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Effect of dose and frequency of exposure to infectious stages on trematode infection intensity and success in mussels

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ABSTRACT: Marine parasites such as trematodes often compromise the fitness of their hosts. Such effects are generally considered to be density-dependent, i.e. the greater the infection intensity in the host, the greater the detrimental impact on host fitness. However, the mechanisms determining infection in marine hosts are still poorly understood. Here, we investigated the effect of cercarial dose and exposure frequency (single vs. trickle infections) of a marine trematode parasite, *Himasthla elongata* (Trematoda: Echinostomatidae), on infection intensity and success in its second intermediate host, the blue mussel *Mytilus edulis*, an abundant and widely distributed bivalve in European coastal waters. In our laboratory experiment, we tested 4 levels of parasite doses and showed that mussels faced higher parasite infection intensity at higher doses of cercarial exposure and that they acquired more infections when repeatedly exposed to smaller doses compared to a single high dose. However, the infection success of cercariae did not differ among 4 dose levels but was only significantly different between trickle and single exposures. This indicates that cercariae were not subjected to a dose-dependent regulation of their infectivity, suggesting that infection intensity in mussels is largely driven by factors mediating the abundance of infective stages. With the combined investigation of the effect of cercarial dose and exposure frequency at realistic dose levels, our study contributes to our currently very limited understanding of the determinants of infection intensity in marine hosts and highlights the usefulness of experimental studies in advancing our knowledge in this field.

KEY WORDS: Parasitism · Trematodes · Transmission · Bivalves · Dose-dependence · Infectivity · *Mytilus edulis* · *Himasthla elongata*

INTRODUCTION

Parasites such as trematodes are ubiquitous in marine coastal environments and are known to modify the phenotype of their hosts by interfering with growth rates, behaviour, reproduction and survival (Mouritsen & Poulin 2002). As such, they are capable of substantially affecting host individuals and entire host communities (Mouritsen & Poulin 2002, Kuris et al. 2008). In the most common life cycle of trematodes, molluscs are used as first intermediate hosts, from which infective stages (cercariae) are released that infect second intermediate hosts (invertebrates or fish). Here, the parasites encyst as metacercariae until the second intermediate host is ingested by their definitive vertebrate hosts within which the parasites mature and release eggs. These hatch into miracidia that go on to infect first intermediate hosts, closing the complex life cycle. As in other parasites, the effects of trematode infections on second intermediate and definitive hosts are generally considered to be density-dependent, i.e. the greater the

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intensity of infection in the host, the greater the detrimental impact on host fitness (Anderson & May 1978, May & Anderson 1978, Fredensborg et al. 2004, Thieltges 2006). Here, we define infection intensity according to Bush et al. (1997) as the number of individuals of a specific species of parasite within a single host. The mechanisms underlying varying infection intensity in trematode hosts thus have high relevance in determining the impact of parasites on their hosts.

As a general principle, infection intensity in hosts is mediated by the abundance of infective stages that a host is exposed to, the so-called dose. Such dose effects have been studied particularly in the free-living cercarial life cycle stage of trematodes due to the experimental tractability of this stage. In general, the infection intensity in terms of the total number of metacercariae encysted in downstream hosts increases with the cercarial dose administered (Poulin 2010). However, the actual infection success (i.e. proportion of cercariae successfully encysting within their host) may decrease with dose due to density-dependent regulation in the form of intra-specific interference among parasites or increased anti-parasitic responses by the host (Ebert et al. 2000, Poulin 2010). Dose effects will be further mediated by the frequency of exposure. A meta-analysis of trematode infection studies found that in most typical experimental designs, the parasite dose is administered in a single event (Poulin 2010). However, under natural conditions, repeated sequential infection events ('trickle infection') are probably more realistic but have rarely been applied in infection studies (Poulin 2010). Another phenomenon observed in this meta-analysis is the fact that the experimental dose levels often seem to be selected arbitrarily, with a tendency to administer higher doses to larger hosts (Poulin 2010). This may be logical, as field studies have revealed that larger organisms do indeed harbour greater numbers of parasites (Poulin 2010); however, infection intensity may actually often depend on temporal exposure and as such on host age rather than directly on host size (Thieltges 2008). Hence, applying dose levels that mimic levels likely to occur in the field would be preferable.

