells (FW) were the most active. Rediae looked bloated, probably as consequence of osmotic regulation because of the freshwater conditions they were exposed to. Regardless of their swollen condition, they were the most active after >50 days of culture. Although less active than rediae in the Bge cell (FW) treatment, rediae in the Bge conditioned treatment had the healthiest appearance. They maintained size and tegument integrity and were still bending, contracting and extending at the time when the experiments were terminated

The role that other factors, such as trematode densities and the need for additional nutrients, play in determining the success of culture systems is worth investigating. For example, Ivanchenko et al. (1999) found that proliferating cultures in the absence of *Bg*e cells could be obtained if sporocysts were maintained at high density in *Bg*e-conditioned medium and held under reduced oxygen. In such cases, growth and development were similar to those of sporocysts grown with *Bg*e cells, but daughter sporocyst proliferation was somewhat reduced. Ivanchenko et al. (1999) noted that medium F was essential to schistosome indefinite proliferation. Exactly which components of Medium F are necessary for continuous propagation of the cultures has not been examined. However, if medium F was not included in the basal nutrient mix, cultures could be initiated and maintained for long time

periods, but continuous sporocyst proliferation could not be maintained. Thus, regulating trematode density and investigating the role of various nutrients might provide ways to improve culture of marine trematodes.

Progeny rediae or cercariae are not produced in culture conditions

All colonies examined here were obtained from mature colonies that were actively shedding cercariae. However, because the colonies originated from field-collected snails that were naturally infected, the exact age of the infection could not be known. At the onset of culture, reproductive rediae contained developing cercariae. Cercariae would often emerge only for the first few days of culture experiments. As far as we can tell, the released cercariae were already fully developed (or very close to full development) when culture conditions started. Although reproductive rediae were observed to contain embryonic cercariae throughout the entire culture periods, it did not appear to us that those embryonic cercariae underwent further development. The lack of continual reproduction observed in the reproductive rediae indicates that we have not yet solved the problem of creating media that permit continuous proliferation of trematodes.

Rediae of HIMA consume rediae and cercariae of a heterospecific species but not Bge cells.

We had not expected rediae to perform well in co-culture with Bge cells given the hypotonic nature of the freshwater conditions. Rediae appeared swollen, but otherwise were very active and exhibiting normal behavior. The exact mechanism by which Bge cells contribute to rediae health and performance has yet to be

determined. Two suggested mechanisms have been predation and direct contact with cells. For instance, Loker et al. (1999) report that rediae readily ingested Bge cells in culture and fared better than rediae from cultures lacking cells (although the beneficial effects of Bge cells were not derived from eating cells alone, as some rediae consistently had guts packed with Bge cells but did not undergo germinal cell development and others were observed to eat few cells yet produced progeny). We, however, did not observe any predation on Bge cells. Further, because Bge cells have been observed to attach to and encapsulate some trematode species (Yoshino & Laursen, 1995) it has been suggested that the intimate contact with cells is a requirement for development of daughter sporocysts. In our experiments, Bge cells did not attach to or encapsulate rediae, similar to what has been reported for *Fascioloides magna* rediae (Laursen & Yoshino, 1999) and *E. caproni* (Loker & Adema, 1995). Further, rediae survived in the Bge-conditioned medium to about the same degree as when co-cultured with the cells, suggesting that the Bge cells release factors that promote survival.

Several studies have found that consumption of moribund or dead cercariae and rediae might be common among the Echinostomatidae. For instance, Gorbushin & Shaposhnikova, (2002) report that nutrients derived from this sort of predation were enough to allow continual survival of rediae for over 5 months and to continue the development of progeny that had already started growing in vivo, but not formation of new embryos *in vitro*. Loker et al. (1999) observed rediae feeding on dead cercariae, but not dead rediae. We, however, did not observe consumption of dead or dying rediae and cercariae. Similarly, and in agreement with Basch &

DiConza (1975), we noted no tendency for cultured rediae to attack live colony members.

Predation on conspecifics and heterospecifics could provide a valuable source of nutrients for cultured rediae- if the cultured species is the dominant one in the interaction. It has long been known that there are strong antagonistic interactions among trematodes. Typically, when two trematode species infect the same host, one kills the other. Dominant species displace established colonies of subordinate species via predation by rediae (Lim and Heyneman, 1972; Lie, 1973; Combes, 1982; Kuris, 1990) This has also been observed *in vitro* (Basch & Diconza, 1975; Garcia-Vedrenne et al. 2016). With this in mind, it is not surprising that HIMA soldiers and reproductives both preyed on EUHA heterospecifics. HIMA soldiers attacked and completely consumed heterospecifics at higher rates than did their reproductive colony mates. The pool of heterospecific rediae was replenished along with the medium change. Heterospecifics were completely consumed the first couple of weeks, but predation rates decreased after that.

There might be disadvantages associated with adding heterospecifics as a food source. First, this technique would require weekly snail dissections (including sterilizing space, dissecting and isolating parasites, rinses and time to let sit in antibiotics). Even with all these measures taken into consideration, there is an inherent higher risk of introducing bacterial or fungal contaminants when adding freshly dissected rediae to a well.

Guidelines on whether to use L-15 or Bge-related media

Our results indicate that trematodes performed best in Bge-related media. However, there are distinct advantages to using the L-15 under certain circumstances. The L-15 medium is a chemically defined medium, entirely free of animal-derived components. By contrast, the other media tested here contain fetal calf serum and/or Bge cell derived factors. These components might provide a valuable source of nutrients, but the amounts of such nutrients and other animal-derived factors cannot be established or controlled. Further, there are technical disadvantages to using serum that include the undefined nature of serum, batch-to-batch variability in composition, and the risk of contamination. Thus, the L-15 medium provides a suitable chemically defined option to study larval trematodes for long periods of time when a pure and consistent culture environment is required.

On the other hand, we note that the presence of Bge cells or Bge released factors has a distinctly beneficial effect on both survivorship and overall health of cultured redial stages. This is the first reported cultivation of marine trematodes that includes co-culture with Bge cells and media with Bge factors. Our results highlight the promise of using these methods for cultivation of marine trematodes. Additional studies to pinpoint what factors are essential for increased trematode survivorship and performance should be undertaken because the prospects for considerably improving culture conditions are high. Development of long-term *in vitro* techniques with continuous propagation of larval stages would provide valuable tools for the study of interactions between trematodes of the same or different species.

Acknowledgements

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Table S1. Statistics for each individual colony examined.

Species	Colony ID	N (Soldier, Immature+ Reproductive)	Proportion immatures	Average body size (µm³)		Average pharynx size (µm³)		Ratio (Repro/Soldiers)	
				Reproductives	Soldiers	Reproductives	Soldiers	Body size	Pharynx size
Cloacitrema michiganensis	1	102 (62, 3+37)	2.9%	61075201	946299	153132	58630	64.5	2.6
	2	124 (32, 3+89)	2.4%	85216179	523301	97266	33441	162.8	2.9
	3	171 (103, 3+65)	1.8%	191530782	452508	88238	27930	423.3	3.2
	4	128 (72, 13+43)	10.2%	81314098	746371	234972	41246	108.9	5.7
	5	111 (77, 1+33)	0.9%	153339148	642642	124223	40567	238.6	3.1
Parorchis acanthus	1	112 (36, 7+69)	6.3%	226163136	691790	72439	38640	326.9	1.9
	2	56 (38, 0+18)	0.0%	68547602	796471	49835	51318	86.1	1.0
	3	89 (62, 2+25)	2.2%	261366386	793941	66710	51333	329.2	1.3
	4	131 (76, 2+53)	1.5%	185088913	479116	65172	32005	386.3	2.0
	5	97 (58, 9+30)	9.3%	352471081	523840	64833	14027	672.9	4.6
Himasthla rhigedana	1	109 (62, 14+33)	12.8%	194345881	5775712	443638	198895	33.6	2.2
	2	121 (73, 2+46)	1.7%	152146906	8358058	568971	367397	18.2	1.5
	3	87 (31, 6+50)	6.9%	261054737	6671661	720283	309848	39.1	2.3
	4	78 (35, 2+41)	2.6%	150578725	4404424	306873	185233	34.2	1.7
	5	52 (18, 5+29)	9.6%	111569550	8068932	534604	381706	13.8	1.4
Acanthoparyphium spinulosum	1	78 (50, 1+27)	1.3%	47964451	648868	79436	36356	73.9	2.2
	2	155 (126, 2+27)	1.3%	41268256	1077503	66025	53229	38.3	1.2
	3	120 (98, 0+22)	0.0%	39228501	948314	72096	49414	41.4	1.5
	4	131 (92, 0+39)	0.0%	54729660	934081	72569	54298	58.6	1.3
	5	94 (61, 2+31)	2.1%	13662112	831869	54609	34402	16.4	1.6
Echinostoma liei	1	66 (0, 15+51)	22.7%	9196167		62333			
	2	18 (0, 4+14)	22.2%	13287702		62572			
	3	56 (0, 12+44)	21.4%	11410266		41947			
	4	122 (0, 4+118)	3.3%	26450994		71490			
	5	115 (0, 20+95)	17.4%	18083199		61380			
	6	93 (0, 2+91)	2.2%	20624018		53321			

* Reported averages for reproductive rediae include measurements obtained for immature rediae

Table S2. Logistic regression analysis of attack rates for each species from the California horn snail.

