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Larval helminths in intermediate hosts: Does competition early in life determine the fitness of adult parasites?

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Abstract

Density-dependent effects on parasite fitness have been documented from adult helminths in their definitive hosts. There have, however, been no studies on the cost of sharing an intermediate host with other parasites in terms of reduced adult parasite fecundity. Even if larval parasites suffer a reduction in size, caused by crowding, virtually nothing is known about longer-lasting effects after transmission to the definitive host. This study is the first to use in vitro cultivation with feeding of adult trematodes to investigate how numbers of parasites in the intermediate host affect the size and fecundity of adult parasites. For this purpose, we examined two different infracommunities of parasites in crustacean hosts. Firstly, we used experimental infections of *Maritrema novaezealandensis* in the amphipod, *Paracalliope novizealandiae*, to investigate potential density-dependent effects in single-species infections. Secondly, we used the crab, *Macrophthalmus hirtipes* (Ocypodidae), naturally infected by the trematodes, *M. novaezealandensis* and *Levinseniella* sp., the acanthocephalan, *Profilicollis* spp., and an acuariid nematode. These four helminths all develop and grow in their crustacean host before transmission to their bird definitive host by predation. In experimental infections, we found an intensity-dependent establishment success, with a decrease in the success rate of cercariae developing into infective metacercariae with an increasing dose of cercariae applied to each amphipod. In natural infections, we found that *M. novaezealandensis*-metacercariae achieved a smaller volume, on average, when infrapopulations of this parasite were large. Small metacercariae produced small in vitro-adult worms, which in turn produced fewer eggs. Crowding effects in the intermediate host thus were expressed at the adult stage in spite of the worms being cultured in a nutrient-rich medium. Furthermore, excystment success and egg-production in *M. novaezealandensis* in naturally infected crabs were influenced by the number of co-occurring *Profilicollis* cystacanths, indicating interspecific interactions between the two species. Our results thus indicate that the infracommunity of larval helminths in their intermediate host is interactive and that any density-dependent effect in the intermediate host may have lasting effects on individual parasite fitness.

Keywords: *Maritrema novaezealandensis*; Trematode; *Profilicollis*; Competition; Acanthocephalan; Crowding; Fecundity; Host-sharing

1. Introduction

In ecological studies of free-living organisms, competition for space and/or food amongst species sharing the same habitat is recognized as an important regulatory process (Begon et al., 1996). Competition usually results in reduced rates of food intake, and eventually decreased fitness in competing individuals by reducing their lifetime reproductive output. Classical examples on the effect of competition on fitness come from studies on the effect of larval competition on growth and reproductive output in adult insects (e.g. Peckarsky and Cowan, 1991; Olson and Andow, 1998). Just as free-living organisms, parasites occupy suitable habitats (hosts) that are limited with regard to the amount of space and resources available. Competition between parasites is a well-known phenomenon from studies on adult helminths in their vertebrate hosts (Hesselberg and Andreassen, 1975; Shostak and Scott, 1993; see Poulin, 1998 and references therein) and also from larval trematodes in their snail first intermediate host (Touassem and Théron, 1989; Kuris, 1990; Sousa, 1993; Kuris and Lafferty, 1994). In all these cases parasites use their host as a source of energy for parasite replication,

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and the regulatory processes are therefore similar to the well-studied larval competition in free-living insects.

Studies of larval helminths in intermediate hosts, where further transmission relies upon ingestion by a definitive host have received less attention. It is generally assumed that once inside the intermediate host, parasites enter a dormant stage, awaiting transmission to the definitive host. More recently, however, some studies have shown that parasites sharing an intermediate host reach smaller sizes (Dezfuli et al., 2001; Brown et al., 2003; Parker et al., 2003; Poulin et al., 2003a) and probably have a reduced future fecundity than parasites in single infections (Wang et al., 2002; Brown et al., 2003). These studies indicate that parasites may interact in hosts, where the parasitic exploitation of host resources is thought to be minimal. Density-dependent reduction in individual parasite size has been most obvious in host-parasite associations where the size of the parasite is large relative to that of the host. Examples of density-dependent effects on parasites in their intermediate host include the development of cestode cysticercoids or procercoids in arthropods, and acanthocephalan cystacanths in amphipods (Gordon and Whitfield, 1985; Wedekind, 1997; Wedekind et al., 2000; Dezfuli et al., 2001).

Two hypotheses have been proposed for the observed density-dependent interaction. First, a negative relationship between size and intensity may be due to competition for host resources (i.e. nutrients and/or space) (see Esch and Fernandez, 1993). If this hypothesis is valid for parasites in intermediate hosts, where transmission relies upon predation, it means that the regulatory mechanisms are similar to parasites using their host as a source of energy for replication. Second, parasite interactions within the intermediate host will not only affect their respective fitness, but possibly also that of their host. Intensity-dependent effects of parasites on their hosts may occur either as parasite increased trophic transmission (PITT, see Lafferty, 1999), and/or as parasite-induced host mortality. Parasite fitness is critically dependent on the successful transmission to the next host in the life cycle. In other words, if the intermediate host dies before transmission, so do all its parasites. Therefore, the optimum exploitation of hosts by parasites will be a balance between the positive effect of con- and heterospecifics on PITT, and the negative effect on host mortality. At least in some circumstances, lowering their own exploitation of the host (i.e. lowering their virulence and growth) may be a beneficial and evolutionary stable parasite strategy to increase the chance of transmission to the next host (Parker et al., 2003). The effects of host-sharing on parasite fitness are important because they provide a greater understanding of host-parasite co-evolution and the evolution of virulence.