Despite the ubiquitous presence and effects of trematodes in marine ecosystems, surprisingly few studies have investigated the effects of dose on infection intensity in hosts. To our knowledge, published data on replicated experiments are only available for a marine amphipod from New Zealand (Fredensborg et al. 2004, Fredensborg & Poulin 2005). However, these studies did not investigate the effect of exposure frequency (trickle vs. single) and did not give an indication of how realistic the choice of dose levels was, both of which are likely to influence parasitic infection success and infectivity. Specifically, one would expect the total number of cercariae as well as the proportion of cercariae that infect a host to be greater for trickle than single exposure, because intraspecific competition between parasites for entry into the host will be lower, as will the anti-parasitic response of the host. Subsequently, infection intensity in terms of total number of metacercariae encysting within a host is likely to reach saturation at the higher doses of parasites, while the success of infection in the host may even decrease (Poulin 2010). Given the abundance and impacts of trematodes in marine organisms, a better understanding of the basic mechanisms driving infection intensity is desirable.

In this study, we investigated the effect of cercarial dose and exposure frequency (single vs. trickle infections) of a marine trematode parasite, *Himasthla elongata*, on infection intensity in its second intermediate host, the blue mussel *Mytilus edulis,* an abundant and widely distributed bivalve in European coastal waters. *H. elongata* infects the gonad-digestive gland complex of its first intermediate host, the common periwinkle *Littorina littorea,* from which cercariae are released and infect various second intermediate bivalve hosts. Here, the parasite encysts as metacercariae and is known to impair the production of byssus threads in blue mussels, and to alter the burrowing ability of common cockles *Cerastoderma edule* (Lauckner 1984). In our controlled laboratory experiments, mussels were exposed to a number of different realistic dose levels of cercariae which were administered either in single events or as trickle infections over time to determine whether either or both affect the infection intensity and infection success in downstream mussel hosts. This experiment is the first to investigate the combined effects of dose and exposure frequency on infection in a marine host and thus significantly contributes to our limited understanding of the determinants of parasite infection in marine organisms.

MATERIALS AND METHODS

Parasites and hosts

Cercariae of *Himasthla elongata* were obtained from common periwinkles collected from the vicinity of the NIOZ Royal Netherlands Institute for Sea

Research on the Dutch island of Texel (Wadden Sea). Snails known to be infected from shedding trials were kept in the dark in aerated flow-through aquaria and fed regularly with sea lettuce *Ulva lactuca* until cercariae were required for experiments. Shedding of cercariae by snails was then induced by incubating around 30 snails in 2.7 l of seawater at 27°C under light for 3 h. Subsequently, within 1 h, the necessary numbers of cercariae were pipetted into labelled pots and administered to the appropriate replicates within the experiment. The maximum age of cercariae at the start of the experiment was therefore 4 h.

Mytilus edulis hosts of 25–30 mm shell length (about 2 yr old) were collected from beach groynes on the west coast of the island of Texel. We chose a relatively small size range to avoid potentially confounding effects of age and size ((Nikolaev et al. 2006, Thieltges 2008). The population of mussels at this location is known to be uninfected with trematode parasites due to the absence of first intermediate hosts (such as the common periwinkle). The dissection of 50 *M. edulis* confirmed the lack of infection. Mussels were placed in the experimental set-up for 24 h prior to the experiments to acclimatise.

Experimental set-up

Plastic containers (25 × 11.0 × 9.5 cm height × width × depth) were filled with 500 ml of seawater, constantly aerated and placed on a bench in a climate-controlled chamber (18°C) in a completely randomised design. Two mussels were placed in each container, and the assigned parasite dose was administered daily. We chose 2 mussels to compensate for possible variation in filtration rates which would affect the uptake and subsequent infection with cercariae. The experiment was run in a 2-factorial design with exposure frequency (single vs. trickle) and total dose (20/60/100/300 free-living cercariae) as fixed factors. Each treatment combination was replicated 8 times.

