LOST IN TRANSMISSION

Unravelling the mechanisms of parasite removal by non-hosts

Royal Netherlands Institute for Sea Research

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General Introduction

Jennifer E. Welsh

Community structure and disease risk

In recent years the idea that an increase in biodiversity can reduce disease risk, a phenomenon which has been coined the "dilution effect" (Keesing et al. 2006, Clay et al. 2009, Pongsiri et al. 2009, Johnson et al. 2013, 2015a, Pfäffle et al. 2015), has gained a lot of attention. Originally, the dilution effect theory assumed that an increase in biodiversity (measured via species richness) would result in fewer highly susceptible or 'competent' host species being present in a given system and thus disease incidences would be reduced (van Buskirk and Ostfeld 1995). The idea is thought to derive from zooprophylaxis, whereby vectors containing infective disease agents are diverted from biting their target host (typically humans) and bite other organisms instead (World Health Organization 1982, Dobson et al. 2006), and thus zooprophylaxis and the original dilution theory primarily focused on vector-borne, human related diseases. However, differing interpretations of the dilution effect led Keesing et al. (2006) to expand on the original theory, defining it as 'the net effect of species diversity reducing disease risk by any of a variety of mechanisms, and for both vector-borne and non-vector-borne diseases'. This new definition broadened the theory to include five key mechanisms in which changes in species diversity drive the reduction in disease risk. *Susceptible host regulation* occurs when compounding factors such as starvation and hunting regulate host populations and thus reduce the number of hosts available for infection. *Infected host mortality*, is when the death of the infected host leads to the death of the disease agent and thus its removal from the system. *Recovery augmentation* occurs when an increase in the recovery rate of the host results in them no longer being infected and potentially immune. *Transmission reduction* is the result of a reduction in successful transmission despite disease-host encounters. This thesis focuses on the fifth mechanism, *encounter reduction*, which occurs when changes in the diversity of non-host organisms result in a reduction in encounters between infective stages and uninfected host species.

Since Keesing et al. (2006), results of studies testing the dilution theory have been contradictory, fueling an intense but valid debate on whether biodiversity does actually reduce disease risk (Randolph and Dobson 2012, Lafferty and Wood 2013, Ostfeld and Keesing 2013). The varying studies have shown that an increase in biodiversity can either decrease disease risk (Civitello et al. 2015), cause no effect, or may even amplify disease risk ('amplification effect'; Randolph and Dobson 2012), thus the effects of biodiversity on disease may not be universal (Ostfeld and Keesing 2013).

Mechanisms of encounter reduction

Of the five types aforementioned mechanisms mentioned, encounter reduction has arguably received the most interest to date (Keesing et al. 2006, Clay et al. 2009). This is perhaps not surprising as all parasites, at one point or another, have a transmission stage when they move from one host to another. Many studies testing the effects of encounter reduction have focused on Lymes disease, a vector borne parasite which infects multiple different hosts. The Borrelia type bacterium which causes Lyme disease is not host specific and can infect multiple species, however some infected species are able to transmit the bacteria to the main vector, a tick, better than other infected species (highly competent and less competent hosts respectively; Johnson et al 2013; Wood and Lafferty 2013). The composition of the community in which the Borrelia type bacterium is present can affect the disease prevalence, that is, the composition of high and low competence hosts within a given system. If the community has a higher proportion of well distributed vectors and highly competent hosts (hosts which are efficient at transmitting the disease), then the chance of encounters between highly competent hosts and the subsequent spread of the bacteria is greater (Keesing et al. 2006, Levi et al. 2012; Fig. 1.1 A). In the Lyme disease system, host competency plays an important role and is a major factor in determining disease prevalence. Highly competent hosts allow propagation, maintenance and spread of the disease by transmitting the bacteria more easily and being geographically and temporally distributed in a way that enables host-to-host contact. Contrarily, low competency hosts are hosts which do not propagate, maintain and spread the disease as readily (Hatcher and Dunn 2011). Low competence host include hosts which are, in the case of Lyme disease, infected but the low competence host's immunity kills the bacteria so that cannot be passed on to subsequent uninfected ticks (Gray 1998, Keesing et al. 2006, Hatcher and Dunn 2011). Therefore, the bacteria cannot go on to infect other hosts and thus, a more diverse system which contains a high proportion of low competency hosts compared to a low diversity system with fewer low competency hosts is expected to result in a lower disease prevalence (van Buskirk and Ostfeld 1995, Ostfeld and Keesing 2000, LoGiudice et al. 2003, Keesing et al. 2010).

Differences in host competency also drive a second type of encounter reduction which does not include vector-borne diseases but, alternatively, non-vector borne diseases. The life strategy of non-vector borne diseases, such as helminth parasites, are typically complex with multi-stage life cycles containing free-living infective stages. The success of the parasite transmission is determined by the free-living stages infecting competent hosts and thus the community composition and ratio of highly

competent to low competent hosts influences the success of infection (Fig. 1.1 B; Johnson et al. 2008, 2013, Hall et al. 2009). For example, the trematode parasite *Ribeiroia ondatrae* infects the larvae of a variety of freshwater amphibians leading to malformations in metamorphosed adult amphibians (Johnson and Sutherland 2003). Similar to the previous vector-borne example, some amphibian hosts are more competent than others (Johnson et al. 2008) and species poor communities, which have been shown to contain more highly competent amphibian hosts compared to species rich communities, result in 78.4% more successful *Ribeiroia ondatrae* infections and a higher percentages of malformations (Johnson et al. 2013). However, unlike the previous vector-borne example, host competency does not only affect disease prevalence (the proportion of infected individuals with in a system) but also infection intensity (the number of parasites within an infected host; Johnson et al. 2008). Infection intensity is important as the host is very rarely affected by a few parasites alone and host health only tends to be affected when infection intensity is high enough to deplete host resources or when combined with other factors such environmental stress (De Montaudouin et al. 1998, Wegeberg and Jensen 1999, Fredensborg et al. 2004, Kelly et al. 2010).

In addition to encounter reduction effects caused by differential host susceptibility in vector-borne and non-vector-borne disease systems, there is a third mechanism which may also result in encounter reduction effects: transmission interference via parasite removal by non-hosts (Johnson and Thieltges 2010). Transmission interference occurs in non-vector borne diseases when organisms which do not serve as hosts for a pathogen or parasite (non-hosts) prevent infective stages from successfully transmitting from one host to another, resulting in a reduction in infection intensity in the downstream host. Non-host organisms can affect transmission via toxic excretions, physical barriers and predation (for review see Thieltges et al. 2008b). Thus, an increase in biodiversity is thought to increase the number of non-host organisms which cause transmission interference, specifically predators of parasites, resulting in more free-living infective stages being removed and therefore, and overall decrease in infection intensity in downstream hosts (Fig. 1.1 C). Until now, this third mechanism has been extremely understudied in regards to biodiversity effects on disease risk but is postulated to significantly contribute to encounter reduction and the overall effects of biodiversity on disease risk (Thieltges et al. 2008b, Johnson et al. 2010).

