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ORIGINAL PAPER

Non-host organisms impact transmission at two different life stages in a marine parasite

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Abstract

The potential for local biodiversity to 'dilute' infection risk has been shown to be particularly important in aquatic trematodes, where non-host organisms can feed on free-living infective stages (cercariae) and reduce transmission rates to target hosts. Non-host organisms could also impact transmission during other stages of the trematode life cycle. In *Philophthalmus* spp., cercariae encyst as metacercariae on external surfaces, where they remain exposed to the adverse effects of non-host organisms. In laboratory experiments, we tested the potential for a range of non-host organisms to (i) prey on cercariae, (ii) induce early (i.e., faster) encystment and (iii) prey on or destroy metacercariae. Our results show that intertidal anemones, and to a lesser extent clams, can consume substantial numbers of cercariae. However, we found no strong evidence that the presence of these predators causes cercariae to encyst faster as a way to escape from predation. We also found that grazing snails can reduce numbers of encysted metacercariae, either by eating or crushing them. Our findings add to the growing evidence that trematode transmission success can be strongly affected by the local diversity of non-host organisms. They also reinforce the notion that parasites are potentially important food items for many organisms, thus playing roles other than consumers in many food webs.

Keywords Biodiversity · Cercariae · Dilution effect · Metacercariae · *Philophthalmus* sp. · Predation · Transmission success

Introduction

Recent evidence indicates that one of the consequences of biodiversity loss may be the increased risk of infectious disease for focal host species (Keesing et al. 2006, 2010; Johnson et al. 2015). For instance, high local species diversity can reduce infection risk for a given host species by providing a large pool of non-competent hosts that reduce the encounter rate between disease propagules or vectors and target hosts (Ostfeld and Keesing 2000; Keesing et al. 2006). This 'dilution effect' is one of the many mechanisms through which local biodiversity can impact infection risk. There is still much debate regarding the universality of the biodiversity-disease relationship; whether local diversity amplifies or reduces infection risk may depend on species composition more than on species richness, and it may also vary depending on the nature of the host-parasite system under consideration (Keesing et al. 2006, 2010; Randolph and Dobson 2012; Salkeld et al. 2013; Wood and Lafferty 2013; Johnson et al. 2015; Welsh et al. 2017).

In the case of trematode parasites in aquatic ecosystems, there is no debate: the impact of local diversity on transmission success is well established (e.g., Welsh et al. 2014, 2017). Typically, trematodes use a snail as their first intermediate host. Within the snail host, they multiply asexually to produce large numbers of cercariae, i.e., free-living and short-lived dispersal stages that leave the snail to seek and infect the next host in the life cycle (Galaktionov and Dobrovolskij 2003). During their short active life, typically a few to several hours, cercariae may encounter a number of non-host organisms that can act as physical barriers, decoy or dead-end hosts or simply as predators of cercariae, the net result being decreased transmission rates to target hosts (Thieltges et al. 2008a, b). Predation on cercariae is common, with a wide range of

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organisms readily and frequently feeding on these transmission stages, including filter-feeding bivalves and barnacles (Prinz et al. 2009; Welsh et al. 2014), benthic arthropods (Welsh et al. 2014; Orlofske et al. 2015), zooplankton (Mironova et al. 2018) and small fish (Kaplan et al. 2009; Orlofske et al. 2015).