Effect Tests				Parameter Estimates				
Source	DF	χ^2	P-value	Term	Estimate	Std Error	χ^2	P-value
Cloacitrema michiganensis								
				Intercept	-10.95688	1024.2721	408.16332	<.0001
Colony ID	2	9.3212336	0.0095	**Colony ID[CLOA 1]**	-0.414536	0.1955433	4.7070057	0.03
Redia type	1	145.38291	<.0001	**Colony ID[CLOA 2]**	0.5352598	0.1815602	8.646672	0.0033
Heterospecific ID	1	0.3511263	0.5535	**Redia type[reproductive]**	-10.11098	1024.2721	145.38291	<.0001
				Heterospecific ID[EUHA 1]	-0.07857	0.1325851	0.3511263	0.5535
Parorchis acanthus								
				Intercept	-12.51156	1327.854	614.64158	<.0001
Colony ID	2	18.238563	0.0001	**Colony ID[PARO 1]**	1.1826114	0.3207008	16.955043	<.0001
Redia type	1	48.845638	<.0001	**Colony ID[PARO 2]**	-1.26195	0.5043033	9.7322245	0.0018
Heterospecific ID	1	17.412844	<.0001	**Redia type[reproductive]**	-9.763434	1327.854	48.845638	<.0001
				Heterospecific ID[EUHA 1]	0.932866	0.2567905	17.412844	<.0001
Acanthoparyphium spinulosum								
				Intercept	-2.500524	0.2301156	333.06623	<.0001
Colony ID	2	35.852681	<.0001	**Colony ID[ACAN 1]**	-1.157106	0.2241269	33.425597	<.0001
Redia type	1	134.71694	<.0001	**Colony ID[ACAN 2]**	0.2938814	0.1805609	2.6670131	0.1024
Heterospecific ID	1	8.5480185	0.0035	**Redia type[reproductive]**	-1.770934	0.2215831	134.71694	<.0001
				Heterospecific ID[EUHA 1]	0.3836494	0.132907	8.5480185	0.0035
Himasthla elongata								
				Intercept	-2.066872	0.5112975	58.687095	<.0001
Colony ID	2	13.732065	0.001	**Colony ID[HIMA 1]**	-0.704028	0.2445346	8.4567049	0.0036
Redia type	1	485.65163	<.0001	**Colony ID[HIMA 2]**	0.9047083	0.2956052	11.286998	0.0008
Heterospecific ID	1	7.3763771	0.0066	**Redia type[reproductive]**	-3.934921	0.5281867	485.65163	<.0001
				Heterospecific ID[EUHA 1]	-0.51122	0.1969023	7.3763771	0.0066

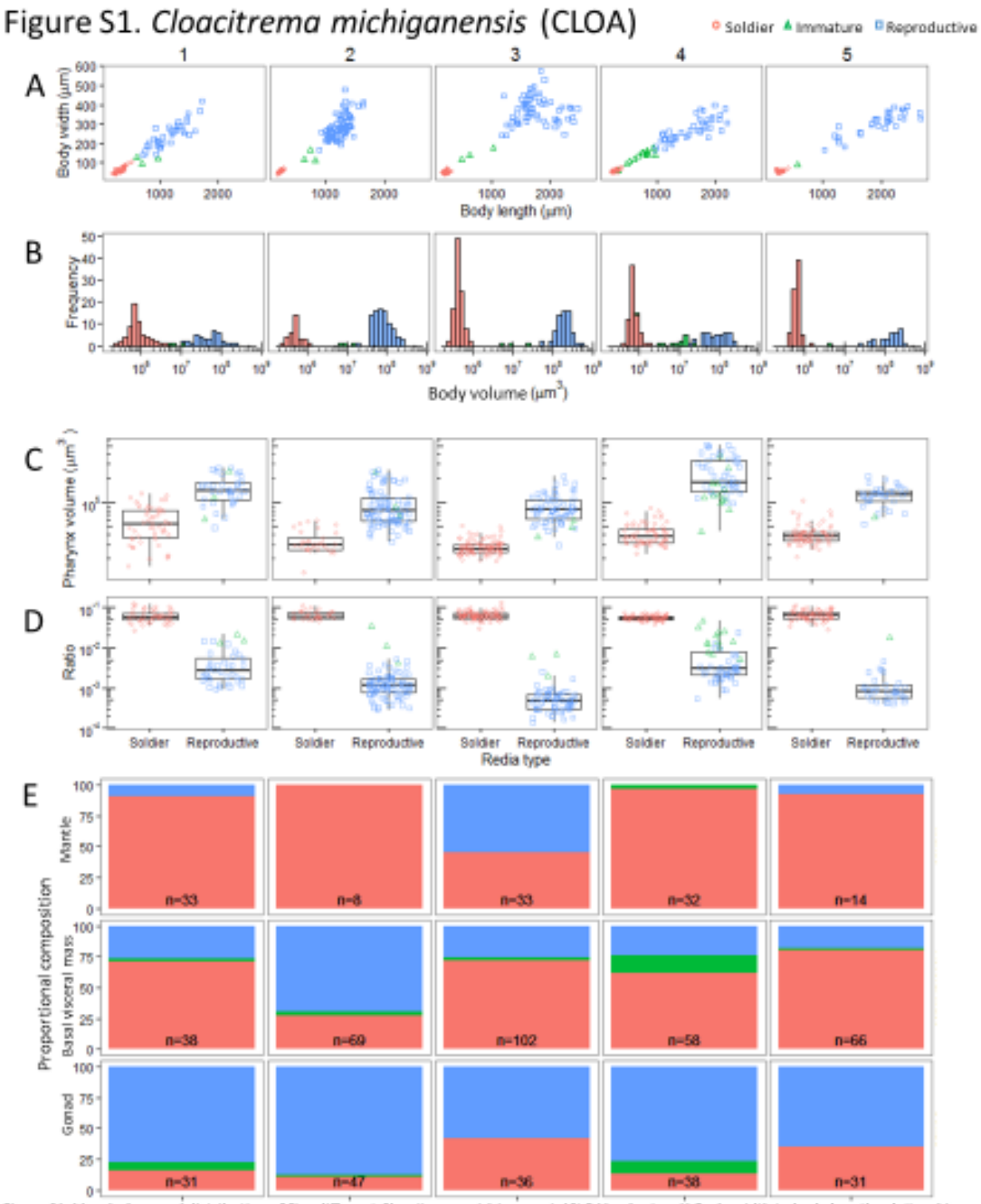


Figure S1. Morphology and distribution of five different *Cloacitrema michiganensis* (CLOA) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single CLOA colony. B) Frequency distributions of total body volume for randomly sampled rediae. X-axis is $\log_{10}$ scale. C) Pharynx volume, and D) Pharynx volume relative to body volume. Points represent raw data for randomly sampled rediae, boxplots indicate median (line), interquartile range (box) and range of data (whiskers). Note the $\log_{10}$ scale for axes. E) Proportion of reproductive, immature and solider rediae found in mantle, basal visceral mass and gonad regions for each CLOA colony examined. Numbers in boxes indicate total number of individuals sampled from each region.

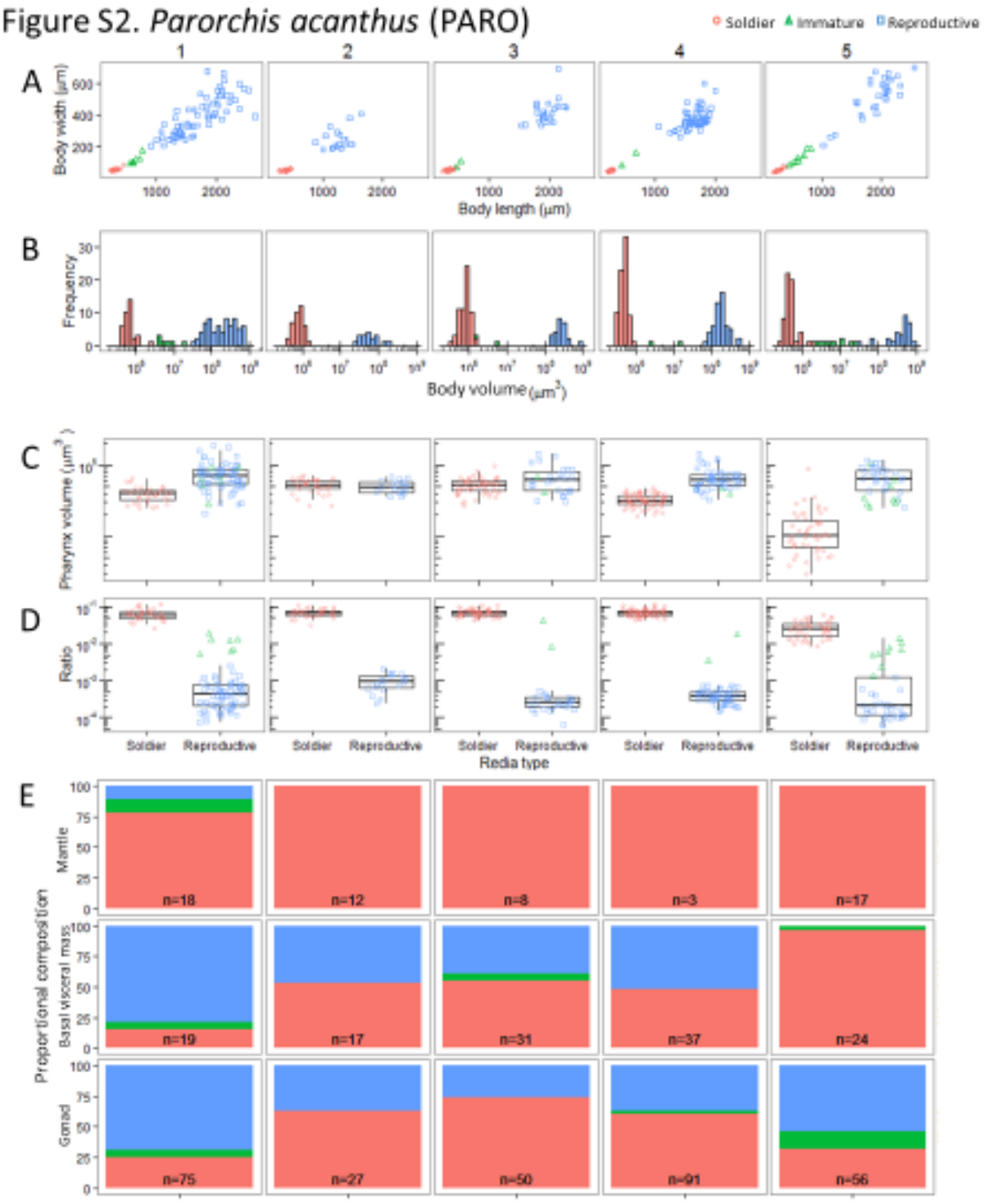


Figure S2. Morphology and distribution of five different Parorchis acanthus (PARO) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single PARO colony. B) Frequency distributions of total body volume for randomly sampled rediae. X-axis is log10 scale. C) Pharynx volume, and D) Pharynx volume relative to body volume. Points represent raw data for randomly sampled rediae, boxplots indicate median (line), interquartile range (box) and range of data (whiskers). Note the log10 scale for axes. E) Proportion of reproductive, immature and solider rediae found in mantle, basal visceral mass and gonad regions for each PARO colony examined. Numbers in boxes indicate total number of individuals sampled from each region.