Transmission interference and its implications

Transmission interference in parasite-host interactions is likely to be a common phenomenon, mainly due to the life strategy of many parasites and the vulnerability of free-living stages during the transmission process. Free-living stages of parasites, such as trematode cercariae, have limited time in which they can find and infect their hosts. During this transmission period, the free-living stages are exposed to multiple environmental stressors (Lowenberger 1995, Pietrock and Marcogliese 2003, Studer and Poulin 2013a, 2013b). In addition to abiotic environmental conditions (Pietrock and Marcogliese 2003), the free-living infective stages are also subjected to biotic factors such as predation by non-host organisms (Mouritsen and Poulin 2003b, Hopper et al. 2008, Prinz et al. 2009, Kaplan et al. 2009). Predation of infective stages results in fewer parasites reaching their downstream hosts and an overall lower infection intensity (Thieltges et al. 2009a, 2008a, Liddell et al. 2015). Predation of free-living infective stages has been known to occur in terrestrial (Ridgway et al. 1967, Barron 1977), freshwater (Schotthoefer et al. 2007) and marine (Hopper et al. 2008, Prinz et al. 2009, Thieltges et al. 2013) systems. Given their high nutritional value, it is perhaps not surprising that infective stages such as cercariae are used as a resource by many species and significantly contribute to food webs (Negus 1968, Lafferty et al. 2006, Kaplan et al. 2009, Johnson et al. 2010). Despite the likelihood of predation of free-living infective stages being extremely common, there have been very few studies focusing on the detailed mechanisms of encounter reduction. For example, there is a lack of information regarding the types of predators, the conditions under which predation occurs and the mechanisms affecting the removal of infective stages and thus it remains unknown whether specific traits, such as non-host organism size and specific predator feeding methods, make a 'good' transmission interferer. There is also limited information regarding external factors, such as temperature and food availability, and how they affect the rate of parasite removal. Furthermore, and essential to the biodiversity and disease risk debate, the simultaneous effects of multiple transmission interferers on free-living infective stages remains undetermined. Without an understanding of these fundamental aspects it would be difficult to predict the extent at which transmission interference occurs or the full effects of changes in diversity on disease risk, thus, deciphering the mechanisms which can alter transmission interference (and encounter reduction) is paramount to ecology and understanding ecosystem health (Johnson et al. 2015b).

Aim and outline of the thesis

The aim of this thesis was to test and analyse different key mechanisms influencing parasite removal by non-host organisms (Box 1). The thesis focuses on two marine parasite-host model systems, marine intertidal trematodes and pelagic viruses. The two systems were considered to be representative of typical micro- and macroparasite groups and commonly occur in many aquatic ecosystems (Box 2).

Throughout the thesis laboratory based multi-factorial experiments were used to test the effects of different mechanisms and species interactions on parasite removal. Laboratory based experiments were chosen to ensure that all experimental factors can been strictly controlled to guarantee robust results and conclusions. All experiments were conducted at the NIOZ Royal Netherlands Institute for Sea Research, Texel (the Netherlands) and used species which are commonly found throughout the Wadden Sea and/or the North Sea.

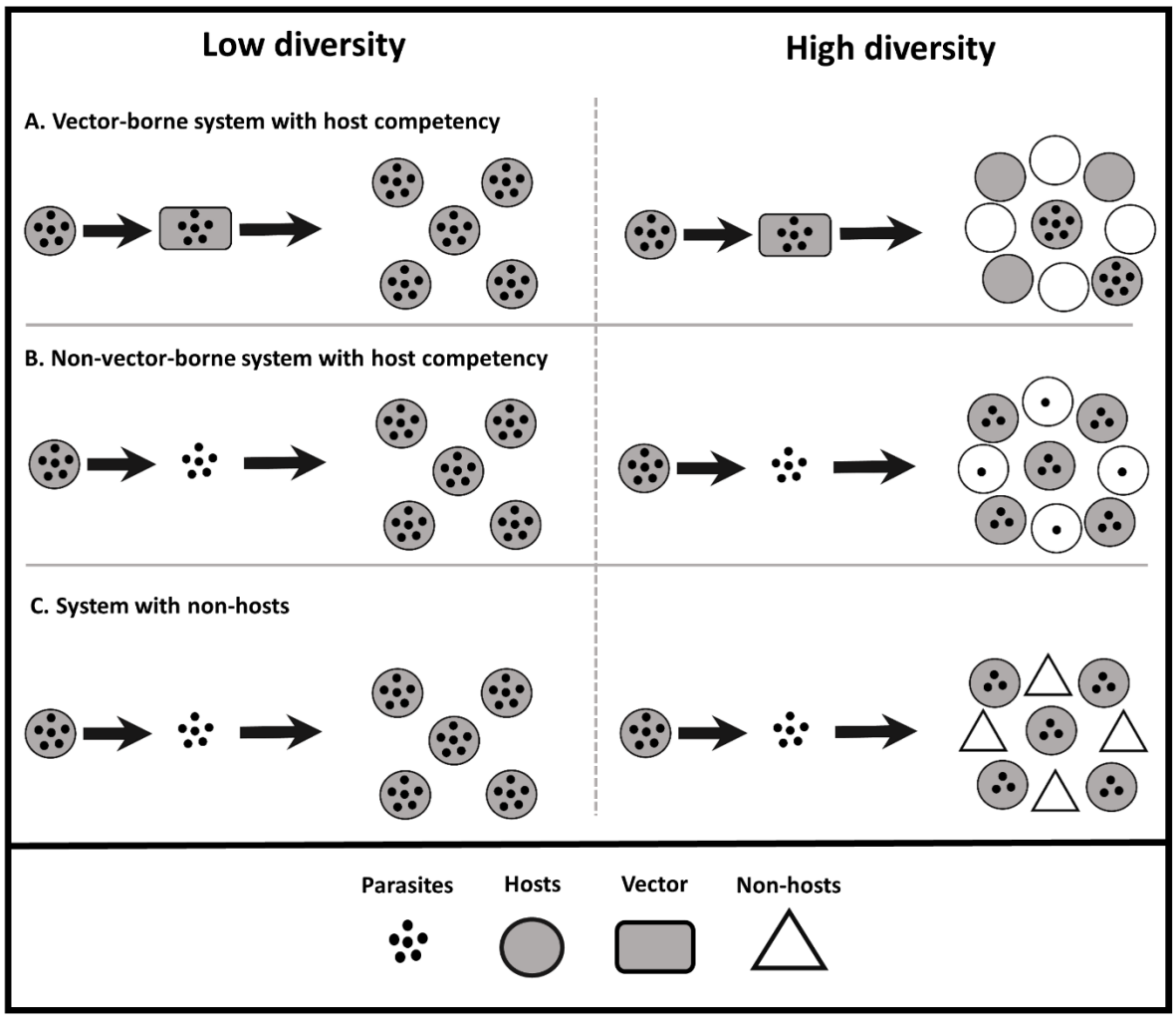
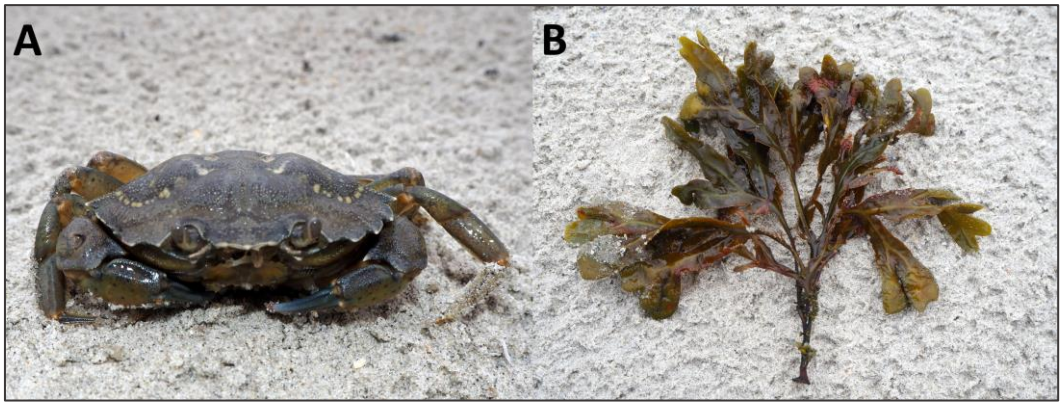