One of the best measures of individual fitness is the number of offspring successfully passed on to the next generation (Fox et al., 2001). An estimate of egg-production would therefore be a more appropriate measure of fitness than size alone. In vitro studies of helminths have usually been carried out on species of potential importance to medical or veterinary research. However, if it allows the successful production of eggs, this method can also be useful in studies of the effect of ecological interactions between parasites sharing a host (Wang et al., 2002; Brown et al., 2003).

In this study we used two approaches to investigate the relationship between the number of co-occuring parasites in second intermediate crustacean hosts and three important fitness-components: excystment success of metacercariae, body size and egg-production. The emphasis was on the microphallid trematode, *Maritrema novaezealandensis*, for which a method to culture in vitro-adult worms to sexual maturity has been developed (Fredensborg and Poulin, 2005). Firstly, we investigated the intensity-dependent effect on the fitness of individual *M. novaezealandensis* by using experimental infections of the amphipod, *Paracalliope novizealandiae*. Secondly, we examined the relationship between four different species of larval helminths in naturally infected crab hosts, and their impact on the fitness of in vitro-adult *M. novaezealandensis*. Here, both intra- and interspecific interactions were possible. The effect of host size on density-dependent effects on parasite volume is discussed in relation to the resource-limitation and the optimal growth-hypotheses. To our knowledge, this is the first study to implement in vitro cultivation with feeding of adult helminths to study ecological effects of crowding during larval stages.

2. Materials and methods

2.1. Description of the host-parasite system

The stalk-eyed mud crab, *Macrophthalmus hirtipes*, is a common and widespread inhabitant of lower regions of intertidal mud flats throughout New Zealand (McLay, 1988). The amphipod, *P. novizealandiae*, is an abundant inhabitant of the soft-bottom littoral zone, where it is mainly found in connection with patches of eel grass (*Zostera novazelandica*) and sea lettuce (*Ulva lactuca*).

The most abundant parasite in *M. hirtipes* and the only helminth recorded from *P. novizealandiae* is the microphallid trematode, *M. novaezealandensis*. Under natural conditions, the prevalence of *M. novaezealandensis* in *P. novizealandiae* is usually $>50\%$ and intensity of infection reaches a maximum of 24 metacercariae per host (Fredensborg et al., 2004b). In contrast, a prevalence close to 100% and more than 300 metacercariae have been recorded in *M. hirtipes* (B. Fredensborg, unpublished data). *Maritrema novaezealandensis* has a complex life cycle including three hosts (Martorelli et al., 2004). Sexual reproduction of the parasite takes place within the intestine of the bird definitive host. Trematode eggs are released into the environment with the feces of the bird, where their

further transmission is dependent upon ingestion by the first intermediate snail host, *Zeacumantus subcarinatus*. In the snail, the trematode multiplies asexually and when the temperature reaches approximately 20 °C, free-swimming cercariae are released from the snail. Cercariae seek out and penetrate the cuticula of a crustacean in which they encyst in the body cavity. Completion of the life cycle depends on ingestion by a bird definitive host. Due to its abundance and negative effect on intermediate host ecology, *M. novaezealandensis* is an important species in the soft-bottom intertidal community (Fredensborg et al., 2004b, 2005; Thompson et al., 2005).

Metacercariae of another microphallid, *Levinseniella* sp., occur with a high prevalence of infection in *M. hirtipes* in the study area (B. Fredensborg, unpublished data). The other hosts in its life cycle were not determined, but based on studies of trematodes of this genus from other geographical areas, it is predicted to have a life cycle very similar to *M. novaezealandensis*.

The acanthocephalans, *Profilicollis antarcticus* and *P. novaezelandensis* have both been found in previous studies of *M. hirtipes* in the study area. They have an identical two-host life cycle with the adult parasite occupying the gastrointestinal tract of shorebirds. A previous study showed that less than 1% of cystacanths found in *M. hirtipes* were *P. antarcticus* (Latham and Poulin, 2002a), and data on the two species in the present study, were therefore pooled and referred to as *Profilicollis* spp.

Two species of third-stage juvenile nematodes are known from the body cavity of *M. hirtipes* (Moravec et al., 2003). The majority of infections ($\approx$95%) belong to a species in the Acuariidae, which matures in birds, whereas the other species, *Ascarophis* sp. matures in fish. Because the vast majority of the juvenile nematodes belonged to Acuariidae, all data for juvenile nematodes were pooled prior to analysis.

2.2. Field sampling

On a single occasion in February 2004, a large sample of *P. novizelandiae* ($\geq$2000 individuals) was collected in Hoopers Inlet, Otago Peninsula (45°50′S, 170°39′E), South Island, New Zealand. The snail first intermediate host does not occur at that locality and local amphipods, therefore, do not harbour any *M. novaezealandensis*-infections, making them suitable for experimental infection (see Fredensborg et al., 2004b). Amphipods were collected during low tide from shallow tidal pools ($\leq$5 cm) using a dip-net and transferred to the laboratory in plastic containers (12.5$\times$30 cm) half-filled with seawater.