Figure S3. *Acanthoparyphium spinulosum* (ACAN)

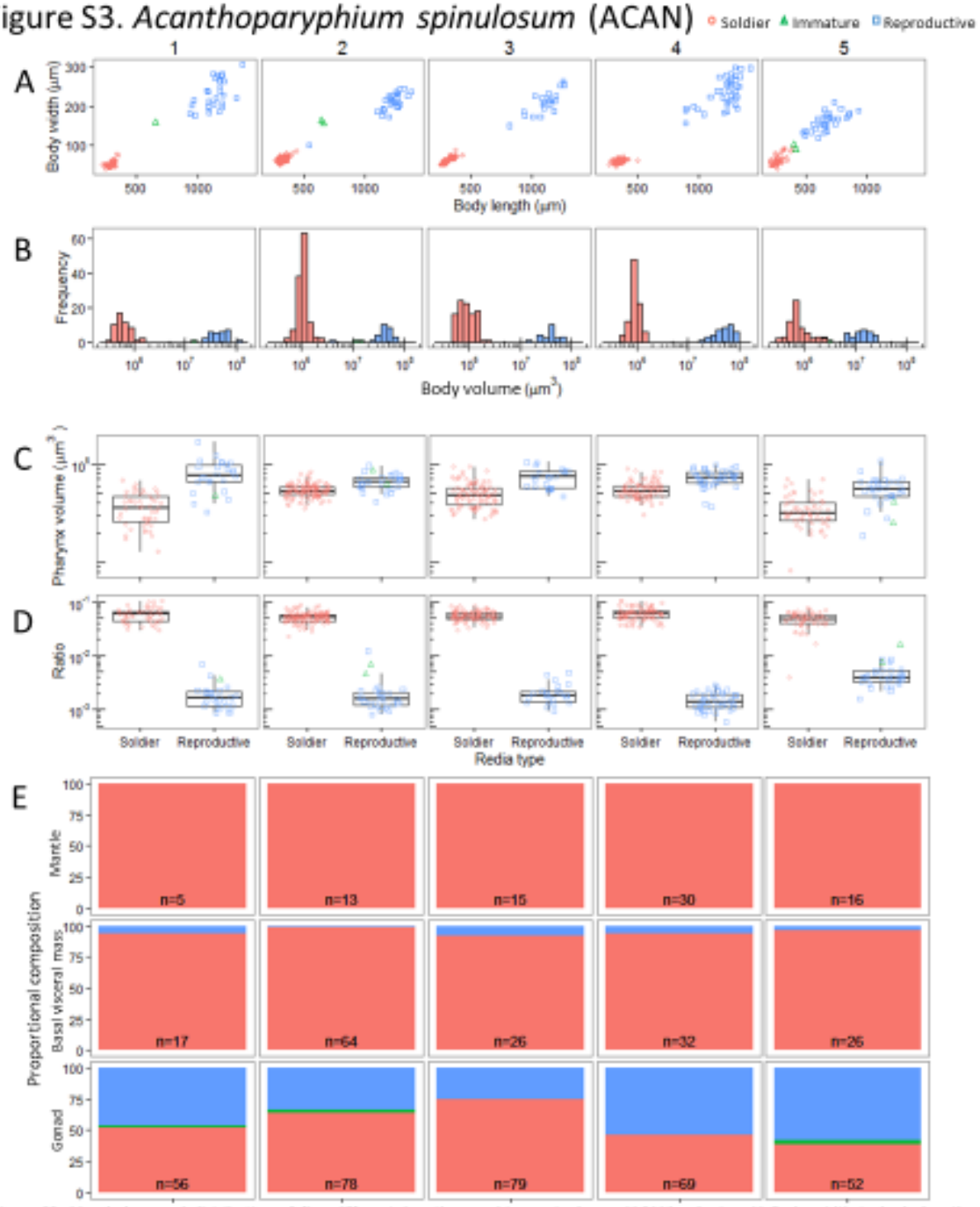


Figure S3. Morphology and distribution of five different Acanthoparyphium spinulosum (ACAN) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single ACAN colony. B) Frequency distributions of total body volume for randomly sampled rediae. X-axis is log10 scale. C) Pharynx volume, and D) Pharynx volume relative to body volume. Points represent raw data for randomly sampled rediae, boxplots indicate median (line), interquartile range (box) and range of data (whiskers). Note the log10 scale for axes. E) Proportion of reproductive, immature and solider rediae found in mantle, basal visceral mass and gonad regions for each ACAN colony examined. Numbers in boxes indicate total number of individuals sampled each given region.

Figure S4. *Himasthla rhigedana* (HIMA)

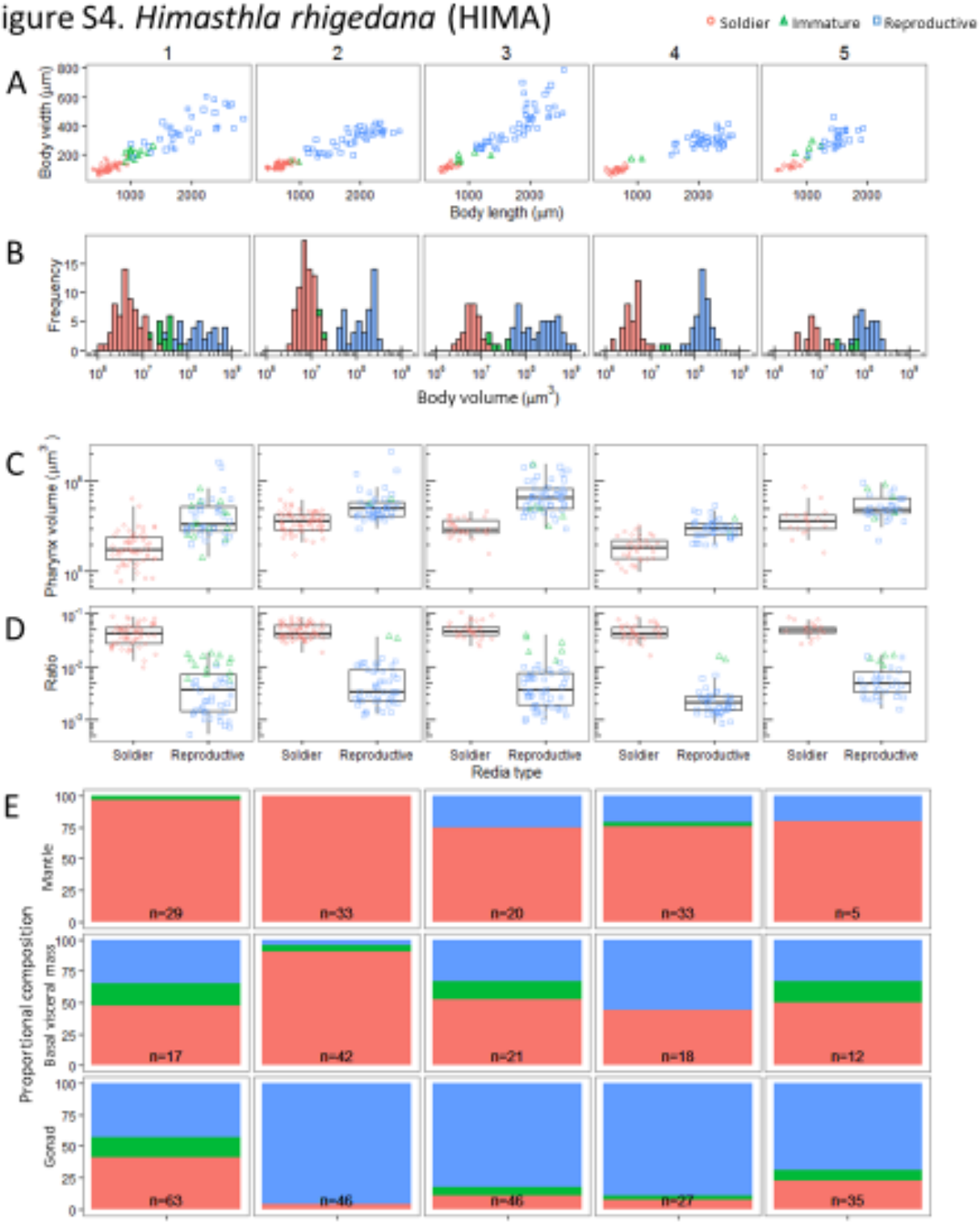


Figure S4. Morphology and distribution of five different Himasthla rhigedana (HIMA) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single HIMA colony. B) Frequency distributions of total body size for randomly sampled rediae. X-axis is log10 scale. C) Pharynx volume, and D) Pharynx volume relative to body volume. Points represent raw data for randomly sampled rediae, boxplots indicate median (line), interquartile range (box) and range of data (whiskers). Note the log10 scale for axes. E) Proportion of reproductive, immature and solider rediae found in mantle, basal visceral mass and gonad regions for each HIMA colony examined. Numbers in boxes indicate total number of individuals sampled from each region.