Dose selection was based on estimated cercarial shedding rates of *H. elongata* parasites from their first intermediate host, the common periwinkle, as described by Thieltges et al. (2008a) and Nikolaev et al. (2006). A single infected snail sheds between 642 and 672 cercariae d^{-1} (Nikolaev et al. 2006, Thieltges et al. 2008a, respectively). The density of these snails can be very high, with about 100 adult ind. m^{-2} recorded in parts of the Wadden Sea (Thieltges et al. 2008a). However, the infection prevalence among periwinkles in the study area is usually below 1% (our unpublished data). With semi-diurnal tides in the study area, a maximum shedding of about 300 cercariae in the vicinity of an infected snail per tide seemed to be realistic and we thus used this as the maximum cercarial dose administered. This maximum cercarial concentration can be expected to be diluted by the water body as well as by the density of down-stream hosts (Thieltges & Reise 2007). The latter effect can be potentially strong, as blue mussels, the main hosts for *H. elongata*, can reach densities of 500 ind. m^{-2} in the Wadden Sea (Drent & Dekker 2013). Hence, we used several lower cercarial doses (100, 60 and 20 cercariae) to mimic various levels of cercarial dilution.

For single-dose treatments, the total dose was administered on a single day (2 replicates on Day 1, 2 on Day 2 and so forth) while for trickle infection treatments, the total dose was administered in sub-doses over 4 d, resulting in the same cumulative number (total dose) of cercariae as the single dose treatments. Cercarial infections of bivalve hosts are known to occur within about half a day due to the short life expectancy of cercariae (Thieltges & Rick 2006). Hence, infections of subsequent trickle infections were unlikely to affect previous infections. The mussels of both single and trickle infection trials remained in their containers for a further 48 h after the last dose had been administered (i.e. 6 d in total) to allow for encystment of metacercariae, after which the mussels were removed and frozen. The mussels were later dissected, their soft tissue squeezed between 2 glass plates and the number of metacercariae in their tissue counted under a light microscope. We define infection success as the proportion of cercariae found encysted (as metacercariae) in the mussel tissue but use the proportion of remaining free-living cercariae, i.e. the unsuccessful infections, for our statistical analyses.

Statistics

The relationship between parasite dose (20, 60, 100 or 300 cercariae) and exposure frequency (single vs. trickle) on the infection intensity of *H. elongata* in mussel hosts was analysed using a binomial generalized linear model (GLM) with a log-link. Assuming a so-called linear pure death process, which means that all infections are independent events, the number of free-living cercarial stages remaining at the end of the experiment, i.e. those that did not successfully infect their mussel host, follows a binomial dis-

tribution. The parameters of the distribution are given by the initial number of parasites and by the probability that a parasite is still free-living at the end of the experiment. This probability equals

$$p = e^{-\theta} \quad (1)$$

where θ is the infection rate per unit of experimental time. It is further assumed that this infection rate is a function of parasite dose, exposure frequency and their interaction. Thus

$$\theta = \mu + \alpha_i + \beta_j + \gamma_{ij} \quad (2)$$

where μ is the intercept, α is the effect of dose, β is the effect of exposure frequency, and γ is their interaction. The model used the absolute number of remaining parasites after the 3 h experimental time period (the number of cercariae added minus the total number of metacercariae counted in the tissue of 2 mussel hosts).

A series of GLMs from the most complex to the least complex were fitted (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d125p085_supp.pdf). The most complex model included all explanatory variables (dose, exposure frequency and the dose × exposure frequency interaction) whereas the simplest model (the null model) excluded all explanatory variables and only included the intercept. Testing for the best fitting model by identifying significant differences between models of descending complexity was carried out using an analysis of deviance. For example, model 1, which included the interaction (dose × exposure frequency) was tested against model 2, which included only the main factors. The delta deviance (the difference in deviance between the 2 models) was subsequently divided by the dispersion factor from the most complete model (Δ Dev/ϕ) and compared to the delta degrees of freedom χ^2 at 0.05. The dispersion factor (ϕ) was calculated by dividing the residual deviance for the most complex model by the degrees of freedom. A significant difference between 2 models reveals that the most complex model of the 2 is the better fit. All analyses were carried out using R (R Development Core Team 2013) version 3.0.2 in R Studio (version 0.98.1103; RStudio Team 2014).

