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Fitness benefits of a division of labour in parasitic trematode colonies with and without competition

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ABSTRACT

A reproductive division of labour has recently been discovered within polyembryonic colonies of two species of parasitic trematodes infecting snail hosts. In these colonies, one morph expands the colony through asexual reproduction while the other morph never reproduces. As in other polyembryonic species using a division of labour (parasitoid wasps, one species of sea anemone), the non-reproducing morph appears specialized for defense against competing colonies. In this study, we first assessed competition between *Philophthalmus* sp. (which possesses reproducing and non-reproducing morphs) and the most common co-infecting species, *Maritrema novaezealandensis*, by quantifying colony success within snail hosts. Colonies of either species that did not compete within their host were more successful (i.e., produced more transmission stages) than colonies that were competing in a shared host. Second, we cultured individuals of both species in vitro, alone or together, to study the interaction more closely and to measure any advantage obtained by the colony from the non-reproducing morphs. This was done by manipulating the presence and abundance of *M. novaezealandensis* as well as the presence of the non-reproducing 'defensive' morph. *Philophthalmus* sp. colonies with both reproducing and non-reproducing morphs but without *M. novaezealandensis* were most successful. This implies the non-reproducing morphs provide a fitness benefit to *Philophthalmus* sp. colonies even in the absence of competition, although the nature of this advantage remains unclear.

1. Introduction

The asexual multiplication of trematodes within their first intermediate host is a characteristic that sets trematodes apart from other helminths (Galaktionov and Dobrovokskij, 2003). This multiplication amounts to obligate polyembryony, where one egg gives rise to a colony of genetically identical individuals (but genetically distinct from the parents). Although a routine event in the life cycle of thousands of trematode species, this phenomenon is rare and seen in few animal taxa: some flatworms other than trematodes, one order of bryozoan, parasitoid wasps, some hydrozoans and echinoderms, armadillos and one species of sea anemone (Francis, 1973; Craig et al., 1997). Recently, polyembryonic colonies of two trematode species, *Himasthla* sp. and *Philophthalmus* sp. (Hechinger et al., 2011; Leung and Poulin, 2011b), have been shown to consist of two distinct morphs. Although the precise function of each morph still needs to be determined, this polymorphism is strongly suggestive of a potential division of labour within the colony.

Division of labour involves the functional specialisation of individuals or parts of individuals. Defined strictly, division of labour encompasses reproduction, such that not all colony members reproduce (Simpson, 2012). The existence of distinct morphotypes is often associated with division of labour (for example, non-reproducing worker bees and the reproducing queen are distinct morphs). Polymorphism itself is seen in 658 genera across five phyla, but the combination of polymorphism and reproductive division of labour is known for only 164 genera (i.e., one reproducing morph and $\geqslant$1 non-reproducing types) (Simpson, 2012). Across these taxa, there exists a wide diversity of strategies that increase colony size, e.g., parthenogenesis (aphids), arrhenotokous parthenogenesis (bees and ants), and polyembryony (parasitoid wasps, bryozoans, trematodes) (Blackman, 1979; Seeley, 1995; Craig et al., 1997; Brusca and Brusca, 2003). Relatedness of colony members depends on the strategy used for multiplication: only individuals within polyembryonic colonies are 100% related to each other. Therefore, the two species of trematodes in which polymorphism has been reported (Hechinger et al., 2011; Leung and Poulin, 2011b) represent a situation where division of labour should be favoured, as all colony members are clones (resulting from polyembryony) whose individual fitness becomes the same as colony fitness. This is not specific to these two species, as all trematodes form colonies of clones in their first intermediate host, and division

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of labour may be more widespread across trematode species (Hechinger et al., 2011).

This is an unusual situation. Obligate polyembryony resulting in a colony of genetically identical individuals also displaying polymorphism is rare; to our knowledge, this includes some parasitoid wasps, one order of bryozoans, one species of sea anemone, and the two above-mentioned trematodes (Francis, 1976; Craig et al., 1997; Hechinger et al., 2011; Leung and Poulin, 2011b; Simpson, 2012). In these cases, there appears to be a division of labour between reproducing and non-reproducing morphs within a colony. In well-studied cases (parasitoid wasps and sea anemones), the non-reproducing morph is specialized for defense against competing heterospecifics or conspecifics (Francis, 1976; Cruz, 1981, 1986). In these instances, where the colony fitness equals individual fitness, the evolution of division of labour can be favoured under a wider range of conditions.