Predation on cercariae is not the only way in which local biodiversity may affect trematode transmission success. In many trematode species, cercariae do not enter a second intermediate host but encyst as metacercariae on various substrates that include the external surfaces of plants or invertebrates on which the parasite's definitive host may feed. In these trematode species, non-host organisms can have two other types of impact on transmission. First, non-host organisms may induce stress in cercariae and stimulate early encystment as an attempt to avoid predation. For example, predatory organisms, via physical disturbance or chemical exudates, may signal their presence to cercariae in which case rapid encystment could offer protection against predation. There is evidence from the trematode *Parorchis* sp. (Philophthalmidae) that precocious encystment may be triggered by external conditions (O'Dwyer and Poulin 2015). In a predator-rich environment, such behaviour would likely decrease cercarial mortality, increase transmission success and thus fitness, particularly for trematodes that produce few large cercariae. Second, non-host organisms may kill metacercariae, either by actively feeding on them or by destroying them as a by-product of other activities, such as movement. To our knowledge, the only study to address this is that of Prinz et al. (2009); working with the trematode *Parorchis acanthus*, they found that grazing snails reduced the number of encysted metacercariae by feeding on the cysts. The generality of such finding remains to be confirmed by studies on different species, especially since metacercariae of different species vary in size, shape and presumably robustness.

Here, we examine the effects of non-host organisms on three transmission steps involving two life stages (cercarial survival, encystment and metacercarial survival) in the marine trematode *Philophthalmus* sp. (Philophthalmidae) (Martorelli et al. 2008). After emergence from their intermediate host, the mudsnail *Zeacumantus subcarinatus*, cercariae of *Philophthalmus* sp. (about 1 mm long including the tail) swim close to the bottom before encysting relatively quickly on hard surfaces (i.e., within 2–3 h of emergence). In nature, these are mostly the shells of molluscs, like topshells, *Diloma* spp., or bivalves (Neal and Poulin 2012), where they await ingestion by their bird definitive host, usually seagulls *Larus* spp.

Our objectives were to assess the potential impact of non-host organisms on different transmission steps of *Philophthalmus* sp. under laboratory conditions. We addressed three questions: (i) Can two abundant non-host predators feed on the large bottom-dwelling cercariae? (ii) Can the presence of these predators accelerate encystment of cercariae? And (iii) can two species of gastropod grazers, also very abundant in the field, kill encysted metacercariae? Our findings reinforce the notion that local non-host diversity impacts trematode transmission beyond the free-swimming cercarial stage, and add to our growing understanding of the complexity of parasite transmission in biodiverse ecosystems.

Methods

Non-host organisms

Two intertidal invertebrates were considered as potential predators of cercariae. The filter-feeding New Zealand clam, *Austrovenus stutchburyi*, is the most abundant bivalve in intertidal soft-sediment areas in Otago Harbour, our study site. It can reach densities of > 200 per m^2, and together, these clams can filter the entire intertidal water volume at high tide several times per submersion period (Mouritsen et al. 2003). The anemone *Anthopleura aureoradiata* is also a common inhabitant of intertidal areas in our study site. Anemones attach to clam shells or other hard substrate, and in places they can reach densities greatly exceeding 100 per m^2 (Mouritsen and Poulin 2003). They actively capture prey using their tentacles and have previously been shown to feed readily on cercariae of other trematode families (Echinostomatidae and Microphallidae) (Mouritsen and Poulin 2003; Hopper et al. 2008).

Two intertidal gastropod grazers, the mudflat snail *Amphibola crenata* and the topshell *Diloma aethiops*, were considered as potential diluters of encysted metacercariae. Both are very common in intertidal areas, reaching densities of 10–50 per m^2 (Jones and Marsden 2005). They feed on microalgae and organic matter from the substrate. Of note, *D. aethiops* is commonly found grazing from the shell surfaces of other topshells or clams, which are prime locations for metacercarial encystment (Neal and Poulin 2012).

Animal collection and maintenance

We collected mudsnails *Zeacumantus subcarinatus* and all four non-host organisms haphazardly, by hand in Lower Portobello Bay, Otago Harbour, New Zealand (45° 49′ 56.5″ S, 170° 40′ 22.6″ E). All organisms were transported to the laboratory, sorted by species and maintained in 5-L tanks filled with continuously aerated sea water and maintained at room temperature (13–15 °C) for 2 weeks. During this time, all organisms had ad libitum access to suitable food, and natural seawater was gradually replaced with artificial seawater of the same salinity. In order to identify *Philophthalmus*-infected snails, individuals were separated into wells of 12-well plates with 5 mL artificial seawater and incubated at 25 °C under constant illumination for a period of 2 h. Cercariae of *Philophthalmus* sp. were identified based on morphological

features using the descriptions of Martorelli et al. (2008). *Philophthalmus*-infected snails were separated and maintained as described above to build a stock for the experiments. Non-host organisms were fasted for 2 days prior to their use in experimental trials.