RESULTS

Infection intensity in mussels increased with cercarial dose and was generally higher when the same dose was administered in a trickle compared to a single exposure (Fig. 1). Furthermore, the increase in infection intensity appears to be proportional to dose, i.e. the relationships seem well described by linear slopes through the origin. This suggests that the infection rate θ only depends upon the factor exposure frequency. Indeed, the GLMs and subsequent model selection identified model 4 — which included only exposure frequency — to be the best fitting model (Tables 1 & 2; for raw data see Thieltges et al. 2017). This means that the infection success of cercariae did not differ among the 4 dose levels but was only significantly different between trickle and single exposures. This pattern could also be seen when plotting the relationship between cercarial dose and the number of free-living remaining cercarial stages at the end of the experiment, i.e. those that did not encyst within their mussel host (Fig. S2 in the Supplement). For both single and trickle exposures, the relationship was a linear one through the origin, i.e. the proportion of cercariae making it into the mussels was principally the same among all doses. However, the slope of the relationship was slightly lower for trickle exposure, indicating a higher infection suc-

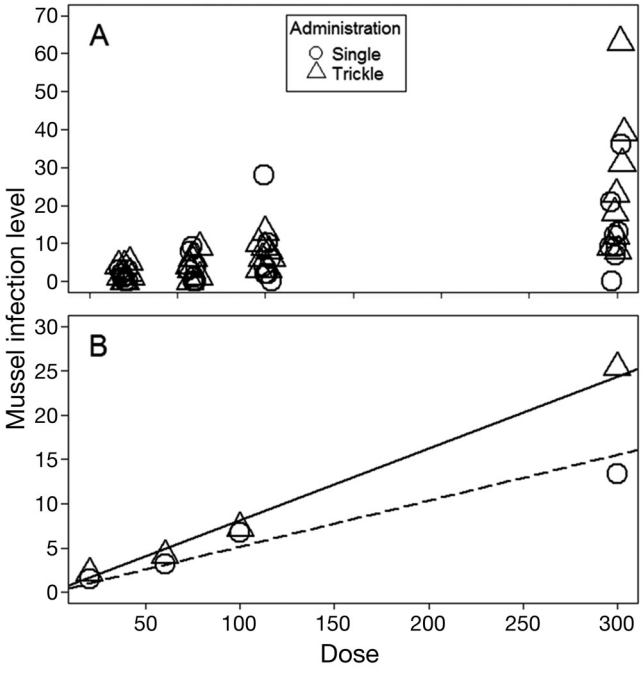

Fig. 1. Infection intensity in blue mussels *Mytilus edulis* (number of metacercariae recovered from 2 mussels) across the 4 cercarial doses (20, 60, 100, 300 cercariae of *Himasthla elongata*) administered for both single (circles) and trickle (triangles) exposure frequencies. (A) Individual results from each replicate. (B) Means for both administration treatments at the various doses. Regression lines are forced through the origin, given that infections are not possible at dose 0 (no parasites)

Table 1. Results of the binomial generalized linear models, ranging from the most complete (1) to the simplest (5) model. Given are the residual deviance and the degrees of freedom (df)

Model code	Model	Deviance	df
1	Dose + Freq. + Dose:Freq.	331.502	55
2	Dose + Freq.	337.875	58
3	Dose	366.024	59
4	Freq.	342.706	61
5	1	369.961	62

Table 2. Results of all model comparisons (i.e. model 1 vs. model 2, model 2 vs. model 3, etc.). The difference in deviance (Δ Deviance) divided by the dispersion factor (ϕ), which equalled 6.027, is compared to a χ^2 distribution with Δ df degrees of freedom. For more details, see the 'Materials and methods'. For model descriptions, see Table 1. *$p < 0.05$