Fig. 1.1 The dilution effect theory states that biodiversity reduces disease risk. This reduction in disease risk can occur via one of three key ways. In vector-borne disease systems an increase in diversity is thought to reduce the relative abundance of high competency hosts (hosts which readily transmit the disease; A). Therefore, an increase in biodiversity reduces the chance of encounters between an infected host and an uninfected vector, resulting in an overall reduction in disease prevalence. In a non-vector borne system with hosts of differential competence, an increase in biodiversity, and thus an increase in the proportion of low competence hosts, is likely to result in the free-living stages of parasites being diverted to the low competence hosts and therefore, result in a decrease in infection intensity in high competence hosts (B). However, an increase in diversity may also lead to a reduction in free-living infective stages due to an increase in the proportion of non-host organisms, that is, organisms which cannot become infected (C). In this instance the non-host organisms may, amongst other mechanisms, predate upon or act as a physical barriers resulting in the removal of the free-living infective stages before they are able to infect their downstream hosts.

Box 1. Non-host organisms

The term 'non-host organism(s)' or, in short, 'non-hosts' refers to organisms which do not become infected by the specified parasites and thus, do not act as hosts. In this thesis it specifically refers to the organisms which also have the ability to interfere with the transmission of free-living parasites. It is important to clearly discriminate non-host organisms from low and highly competent hosts as the latter can become infected and have the potential to allow the parasite to persist within a given system. Some non-host organisms do not predate on the free-living infective stages but, instead, create a physical barrier in which the infective stages can become entangled and thus, also prevent infection of downstream hosts.

Throughout this thesis I used a variety of non-host organisms which were chosen for the experiments based on their habitats coinciding with that of the parasites as well as their relatively high abundance. The specific non-host organism species used in this thesis can be found in intertidal to subtidal areas of the Wadden and North Sea and are typically found in areas of hard to muddy substrate, however, similar species can be found in most marine areas making them good model species. For predatory non-hosts, I used organisms with different types of feeding strategies. The key feeding strategies included suspension feeders (barnacles), filter feeders (oysters) and active predators/scavengers (crabs and shrimps; Box 1.1.). A variety of ecologically relevant non-host organisms and biological material, which had the potential to act as physical barriers, were chosen due to their variation in physical structure and form.

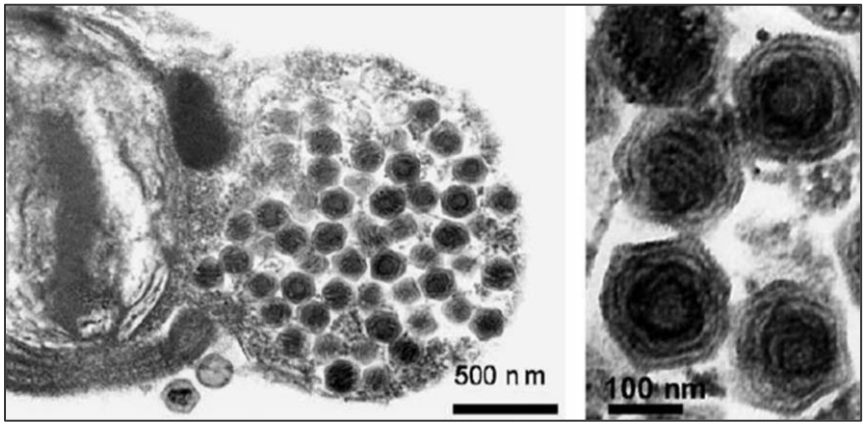


Box 1.1. Predators such as crabs (A), shrimps, oysters and barnacles were chosen for their different feeding mechanisms whereas organisms acting as physical barriers such as seaweed (B) and shells were chosen due to their structure.

Box 2. Model species and their life-cycles

Microparasite – *Phaeocystis globosa* virus

Viruses are the world's smallest entities but are extremely abundant (Suttle 2007). In order to exist and replicate viruses must use hosts and thus fall under the same umbrella as parasites. Typically, viruses have direct lifecycles and only require one host in order to replicate. The lytic virus used in this thesis, *Phaeocystis globosa* virus strain PgV-07T (NIOZ culture collection; Baudoux and Brussaard, 2005; Box 2.1.), is commonly found in marine intertidal and pelagic zones of temperate regions and is highly host specific with free-living viral particles only infecting the cells of the marine algae *P. globosa.* When *Phaeocystis* spp. form large blooms it is known to be problematic to other marine organisms (Peperzak 2002), however, PgV-07T infections can result in the rapid termination of such blooms (Brussaard et al. 2007).

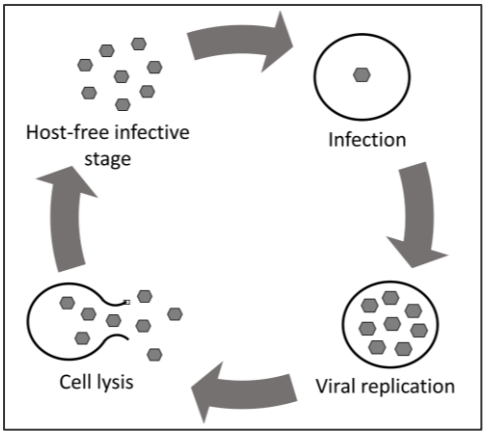


Box 2.1. PgV infected *P. globose* algal cell showing each individual hexagonal PgV particle (left) and a magnified image of PgV particles (right). Electron microscopy images replicated from Baudoux and Brussaard (2005).

The direct life cycle strategy of PgV-07T means that they have one free-living life cycle stage. After infection the PgV-07T virus replicates inside the algal cell until it reaches between 100- 300 particles, which takes approx. 24 hours (Brussaard et al. 2005, Baudoux 2007). Once the virus has replicated, the host *P. globosa* cell bursts releasing the viral particles into the water column, a process known as 'cell lysis' (Box 2.2). PgV viral infection results in the death of the host and thus viral prevalence and ambient populations are important measures for attaining disease risk.