Experiment 1: cercarial survival and encystment

In this experiment, we tested the effects of two potential predator species on free-swimming *Philophthalmus* cercariae and their encystment rate: the filter-feeding clam *Austrovenus stutchburyi* and the predatory anemone *Anthopleura aureoradiata*. To obtain freshly released cercariae, infected *Z. subcarinatus* snails were placed into wells of 6-well plates (one haphazardly chosen snail per well) with 7 mL of artificial seawater and incubated at 25 °C for 1 h under constant illumination. After this time, snails were removed and the number of free-swimming cercariae were counted under a stereomicroscope; excess cercariae were removed using a pipette so wells contained 10 to 20 cercariae each, with the exact number of cercariae recorded for each individual well. We then added either one of the two predators to each well, or no predator for control replicates. Only predators of similar sizes were used in the experiment (mean ± SE: *A. stutchburyi*, shell length 25.6 ± 1.2 mm; *A. aureoradiata*, basal diameter 5.3 ± 0.5 mm). Sea anemones were attached to small rocks of similar sizes for easier handling of specimens. All plates were then left in an incubator at 15 °C and constant illumination for 24 h. After this time, the predators were carefully removed from the well, inspected for any encysted metacercariae and the remaining cercariae or metacercariae (encysted on the bottom or sides of wells) in each well were counted under a stereomicroscope.

Infected *Z. subcarinatus* snails release unpredictable numbers of cercariae on any given day. Additional cercariae were not transferred into wells to avoid confounding effects of handling that might speed up encystment. For this reason, not all wells contained a sufficient number of cercariae. Therefore, this experiment was performed in multiple batches, over several days and with equal numbers of replicate wells assigned to each treatment (clam, anemone, control) in each batch. Each treatment consisted of 15 replicate trials.

Experiment 2: metacercarial survival

In this experiment, we tested the effects of two grazing snail species, *Diloma aethiops* and *Amphibola crenata,* on encysted *Philophthalmus* metacercariae. Fresh *Philophthalmus* cercariae were obtained as described above. We then transferred 15–20 cercariae into individual plastic Petri dishes (diameter 6.8 cm) and left them at room temperature for 48 h to encyst and form metacercariae. We then screened each petri dish to ensure it contained at least 10 fully formed cysts and recorded their number per well; the location of all cysts was marked on the underside of the Petri dish with a small dot. One of the two snail grazers, or no snail for control replicates, was then added to each Petri dish. Only snails of similar sizes were used in the experiment (mean ± SE: *D. aethiops*, shell height 12.5 ± 1.4 mm; *A. crenata*, shell diameter 14.3 ± 1.0 mm). All Petri dishes were placed on a tray and covered with a raised plexiglass sheet that allowed snails to move freely in the individual dishes but prevented them from escaping. The dishes were then kept in an incubator for 48 h at 15 °C. After this time, we carefully removed all snails and counted the numbers of intact, ruptured/empty and missing (i.e., no longer found in the dish) metacercarial cysts in each Petri dish. Again, because of the unpredictable availability of cercariae on any given day, this experiment was performed in multiple batches, over several days and with equal numbers of replicates assigned to each treatment (*D. aethiops* snail, *A. crenata* snail, control) in each batch. Each treatment consisted of 16 replicate trials.