Model Comparison	Δ Deviance	Δ df	Δ Dev/ ϕ	Pr ($<\chi^2$)
1 vs. 2	6.373	3	1.057	0.787
2 vs. 3	28.15	1	4.670	0.031*
2 vs. 4	4.831	3	0.802	0.849
3 vs. 5	3.937	3	0.653	0.884
4 vs. 5	27.26	1	4.522	0.033*

cess of cercariae compared to single exposure. In general, infection success of cercariae as calculated from the final model was relatively low, with the average proportion of cercariae not encysted in mussel tissue at the end of the experiment being 0.948 for single infections and 0.919 for trickle infections. This resulted from relatively low instantaneous infection rates of cercariae, with 0.051 per experimental period (i.e. 6 d) at single exposure and 0.081 at trickle infection exposure.

Metacercariae were recovered primarily from the foot of their mussel hosts (79.9%), followed by the gills (9.8%) and mantle (8.4%). The remaining metacercariae were found in muscle tissue and digestive glands.

DISCUSSION

Our experiment showed that infection intensity in mussels increased in proportion with the number of cercariae they were exposed to, and infection success of cercariae was thus independent of dose. However, infection success significantly differed between the 2 exposure frequency treatments and was higher in trickle compared to single infections, leading to higher infection intensity in the mussels exposed to trickle infections.

Adding higher doses of infective stages to the experimental containers resulted in higher numbers of metacercariae in mussel tissue, i.e. infection intensity in mussels increased with parasite dose. Such an increase in infection intensity in hosts with increasing parasite dose is generally explained by the mass-action principle, which assumes that the number of susceptible host individuals which become infected over time and the individual infection intensity are simply related to the density of hosts and the concentration of parasites to which they are exposed (Ben-Ami et al. 2008). The infection success of cercariae in our experiment was not affected by cercarial dose, which means that infection intensity in mussels continually increased with increasing dose and did not level off at higher doses to show a saturation of infection intensity. Such a saturation has been observed in some studies and is considered to be a deviation from the basic frequency-dependent mass-action principle, due to, for instance, density-dependent regulation of infections which includes intra-specific competition between parasites as well as behavioural and immunological anti-parasitic responses by hosts (Ebert et al. 2000, Karvonen et al. 2003, Poulin 2010). The fact that we did not observe a saturation at higher doses in our experiment suggests that density-dependent regulation of trematode infections in mussels may not exist, at least not at the realistic doses that mussels were exposed to in our experiment. Other studies have used much higher doses as indicated by a recent meta-analysis of published studies on cercarial infection success under experimental conditions (Poulin 2010). This meta-analysis indicates that the average median dose of cercariae administered in trematode infection studies has been 435.32 ± 1249.51 SD, with a wide range from 4 to 10 000 (Poulin 2010). In addition, this analysis also revealed that density-dependent regulation of infection intensity within parasite–host systems increases with the median cercarial dose administered (Poulin 2010). Hence, we cannot exclude the possibility that mussels also experience a dose-dependent regulation of infection intensity at very high doses. However, given the unlikeliness of such high dose levels under natural conditions, such an effect would be of little ecological relevance. The absence of dose-dependent regulation at realistic doses has important implications, as the resulting proportional relationship between cercarial dose and infection intensity suggests that vary-

ing densities of infective stages will directly translate into varying intensities of infection in mussel hosts. Hence, infection intensity in mussels (and potentially other second intermediate trematode hosts) will be largely driven by factors mediating the abundance of infective stages instead of intraspecific competition between parasites or behavioural and immunological anti-parasitic responses by the hosts. Potential factors mediating the abundance of infective stages may be abiotic environmental conditions such as temperature and salinity (Pietrock & Marcogliese 2003) or biotic interactions such as consumption of cercariae by ambient organisms (Thieltges et al. 2008b, Welsh et al. 2014). However, more studies are needed to confirm whether this is a general pattern in second intermediate trematode hosts.