However, the apparent division of labour seen within polyembryonic colonies of the trematodes *Himasthla* sp. and *Philophthalmus* sp. (Hechinger et al., 2011; Leung and Poulin, 2011b) is yet to be validated. In these colonies, large asexually reproducing individuals (rediae) exist alongside developmentally and morphologically distinct small, non-reproducing rediae. In *Philophthalmus* sp., cytochrome oxidase subunit 1 (COI) sequencing has confirmed that large and small rediae are of the same species (Leung and Poulin, 2011b) and preliminary analysis using microsatellite markers confirms small and large rediae of the same infection are clonal (M. Lloyd, unpublished data). Furthermore, over 41 days within an in vitro culture system, the small rediae do not grow or develop into large ones, while in parallel cultures, underdeveloped large rediae develop normally to asexual reproduction (M. Lloyd, personal observation). In *Himasthla* sp., morphological differences (small rediae, while much smaller in body size, have muscular pharynges equal in size to those of the large rediae) suggest that small rediae are specialized for defense against co-infecting trematode species within the host where competition for resources of space and food is intense (Hechinger et al., 2011). The small rediae of both *Philophthalmus* sp. and *Himasthla* sp. have been seen feeding on competing heterospecific trematodes in freshly dissected snails (Hechinger et al., 2011; Leung and Poulin, 2011b). Small rediae of both species are found throughout the colony, but much more commonly than large rediae in the mantle of the snail which would be infected by a secondary species (M. Lloyd personal observation; Hechinger et al., 2011). However, the benefits of this division of labour for the colony may also involve non-defensive functions such as nutrient exchanges, since small and large morphs are often in physical contact (M. Lloyd, personal observation), or protection against microbial infection, as is seen in the colonies of several social insects (Turnbull et al., 2012).

The likelihood of co-occurring with a competing colony, and the intensity of the resulting competition, are possibly the main selective forces acting on the evolution of division of labour in trematode colonies. Trematode colonies use molluscs (usually gastropods) as the first host in their complex lifecycle, and each snail may be host to multiple trematode species. When a snail host is infected by more than one colony, each of which is capable of multiplying to occupy 15–30% of the volume within the host shell (Hechinger et al., 2009), competition for space and food is expected (Sousa, 1992; Poulin, 2001). Species interactions between trematodes have generally been tested indirectly by comparing the frequency of observed double species infections with those expected from a null model assuming random assemblages, where a lower-than-expected frequency would indicate competition (Sousa, 1993; Kuris and Lafferty, 1994; Keeney et al., 2008). Evidence of competition can also be obtained from observations of changes in parasite niche within the host in cases of double infection (Leung and Poulin, 2011a), or from comparisons of snap-shot counts of individuals between doubly- and singly-infected snails (Hendrickson and Curtis, 2002; Lagrue et al., 2007; Keeney et al., 2008). Direct evidence has been gained from observations of trematode pairs sharing a snail in which an obviously dominant species attacks and eats the larvae of the co-infecting species (Lie et al., 1965). Competitive interactions that occur in the snail host should have lasting effects throughout the rest of the parasite's complex lifecycle (Fredensborg and Poulin, 2005). Ultimately, the impact of competition within the snail host should be measured by its effect on the colony's output of infective stages (cercariae) produced for transmission to the next host.

Philophthalmus sp. infects the common New Zealand mudsnail, *Zeacumantus subcarinatus*, as well as another trematode, *Maritrema novaezealandensis* (Martorelli et al., 2004). Infection prevalence of each species varies seasonally; in our study site, *M. novaezealandensis* infect 50–80% of snails and *Philophthalmus* sp. from 3–8% of snails, with double infections by both species occurring in up to an additional 11% of snails (Keeney et al., 2008; Bates et al., 2011). In a previous study, species interactions between *M. novaezealandensis* and *Philophthalmus* sp. within their snail host have been indirectly assessed, with no competition detected (frequencies of double species infections were not significantly different from the null model and the number of *Philophthalmus* sp. rediae was not lower in the presence of *M. novaezealandensis*) (Keeney et al., 2008). However, competitive interactions between *M. novaezealandensis* and *Philophthalmus* sp. require further study due to the recent discovery of a division of labour in *Philophthalmus* sp. colonies where a distinct, non-reproducing morph appears specialized for defense in competitive interactions.

We believe that our study is not only one of the first to quantify the fitness effects of division of labour in an obligately polyembryonic species, but it also provides the first experimental test of fitness benefits accruing from division of labour in a parasitic organism. Its aims were to, first, investigate competitive interactions between larvae of *M. novaezealandensis* and *Philophthalmus* sp. in their snail host; second, and more importantly, quantify any advantage to the *Philophthalmus* sp. colony of producing the small, non-reproducing morph. This was done by (i) quantifying colony success in snails with single-species and double-species infections, and (ii) quantifying success of *Philophthalmus* sp. colonies consisting of large, asexually reproducing rediae cultured in vitro with or without the small, non-reproducing redial morph as well as being exposed to varying amounts of co-infecting *M. novaezealandensis*. Our design allowed us to distinguish between a purely defensive role for the small morphs and a non-defensive role yielding benefits to the colony even without competition.

2. Materials and methods

2.1. Study system

Trematodes have complex life-cycles usually involving three hosts. Developmental stages (rediae or sporocysts, depending on the species) reproduce asexually in a first intermediate host, usually a snail, almost filling the snail shell with rediae or sporocysts as well as cercariae, the free-swimming infective stages that leave the snail to encyst in or on a second intermediate host and await ingestion by the definitive host. In the latter, development to adulthood occurs, as well as sexual reproduction and the release of eggs (Galaktionov and Dobrovokskij, 2003).