Figure S5. *Echinostoma liei*

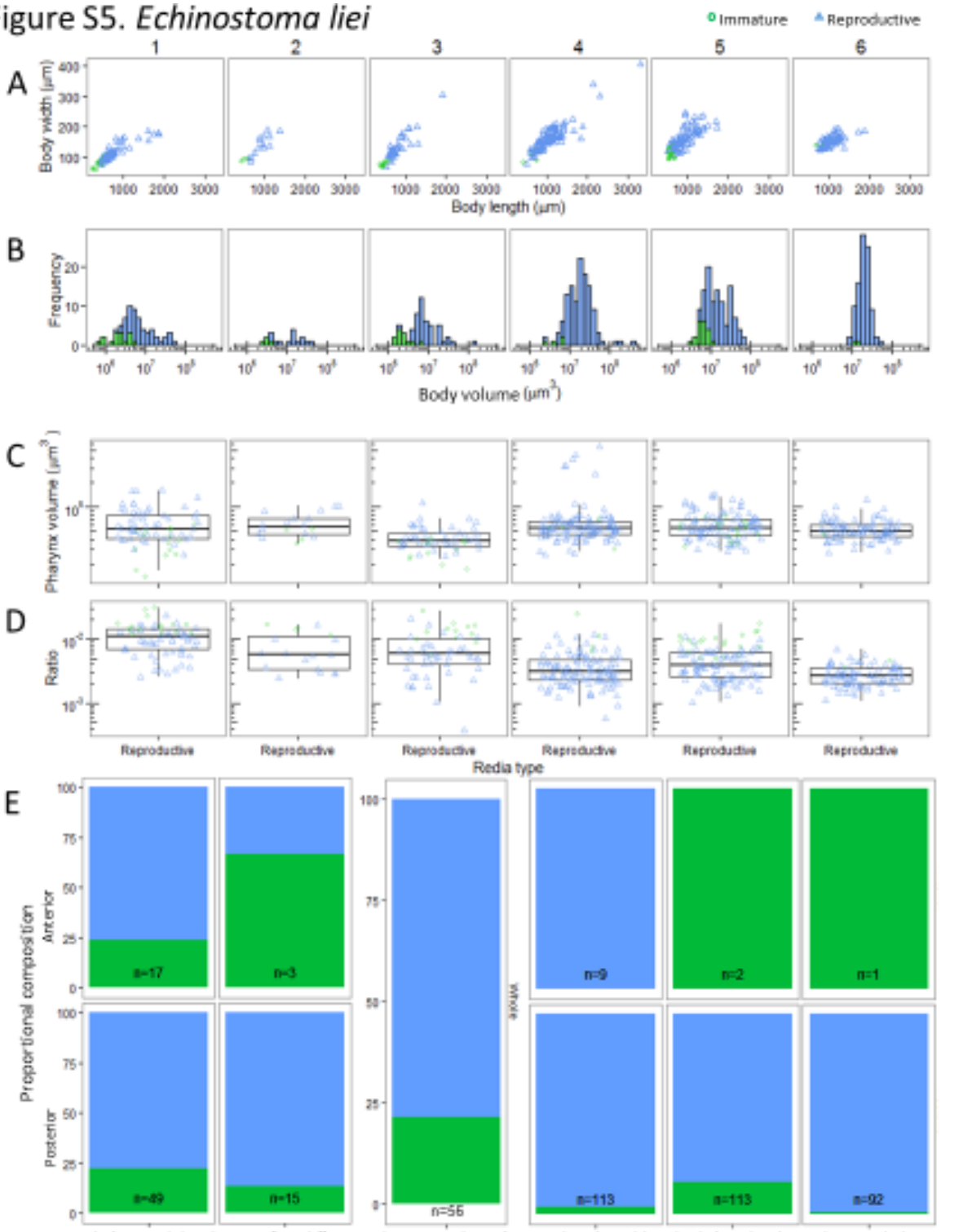


Figure S5. Morphology and distribution of six different Echinostoma liei colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single E. liei colony. B) Frequency distributions of total body volume for randomly sampled redia. X-axis is log10 scale. C) Pharynx volume, and D) Pharynx volume relative to body volume. Points represent raw data for randomly sampled rediae, boxplots indicate median (line), interquartile range (box) and range of data (whiskers). Note the log10 scale for axes. E) Proportion of reproductive and immature rediae found in anterior and posterior regions for each E. liei colony examined. Numbers in boxes indicate total number of individuals sampled from each region.

Figure S6. Early infection with *Himasthla rhigedana* (HIMA)

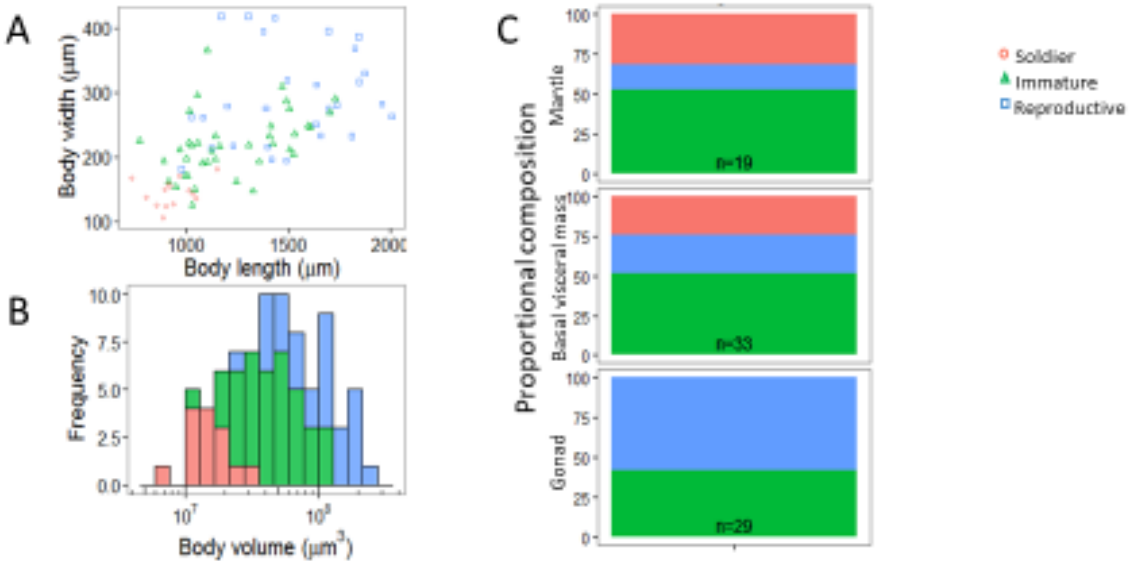
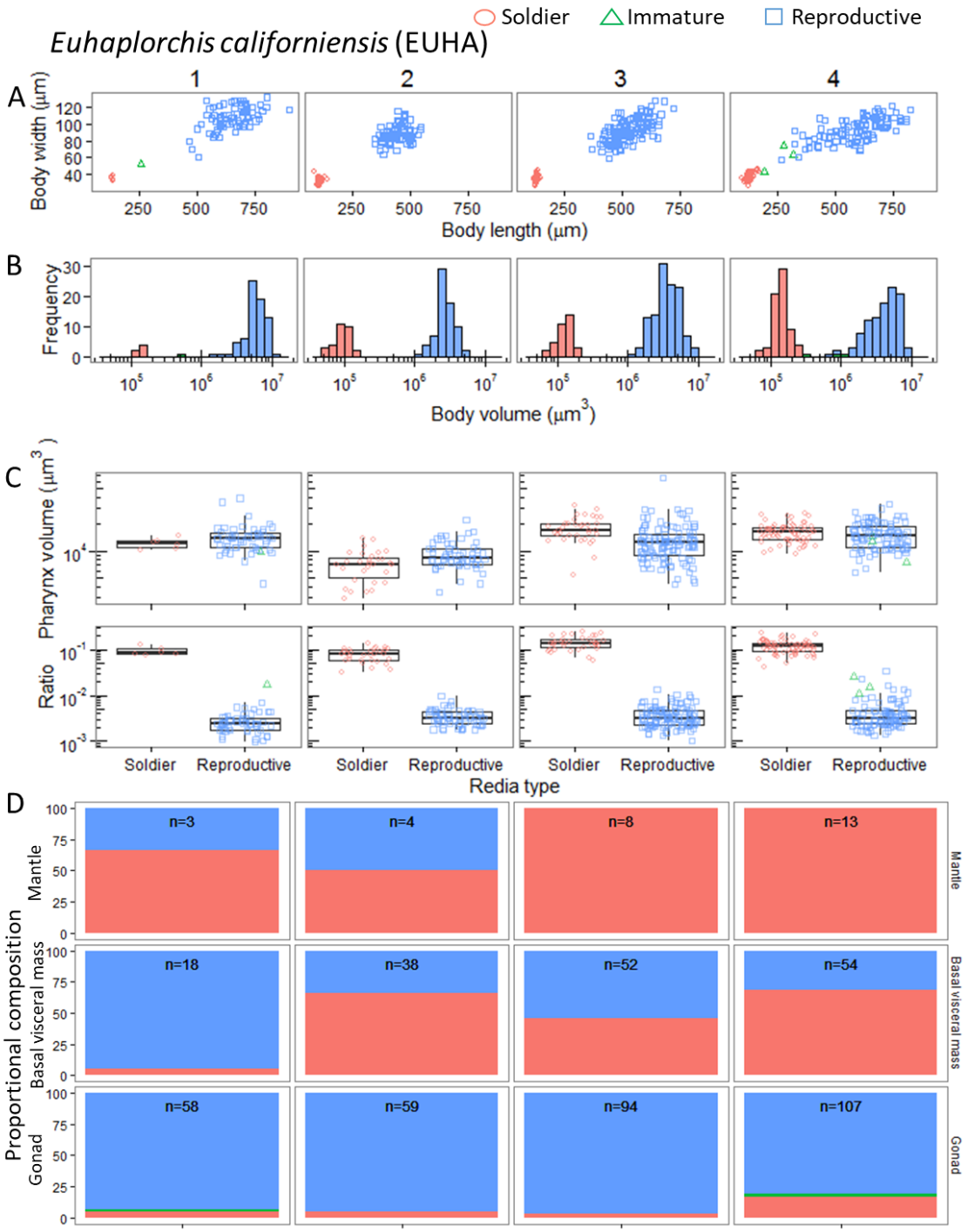