Box 2. Model species and their life-cycle (continued)

Physical parameters such as temperature and UV radiation are known to affect the infectivity of algal marine viruses (for review see Mojica and Brussaard 2014), but little is known about how non-host organisms can interfere with the virus transmission.



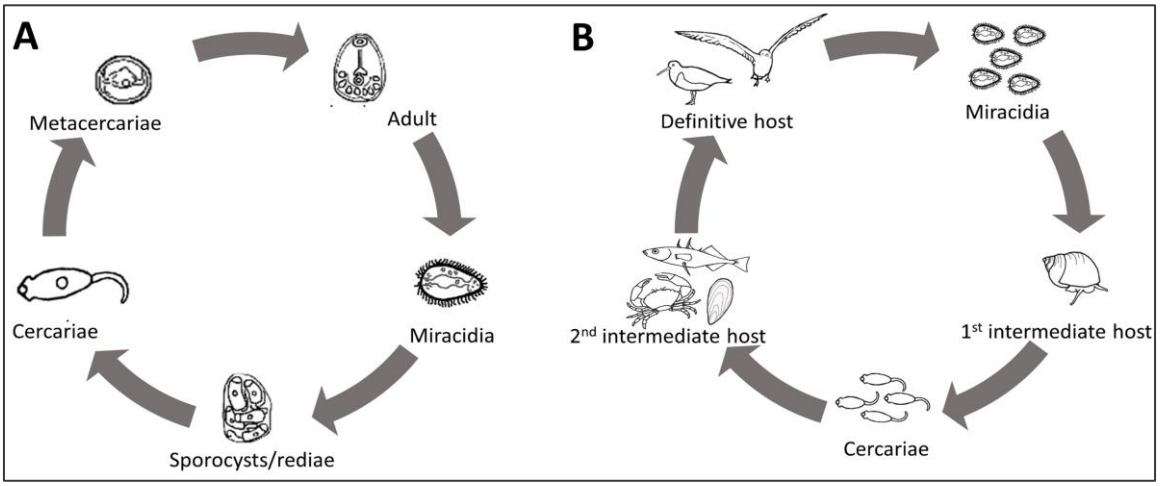
Box 2.2. Life cycle of the microparasite *Phaeocystis globosa* virus from the free-living life stage where the viral particles (grey hexagons) are free in the environment, the infection of a *Phaeocystis globosa* algal host (circle) by a virus, replication of the viruses within the host until burst point, at which cell lysis occurs releasing the viral particles back into the environment.

Macroparasite - trematodes *Himasthla elongata* and *Renicola roscovita*

Trematodes belong to the phylum Platyhelminthes (flatworms) and are ubiquitous throughout marine, freshwater and terrestrial systems. Trematodes exhibit complex life-cycles which require intermediate hosts and a definitive host, typically a vertebrate in which they sexually reproduce. One of the intermediate hosts is usually a mollusc, such as a snail, and in which they asexually reproduce (Box 2.3). Transmission occurs when free-living infective stages are released from one host and go on to infect a different downstream host. However, no free-living infective stages are released from the last intermediate host as these infected hosts are directly consumed by the definitive host. For a successful transmission, indirect lifecycle parasites require an environment where all hosts coincide at the corresponding time. Life-cycle complexity varies amongst trematode species and the complexity depends on the number of host species the parasite requires to fulfill its life cycle to enable reproduction (Mccarthy et al. 2002, Poulin and Cribb 2002).

Box 2. Model species and their life-cycle (continued)

The effects of trematode infection on their hosts are density dependent and hosts infected with more than one trematode can continue to live so long as infection intensity (the number of parasites within an individual host) remains below the hosts threshold. Hence, infection intensity is an appropriate measure and a good disease risk indicator.



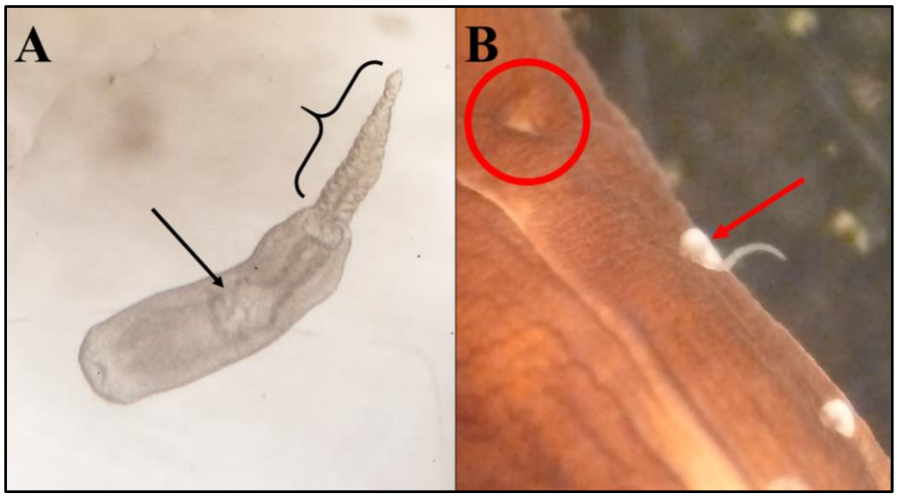
Box 2.3. Typical life cycle of a trematode parasite with the different life-cycle stages (A); and the consecutive intermediate and definitive hosts and free-living infective stages (B).

The two marine trematodes *Himasthla elongata* and *Renicola roscovita* used in this thesis are commonly found throughout the North-Atlantic (Werding 1969, Thieltges et al. 2009). Both trematode species use the intertidal snail *Littorina littorea* (the common periwinkle) as their first intermediate host. In this host they are present as sporocysts or rediae and undergo asexual reproduction. After asexual reproduction and under specific environmental conditions, they are released as free-living infective stages called cercariae, which then infect a second intermediate host (mussels and cockles).

Cercariae are motile and live for a few hours in order to find their next (2nd) intermediate host, most commonly the blue mussel *Mytilus edulis*. The cercariae bore into the tissue of the blue mussel (Box 2.4), lose their tails and encyst as metacercariae.

Box 2. Model species and their life-cycle (continued)

The metacercariae remain encysted until the mussel is consumed by the vertebrate definitive host, typically an oystercatcher or other shore bird. Once the metacercariae are in the digestive tract of the definitive host they mature into adults and sexually reproduce, releasing eggs which are then released into the environment via the definitive hosts feces. If the eggs, from which miracidia hatch, are released into an area such as intertidal mudflats where the first intermediate snail host are present the transmission cycle can continue.



Box 2.4. The free-living infective stage (cercariae) from the marine trematode *Himasthla elongata* (A), showing its glycogen rich tail used for mobility (top right of body) and suction pad for attachment (black arrow). Once a host is located, the cercariae (B, red arrow) bores into the hosts exposed tissue, which leaves a distinguishable bore hole (red circle).