The New Zealand mudsnail, *Zeacumantus subcarinatus*, is the first intermediate host of multiple trematode species including *Philophthalmus* sp. (rediae only within the first intermediate host) and *M. novaezealandensis* (sporocysts only within the first intermediate host) (West, 1961; Martorelli et al., 2008). *Maritrema novae-*

zealandensis cercariae leave the snail to encyst in one of several suitable crustaceans serving as second intermediate hosts (Martorelli et al., 2004). Cercariae of *Philophthalmus* sp. leave the snail and encyst on the shells of various species of gastropods (Neal and Poulin, 2012). Here they await ingestion by the definitive host within which they will migrate to their site of infection: the intestine of a gull in the case of *M. novaezealandensis* (Martorelli et al., 2004) and the gull's orbit in the case of *Philophthalmus* sp. (Howell, 1965). Cercariae of *Philophthalmus* sp. readily encyst on artificial substrates, such as plastic surfaces (Lei and Poulin, 2011), such that their accumulation in experimental cultures is easily monitored.

2.2. Competition within naturally infected snails

2.2.1. Snail collection, screening and maintenance

Zeacumantus subcarinatus snails were collected from Lower Portobello Bay, Otago Harbour, South Island, New Zealand (45°52′ S, 170°42′ E) during December 2010 or February 2011. They were screened for infection so that they could be distinguished as infected by either *Philophthalmus* sp. only, *M. novaezealandensis* only, or both species. This was achieved by forcing cercarial release from colonies by incubating snails overnight at 26 °C in wells of a 12-well culture plate filled with natural seawater. Ten snails of each desired infection type were numbered and colour coded according to infection (with cyanoacrylate glue and numbered plastic tags from Bee Works, Orillia, ON, Canada). Snails in each group ranged between 12.0 and 17.9 mm in shell length; however, shell sizes were evenly matched among the three groups to standardize this variable across groups and eliminate any potential host-size effects. Snails were kept in plastic containers (17 × 17 cm) for up to 2 months prior to the experiment; each container was filled with natural, aerated seawater, 2 mm of sand and ample sea lettuce, *Ulva lactuca*. Containers were cleaned and water was changed weekly.

2.2.2. Counting Philophthalmus sp. cercariae

Colony success within each snail was measured by counting the number of emerging cercariae. To quantify the cercarial output of *Philophthalmus* sp. colonies, snails were kept in wells of six-well culture plates filled with natural seawater and a small piece of sea lettuce. Before the start of the experiment, snails were allocated to wells and incubated at 26 °C overnight to standardize their prior cercarial load to zero. On Day 0 of the experiment, the snails were transferred to clean wells. On Day 1, and every 3–4 days thereafter, *Philophthalmus* sp. cercariae and encysted cercariae were counted in the well and on the sea lettuce. *Philophthalmus* sp. does not infect a second intermediate host, as is common for other trematode species, and the number of cercariae shed from the snail and encysting on substrates is the best available measure of colony success. To ensure that the environment was suitably aerated and clean, on the days when cercariae in the wells were counted, the water was changed, the wells were cleaned, and a fresh piece of sea lettuce was added. For most of the experiment, the six-well plates were kept at room temperature and under ambient light.

2.2.3. Counting M. novaezealandensis cercariae

Unlike *Philophthalmus* sp., *M. novaezealandensis* infects a second intermediate host, therefore its cercariae did not encyst in the wells. Furthermore, accumulated cercariae of *M. novaezealandensis* emerge from infected snails in distinct pulses separated by periods of little or no output (Studer et al., 2010). In the laboratory, this can be induced by incubation at 26 °C and under bright light. Every 2 weeks, all snails in the experiment were forced to shed *M. novaezealandensis* cercariae by incubation overnight. Under exposure to increased heat and light, a greater number of *Philophthalmus* sp. cercariae also emerge from their host. Therefore, snails harbouring only *Philophthalmus* sp. were also exposed to this incubation. Cercariae of *M. novaezealandensis* were counted only on days following an incubation period.

2.2.4. Data analysis

Significant differences in numbers of cercariae (free-swimming and encysted combined) of *Philophthalmus* sp. and cercariae of *M. novaezealandensis* between colonies in snails of different infection types were tested using a repeated measures ANOVA in the statistics programme SPSS. Time (day) was the within-subjects variable and infection type was the between-subjects variable. Sphericity was tested using Mauchly's test. When sphericity could not be assumed, the Greenhouse-Geisser correction was used.

2.3. In vitro culture experiment

Zeacumantus subcarinatus snails were collected from the same site as mentioned in Section 2.2.1 during February 2011 or June 2011. They were screened for single species infections by *Philophthalmus* sp. or *M. novaezealandensis* by forcing cercarial release by incubation (see Section 2.2.1). Infected snails were kept in the laboratory for up to 3 months, under the same conditions as snails from the previous experiment.