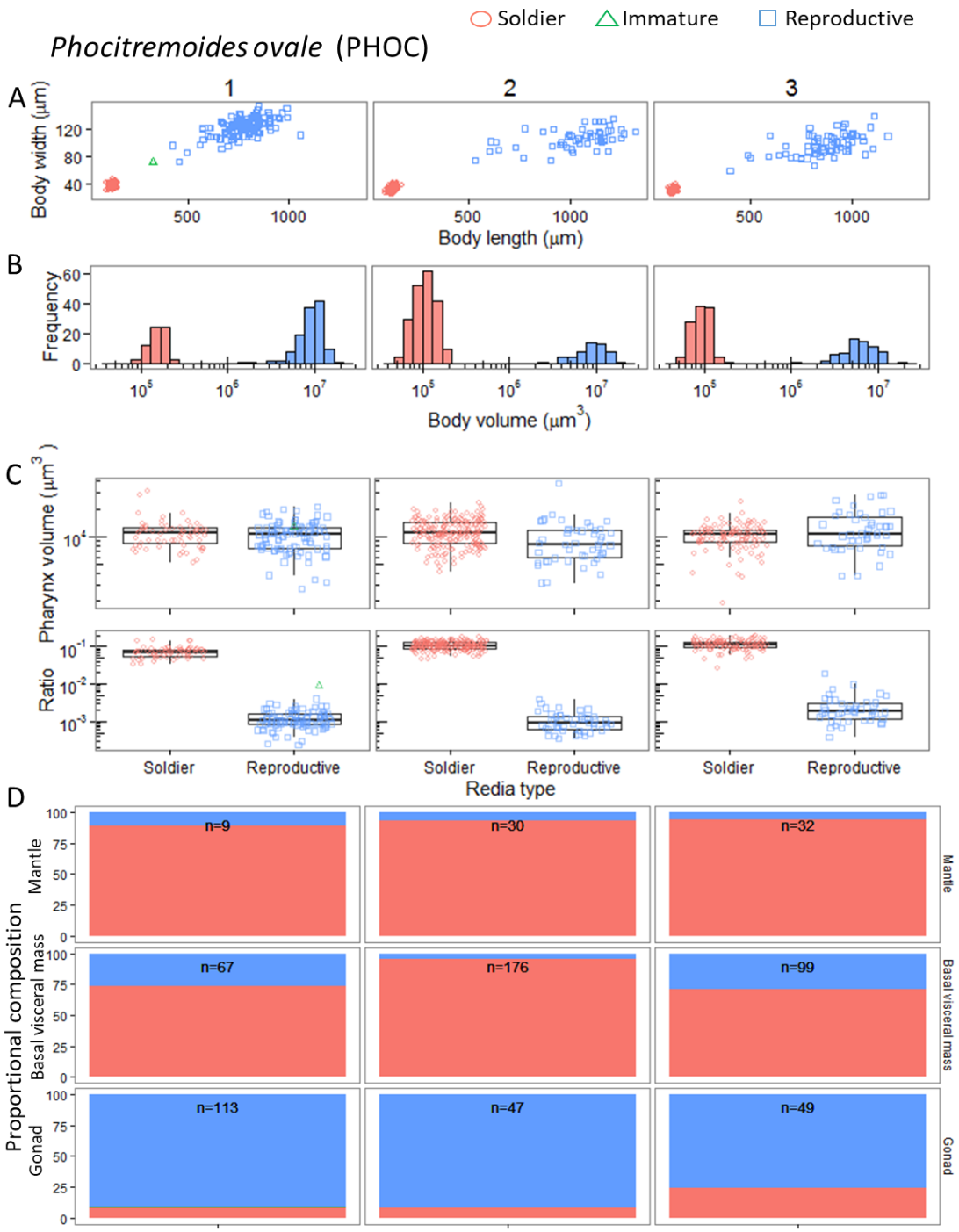
Figure S6. Morphology and distribution of an early infection with Himasthla rhigedana (HIMA). A) Body width to body length relationships. Each data point represents one randomly sampled redia. B) Frequency distributions of total body size for randomly sampled rediae. X-axis is log10 scale. C) Proportion of reproductive, immature and solider rediae found in mantle, basal visceral mass and gonad regions. Numbers in boxes indicate total number of individuals sampled from each region.

Supplementary Table S1. Statistics for each individual colony examined.

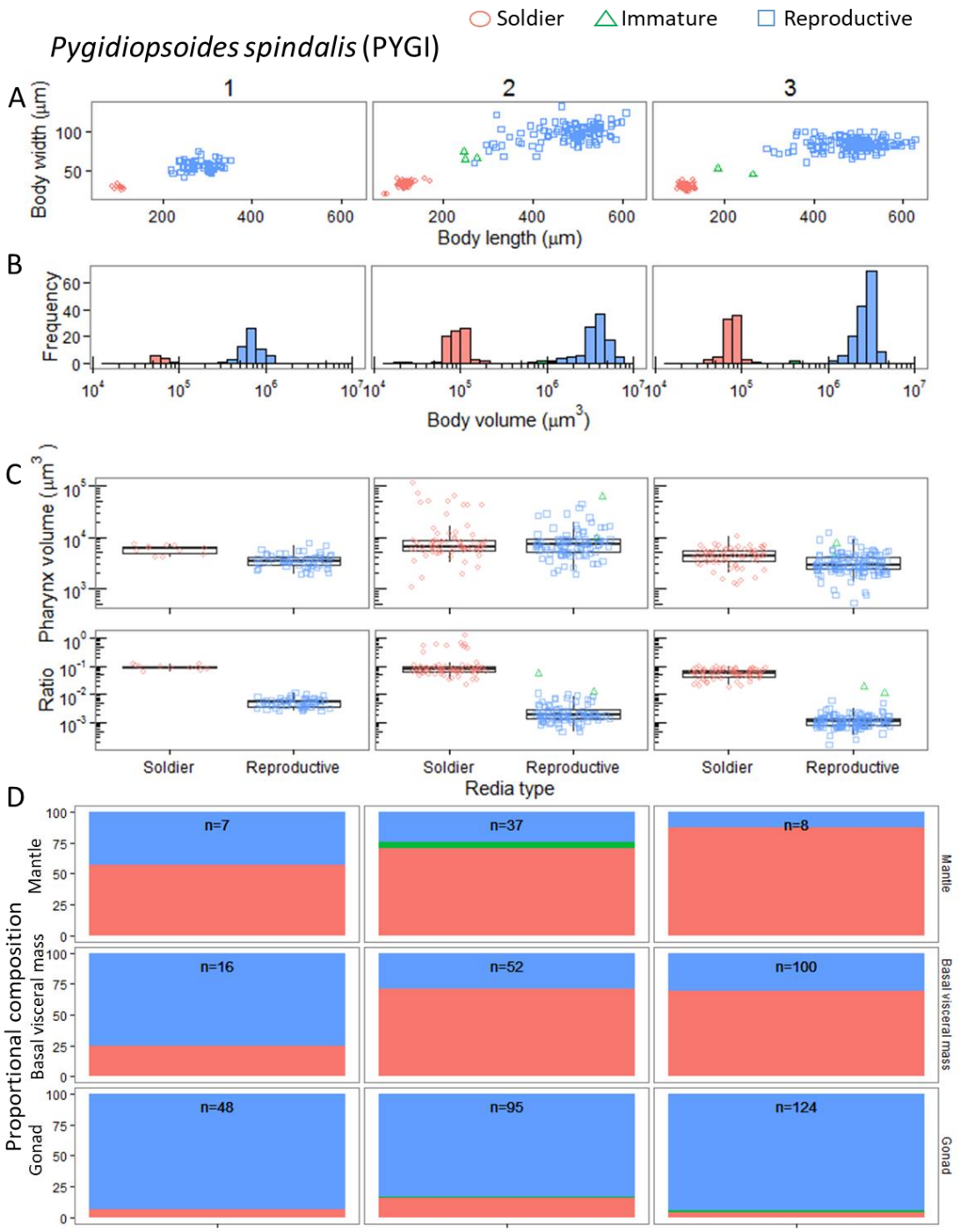
Species	Colony ID	N (Soldier, Immature+ Reproductive)	Proportion immatures	Average body size (μm³)		Average pharynx size (μm³)		Ratio (Repro/Soldiers)	
				Reproductives	Soldiers	Reproductives	Soldiers	Body size	Pharynx size
Euhaplorchis californiensis	1	79 (6, 1+72)	1.3%	6019242	135061	14416	12198	44.6	1.2
	2	101 (30, 0+71)	0.0%	2795769	93222	9293	7177	30.0	1.3
	3	154 (35, 0+119)	0.0%	3787345	130199	13341	17944	29.1	0.7
	4	174 (68, 3+103)	1.7%	4355124	144115	15589	16164	30.2	1.0
Phocitremoides ovale	1	189 (66, 1+122)	0.5%	9478926	156821	10422	11029	60.4	0.9
	2	253 (200, 0+53)	0.0%	9414982	94342	9532	11343	86.0	0.8
	3	180 (112, 0+68)	0.0%	6745647	93539	12685	10336	72.1	1.2
Pygidiopsoides spindalis	1	71 (11, 0+60)	0.0%	716387	61171	3636	5714	11.7	0.6
	2	184 (78, 3+103)	1.6%	3750907	94342	9120	13294	39.8	0.7
	3	232 (81, 2+149)	0.9%	2750525	77552	3334	4395	35.5	0.8
Stictodora hancocki	1	41 (21, 0+20)	0.0%	4963761	162337	10464	10805	30.6	1.0
	2	109 (59, 0+50)	0.0%	3505163	147278	8277	9494	23.8	0.9
	3	55 (14, 0+41)	0.0%	2752334	104934	6811	5780	26.2	1.2
	4	180 (43, 13+124)	7.2%	2009645	187231	9299	8809	10.7	1.1
	5	65 (48, 0+17)	0.0%	1833442	112034	4798	6629	16.4	0.7