In late August 2004, 45 crabs (*M. hirtipes*) were collected by hand from a tidal mudflat in patches of eelgrass (*Z. novazelandica*) in Lower Portobello Bay, Otago Peninsula, South Island, New Zealand. The crabs were all collected within a small area (approximately 50$\times$25 m) and only individuals within a relatively narrow size range (8–16 mm carapace width) (normal size range: 6–40 mm) were retained to ensure approximately equal age and exposure time to helminth infections. Crabs were returned live to the laboratory before dissection for retrieval of larval helminths.

2.3. Experimental infections

In the laboratory, 1500 amphipods were randomly selected from the field-collected sample and divided evenly into three treatment groups of 500 individuals each. Amphipods from the three treatment groups were then transferred individually to 5 ml containers each holding 0.5 ml of sea water, and exposed to five, 10 or 20 cercariae each, respectively (see Fredensborg et al., 2004b for a detailed description of the infection process). To standardise genetic relatedness among parasites sharing the same host, each amphipod was infected by cercariae originating from one snail. A total of 30 infected snail first intermediate hosts were used for the experimental infections (one snail per 50 amphipods). After 6 h of exposure to *M. novaezealandensis*-cercariae, amphipods from the three treatment groups were transferred to 0.3 l containers each holding 25 individuals, and all containing 0.3 l seawater, small rocks and sea lettuce (*U. lactuca*). Amphipods were kept for 10 weeks to ensure that metacercariae would be fully developed and thus infective to the definitive host. During that period, 3/4 of the water was changed every second day and sea lettuce was changed weekly.

After 10 weeks, amphipods from the three treatments were measured (from rostrum to telson), sexed and dissected. The metacercariae were measured (length (L)$\times$width (W) (to the nearest 10 μm)) using a graticule under a compound microscope, transferred to a multi-welled plate and individually incubated in 0.3 ml of culture medium (NCTC-109) at 40 °C, which provide ideal conditions for excystment (see Fredensborg and Poulin, 2005). Metacercariae were checked approximately every 30 min over 5 h. Metacercariae not excysted after 5 h of incubation were assumed non-viable if ingested by a definitive host, and therefore, excluded from further study. Excysted metacercariae were transferred individually to 0.5 ml microtubes containing 0.5 ml culture medium (NCTC-109) and incubated at 40 °C (see Fredensborg and Poulin, 2005 for further details on cultivation method). After 48 h of incubation, survival, size ($L\times W$) (to the nearest 10 μm), and number of eggs in the uterus were recorded for each 'in vitro'-adult trematodes. Adult trematodes were fixed in hot 5% formalin before measurements were taken using a graticule under a compound microscope (to the nearest 10 μm). In addition, a random sub-sample of five eggs from each trematode was measured ($L\times W$) (to the nearest 2.5 μm), and the egg volume estimated using the formula for an ellipsoid of revolution ($V=\pi\times L\times W^2/6$).

2.4. Natural infections

Each of the 45 *M. hirtipes* was measured to the nearest 0.1 mm (carapace width, measured at the level of the second pair of lateral spines), sexed and all soft tissue dissected for retrieval of larval helminths. All parasites from each crab were transferred to 10 ml petri dishes and counted. Twenty *M. novaezealandensis* metacercariae from each crab were randomly collected and used for volumetric measurements and 20 metacercariae from 41 of the 45 crabs were subsequently cultivated. In vitro excystation and cultivation was carried out as described above for experimental infections, except that the cultivation medium was supplemented with 40% chicken serum. A random subsample of five *Levinseniella* sp. metacercariae and five *Profilicollis* spp. cystacanths per crab were measured ($L \times W$) (if fewer than five specimens were present, all were measured). The volume of those parasites was determined using the formula for an ellipsoid as described above. For both species of trematode metacercariae and *Profilicollis* cystacanths, the coefficient of variation in volume (SD × 100/mean) was calculated as an estimate of the relative variation in parasite volume per crab.

2.5. Statistical analyses

All data were examined for normality and homogeneity of variance using Kolmogorov Smirnov and Levene's tests, respectively. Data on intensity of *M. novaezealandensis* and *Levinseniella* sp. in naturally infected crabs were log-transformed (or $\log(x+1)$—transformed if there were 0 s) prior to analysis to mitigate violations of the assumption of normality and heterogeneity of variance. In analyses where transformation of the data did not improve the above assumptions, data were analysed using equivalent nonparametric tests.

For natural infections in *M. hirtipes*, the variables: intensity of *M. novaezealandensis*, *Levinseniella* sp., *Profilicollis* spp. and juvenile nematodes, the mean individual volume of *M. novaezealandensis*, *Levinseniella* sp. and *Profilicollis* spp., and the coefficient of variation in volume of each of those parasites were investigated in relation to the intensities of each other and crab carapace width. For each variable, a hierarchical stepwise multiple regression analysis was performed, sequentially adding variables in order of importance to the dependent variable. Likewise, in vitro measurements obtained only for *M. novaezealandensis*: excystment success of a sub sample of 20 metacercariae per crab, mean size of adult trematodes, mean egg-production per adult trematode and mean egg volume per trematode were investigated in relation to the above variables and each other. Only variables significantly contributing to explaining the variation in the dependent variable were entered in each model. For models with more than one variable, the importance of the individual predictors is presented as partial correlation coefficients. The distribution of residuals was checked and data were investigated for collinearity of predictor variables by inspection of the correlation matrix prior to analysis, and by evaluating the Variance Inflation Factor (VIF) and the distribution of eigenvalues for each variable in each analysis. Collinearity among predictor variables was weak, and did not influence any of the analyses.