While the infection success of cercariae in their downstream mussel hosts was unaffected by cercarial dose, infection successes and infection intensity in mussels were higher with a trickle exposure compared to a single exposure to cercariae. This pattern clearly differed from the few available studies on other parasite–host systems where infection success was not affected by single or trickle exposure (e.g. monogeneans infecting fish: Rubio-Godoy & Tinsley 2002). Although the repeated exposure of hosts to smaller cercarial doses is probably a much more realistic scenario under natural conditions in the field than a single high dose exposure event, there are, to our knowledge, no published studies that have experimentally compared the effect of single versus trickle infections on trematode infection intensity in second intermediate hosts. Hence, it remains to be investigated whether the observed increase in infection intensity at higher exposure frequency is a general phenomenon in trematode second intermediate hosts or is restricted to mussels.

A possible explanation for the higher infection intensity of mussels at trickle exposure may be based on the process of infection. Mussels are filter feeders and become mainly infected via their inhalant current (authors' pers. obs.). As bivalve filter feeding is not a constant process and can be interrupted or altered in magnitude due to a multitude of factors including acoustic or mechanical disturbances (Gosling 2003), it may be that mussels were not always constantly filtering when cercariae were added, e.g. due to minor disturbances caused by the cooling system in the climate chamber or usage of neighbouring chambers. A multiple exposure to cercariae may increase chances that filtration takes place at cercarial exposure and thus increase overall infection intensity. However, there may also be other factors responsible for the observed pattern, such as density-dependent regulation in the form of intra-specific interference among parasites both during and after infection or increased anti-parasitic immune responses by the host (Ebert et al. 2000, Poulin 2010). Whatever the exact mechanisms, the observed pattern has important implications for mussel hosts as they face a higher infection risk when repeatedly exposed to even small doses over time. Field observations and experiments indicate that cercariae are constantly released from their first intermediate gastropod hosts and that infection intensity in downstream second intermediate bivalve hosts slowly increases over time (e.g. de Montaudouin et al. 2016). This suggests that hosts can accumulate high infection intensity over time, even if they are only exposed to relatively small doses at a time.

In general, the infection success of trematode cercariae observed in experimental studies varies by orders of magnitude (Poulin 2010) and was relatively low in our study (5.1% for single and 8.1% for trickle exposure). These values are also lower than the ones observed in the few previous experimental studies on infection success of *Himasthla* species where hosts were exposed to a fixed number of cercariae (instead of the different doses as used in our experiments). De Montaudouin et al. (2005) reported 13 to 97% infection success of *H. quissetensis* in cockles *Cerastoderma edule*, depending on cockle size. Likewise, Wegeberg et al. (1999) reported a host size-dependent infection success of *H. elongata* in cockles ranging from 16 to 60%. For *H. elongata* infecting mussels, Nikolaev et al. (2006) determined an infection success of 55 to 85% in mussels of a similar size range to those used in our study. The generally higher infection success observed in these studies compared to our study most likely results from several differences in the study design. First, there was a large difference in size of the containers used in the various experiments. While in our experiment mussels were placed in 500 ml of sea water and then exposed to a range of parasite doses (20–300 cercariae), the previously described experiments only used 30 to 70 ml of sea water and 10 to 25 parasites. Hence, the density of parasites was much greater in the other experiments, which likely increased the probability of cercariae encountering and thus infecting their hosts (Karvonen et al. 2003). Furthermore, infection efficiency of trematode cercariae has been shown to generally increase with temperature (Thieltges & Rick 2006). The previously described

studies ran their experiments at 24°C while our experiment was conducted at 18°C (the average summer water temperature in our study area; van Aken 2008), which may have contributed to lower infection success. Finally, the studies by Wegeberg et al. (1999) and de Montaudouin et al. (2005) used cockles as downstream hosts which may exhibit a higher susceptibility to infections compared to mussels.

In conclusion, our experiment indicated that mussels face higher parasite infection intensity at higher doses of cercariae as well as when repeatedly exposed to smaller doses compared to a single high dose. At the realistic dose levels applied in our experiment, cercariae did not show a dose-dependent regulation of their infectivity, suggesting that infection intensity in mussels is largely driven by factors mediating the abundance of infective stages. With the combined investigation of the effect of cercarial dose and exposure frequency at realistic dose levels, our study contributes to our currently very limited understanding of the determinants of infection intensity in marine hosts and highlights the usefulness of experimental studies in advancing our knowledge in this respect.

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