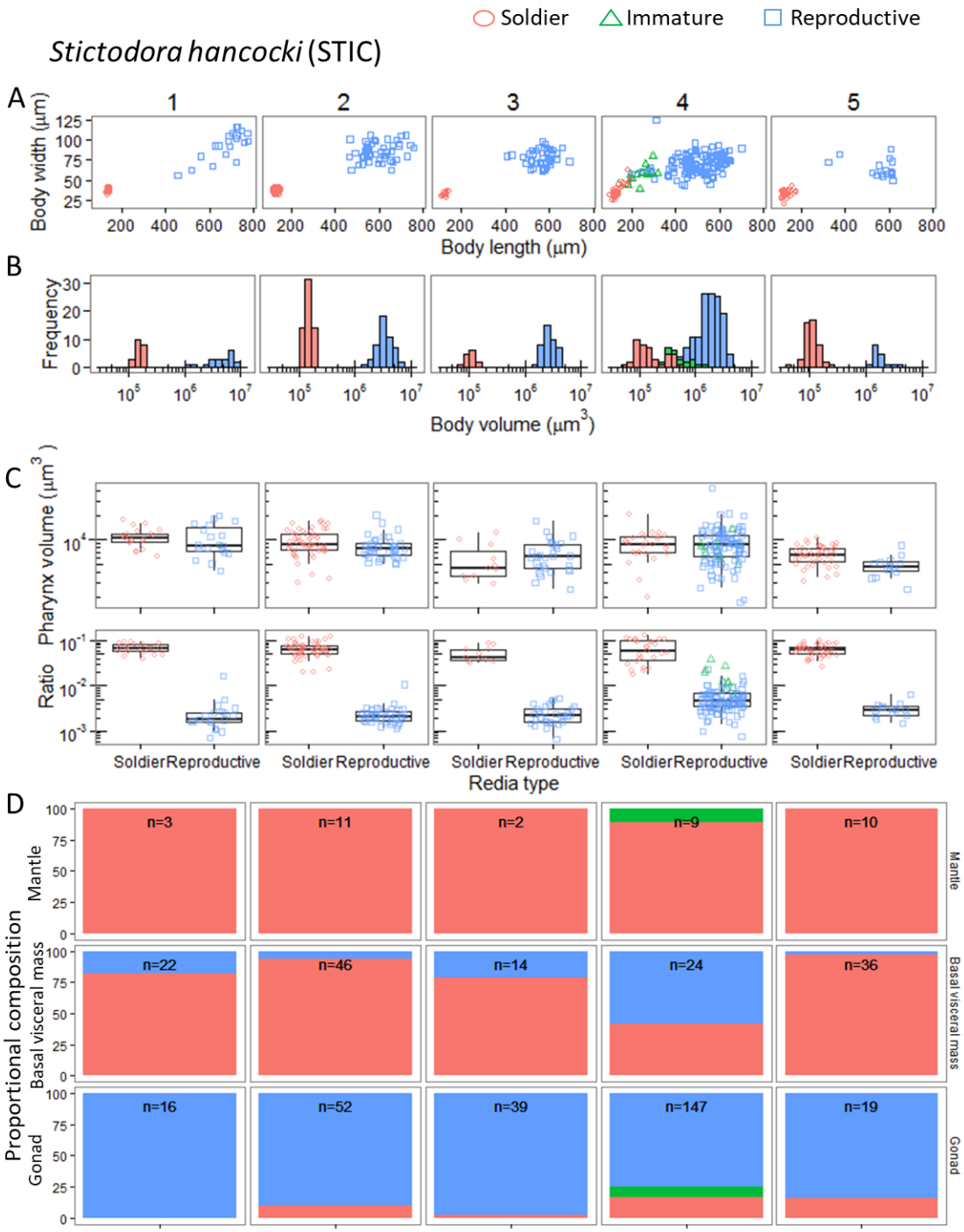
Supplementary Fig. S1. Morphology and distribution of four different *Euhaplorchis californiensis* (EUHA) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single EUHA colony. B) Frequency distributions of body volume for randomly sampled rediae. X-axis is $\log_{10}$scale. (C-D) Boxplots indicating median (line), interquartile range (box) and values that are within 1.5 * interquantile range (whiskers) of data for (C) absolute pharynx volume and (D) pharynx volume relative to body volume. Note the $\log_{10}$ scale for Y-axes. (E) Proportion of reproductive, immature and solider rediae found in mantle, basal visceral mass and gonad regions for each EUHA colony examined. Numbers in boxes indicate total number of parthenitae sampled from each given region.



Supplementary Fig. S2. Morphology and distribution of three different *Phocitremoides ovale* (PHOC) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single PHOC colony. B) Frequency distributions of body volume for randomly sampled rediae. X-axis is $\log_{10}$scale. (C-D) Boxplots indicating median (line), interquartile range (box) and values that are within 1.5 * interquantile range (whiskers) of data for (C) absolute pharynx volume and (D) pharynx volume relative to body volume. Note the $\log_{10}$ scale for Y-axes. (E) Proportion of reproductive, immature and solider rediae found in mantle, basal visceral mass and gonad regions for each PHOC colony examined. Numbers in boxes indicate total number of parthenitae sampled from each given region.



Supplementary Fig. S3. Morphology and distribution of three different *Pygidiopsoides spindalis* (PYGI) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single PYGI colony. B) Frequency distributions of body volume for randomly sampled rediae. X-axis is $\log_{10}$scale. (C-D) Boxplots indicating median (line), interquartile range (box) and values that are within 1.5 * interquantile range (whiskers) of data for (C) absolute pharynx volume and (D) pharynx volume relative to body volume. Note the $\log_{10}$ scale for Y-axes. (E) Proportion of reproductive, immature and solider rediae found in mantle, basal visceral mass and gonad regions for each PYGI colony examined. Numbers in boxes indicate total number of parthenitae sampled from each given region.

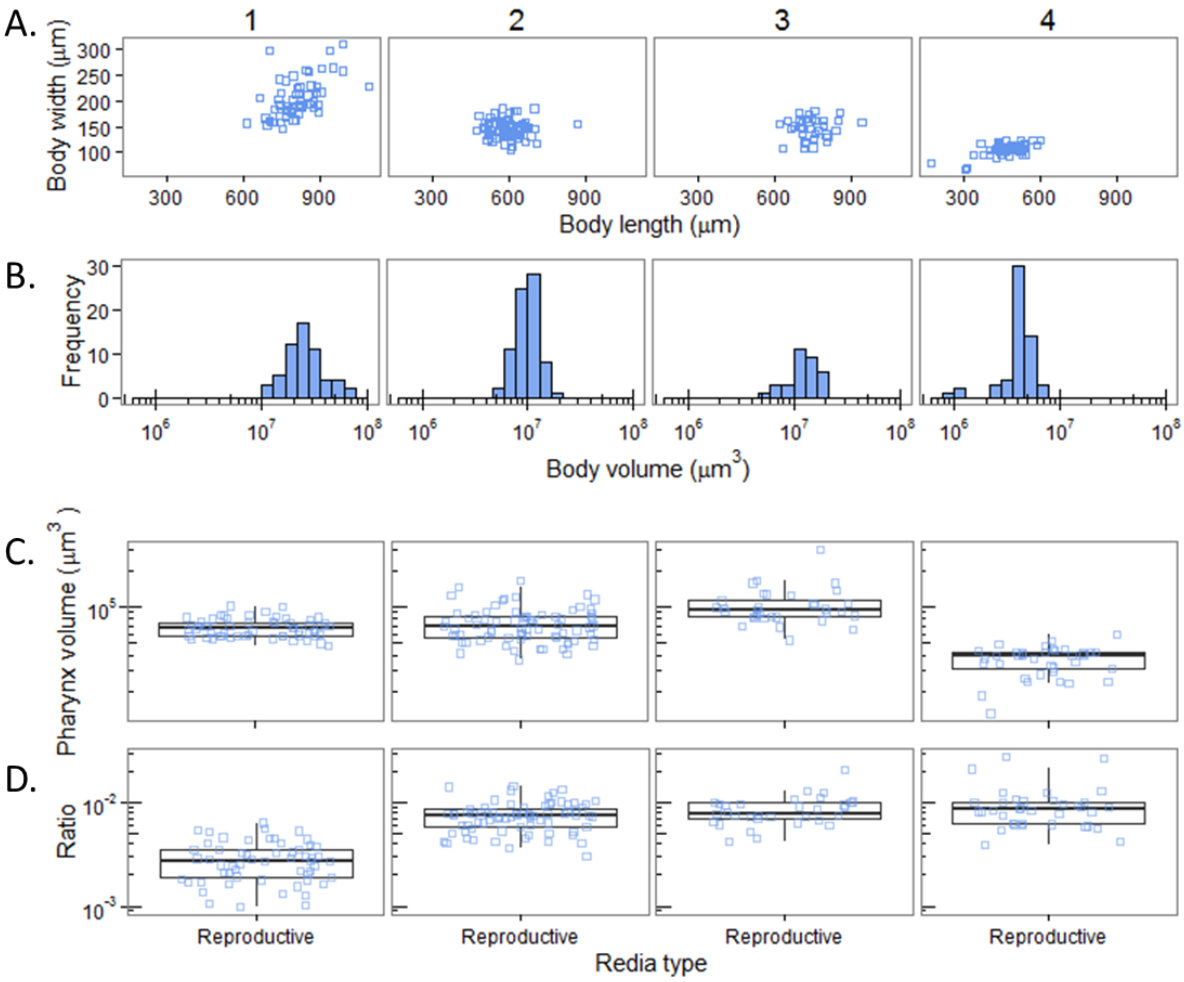


Supplementary Fig. S4. Morphology and distribution of five different *Stictodora hancocki* (STIC) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single STIC colony. B) Frequency distributions of body volume for randomly sampled rediae. X-axis is $\log_{10}$scale. (C-D) Boxplots indicating median (line), interquartile range (box) and values that are within 1.5 * interquantile range (whiskers) of data for (C) absolute pharynx volume and (D) pharynx volume relative to body volume. Note the $\log_{10}$ scale for Y-axes. (E) Proportion of reproductive, immature and solider rediae found in mantle, basal visceral mass and gonad regions for each STIC colony examined. Numbers in boxes indicate total number of parthenitae sampled from each given region.

Supplementary Table S1. Statistics for each individual colony examined.