Data on experimentally infected amphipods were analysed using multiple regression analysis on the actual intensity of infection as described above. A separate analysis tested the effect of treatment (number of cercariae added) on mean individual metacercarial volume, excystment success and mean egg-production per in vitro-adult trematode.

Two approaches were taken to evaluate the effect of intensity of infection by *M. novaezealandensis* on the total volume of this trematode per crab. First, a strong intensity-dependent effect of metacercariae on mean total volume may produce a non-linear relationship between total volume and intensity of infection because the total volume of metacercariae may eventually reach an asymptote with no or very little increase with increasing intensity of infection. The observed relationship between those two factors was therefore tested with a linear regression analysis. Second, even if the relationship between intensity of infection and total volume of *M. novaezealandensis* does not reach an asymptote, there may still be a more subtle effect of intensity of infection on total volume of *M. novaezealandensis*. A predicted total volume of *M. novaezealandensis* metacercariae was therefore estimated, based on the mean volume of metacercariae from the five crabs with the lowest intensity of infection (<40 metacercariae per crab) multiplied by the actual intensity of infection in all other crabs. The discrepancy between the predicted and the observed total volume depicts the effect of crowding on the total volume of *M. novaezealandensis* per crab.

Statistical analyses were performed using the statistical packages SPSS for Windows (10.1) and STATISTICA (6.0). All tests were two-tailed and used the 5% level of significance.

3. Results

3.1. Experimental infections of P. novaezealandiae

Amphipod mortality over the 10-week experiment was considerable, and thus data are only available for the surviving amphipods. The intensity of infection in the amphipods for the three treatments was: five cercariae applied: Mean ($\pm$SE) number of metacercariae per amphipod $= 1.60 \pm 0.27$, $n = 10$; 10 cercariae: 2.68 ± 0.38, $n = 19$; 20 cercariae: 3.03 ± 0.38, $n = 32$. Increasing the dosage did not significantly increase the intensity of infection in the amphipods ($F(2,60) = 1.992$, $P = 0.146$).

The three treatment groups did not differ with regard to mean volume of individual *M. novaezealandensis* metacercariae (One way ANOVA: $F(2,59)=1.657$, $P=0.200$), excystment success (Chi-square test: $\chi^2=2.339$, df $=2$, $P=0.311$), and mean egg-production per trematode (One way ANOVA: $F(2,33)=0.709$, $P=0.500$).

Male amphipods were significantly larger than females (♂: mean ($\pm$SE) $=3.46\pm0.08$ mm, $n=22$; ♀: 2.65 ± 0.04 mm, $n=39$, Mann–Whitney U-test: $Z=5.737$, $P<0.001$). There was, however, no difference in intensity of infection between the two sexes (♂: mean ($\pm$SE) $=2.64\pm0.48$, $n=22$; ♀: 2.72 ± 0.27, $n=39$; Mann–Whitney U-test: $Z=-0.883$, $P=0.377$). Within treatments, there was no relationship between amphipod length and intensity of infection (all three treatments, $r<0.14$, $P>0.700$). Across treatments, female amphipod length was positively correlated with the intensity of infection ($r_s=0.337$, $P=0.036$). No such correlation was observed in males ($r_s=-0.020$, $P=0.933$). Neither intensity of infection nor amphipod length explained a significant amount of the variation in mean volume of individual metacercariae across *P. novizealandiae* (both variables, $P>0.20$). The coefficient of variation in volume of metacercariae was not significantly related to amphipod length or intensity of infection (no variables entered in regression model, both variables, $P>0.18$).

Mean individual volume of metacercariae did not appear to be a major determinant of excystation success. The volume of excysted metacercariae was not significantly larger than that of metacercariae that did not excyst (excysted: mean $=0.062$ mm^3, unexcysted: mean $=0.060$ mm^3, Student's t-test: $t=-1.435$, $P=0.153$).

Egg-production in in vitro-adult trematodes was negatively influenced by intensity of infection (all trematodes: $r_s=-0.31$, $n=61$, $P=0.016$ (Fig. 1). This relationship was mainly due to a decrease in the variation in trematode egg-production with increasing intensity of infection (Fig. 1).

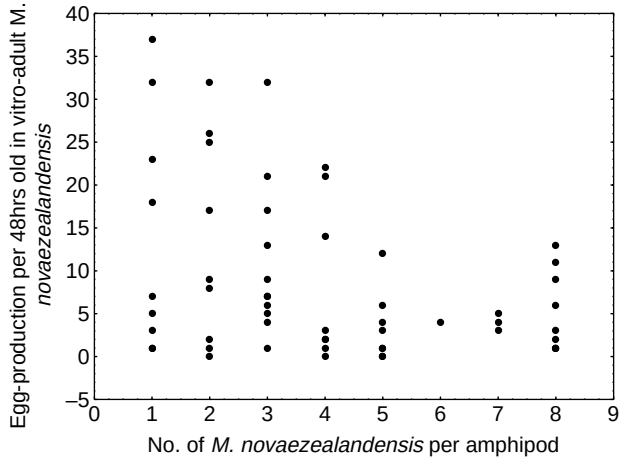

Fig. 1. Relationship between numbers of metacercariae per amphipod and egg-production of in vitro-adult *Maritrema novaezelandensis* after 48 h of cultivation.