Chapter 2 of this thesis examines how a reduction in free-living parasite stages affects infection intensity in downstream hosts. This study also deliberates the effects of an intense, high parasite density, one-time infection event as used in experiments compared to a slower, lower parasite density, trickle exposure which is probably more commonly seen in nature. The results confirmed that infection intensity was higher at high doses of infective stages and that the number of infections were greater when hosts were exposed to slower, lower dose, trickle exposures. However, due to cercarial infection success not varying over the parasite dose levels there was no indication of dose-dependent regulation of infectivity. Hence, any reduction in the numbers of free-living stages by parasite removal through non-hosts are likely to result in lower infection levels in downstream hosts and thus a reduction in disease risk.

Chapter 3 describes laboratory based experiments used to test a wide range of non-host organisms to determine their capability for interfering with trematode parasite transmission via parasite removal. I show that transmission interference is a wide spread phenomenon and is not necessarily restricted to certain taxa or mechanisms.

Chapter 4 investigates the mechanisms driving transmission interference by testing the effect of non-host organism size on parasite removal. Experiments tested five different size categories of three non-host species known to interfere with parasite removal and one alternative host. I conclude that consumer and host size does have a significant effect on parasite removal and thus, the size of consumer and hosts have the potential to alter infection levels in down-stream hosts. Interestingly, many parasite stages removed by the alternative hosts did not infect the host suggesting that hosts may also cause a significant loss of infective stages from the environment.

Chapter 5 addresses the capability of parasite removal by non-host organisms when the density of free-living infective stages within a system increases, potentially leading to a saturation of transmission interference. The additional factor of alternative food was added to the experiments to evaluate its effect on the removal of free-living infective stages. In some species, significant interactive effects of cercarial density and alternative prey on cercarial predation occurred while in others cercarial removal rates were unaffected by both factors.

Chapter 6 investigates the effect of temperature on parasite removal by non-host organisms. Both the emergence and infection success of free-living trematode cercariae has previously shown to be positively correlated with an increase in temperature, potentially resulting in an increase in disease risk with global climate change. However, increasing temperature will also increase the metabolism and feeding rates of non-host organisms known to remove infective stages, potentially buffering the effects of increased parasite emergence with increased temperature. This chapter used laboratory based experiments and three non-host organisms to assess the effects of temperature on both infection success and transmission interference. Our results suggest that some non-host organisms may play an important role in mediating the effects of disease risk under climate change scenarios.

Chapter 7 looks at the effect of non-host diversity on parasite removal. A large, full factorial response surface experiment consisting of multiple non-host species at different densities examined whether an increase in diversity leads to a reduction in disease risk. I show that the effects non-host species on cercarial removal is specific to both the non-host organisms present as well as their density. Depending on the combination of non-host species and their densities, parasite removal was either

neutralised, amplified or reduced and thus the results contradicted the umbrella idea that an increase in biodiversity results a reduction in disease risk.

Chapter 8 uses a virus model system to test for the occurrence of parasite removal effects in a micro-parasite system. In this chapter, multiple inter- and subtidal non-host species were tested for their capabilities in reducing the abundance of infective virus PgV-07T particles. The experiments then focused on the most efficient virus removing organism to assess its ability to remove viral particles over longer periods of time and at consistent viral abundance. The results showed that many different non-host organisms can interfere with virus-host transmission, resulting in a reduction in viral particles. Furthermore, the chapter highlights how rapid reductions in viral particles can occur and that reductions can occur over extended periods of time.

Chapter 9 reviews the potential practical uses of transmission interference as a method of disease control. Climate change is predicted to increase the number of disease incidences which may pose a threat to both wild and commercially valuable organisms. This review focuses on the highly efficient transmission interference filter feeders to assess their potential in mitigating the predicted increase in diseases which is expected with the increase in global temperatures. The review highlights the current commercial value, future possibilities and pitfalls of using filter feeders as a biological cleaning mechanism.

Chapter 10 collates the results of all the experiments and places the fundamental discoveries from this thesis into context with how biodiversity affects disease risk. The occurrence of parasite removal caused by non-host organisms is clearly defined in terms of infection intensity and prevalence and compared with examples from the literature. The mechanisms affecting parasite removal by non-host organisms are discussed and, using existing studies, the chapter goes on to highlight how extensive transmission interference is and culminates in illustrating the contribution this thesis makes to the field of disease ecology. Further, the implications and limitations of the results are discussed with suggestions for the potential to extend on key ideas highlighted this thesis through future research. Finally, I conclude with a statement summarizing why this research is important and how it can contribute to the understanding and management of ecosystems.

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

Caroline Liddell, Jennifer E. Welsh, Jaap van der Meer and David W. Thieltges

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.

Diseases of aquatic organisms (2017) 125:85-92

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 and Poulin 2002). As such, they are capable of substantially affecting host individuals and entire host communities (Mouritsen and 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 intensity of infection in the host, the greater the detrimental impact on host fitness (Anderson and May 1978, May and Anderson 1978, Fredensborg et al. 2004, Thieltges 2006a, 2006b). 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 and 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 intra-specific 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. (2008c) and Nikolaev et al. (2006). A single infected snail sheds between 642 and 672 cercariae d^{-1} (Nikolaev et al. 2006, Thieltges et al. 2008c, 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. 2008c). 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 and 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 and 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 subdoses 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 and 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.

Statistical analyses

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 distribution.

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

$$p = e^{-\theta}$$

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

$$\theta = \mu + \alpha_i + \beta_j + \gamma_{ij}$$

where μ is the intercept, α is the effect of dose, β is the effect of exposure frequency, and γ is their inter- action. 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 (Fig. 2.1). 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 X^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; R Studio Team 2014).