2.3.1. Culturing

Philophthalmus sp. rediae and *M. novaezealandensis* sporocysts were dissected out of separate, singly-infected snails. They were cultured in vitro using a culture system previously tested for both species (Lloyd and Poulin, 2011). Briefly, the culture medium consists of 400 mg of commercially availavle Leibovitz-15 powder (Sigma L4386, a culture media containing inorganic salts, amino acids, and vitamins), 1.56 g of Instant Ocean powder (www.instantocean.com, USA) and 1 ml of penicillin–streptomycin–neomyacin solution (Sigma P4083) in 50 ml of autoclaved water (more details in Lloyd and Poulin, 2011). Dissected rediae and sporocysts were separated into eight different treatments in individual wells of a 12-well plate (Fig. 1). Importantly, *Philophthalmus* sp. rediae (small and large) from the same snail were used for one replicate of each treatment, creating a blocked design in which any effect of genetic variation among colonies was neutralised. It is possible that the individual snails harboured not one, but two or three genetically distinct *Philophthalmus* sp. colonies, each issued from a different egg. However, this is unlikely to be a problem for three reasons.

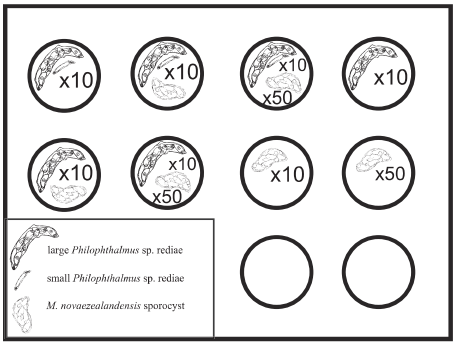

Fig. 1. Design of the in vitro experimental culture of *Philophthalmus* sp. and *Maritrema novaezealandensis* colonies. For one set of replicates, as illustrated here, all *Philophthalmus* sp. rediae came from the same snail and all *M. novaezealandensis* sporocysts came from the same snail. The number of each type of rediae or sporocysts per replicate well (circle) on a culture plate is indicated for each of the eight treatments. The approximate sizes of the different types of rediae or sporocysts are shown in relation to each other and all are an order of magnitude smaller than the well.

First, previous studies on other trematode species indicate that the majority of infections consist of a single trematode clone per snail (Theron et al., 2004; Keeney et al., 2007; Lagrue et al., 2007), with rare exceptions (Rauch et al., 2005). Second, a preliminary analysis of *Philophthalmus* sp. infections in snails from our study site using microsatellite markers also indicates that single-clone infections are numerically much more common than multi-clone infections; and small and large rediae of the same infection are clonal (M. Lloyd, unpublished data). Third, even if a snail harbours two or three clones, the random allocation of sets of individual rediae from the same snail to different treatments ensured homogenisation of genotypes across treatments.

The treatments were designed to allow the measurement of the separate and combined effects of the presence of small non-reproductive rediae and heterospecific competitors on *Philophthalmus* sp. colony success. They were: (i) 10 large *Philophthalmus* sp. rediae and 10 small rediae; (ii) 10 large *Philophthalmus* sp. rediae, 10 small rediae and approximately 10 *M. novaezealandensis* sporocysts (sporocysts are very difficult to separate without breaking the tegument, thus counts were approximate); (iii) 10 large *Philophthalmus* sp. rediae, 10 small rediae and approximately 50 *M. novaezealandensis* sporocysts; (iv) 10 large *Philophthalmus* sp. rediae; (v) 10 large *Philophthalmus* sp. rediae and approximately 10 *M. novaezealandensis* sporocysts; (vi) 10 large *Philophthalmus* sp. rediae and approximately 50 *M. novaezealandensis* sporocysts; (vii) 10 *M. novaezealandensis* sporocysts; and (viii) 50 *M. novaezealandensis* sporocysts (Fig. 1). Although the total number of individual rediae and sporocysts is not constant across treatments, this 'additive design' is ideally suited to evaluate the effects of competitors or helpers (small rediae) on the performance of the 'focal' large rediae (Inouye, 2001). Sporocysts and rediae dissected out of their respective host snails contributed to all treatments within one plate (one of each of the above listed treatments per plate), thus each pair of snails acted as one genetically homogeneous replicate, allowing for robust comparisons between treatments. In total there were 13 replicates, i.e., 13 initial *Philophthalmus* sp. colonies split into smaller experimental cultures. Cultures were always started on a Monday and media changed every Friday and Monday thereafter.

To measure *Philophthalmus* sp. colony success, encysted cercariae were counted every weekday (Monday–Friday) for up to 30 days after the culture was started. Cultures were kept in the dark at room temperature except for the time taken to record data and change culture media. Counted cysts were removed from the cultures by pipetting to ensure they were not recounted on subsequent days. *Maritrema novaezealandensis* sporocysts were scored as alive if they continued to release cercariae. Initially, *Philophthalmus* sp. rediae were predicted to kill and eat *M. novaezealandensis* sporocysts; therefore, cultures with only *M. novaezealandensis* sporocysts served as a control to compare the health of sporocysts co-cultured with *Philophthalmus* sp. rediae.

2.3.2. Data analysis

Counts of encysted *Philophthalmus* sp. cercariae per well per day were recorded and compared between treatments. To achieve this, a generalised linear mixed model GLMM was performed in R version 2.14.0 (R Development Core Team, 2011. R: a language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria) using the package glmmADMB version 0.6.5 (Skaug, H., Fournier, D., Nielsen, A., Magnusson, A., Bolker, B. 2011. glmmADMB: generalised linear mixed models using AD Model Builder.).