Mean egg-production per amphipod was not related to any of the variables entered in the multiple regression analysis (all variables, $P>0.19$). The volume of trematode eggs was significantly and positively related to egg-production (model: $F(1,31)=10.250$, $r=0.503$, $P=0.002$). The coefficient of variation in volume of trematode eggs was not related to either host size, intensity of infection or egg-production (all variables, $P>0.42$).

3.2. Natural infections in *M. hirtipes*

All four parasites occurred with a high prevalence in the *M. hirtipes* examined, although intensity of infection (number of parasites per host) varied greatly between crabs (Table 1). There was a large degree of overlap with regard to site of infection, however, some preferences were observed. Metacercariae of *Levinseniella* sp. were mainly found attached to the hepatopancreas, cystacanths of *Profilicollis* spp. were found in the hemocoelomic cavity and juvenile nematodes were mainly residing centrally in the body cavity in the vicinity of the stomach of the crab. Metacercariae of *M. novaezealandensis* had the least specificity and occupied all soft tissue within the crab as well as the hemocoelomic cavity.

Male and female crabs did not differ in size (unpaired t-test: $t=-1.380$, df $=43$, $P=0.175$), or intensity of infection with *M. novaezealandensis*, *Profilicollis* or juvenile nematodes (Mann–Whitney U-test, all $P\geq0.24$). Female *M. hirtipes* did, however, harbour more *Levinseniella* metacercariae than males (♀ $=13.47\pm4.29$, $n=19$; ♂ $=6.96\pm1.62$, $n=25$; Mann–Whitney U-test: $Z=-2.259$, $P=0.024$). Of the variables: crab carapace width and the intensities of *Profilicollis* sp., *Levinseniella* sp., and nematodes, entered into a hierarchical multiple regression, only crab carapace width was significantly related to the intensity of *M. novaezealandensis* (model: $F(1,44)=14.782$, $\beta=-0.51$, $P<0.001$). Hence, intensity of infection with *M. novaezealandensis* decreased significantly with increasing size of crabs.

The mean volume of individual *M. novaezealandensis* metacercariae was only significantly related to the intensity of infection of this trematode (model: $F(1,44)=4.508$,

Table 1
Infection parameters of *Macrophthalmus hirtipes* ($n=45$) naturally infected with four helminth species

Parasite species	Prevalence (%)	Mean intensity	Range
Maritrema novaezealandensis	100.0	110.3	24–337
Levinseniella sp.	91.1	10.7	1–87
Profilicollis spp.	97.8	9.0	1–27
Juvenile nematodes	57.8	2.2	1–5

$\beta = -0.31$, $P = 0.040$) (Fig. 2a). Although the relationship is not very strong, the negative relationship between the intensity and mean volume of *M. novaezealandensis* suggests that infrapopulation size negatively affects the size at which *M. novaezealandensis* is transmitted to the bird definitive host. The mean total volume of *M. novaezealandensis* metacercariae per crab increased linearly with numbers of metacercariae ($F(1,43) = 1846.802$, $\beta = 0.99$, $P < 0.001$) (Fig. 2b). However, the observed total volume of *M. novaezealandensis* metacercariae per crab was consistently below the expected total volume at intensities larger than 100 metacercariae per crab. This result suggests that the reduction in individual parasite size may have a negative effect on the total volume of *M. novaezealandensis* in the crab host (Fig. 2b).

The coefficient of variation in volume of metacercariae per crab was significantly related to crab carapace width and number of *M. novaezealandensis* (model: $F(2,44) = 5.702$, $P = 0.006$, $R^2 = 0.21$; partial correlation coefficients: number of *M. novaezealandensis* metacercariae: $r = -0.44$, $P = 0.003$; crab carapace width: $r = -0.38$, $P = 0.011$). The variation in volume of *M. novaezealandensis* metacercariae per crab thus decreases with increasing number of conspecifics and crab carapace width.

The mean excystment success of a sub sample of 20 metacercariae per crab (±SE) was 74 ± 14%, $n = 41$. There was no difference in volume between metacercariae that excysted successfully and those that did not (Student's *t*-test: $t = 0.062$, df = 838, $P = 0.951$, data pooled across all crabs). Mean excystment success was positively influenced by the number of acanthocephalan cystacanths and negatively related to crab carapace width (model: $F(2, 40) = 5.722$, $P = 0.007$, $R^2 = 0.23$; partial correlation coefficients: number of acanthocephalan cystacanths: $r = 0.46$, $P = 0.003$; crab carapace width: $r = -0.35$, $P = 0.027$).

Only the mean volume of individual metacercariae per crab was significantly and positively related to the mean size of 48 h old in vitro-adult *M. novaezealandensis* per crab (model: $F(1,40) = 8.470$, $r = 0.42$, $P = 0.006$, Fig. 3). This suggests that the effect of metacercarial volume on size of the in vitro-adult trematodes is evident for at least the first 48 h of cultivation.