Statistical analysis

In the first experiment, our main goal was to test whether the presence of predators caused a reduction in the number of surviving transmission stages, and whether it affected the number that successfully encysted before the end of the trial. Therefore, (i) the total number of all surviving transmission stages (combining still active cercariae and encysted metacercariae) and (ii) the number of encysted metacercariae were analysed using two separate mixed effects models with Poisson error structure implemented in JMP version 11.0 (SAS Institute Inc., Cary, NC, USA). Predator treatment (control, clam or anemone) and the number of cercariae per replicate at the start of the trials were included as fixed factors in the models. In both models, cercarial batch was included as a random factor to account for any variation in cercarial quality among batches or experimental days, and for the non-independence in the data arising from the fact that multiple data points come from the same batch. We calculated the proportion of the total variance unexplained by the fixed effects that could be accounted for by the random factor (Nakagawa and Schielzeth 2013). In addition, we used Pearson's correlation coefficients to test for relationships between predator size (clam: shell length; anemone: basal diameter) and the percentage of initial cercariae that survived (either as active cercariae or encysted metacercariae), separately for each predator.

For the second experiment, we again used a mixed effects model with Poisson error structure to analyse the total number of surviving metacercariae per trial. Snail treatment (control, *Amphibola* or *Diloma*) and the number of metacercariae per replicate at the start of the trials were included as fixed factors in the model. Cercarial batch was again included as a random factor, for the same reasons as above, and we calculated the proportion of the total variance unexplained by the fixed effects

that could be accounted for by this factor. Finally, we used Pearson's correlation coefficients to test for relationships between snail size (shell height or diameter) and the percentage of initial metacercariae that survived, separately for each snail species.

Results

Cercarial survival and encystment

In our first experiment, cercarial survival was reduced through the action of predators. As the initial number of cercariae varied among replicates, not surprisingly, the more cercariae were present at the start, the more survived (either as still active cercariae or encysted metacercariae) at the end (Table 1). However, the presence of predators caused a significant reduction in the number of surviving cercariae (Table 1). Anemones were particularly efficient predators, snatching cercariae from the water with their tentacles (see video in online resource 1). Predation by anemones resulted in the removal of almost two-thirds of the initial number of cercariae (Fig. 1). In contrast, predation by clams caused the loss of slightly less than half of cercariae, though this did not differ quite significantly from control treatments (Table 1). In control trials, virtually all cercariae survived either encysted or as cercariae. There was no correlation between predator size and the percentage of initial cercariae that survived, for either predator (Pearson's coefficients: both $P > 0.55$).

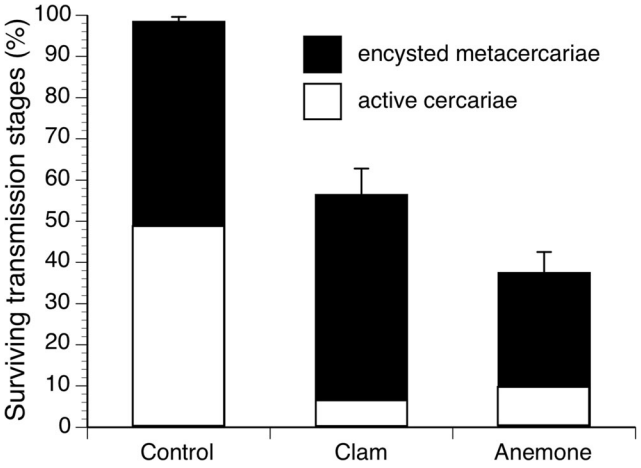

Fig. 1 Mean (± SE) percentage of *Philophthalmus* sp. cercariae surviving at the end of the trials following exposure to either no predator (control), the clam *Austrovenus stutchburyi* or the anemone *Anthopleura aureoradiata*. The relative proportions of survivors that were either still actively swimming cercariae or recently encysted metacercariae are indicated by different shading. $N = 15$ replicate trials for each treatment

At the end of these trials, the number of encysted metacercariae also varied among treatments. In addition to being influenced by the number of cercariae at the start of trials, the number of encysted metacercariae was much lower in trials with anemones, but only marginally significantly different in trials with clams compared to control trials (Table 1). However, among surviving transmission stages, the proportion of encysted metacercariae relative to free-swimming cercariae was greater in both predator treatments than in control trials (Fig. 1).