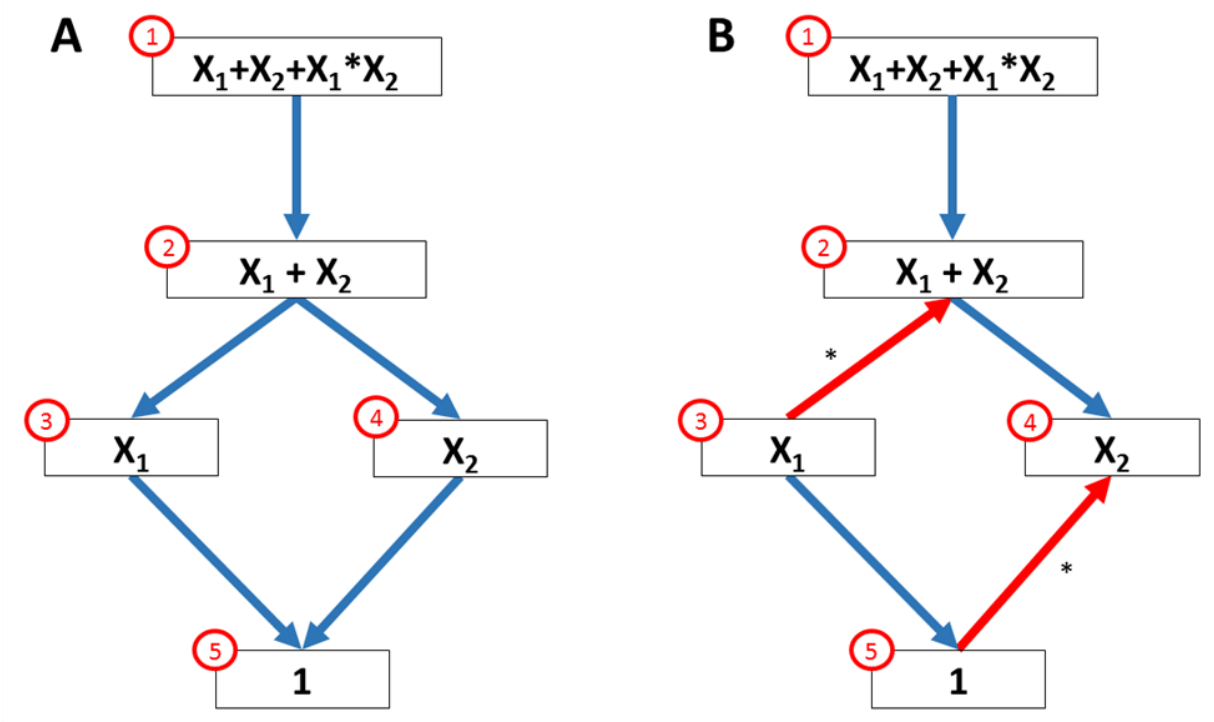


Fig. 2.1. (A) Schematic representation of the model selection procedure based on the best fitting model. Numbers in red circles represent model number from the most complex (1) to the least complex (5) model. X indicates explanatory variables with X_1 representing dose and X_2 representing exposure frequency. Blue arrows indicate which model was tested with which. The testing procedure started with testing the most complex model to the next, less complex model, and so on (i.e., model 1 was tested against model 2, model 2 against model 3 and 4, model 3 against model 5 and model 4 against model 5). When a significant difference between two models occurred it was not necessary to continue (as indicated by the red arrows in B) with a reversed direction). (B) Actual model selection procedure for the effect of dose and exposure frequency on trematode infection intensity in mussels. Significant differences (indicated by red arrows; * denoting a significance level of 0.05) occurred between model 2 and 3, as well as model 4 and 5; therefore, model 2, which included both dose and exposure duration (but no interaction), was significantly different from model 3, which only included dose. Model 4, which included exposure duration only, was significantly different from the null model (model 5). This concludes that model 4 was the best fitting, and thus, exposure duration (single vs. trickle) had a significant effect on the trematode infection intensity in mussels.

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. 2.2).

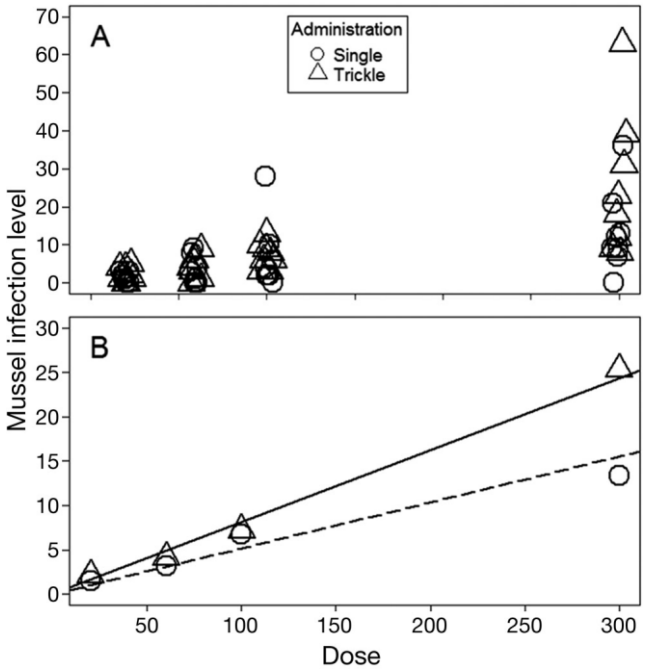


Fig. 2.2 Infection intensity in blue mussels *Mytilus edulis* (number of metacercariae recovered from 2 mussels) across the 4 cercarial doses (20, 60, 100, 300 cercariae of *Himasthla elongata*) administered for both single (circles) and trickle (triangles) exposure frequencies. (A) Individual results 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).

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 2.1 and 2.2; 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. 2.3).

Table 2.1. 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 2.2. Results of all model comparisons (i.e. model 1 vs. model 2, model 2 vs. model 3, etc.). The difference in de- viance (Δ Deviance) divided by the dispersion factor (Φ), which equalled 6.027, is compared to a X^2 distribution with Δ df degrees of freedom. For more details, see the 'Materials and methods'. For model descriptions, see Table 2.1. *$p < 0.05$.

Model Comparison	Δ Deviance	Δ df	Δ Dev/ Φ	Pr ($<X^2$)
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*

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 success of cercariae compared to single exposure.

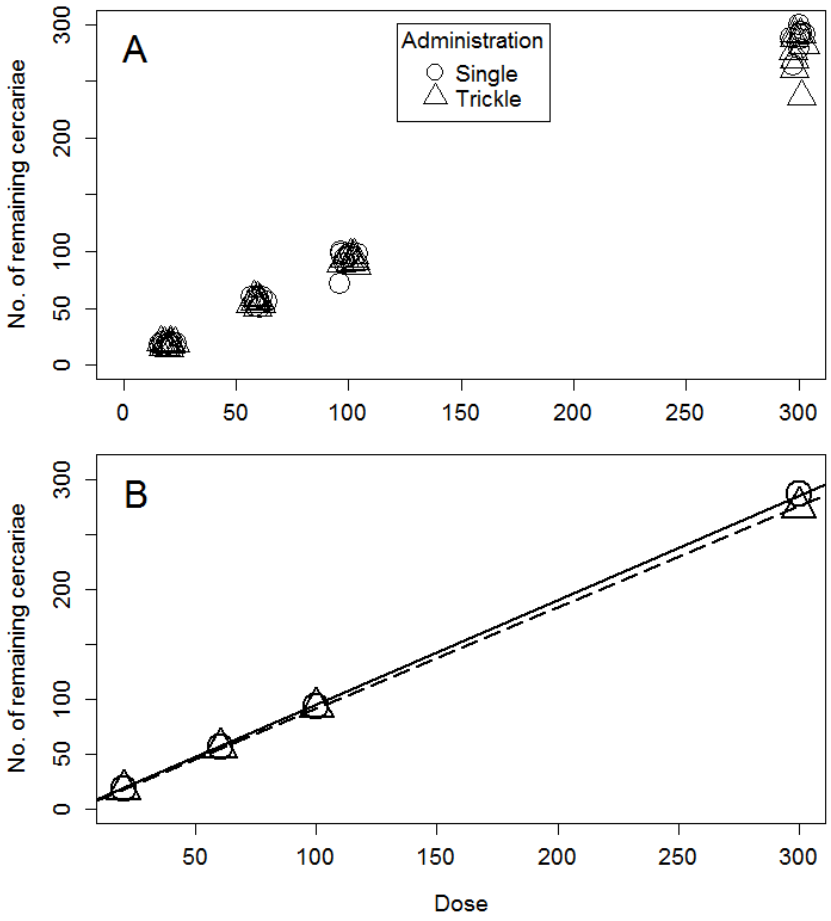


Fig. 2.3. Relationship between cercarial dose and the number of remaining cercariae as a measure of infection success (infection success increases with decreasing proportion of remaining cercariae). (A) shows the individual results from each replicate and (B) the means for both administration treatments at the various doses. Regression lines are forced through the origin as at dose 0 (no parasites) infections are not possible.