The main factors included the presence or absence of small *Philophthalmus* sp. rediae, the number of *M. novaezealandensis* (0 sporocysts, ~10 sporocysts or ~50 sporocysts), and the interaction between the two. Random factors included the plate number representing the snail identity from which the rediae and sporocysts were dissected (one *Philophthalmus* sp. infected snail contributed all of the rediae across treatments of one plate and one *M. novaezealandensis* infected snail contributed all of the sporocysts to that plate) and well position nested within plate (to account for the repeated measures of each culture on each day). Data were zero inflated (i.e., many cysts were counted in cultures on a few days, but few or zero cysts were seen on most days) and over-dispersed. To reduce the number of zeros in the data, daily counts were pooled across three consecutive days, and these 3-days counts were used in the models. Furthermore, models were compared which incorporated negative binomial and Poisson error structures as well as zero inflation. The model with a negative binomial error structure and without zero inflation was chosen as the best fit model based on the lowest Akaike Information Criterion (AIC) value.

3. Results

3.1. Competition within naturally infected snails

Of the 10 snails with both infections (*M. novaezealandensis* and *Philophthalmus* sp.) and the 10 with only *M. novaezealandensis* infections, nine of each survived until day 70. Of the 10 snails with only *Philophthalmus* sp. infections, six survived until day 70. Cercariae of both species continued to emerge from all double infections over the 70 days (i.e., neither species was excluded from the host over 70 days). In the case of single infection snails, only cercariae of the species type in which they had been initially categorised emerged. Furthermore, at the end of the experiment, snails were dissected to confirm that all had been correctly classified as strictly singly- or doubly-infected.

Counts of *Philophthalmus* sp. cercariae (free-moving and encysted combined) from colonies in snails infected with both species were significantly lower than from colonies in snails with only *Philophthalmus* sp. (Fig. 2, Table 1). Similarly, colonies in snails harbouring both infections released significantly fewer *M. novaezealandensis* cercariae than colonies in snails harbouring only *M. novaezealandensis* (Fig. 3, Table 2). Average counts of cercariae following incubation events from *Philophthalmus* sp. colonies decreased over the length of the experiment (Fig. 2). This could be because cercarial release decreased as the snail approached death (Karvonen et al., 2004). Infection by either *Philophthalmus* sp., *M. novaezealandensis*, or both has not been proven to shorten the life of the snails. However, these snails were perhaps kept under less than ideal conditions (small wells, limited space and water), therefore some mortality was expected.

3.2. In vitro culture experiment

The longest surviving cultures remained free of contamination for 37 days. The majority of cultures were terminated at 25 or 28 days due to bacterial or fungal contamination. If a culture did not survive until the 37th day it was not because the rediae or sporocysts died, but because the culture became contaminated. Colonies tended to produce large numbers of cercariae/cysts during the first days after initiation (up to 43 in the first 3 days) followed by fewer on subsequent days (generally 0–5) but continued to produce cercariae/cysts throughout the experimental culture period. Colonies which included both small and large *Philophthalmus* sp. rediae and excluded *M. novaezealandensis* sporocysts were most successful, producing the highest numbers of accumulated cysts over the culture period (Fig. 4). According to the GLMM, significant factors included the presence of small *Philophthalmus* sp. rediae and the interaction between the presence of small *Philophthalmus* sp. rediae and the presence of 10 *M. novaezealandensis* sporocysts (Table 3).