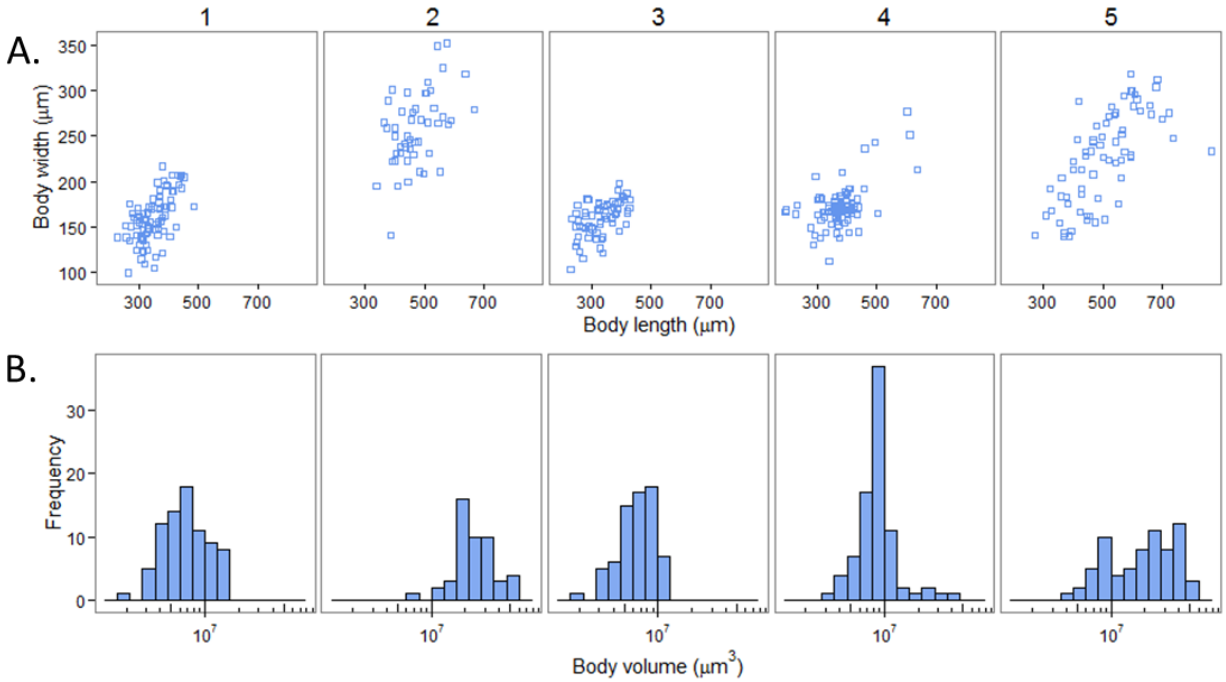
Species	Colony ID	N (Soldier, Immature+Reproductive)	Proportion immatures	Average body size (μm³)	
				Reproductives	Soldiers
Catatropsis johnstoni	1	58 (0, 0 + 58)	0.0	27,824,579	N/A
	2	75 (0, 0 + 75)	0.0	10,110,210	N/A
	3	33 (0, 0 + 33)	0.0	12,780,123	N/A
	4	57 (0, 1 + 56)	1.8	4,258,115	N/A
Probolocoryphe uca	1	78 (0, 1 + 77)	1.3	7,276,544	N/A
	2	70 (0, 1 + 69)	1.4	6,761,718	N/A
	3	84 (0, 0 + 84)	0.0	9,286,241	N/A
Small microphallid	1	82 (0, 1 + 81)	1.2	12,833,127	N/A
	2	81 (0, 0 + 81)	0.0	18,068,054	N/A
Renicola buchanani	1	70 (0, 0 + 70)	0.0	211,380,137	N/A
	2	87 (0, 2 + 85)	2.3	185,511,765	N/A
	3	52 (0, 0 + 52)	0.0	298,728,602	N/A
	4	40 (0, 0 + 40)	0.0	353,707,005	N/A
	5	45 (0, 0 + 45)	0.0	227,578,677	N/A
	6	57 (0, 0 + 57)	0.0	263,688,028	N/A
***Renicola* sp. "polychaetophila"**	1	80 (0, 0 + 80)	0.0	5,874,682	N/A
	2	28 (0, 2 + 26)	7.1	2,328,015	N/A
Mesostephanus appendiculatus	1	27 (0, 2 + 25)	7.4	22,725,650	N/A
	2	43 (0, 0 + 43)	0.0	89,078,540	N/A
	3	36 (0, 2 + 34)	5.6	110,858,903	N/A
	4	62 (0, 4 + 58)	6.5	55,620,019	N/A
	5	37 (0, 0 + 37)	0.0	19,652,982	N/A
Small cyathocotylid	1	53 (0, 1 + 52)	1.9	15,112,085	N/A
	2	37 (0, 0 + 37)	0.0	14,931,462	N/A
***Austrobilharzia* sp.**	1	68 (0, 2 + 66)	2.9	27,289,898	N/A
	2	46 (0, 0 + 46)	0.0	13,162,857	N/A
Himasthla rhigedana	1	85 (21, 31 + 33)	36.5	25,411,404	4,318,651
	2	31 (8, 19 + 4)	61.3	13,512,043	4,643,556

Figure S1. *Catatropsis johnstoni* (CATA)



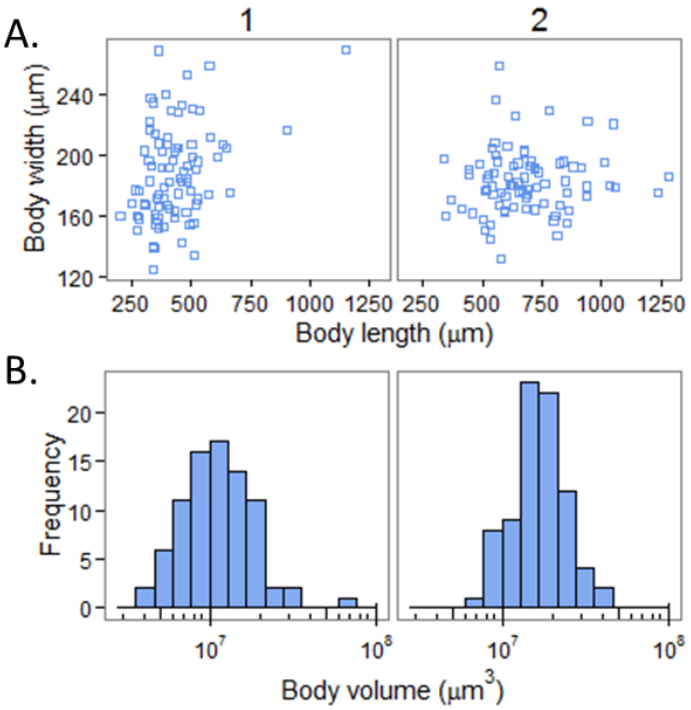
Supplementary Figure S1. Morphology and distribution of four different *Catatropis johnstoni* (CATA) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled redia from a single colony. B) Frequency distributions of body volume for randomly sampled rediae. X-axis is $\log_{10}$scale. (C-D) Boxplots indicating median (line), interquartile range (box) and values that are within 1.5 * interquantile range (whiskers) of data for (C) absolute pharynx volume and (D) pharynx volume relative to body volume. Note the $\log_{10}$ scale for Y-axes.

Figure S2. *Probolocoryphe uca* (PROB)



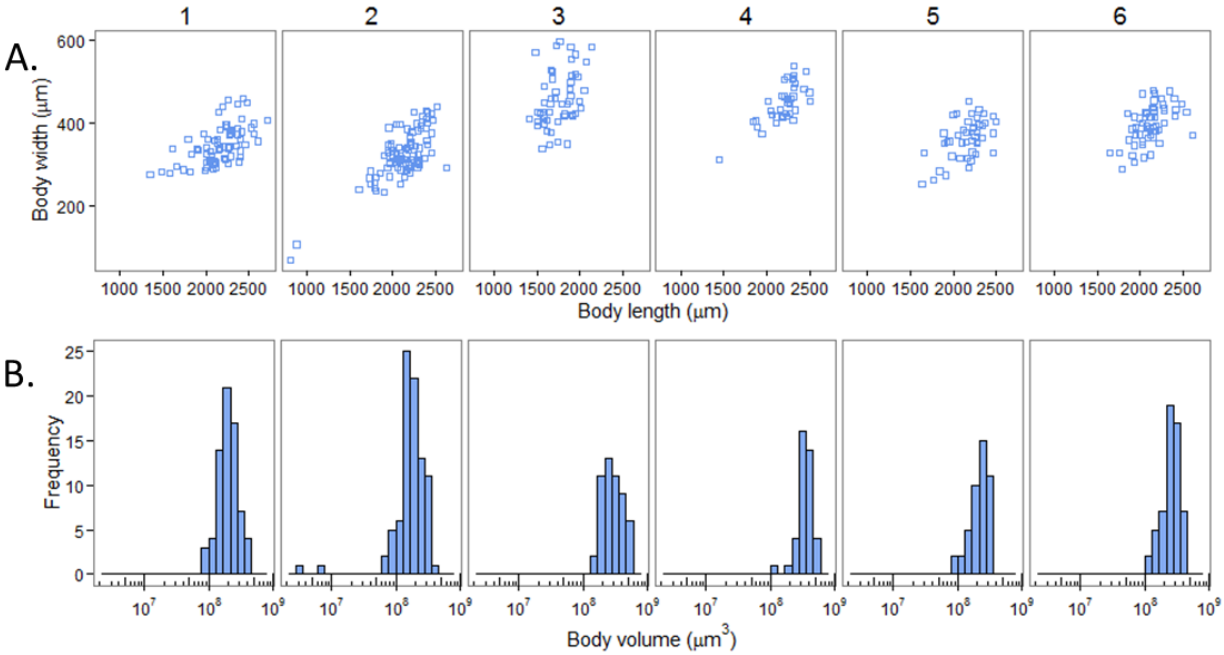
Supplementary Figure S2. Morphology and distribution of five different *Probolocoryphe uca* (PROB) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled sporocyst from a single colony. B) Frequency distributions of body volume for randomly sampled sporocysts. X-axis is $\log_{10}$scale.

Figure S3. Small microphallid (SMMI)



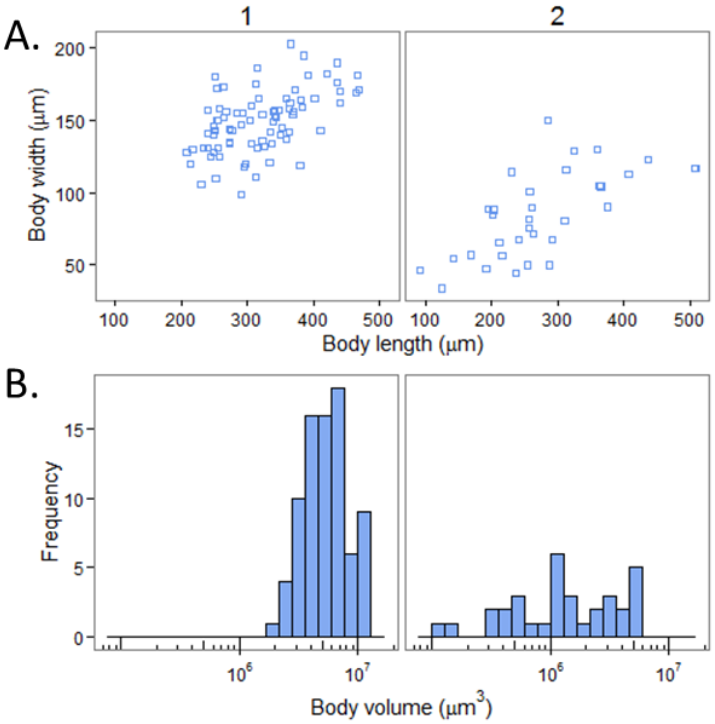
Supplementary Figure S3. Morphology and distribution of two different Small microphallid (SMMI) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled sporocyst from a single colony. B) Frequency distributions of body volume for randomly sampled sporocysts. X-axis is $\log_{10}$scale.