Table 1 Results of the mixed effects model with response variables (a) total number of surviving transmission stages (combining still active cercariae and encysted metacercariae), and (b) the number of encysted metacercariae from the first experiment and (c) the total number of intact metacercariae that survived trials from the second experiment showing the effects of the main predictors and the proportion of the remaining variance accounted for by the random factor

Fixed effects	Estimate	Std error	*t*-value	*P*	Random factor	% variance
(a) Total number surviving						
Intercept*	− 1.566	2.921	0.54	0.5947	Batch	1.9%
Initial number of cercariae	0.834	0.171	4.87	< 0.0001		
Predator (clam)	− 1.185	0.602	1.97	0.0559		
Predator (anemone)	− 4.129	0.600	6.88	< 0.0001		
(b) Number encysted metacercariae						
Intercept*	− 2.082	3.426	0.61	0.5468	Batch	0.6%
Initial number of cercariae	0.637	0.198	3.22	0.0025		
Predator (clam)	1.459	0.695	2.10	0.0422		
Predator (anemone)	− 2.631	0.694	3.79	0.0005		
(c) Number intact metacercariae						
Intercept*	1.393	1.517	0.92	0.3658	Batch	3.6%
Initial number of metacercariae	0.876	0.069	12.59	< 0.0001		
Snail (*Diloma*)	− 0.727	0.279	2.60	0.0126		
Snail (*Amphibola*)	− 0.133	0.278	0.48	0.6352		

*Control treatment is included in the intercept

Metacercarial survival

In our second experiment, most metacercariae were recovered intact and alive (with the worm moving inside the cyst) at the end of trials in all treatments (Fig. 2A). However, in trials with grazing snails, several metacercariae were lost; of these, almost two thirds of the cysts were simply no longer present anywhere in the dishes, whereas the other cysts were still present but no longer contained a worm. In rare cases, the cyst was damaged (Fig. 2B), but most often the empty cyst was intact but the plug meant to keep it closed had been removed and the worm was gone.

The number of intact and live metacercariae at the end of a trial depended on how many were used at the start of that trial; more importantly, there was a significant effect of the main treatment on how many live metacercariae were found at the end of trials (Table 1). All metacercariae survived in control treatments with no snail. In contrast, some cysts were lost or destroyed when maintained with snails, although the decrease in metacercarial survival was only significant for *Diloma aethiops* (Fig. 3). This snail caused the destruction or removal of about 10% of metacercariae, about twice the mortality caused by the other snail species, *Amphibola crenata*. There was no correlation between predator shell size and the percentage metacercariae that survived for either snail species (Pearson's coefficients: both $P > 0.75$).

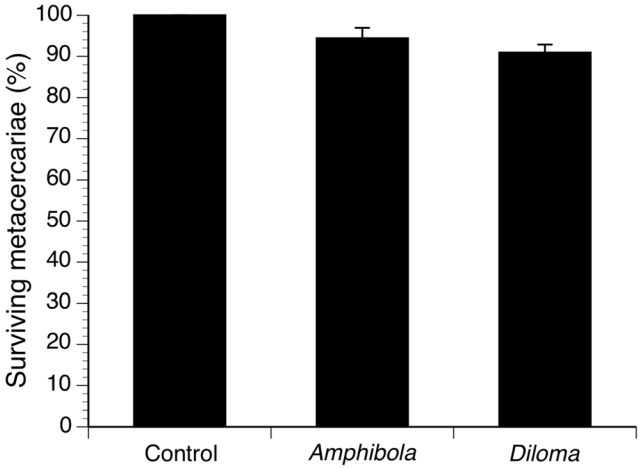

Fig. 3 Mean (± SE) percentage of intact and surviving *Philophthalmus* sp. metacercariae at the end of the trials following exposure to either no snail (control), the mudflat snail *Amphibola crenata* or the topshell *Diloma aethiops*. $N = 16$ replicate trials for each treatment