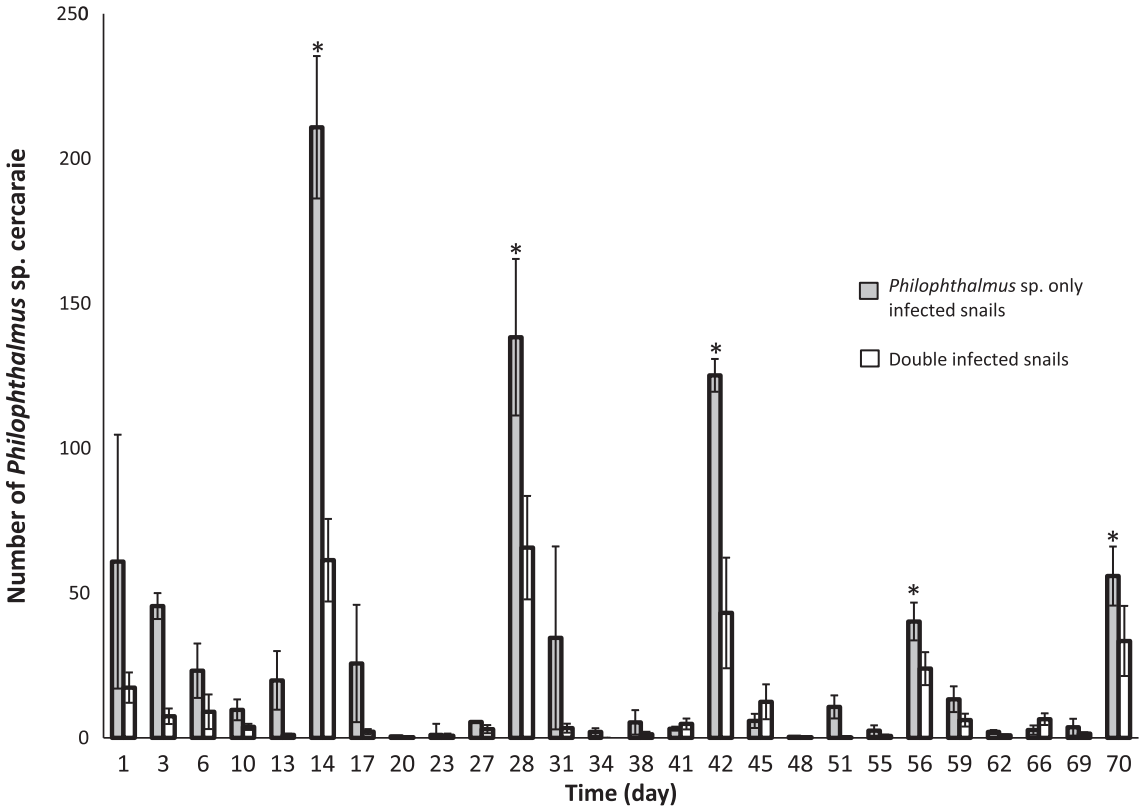

Fig. 2. Average (±S.E.) number of *Philophthalmus* sp. cercariae (encysted and free-swimming combined) released daily by snails with single versus double infections. Asterisks indicate days after incubation (coinciding with days on which cercariae from *Maritreama novaezealandensis* were counted).

Table 1
Competition within naturally infected snails: results of a repeated measures ANOVA comparing cercarial output of snails infected only with *Philophthalmus* sp. versus snails infected with both *Philophthalmus* sp. and *Maritrema novaezealandensis*.

Factor	df	Mean Squares	*F*	*P*
Between subjects				
Infection	1	40,833.600	6.355	0.026
Error	13	6,425.682		
Within subjects				
Time	2.909	147,117.460	9.088	<0.0001
Time * Infection	2.909	36,807.608	2.274	0.097
Error	37.820	16,188.902		

4. Discussion

The recent discovery of polymorphism in trematode colonies, involving the co-occurrence of a small, non-reproducing morph and a larger reproductive one, suggested a division of labour within the clonal colonies of these parasites in their snail host (Hechinger et al., 2011; Leung and Poulin, 2011b). Until now, this suggestion remained untested. Here, it was demonstrated that interspecific competition can markedly reduce colony success in *Philophthalmus* sp., providing sufficient selective pressure to favour adaptive strategies to counter its effects. More importantly, it was shown experimentally that the presence of the small non-reproductive morph may not only mitigate the impact of interspecific competition, but also provide benefits to the colony in the absence of competitors.

Competitive interactions between parasite species co-infecting their intermediate host are often species-specific and involve a wide range of mechanisms. Multiple factors lead us to expect a negative association between *Philophthalmus* sp. and *M. novaezealandensis*: infection prevalences of both species are relatively high, cercarial production is probably limited by the resources that the colony can obtain from the snail, as suggested earlier (Karvonen et al., 2012), and both species benefit by maximising cercarial output to increase the odds of reaching a similar definitive host.

However, prior evidence suggested a neutral interaction between *Philophthalmus* sp. and *M. novaezealandensis* within their snail host based on observed frequencies of double-infections from three field surveys (Keeney et al., 2008; Bates et al., 2011). The interaction between colonies in a laboratory setting was examined due to the recently observed non-reproducing morph within *Philophthalmus* sp. colonies that may be specialised for defense against co-infecting colonies (the most common being *M. novaezealandensis*). Our results showed reciprocally decreased colony success, as measured by the production of transmission stages (cercariae), during co-infection, suggesting a negative competitive interaction between the two species. Perhaps not surprisingly, in a study of amphibians and their competing trematode parasites, Johnson and Buller (2011) also found contrasting outcomes in their field survey and laboratory experiment. Furthermore, in a review of the effectiveness of methods used to investigate species interactions, Fenton et al. (2010) concluded that strong interactions actually occur in half of the cases where none are predicted based on observed infection rates.

The results of the field study of Keeney et al. (2008) showed that both the number of *Philophthalmus* sp. rediae and the number of genotypically distinct *M. novaezealandensis* colonies were not affected by co-infection. Perhaps competition is not acting on that stage of the life-cycle but on the transmission stage only (i.e., there is enough food and space to adequately provide for rediae and sporocysts of both species, but not for the production of cercariae). This could certainly favour the evolution of a non-reproducing morph within trematode colonies. In the case of *Philophthalmus*