The mean egg-production of in vitro-adult *M. novaezealandensis* per crab was best explained by the three variables: number of *Profilicollis* cystacanths, crab carapace width and mean size of in vitro-adult *M. novaezealandensis* (model: $F(3,40) = 4.537$, $P = 0.008$, $R^2 = 0.27$). However, only the number of *Profilicollis* spp., and crab carapace width were significantly related to mean egg-production (partial correlation coefficients: *Profilicollis* spp.: $r = -0.35$, $P = 0.030$; crab carapace width: $r = 0.40$, $P = 0.012$; mean in vitro-adult size: $r = 0.28$, $P = 0.086$).

There was no difference in egg-production between trematodes from male and female crabs (♂: Mean (±SE)

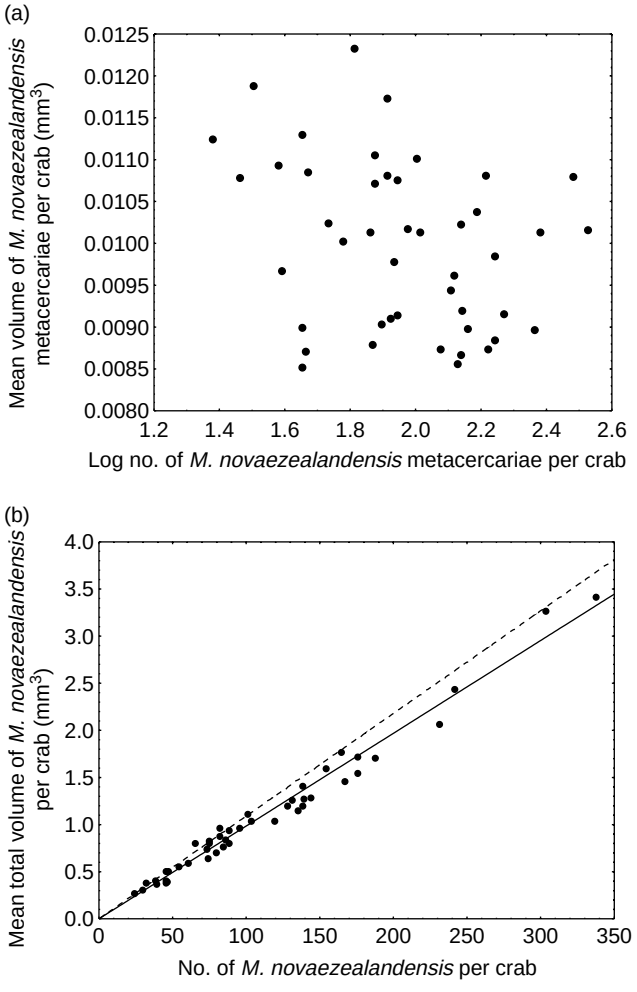

Fig. 2. Relationship between number and (a) mean individual size of *Maritrema novaezealandensis* metacercariae among crab intermediate hosts, *Macrophthalmus hirtipes*, (b) mean total observed (solid line), and predicted (broken line) volume of *M. novaezealandensis* ($n = 45$). Mean total predicted volume of *M. novaezealandensis* per crab was estimated based on the mean volume of metacercariae in the five crabs with the lowest intensity of infection multiplied by the actual intensities in the other crabs.

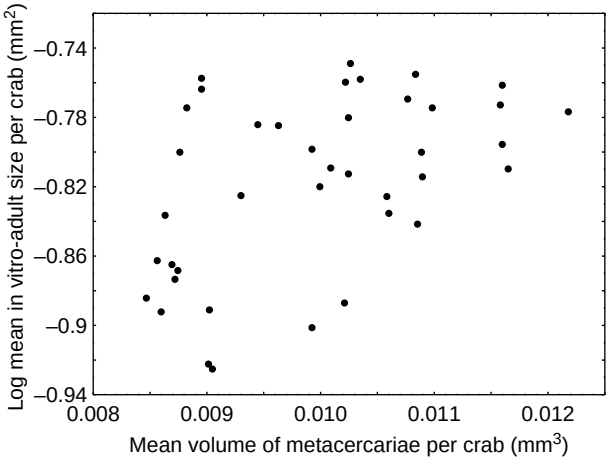

Fig. 3. Relationship between mean size of metacercariae and mean size of in vitro-adult *Maritrema novaezealandensis* after 48 h of cultivation. ($n = 41$ crabs).

$=48.00\pm2.06$, $n=248$; ♀: 47.03 ± 1.98, $n=234$; Mann–Whitney U-test: $Z=0.194$, $P=0.846$).

The volume of trematode eggs showed a significant, but very weak positive relationship with both egg-production and volume of metacercariae (model: $F(2,436)=25.868$, $P=0.001$, $R^2=0.11$; partial correlation coefficient: volume of metacercariae: $r=0.26$, $P<0.001$; egg-production: $r=0.19$, $P<0.001$). The coefficient of variation in volume of trematode eggs was not significantly related to host size, parasite intensity or egg-production of *M. novaezealandensis* (all variables, $P\geq0.18$).