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 meta- cercariae 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 varying

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 intra-specific 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 and Marcogliese 2003) or biotic interactions such as consumption of cercariae by ambient organisms (Thieltges et al. 2008a, 2008b, Welsh et al. 2014). However, more studies are needed to confirm whether this is a general pattern in second inter- mediate 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 and 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 and 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 ex- posed 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 under- standing 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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Be careful with water !
Voorzichtig met water !
NEW
Himasthla
Autumn 2011
NEW
Cryptocot.
NEW
Himasthla
Autumn '11
Cryptocotyk
Summer 2012
Summer 2012
Himasthla

Inventory of organisms interfering with transmission of a marine trematode

Jennifer E. Welsh, Jaap van der Meer, Corina P. D. Brussaard and David W. Thieltges

Abstract

It has increasingly been recognized that organisms can interfere with parasitic free-living stages, preventing them from infecting their specified host and thus reducing infection levels. This common phenomenon in freshwater and terrestrial systems has been termed the 'dilution effect' and, so far, is poorly studied in marine systems. Ten common intertidal organisms found in the Dutch Wadden Sea (North Sea) were tested to establish their effects on the free-living cercarial stages of the trematode parasite *Himasthla elongata*. Most species tested resulted in a significant reduction in cercariae over a 3 hr time period. The amphipod *Gammarus marinus* removed 100% of the cercariae, while other effective diluters were *Crangon crangon* (93%), *Sargassum muticum* (87%), *Semibalanus balanoides* (71%), *Crassostrea gigas* (67%), *Hemigrapsus takanoi* (54%), *Crassostrea gigas* shells (44%) and *Idotea balthica* (24%). In contrast, mixed shells (*Cerastoderma edule*, *Mytilus edulis*, *Ensis americanus* and *Littorina littorea*) and *Fucus versiculosus* had no significant effect. These results suggest that dilution effects are widespread in the trematode of *H. elongata*, with potentially strong effects on its population dynamics.

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Introduction

Parasites are ubiquitous in marine ecosystems and can have effects on individual hosts, host populations, communities and entire ecosystems (Mouritsen and Poulin, 2002). For example, they can manipulate the behaviour of their host (Swennen, 1969; Edelaar et al., 2003; Thomas et al., 2005; Bates et al., 2011), reduce fecundity and reproduction (Lafferty, 1993; Mouritsen and Poulin, 2002; Fredensborg et al., 2005) and contribute to mortality (Jensen and Mouritsen, 1992; Mouritsen and Jensen, 1997), which can subsequently alter competition and predation interactions among hosts. Furthermore, parasites can play a vital role in marine ecosystems by significantly contributing to biomass (Kuris et al., 2008), as well as affecting the topology and stability of food webs (Lafferty et al., 2008; Dunne et al., 2013). However, more recently it has been shown that parasites can also act as a resource themselves; thus, local community composition of consumers of parasites can strongly influence parasite dynamics by interfering with transmission pathways (Johnson et al., 2010). This interference, which removes parasites from the system and prevents successful host infection, has been termed the 'dilution effect', and can lead to a reduction in disease risk (Keesing et al., 2006; Johnson and Thieltges, 2010).

Dilution effects on macroparasite transmission are widespread and have been particularly well studied in trematode parasites from freshwater ecosystems (for review see Thieltges et al., 2008b). Trematodes have complex lifecycles, with vertebrates being used as definitive hosts. Miracidia, a free-living stage released from the definitive host, infect the first intermediate host (a mollusc) from which a second free-living stage (termed cercariae) is released. These cercariae have a short lifespan (usually <1 d) and infect a second intermediate host (invertebrates or fish, depending on the parasite species). When the second intermediate host is consumed by a definitive host the cycle is closed allowing the parasite to sexually reproduce and for the cycle to start again (Galaktionov and Dobrovolskij, 2013). Recent studies have shown that the free-living stages of trematodes are also subjected to dilution effects in marine ecosystems (Mouritsen and Poulin, 2003; Hopper et al., 2008; Thieltges et al., 2008a, 2008b; Kaplan et al., 2009; Prinz et al., 2009; Studer et al., 2013). A particularly strong factor seems to be predation by non-host species, either through active or passive predation. For example, it is postulated that some marine predators such as shrimps, may actively seek and consume free-living stages, whilst others, such as filter feeders, may not selectively but accidentally ingest cercariae. Another mechanism can be physical obstacles, e.g. algae, which may obstruct the cercariae from reaching the host. These examples illustrate how ambient fauna and flora may interfere with free-

living trematode cercariae, thus removing infective stages from the system and reducing the disease risk for down-stream hosts in marine ecosystems.

For most marine parasite species and localities we lack an inventory of potential diluting organisms, making it difficult to evaluate the generality of dilution effects. This is also true for trematodes in the Wadden Sea, an extensive area of marine-to-estuarine intertidal mudflats and sub-tidal gullies along the Danish, German and Dutch coast, dominated by benthic molluscs and polychaetes (van der Graaf et al. 2009). The high production of invertebrates is used by a plethora of fish and birds, making it an ideal habitat for trematodes due to the presence of the necessary sequential hosts (Thieltges et al., 2012). Dilution effects have only been studied in one of the dominant local trematodes, *Himasthla elongata*. This trematode uses the periwinkle *Littorina littorea* as first and mussels and cockles as second intermediate and birds as definitive hosts (Werding, 1969). In a field experiment also conducted in the Wadden Sea, dilution effects by Pacific oysters (*Crassostrea gigas*) could be observed which reduced infection levels of *H. elongata* in blue mussels (*Mytilus edulis*) more than three-fold (Thieltges et al., 2009). Laboratory experiments conducted at the Limfjord, a large brackish water system in the north of Denmark, suggest that other species also interfere with transmission of cercariae of *H. elongata*, namely the filter-feeding gastropod *Crepidula fornicata*, the crustacean predators *Crangon crangon* and *Carcinus maenas* and the bivalve *Mya arenaria* (Thieltges et al., 2008a). However, it remains unclear if the experimental results from the study in the Limfjord can be transferred to different ecosystems, and what other species might cause dilution effects in this trematode species.

Here, our aim is to experimentally test the dilution potential of various common species from the Wadden Sea with regard to *Himasthla elongata* cercariae. In addition, from our results and other examples from the literature, we compile an extended inventory of diluters of *H. elongata* in different marine ecosystems.