Discussion

Within a biodiverse community, parasite transmission can be a real challenge, with the presence of multiple non-host organisms reducing the likelihood that parasite infective stages reach their target host (Thieltges et al. 2008b). We tested the effect of non-host organisms on different transmission steps in the marine trematode *Philophthalmus* sp. Although our results are based on experiments conducted in artificial environments, they indicate that common invertebrates have the ability to kill, either through predation or destruction, both the cercarial and metacercarial stages of this trematode, and induce considerable fitness costs on the parasites. These findings have implications regarding both the ecology and evolution of transmission strategies in this parasite, ranging from host-parasite interactions to disease dynamics in ecosystems.

Our results indicate that the anemone *Anthopleura aureoradiata* consumed more cercariae per unit time than the clam *Austrovenus stutchburyi*. This may in part be due to the relatively small sizes of clams used in our experiment; *Philophthalmus* cercariae may be too large to be easily sucked down their inhalant siphon. In contrast, the anemones had no problem snatching cercariae from the water with their tentacles (see video in online resource 1). The anemone *A. aureoradiata* has also previously been shown to feed on the cercariae of two other trematode species, in both cases resulting in greatly reduced infection levels in target hosts under experimental conditions (Mouritsen and Poulin 2003; Hopper et al. 2008). Given the high densities of both clams and anemones (in both cases several hundred per m^2) in many

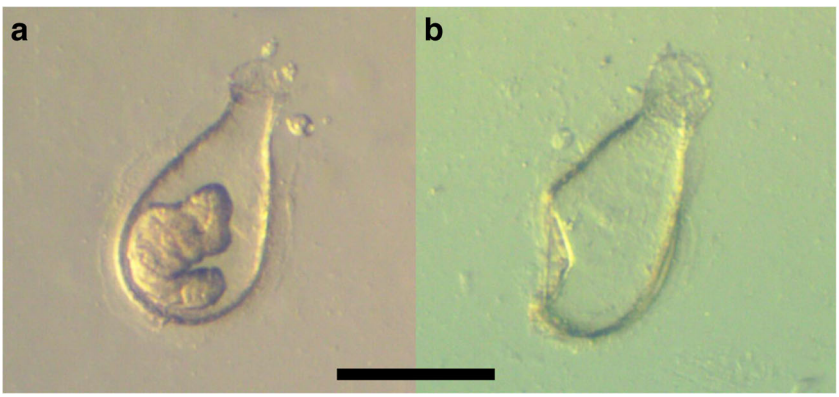

Fig. 2 Metacercarial cysts of *Philophthalmus* sp. (**a**) Intact cyst with the live worm visible inside. (**b**) Empty cyst damaged by a snail; the worm is missing. Scale bar: 0.2 mm

parts of our study area, it is very likely that they represent an important cause of mortality for the bottom-dwelling cercariae of *Philophthalmus* sp. under natural conditions.

Our study was designed to test simultaneously predation on cercariae and its possible indirect effect on encystment rate. The lower absolute number of cercariae that encysted in the presence of anemones (compared to control trials) is probably simply due to the fact that fewer cercariae remained to encyst, many having been eaten. This also explains why among surviving parasites in the anemone treatment, there were relatively more encysted individuals than free-swimming cercariae: those that did not encyst quickly experienced high mortality through predation. Only a higher absolute number of encysted individuals in the predator treatments would have been convincing evidence that predator cues induce faster encystment. Further experiments, in which cercariae are exposed only to chemical or physical cues simulating the presence of a predator, but without the predator itself, will be necessary to categorically rule out the possibility of rapid encystment as a mean to escape predation. However, whether or not they induce faster encystment, anemones may act as a selection force favouring the evolution of a more rapid encystment. Cercarial genotypes that encyst more quickly can reduce their risk of being killed by predators, and transmit more successfully to avian definitive hosts, than those that take longer to form a cyst. Active dispersal in the ecosystem and the potential chance to reach more final hosts, and a longer search for a suitable encystment surface therefore come with a higher risk of being killed.