Figure S4. *Renicola buchanani* (REBU)



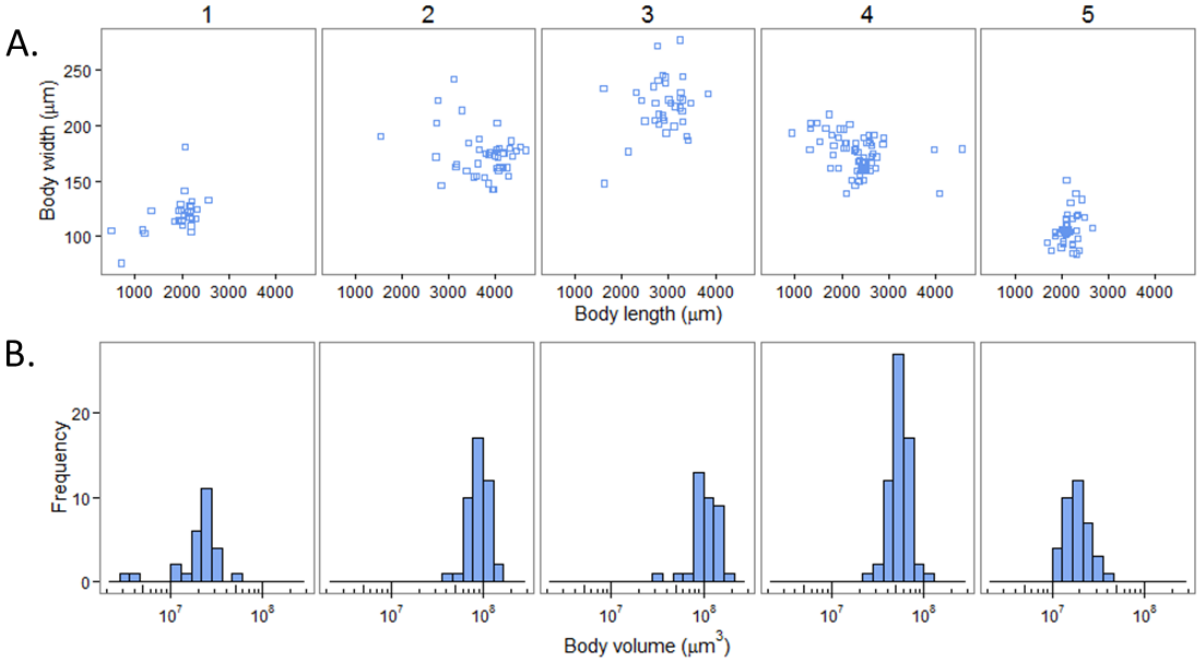
Supplementary Figure S4. Morphology and distribution of six different *Renicola buchanani* (REBU) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled sporocyst from a single colony. B) Frequency distributions of body volume for randomly sampled sporocysts. X-axis is $\log_{10}$scale.

Figure S5. *Renicola* sp. *"polychaetophila"* (REPO)



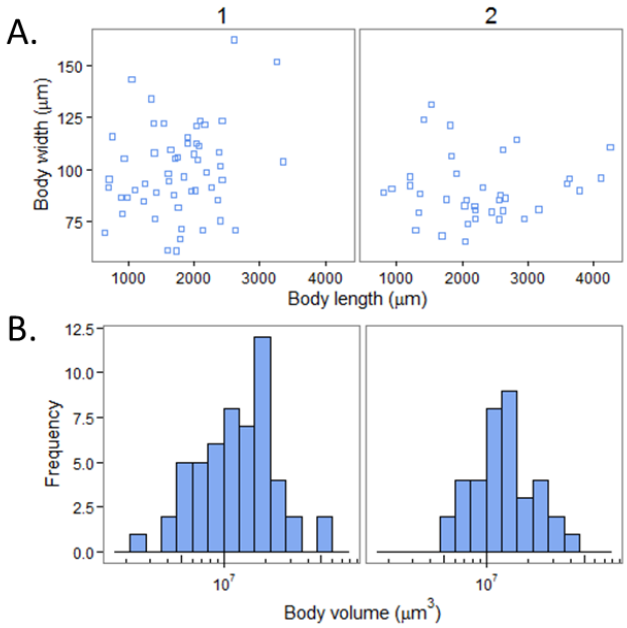
Supplementary Figure S5. Morphology and distribution of two different *Renicola* sp. "*polychaetophila"* (REPO) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled sporocyst from a single colony. B) Frequency distributions of body volume for randomly sampled sporocysts. X-axis is $\log_{10}$scale.

Figure S6. *Mesostephanus appendiculatus* (MESO)



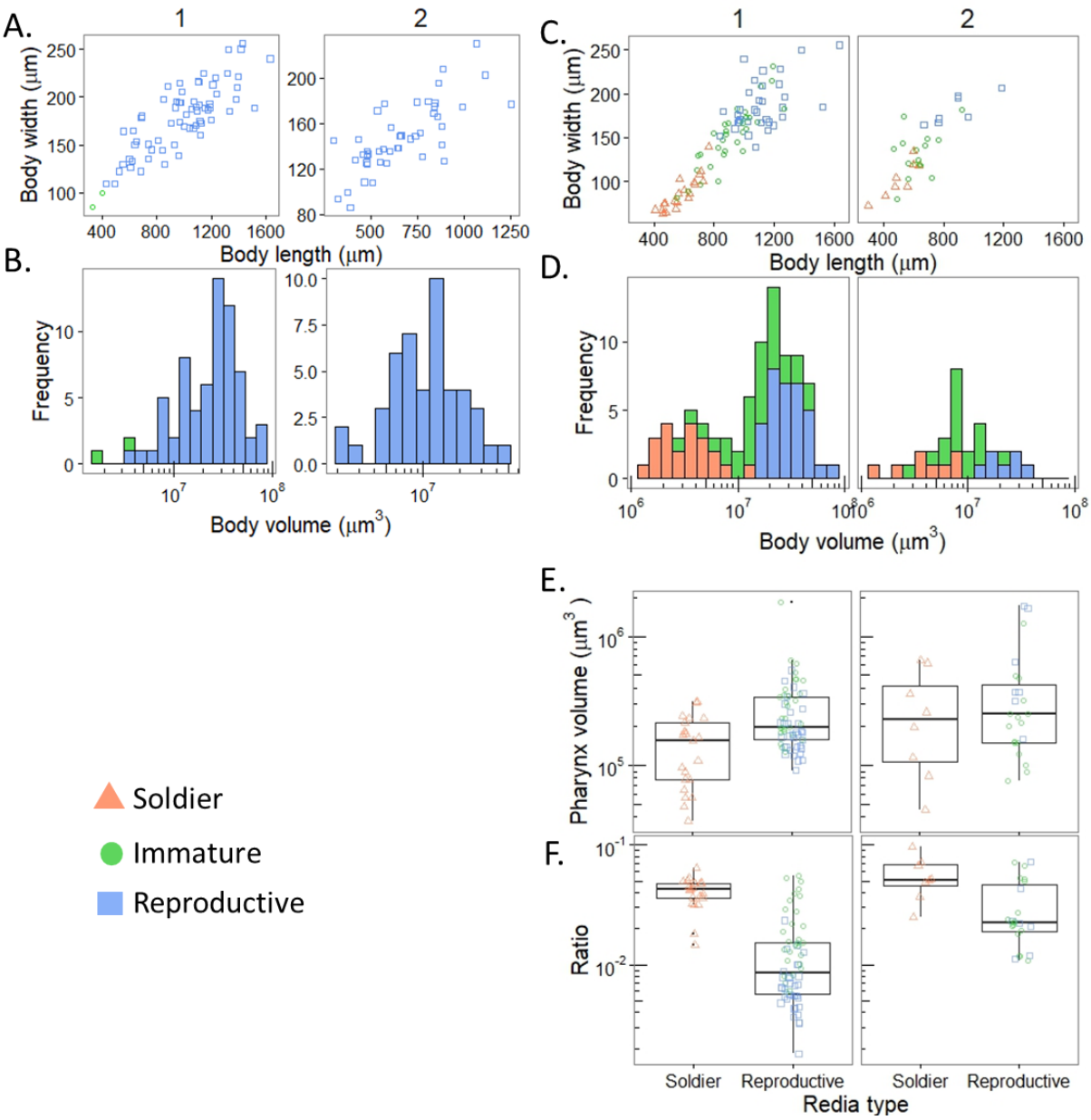
Supplementary Figure S6. Morphology and distribution of five different *Mesostephanus appendiculatus* (MESO) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled sporocyst from a single colony. B) Frequency distributions of body volume for randomly sampled sporocysts. X-axis is $\log_{10}$scale.

Figure S7. Small cyathocotylid (SMCY)



Supplementary Figure S7. Morphology and distribution of two different Small cyathocotylid (SMCY) colonies. A) Body width to body length relationships. Each data point represents one randomly sampled sporocyst from a single colony. B) Frequency distributions of body volume for randomly sampled sporocysts. X-axis is $\log_{10}$scale.

Figure S8. *Austrobilharzia* sp. (AUST) with *Himasthla rhigedana* (HIMA)



Supplementary Figure S8. Morphology and distribution of two different A-B) *Austrobilharzia* sp. (AUST) colonies in co-infection with C-F) *Himasthla rhigedana* (HIMA). A) Body width to body length relationships. Each data point represents one randomly sampled sporocyst from a single colony. B) Frequency distributions of body volume for randomly sampled sporocysts. X-axis is $\log_{10}$scale. C) Body width to body length relationships. Each data point represents one randomly sampled redia from a single colony. D) Frequency distributions of body volume for randomly sampled rediae. X-axis is $\log_{10}$scale. (E-F) Boxplots indicating median (line), interquartile range (box) and values that are within 1.5 * interquantile range (whiskers) of data for (E) absolute pharynx volume and (F) pharynx volume relative to body volume. Note the $\log_{10}$ scale for Y-axes.