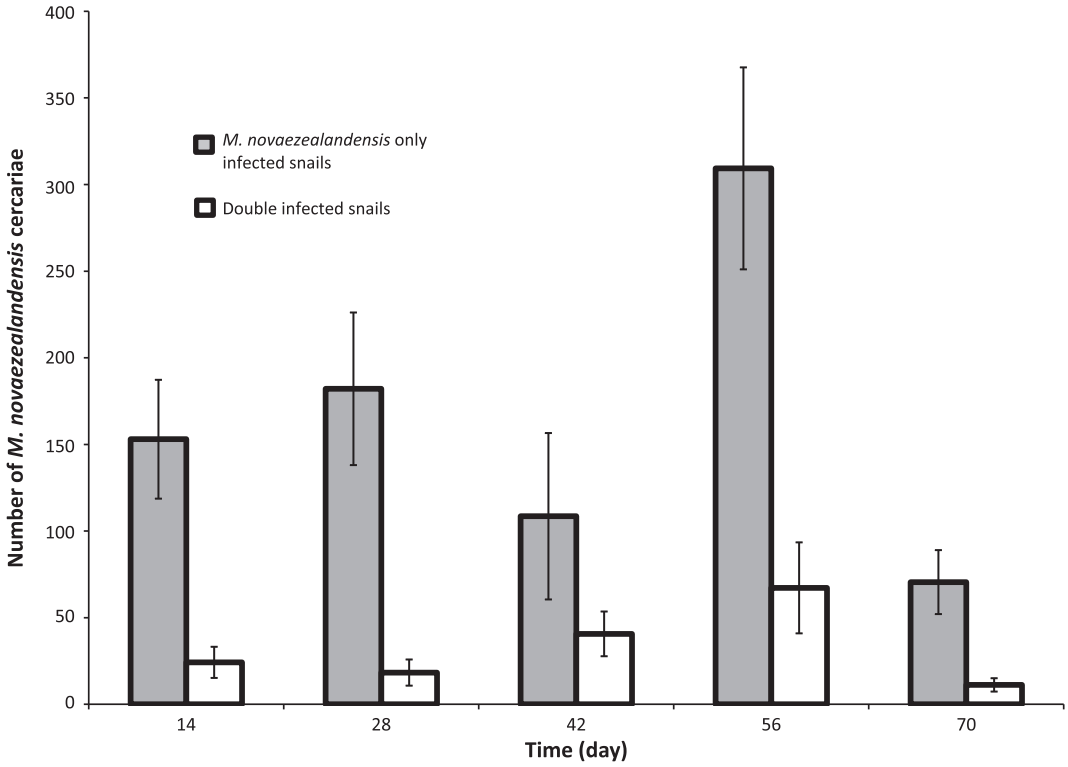

Fig. 3. Average (± S.E.) number of *Maritrema novaezealandensis* cercariae released daily by snails with single versus double infections.

Table 2
Competition within naturally infected snails: results of a repeated measures ANOVA comparing cercarial output of snails infected only with *Maritrema novaezealandensis* versus snails infected with both *Philophthalmus* sp. and *M. novaezealandensis*.

Factor	df	Mean Squares	*F*	*P*
Between subjects				
Infection	1	325,597.601	19.652	<0.0001
Error	16	16,568.353		
Within subjects				
Time	3.068	60,077.560	5.481	0.002
Time * Infection	3.068	32,306.101	2.948	0.041
Error	49.090	10,960.493		

sp., more than half of the snail population harbours *M. novaezealandensis*. This experiment showed that when both species share the same snail, the cercarial output of *Philophthalmus* sp. is reduced by over 50% (see Fig. 2); interspecific competition thus appears very important.

In other examples of species that form genetically identical colonies using a reproductive division of labour, the purpose of the non-reproducing members is for defense in competitive interactions (Francis, 1976; Cruz, 1981, 1986). For this reason, the non-reproducing rediae seen in *Philophthalmus* sp. colonies were expected to be specialised for defense against the very common co-infecting trematode, *M. novaezealandensis*. Earlier, Leung and Poulin (2011b) reported that the small, non-reproducing rediae were seen attacking *M. novaezealandensis* sporocysts or cercariae in freshly-dissected snails. Here, this potential defensive role was tested using in vitro cultures, where the presence of non-reproducing rediae as well as the presence and abundance of competitors could be manipulated, while controlling for the genetic composition of the cultures.

The results of our experiment indicate a clear advantage in having non-reproductive morphs within the colony, but not necessarily with respect to defense against competitors. There was a significant effect of the presence of the non-reproducing rediae, whereby colonies with both small and large rediae produced significantly more cercariae than colonies with only large rediae. This implies the small non-reproducing rediae provide some benefit to the colony, even in the absence of competitive pressure from *M. novaezealandensis*. There was also an effect of the interaction between the presence of the non-reproducing rediae and the presence of 10 *M. novaezealandensis* sporocysts (but not an interaction between the presence of the non-reproducing rediae and the presence of 50 *M. novaezealandensis* sporocysts). This could potentially indicate that the non-reproducing rediae benefit the colony when it is competing with *M. novaezealandensis*, but if this were the case a significant interaction with the presence of 50 *M. novaezealandensis* sporocysts would be expected as well.