The intensity of infection by *Profilicollis* cystacanths was positively correlated with carapace width and the number of *Levinseniella* metacercariae (model: $F(2,44)=8.941$, $P=0.001$, $R^2=0.30$; partial correlation coefficients: number of *Levinseniella*: $r=0.41$, $P=0.006$; crab carapace width: $r=0.32$, $P=0.033$).

The mean volume of cystacanths was not related to any of the variables entered in a stepwise hierarchical multiple regression (all, $P>0.16$). Likewise, the coefficient of variation in mean volume of *Profilicollis* spp. cystacanths was not related to any of the other variables (all, $P>0.13$).

The mean volume of *Levinseniella* metacercariae was not related to intensity of infection by this or any other parasite (all variables, $P>0.20$). However, the coefficient of variation in volume of *Levinseniella* metacercariae was negatively affected by intensity of infection with this trematode, intensity of infection with *M. novaezealandensis*, and crab carapace width in female crabs (model: $F(3,13)=11.943$, $P=0.001$, $R^2=0.78$; partial correlation coefficients: number of *Levinseniella*: $r=-0.69$, $P=0.012$; number of *M. novaezealandensis*: $r=-0.74$, $P=0.006$; crab carapace width: $r=-0.81$, $P=0.002$). *Levinseniella* metacercariae in female crabs are thus increasingly uniform in size with increasing crab size and intensities of both *Levinseniella* itself and *M. novaezealandensis*. No such relationships were observed in male crabs (all variables, $P>0.14$).

The intensity of juvenile nematodes was not related significantly to crab carapace width or any of the co-occuring parasite species (all variables, $P>0.30$).

4. Discussion

The effect of intra- and interspecific competition amongst parasites in intermediate hosts on adult parasite fitness has received little attention in the literature. This study is unique in that it is the first to use in vitro cultivation with feeding of adult helminths to study ecological effects of crowding in parasites co-occuring in crustacean intermediate hosts.

Infrapopulation size of *M. novaezealandensis* affect mean volume of the metacercariae and size of the in vitro-adult specimens (natural infections), and ultimately egg-production by trematodes with an increasing number of conspecifics sharing a host (both studies). Our results concur with those of a previous study, which demonstrated that the number of metacercariae sharing an amphipod host had a negative effect on volume and short-term egg-production of encysted metacercariae in a related trematode (Brown et al., 2003).

The significant negative relationship between intensity and individual mean volume of *M. novaezealandensis* metacaercariae in crabs is an interesting finding because trematodes are not usually thought to compete for resources in their second intermediate host. However, although *M. novaezealandensis* does not replicate inside the second intermediate host, energy is required for a considerable growth of the metacercariae during the first few weeks after infection, as typically seen for trematodes of the family Microphallidae (Galaktionov et al., 1996, 1997; McCarthy et al., 2002; Fredensborg et al., 2004b). During the metacercarial stage, the trematode also undergoes complete development of sexual organs. Both these processes are presumably costly to the host. In crabs, individual parasites probably do not place any constraints on host resources because of the small parasite-to-host size- ratio. However, due to the nature of cercarial release observed in *M. novaezealandensis*, multiple simultaneous infections in crabs are likely to be common (see Fredensborg et al., 2004b). It is therefore possible that the number of conspecifics entering a crab host within a short period of time may have a negative effect on the amount of nutrients available for development during the intense growth phase. Our results suggest that the total volume of *M. novaezealandensis* per crab was also negatively affected by intensity of infection of this trematode (Fig. 2b). Yet, the total parasite volume increased linearly with the intensity of *M. novaezealandensis* metacercariae (Fig. 2b), and it remains unclear whether the slight reduction in observed total metacercarial volume has any impact on host fitness. Thus, the most parsimonious explanation to the observed intensity-dependent reduction in individual and total volume of *M. novaezealandensis*, is that individual *M. novaezealandensis* suffer from a reduced growth inflicted by crowding, and not through an adaptive growth strategy of *M. novaezealandensis* in *M. hirtipes* as in Parker et al. (2003).

The positive correlation between volume of metacercariae and adult parasite size found in natural infections of *M. hirtipes*, indicates that volume of metacercariae at the time of transmission more or less determines adult size after 48 h. As usually observed in helminth parasites (see Trouvé et al., 1998), egg-production of *M. novaezealandensis* was positively correlated with adult size (Fredensborg and Poulin, 2005; this study). Reductions in metacercarial volume caused by high intensity of infection thus lead to a reduction in the egg-production of adult trematodes. Larger metacercariae also produced larger eggs. A positive correlation between egg-production and egg-volume was also found in a related trematode (Brown et al., 2003), and across species of acanthocephalans (Poulin et al., 2003b),

suggesting this pattern may be general in helminths. Besides having a positive effect on egg-production, size may also have other important effects on parasite fitness. Even though we did not observe mean volume to affect excystment success, size has been reported as a determinant of establishment success and survival in the definitive host for the acanthocephalan, *Leptorhynchoides thecatus* (Steinauer and Nickol, 2003).