However, encystment does not in itself guarantee survival against non-host organisms. We found that one of the two snail species used here, *Diloma aethiops*, was a significant cause of cyst disappearance and/or destruction, at least in a laboratory setting. In the field, these snails graze the external surfaces of other molluscs, where *Philophthalmus* metacercariae are mostly found (Neal and Poulin 2012), and thus their negative impact on metacercariae should extend to natural conditions. We found that about 10% of the metacercariae were killed by *D. aethiops*. The only other comparable study, with a different species of philophthalmid trematode and different snail species, used more snails per dish and a longer experimental duration (Prinz et al. 2009). It is therefore difficult to compare the quantitative findings of the two studies. However, whereas Prinz et al. (2009) observed that metacercariae were simply eaten by snails, we found that almost a third of killed metacercariae in our study had been destroyed by being squeezed out of their cyst, presumably under pressure from the snail's foot, leaving behind an empty cyst. In rare cases, empty cysts showed damage consistent with scraping from the snail's radula during grazing. The majority of killed cysts had completely disappeared, presumably consumed by the snails. Although metacercariae can sometimes survive passage through the digestive tract of snails (Latham et al. 2003; Prinz et al. 2009), philophthalmid metacercariae exiting in a snail's faeces would likely die, as they would be unable to reattach to a suitable hard substrate.

In addition to the risk of predation or destruction by non-host organisms, encysted metacercariae of *Philophthalmus* sp. are also at risk from a range of abiotic factors (Pietrock and Marcogliese 2003). Scraping by seaweed during storms or as a result of regular tidal currents seems likely to dislodge or destroy metacercariae in our study site. Also, the long-term survival of *Philophthalmus* sp. metacercariae is significantly reduced at salinities below that of normal seawater (Lei and Poulin 2011), conditions that are common in intertidal areas exposed to freshwater runoff from adjacent terrestrial habitats. In addition, *Philophthalmus* sp. metacercariae also experience lower survival at the slightly lower pH values projected for coastal areas in the near future, as a consequence of ocean acidification (Guilloteau et al. 2016). Therefore, there is a complex interplay of biotic and abiotic factors affecting the survival of these transmission stages and their probability of reaching a bird definitive host.

Earlier research efforts have focused mostly on predation and other ways in which non-host organisms can 'dilute' infection risk by cercariae. However, many other trematode taxa (e.g., Fasciolidae, Paramphistomidae, Zygocotylidae) also have vulnerable metacercariae that encyst on external surfaces rather than inside a second intermediate host both in aquatic or terrestrial habitats (Galaktionov and Dobrovolskij 2003); these most likely also experience significant mortality due to the effects of abiotic factors as well as non-host organisms. Future studies will be needed to reveal whether and to what degree these other taxa suffer from non-host organisms similarly to philophthalmids, and what this implies for the infection dynamics for downstream hosts.

The role of parasites as food for non-host organisms has been increasingly acknowledged in recent years (Johnson et al. 2010; Thieltges et al. 2013). Trematode cercariae appear to be regular components of the diet of many small organisms, in some cases being sufficient to sustain long-term predator survival and reproduction (Mironova et al. 2018). The present and previous studies suggest that cercariae of many trematode species may be regular food items for the abundant intertidal anemone *A. aureoradiata*. Trematode metacercariae are not as widely recognised as potential prey. In our study, it remains unclear whether they are digested by snails or simply destroyed as collateral damage of the grazing activity. Either way, in our study species, both the cercarial and metacercarial stages appear to incur losses due to non-host organisms. In the life cycle of the parasite, these risks add up to the already uncertain transmission from snail first intermediate hosts to the next target hosts. The influence of non-host organisms on parasite transmission must be considered as a key factor in any attempt to predict future disease risk in response to environmental change and the associated changes in biodiversity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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