The in vitro culture system may not have been ideal to study competitive interactions: there was no effect of the presence or abundance of *M. novaezealandensis*. Furthermore, in cultures of both species, the small non-reproducing *Philophthalmus* sp. rediae were not seen attacking and eating *M. novaezelandensis* sporocysts. This could be because competitive pressures were not strong enough in the cultures or the non-reproducing rediae benefit the colony in another way. Competitive pressures expected in this interaction are for space and food; the culture medium for this experiment was supplemented with a food source which has been shown to be necessary to keep cultures alive over a long period of time (Lloyd and Poulin, 2011) and limiting space in culture wells is not possible. Other possible benefits of the non-reproducing rediae need to be considered. Perhaps some form of communication or exchanges take place between morphs, with positive results in terms of cercarial production. Within the cultures, conspecifics of the same or different morphs are frequently seen in physical contact, attached for long periods via a small protuberance on the rear or lateral portion of the redial body, known as the posterior adhesive appendage or lateral adhesive appendage (West, 1961). Those

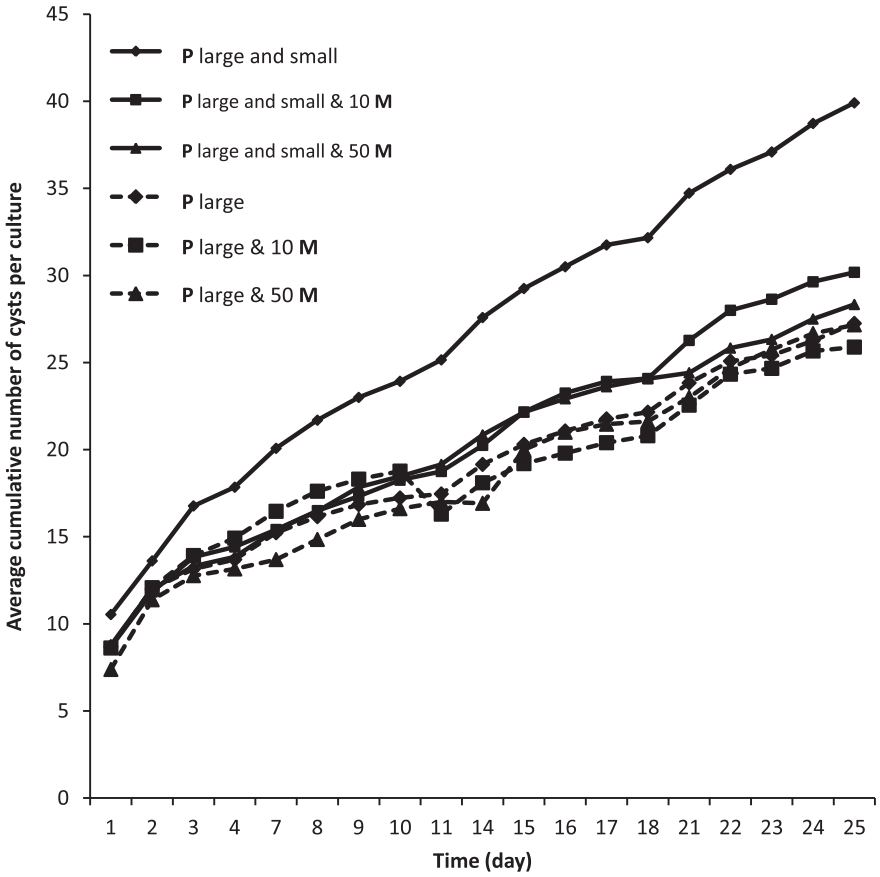

Fig. 4. Cumulative number of *Philophthalmus* sp. cysts in culture over time. P denotes *Philophthalmus* sp., M denotes *Maritrema novaezealandensis*. Cultures with both large and small *Philopthalmus* sp. rediae are indicated with a solid line, cultures without small rediae are indicated with a dashed line. Standard errors are not shown to improve clarity.

Table 3
Results of a generalised linear mixed model evaluating the impact of the presence of small, non-reproducing rediae and/or the presence of *Maritrema novaezealandensis* sporocysts on the cercarial output of *Philophthalmus* sp. colonies in experimental in vitro cultures.

Factor	Estimate	Standard error	*Z* value	*P*
Intercept	1.200	0.190	6.32	<0.0001
Presence of small rediae	0.415	0.160	2.59	0.0096
Presence of 10 M. novaezealandensis	0.152	0.161	0.94	0.3457
Presence of 50 M. novaezealandensis	0.027	0.161	0.17	0.8665
Presence small rediae * 10 *M. novaezealandensis*	−0.470	0.229	−2.06	0.0398
Presence small rediae * 50 *M. novaezealandensis*	−0.344	0.226	−1.52	0.1279

colonies which attached to each other survived longer in culture than those which did not attach (M. Lloyd, personal observation). The possibility of communication or nutrient exchanges thus exists, although the exact nature of what passes among individuals remains to be determined. Another possible way in which the non-reproducing rediae could benefit the colony is by protecting it against microbial infection. Infection by trematodes suppresses host immune function, allowing for secondary, potentially more harmful, infection (Iakovleva et al., 2006). Although the results of the present study show a clear advantage associated with the presence of the non-reproductive morph, testing these alternative hypotheses will be one way forward toward elucidating the underlying mechanisms.

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