It is intriguing that the intensity of infection with *M. novaezealandensis* did not increase with increasing number of cercariae added to the amphipods in the experimental infections. In a previous study, the rate of cercariae penetrating the amphipod host did not decrease with an increasing dose of cercariae (see Fredensborg et al., 2004b). Hence, the reduction in the success rate of *M. novaezealandensis* developing into infective metacercariae is likely due to mortality within the amphipod host. The higher mortality rate of *M. novaezealandensis* with increasing number of simultaneous infections suggests that intensity-dependent death of premature metacercariae may be an important regulatory process of trematode infections in second intermediate amphipod hosts. Such an effect will be overlooked when only examining infective stages of trematodes, and would need to be addressed in future studies.

In addition to the observed crowding effect in *M. novaezealandensis*, this study indicates that helminths of different species interact. For example, a negative relationship was found between egg-production of in vitro-adult *M. novaezealandensis* and the number of *Profilicollis* cystacanths in their common intermediate crab host. The reduction in egg-production was, however, not linked to a reduction in metacercarial volume. The underlying mechanism of this antagonistic effect is unclear. Antagonistic effects between heterospecific parasites have previously been recorded affecting the distribution (Barger and Nickol, 1999; Fauchier and Thomas, 2001), size (Dezfuli et al., 2001), and fecundity (Wang et al., 2002) of helminths sharing the same crustacean intermediate host. The underlying mechanisms in those studies ranged from crowding effects to exclusion or avoidance based on conflicts of interest between parasite species with different next hosts. *Maritrema novaezealandensis* and *Profilicollis* share the same definitive hosts (Fredensborg et al., 2004a), and there is thus no conflict involved in their interaction in the crab. In fact, several studies have indicated that *Profilicollis* affects the behaviour of *M. hirtipes*, making them more susceptible to avian predation (Latham and Poulin, 2001, 2002a, b), thus facilitating the transmission of both parasites to their definitive host. The positive relationship between excystment success in *M. novaezealandensis* and number of *Profilicollis* also suggests an interaction between the two species inside the crab host. Although the underlying mechanism is unknown, this study reveals a hitherto unobserved way in which the presence of one parasite species facilitates the successful transmission of another. Overall, a reduction in egg-production in adult *M. novaezealandensis* with increasing numbers of *Profilicollis* cystacanths may be seen as a relatively small cost to pay if the probability of transmission is significantly enhanced by sharing a crab with the acanthocephalan.

The positive correlation between numbers of *Levinseniella* and *Profilicollis* represents another example of interspecific interactions of parasites in *M. hirtipes*. A similar pattern between the two species was observed in a previous study on larval helminths in *M. hirtipes* (Poulin et al., 2003a). As stated by those authors, the observed relationship between the infection patterns of the two species is not clear. The infection processes of the two species are completely different, in that acanthocephalans are transmitted to the crab by ingestion of eggs, while *Levinseniella* are transmitted by cercariae penetrating and encysting in the crab. One possible explanation for the positive correlation between the two species is that infections originate from bird faeces and that differences amongst crabs may reflect small-scale differences in the distribution of shorebirds on a mud flat. Other possible explanations include a higher exposure to *Levinseniella* cercariae following behavioural modifications of the crab caused by infections with *Profilicollis*, or that *Levinseniella*, somehow, can selectively infect crabs already infected by *Profilicollis*. The latter possibility, known as 'hitch-hiking', would give the 'passenger' the advantage of enhancing its own probability of transmission without paying the cost of host manipulation. This phenomenon has been documented in a related host-parasite association (Thomas et al., 1997).

Previous studies suggest that host sex may play a role in the development and fitness of larval helminths in their crustacean (Wedekind and Jacobsen, 1998; Brown et al., 2003), and vertebrate hosts (Poulin, 1996). In the experimental infection study there was no difference in the intensity of infection between male and female amphipods. Neither did we find any difference in naturally infected crabs in terms of mean volume in any of the parasites or egg-production in trematodes between males and females. However, the variation in volume among *Levinseniella* metacercariae was dependent on intensity of infection only in female crab hosts. Those results suggest that either resources are limited in females compared with males, or that activation and function of the host immune system may differ between sexes. The results also suggest that any host response to parasites is species-specific, because effects differ between parasite species.

A few limitations to this study must be pointed out. One of the major underlying assumptions in this study is that egg-production of in vitro-adult trematodes reflects the reproductive output of adults in the vertebrate host. Adult microphallids can, during their short life span in the definitive host, produce hundreds of eggs, and it is not confirmed whether the observed pattern in this study is truly reflecting natural infections in definitive hosts. Secondly, adult size and egg-production were measured after 48 h of

incubation based on a previous cultivation experiment with *M. novaezealandensis* (Fredensborg and Poulin, 2005). In that study, no growth occurred after 48 h. However, it may be that microphallids in definitive hosts continue to grow and reproduce for another few days. Egg-production after 48 h of in vitro cultivation may therefore not reflect lifetime reproductive output in *M. novaezealandensis*. In addition, there may be variables not included in the analysis that are associated with mean volume and egg-production of helminths. The large amount of scatter in the data points, even for significant relationships (see Figs. 1–3), suggests that other factors influence the fitness of *M. novaezealandensis.*

In conclusion, the results of this study reveal that larval helminths infecting their crustacean intermediate host are not just entering a dormant stage waiting for transmission to their bird definitive host. Instead, the infracommunity of larval helminths in intermediate hosts is dynamic and the number, size and egg-production of parasites are dependent on interactions between conspecifics and heterospecifics, as well as on the host they share.

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