Materials and Methods

Selection of diluters

A range of potential diluters (predators, filter feeders, live biotic obstacles and dead biotic obstacles) were collected from the Wadden Sea, along the eastern coast of Texel, The Netherlands (Table 3.1). The organisms were chosen due to their observed presence and abundance in intertidal areas of the Wadden Sea where all *Himasthla elongata* hosts can be found (primarily on hard bottom structures like dykes, mussel

and oyster beds). Densities used in the experiments were kept at levels observed in the field. Upon returning to the laboratory, all organisms and physical objects were cleaned and any epibionts carefully removed. The organisms were then stored in tanks (60 × 30 × 30 cm) filled with filtered and aerated seawater within a climate controlled room at 15°C (based on air temperatures at time of collection).

Table 3.1. Organisms used in the different treatments, their expected dilution mechanism and densities or fresh weights used in the experiments 1.7 l tanks containing 1.5 l filtered seawater.

Treatment	Mechanism	Density/wet weight/volume
Sargassum muticum	Live biotic obstacle	25 g (+1 g)
Fucus vesiculosus	Live obstacle	25 g (+1 g)
Crassostrea gigas shells	Dead biotic obstacle	40 g (+10 g)
Mixed shells (*Cerastoderma edule, Mytilus edulis, Ensis americanus, Littorina littorea*)	Dead biotic obstacle	85 g (+1 g)
Gammarus marinus	Predator	3 (10 mm each)
Idotea balthica	Predator	4 (12 mm each)
Crangon crangon	Predator	6 (30 mm each)
Hemigrapsus takanoi	Predator	3 (30 mm each)
Semibalanus balanoides	Filter feeder	2.5 cm^2
Crassostrea gigas	Filter feeder	(40 ml +10 ml vol. each)

Source of cercariae

Periwinkles (*Littorina littorea*) collected from the field were screened for the presence of *H. elongata* infections by keeping them in an incubator at 25°C for several hours and checking for shed cercariae. Infected snails were then separated and kept in aquaria. To obtain cercariae for the experiments, 150–200 infected snails were incubated in 1.8 l of filtered seawater at 27°C and under light for 3 h to encourage the release of cercariae. The water and cercariae (hereafter termed 'broth') was drained into a 2 l beaker. The broth was then gently stirred (anticlockwise three times and then clockwise three times using a plastic spoon), and immediately after 50 ml of broth was scooped out of the beaker using a small measuring jug and added to experimental containers (1.7 l), resulting in a uniform infection dose. Eight samples of 50 ml were also taken to get an average of the number of parasites added to each container.

Experimental set-up

Five experiments were carried out, each testing two different diluters versus a control. For each experiment eight replicates were used for each of the three treatments. Each replicate consisted of a 1.7 l container with 1.5 l of filtered seawater randomly placed in a single climate controlled room at 18.5°C (+0.2°C), a typical water temperature which occurs during the summer transmission period. All organisms were starved and kept in the experimental containers for 24 h prior to adding the cercariae for acclimation. At the start of the experiment, 50 ml of cercariae broth was added to each container (see above). After 3 h large dilutors were quickly removed by forceps and discarded; smaller dilutors were fixed along with the cercariae.

Experiments were run for 3 h as survival of cercariae usually starts to decrease after about 10 h (Thieltges and Rick, 2006), to avoid the decrease in cercarial survival confounding the effects of diluters (maximum age of cercariae in our experiments was 6 h, including the 3 h incubation period). At the end of the experiment, all water from the containers was filtered through a 25 mm sieve to retain any remaining cercariae. Containers were then rinsed and filtered twice. Subsequently, cercariae were washed from the sieve with 50–100 ml filtered seawater and fixed using 10 ml 96% ethanol and stained using rose Bengal. Cercariae were then counted in Petri dishes under a light microscope.

Statistical analyses

The effect of a potential diluter versus the respective control was tested using an ANOVA followed by Dunnett's family error post-hoc test. All ANOVAs were conducted on raw, untransformed data as all experiments proved to be normally distributed after checking for normality and homoscedasticity. Finally, the percentage of cercariae removed by each potential diluter (versus the mean of the respective control) was calculated. All results shown are means ± standard error.

Results

Overall, most species tested resulted in a reduction of cercariae of more than 40% (Fig. 3.1; Table 3.2). The grazer *G. marinus*, the predator *C. crangon* and the live biotic obstacles *S. muticum* removed the most amount of cercariae (87–100%). Other species, *H. takanoi*, *C. gigas* shells, *C. gigas, S. balanoides* and *I. balthica*, removed between 24% and 71% (Fig. 3.1; Table 3.2). However, *F. vesiculosus* and mixed bivalve shells did not show a significant reduction (Fig. 3.1; Table 3.2).

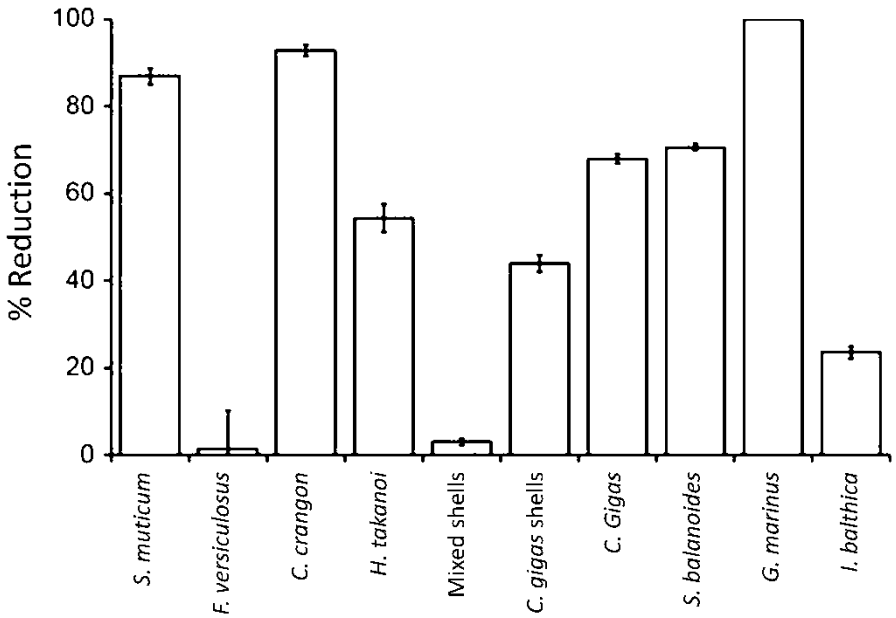

Fig. 3.1. Percentage (+standard error) of cercariae of *Himathla elongata* removed from the experimental containers by the different potential diluters compared to the relevant control.

The average number of cercariae recovered from controls decreased with each experiment (Table 3.2). Whilst an average of 116 cercariae were recovered in the controls of the first experiment (testing *S. muticum* and *F. versiculosus*), an average of only 11 was observed in the final experiment (which tested *G. marinus* and *I. balthica*). This reduction was also shown in the sub-samples (decreasing from 128 ± 6 to 38 ± 4, 23 ± 2, 17 ± 2 and 14 ± 1, respectively) and was presumably due to depletion of the cercarial production by the pool of snails during the repeated shedding procedures. However, as we only compare the respective controls with the treatments within each experiment, such a reduction in production does not affect the overall results.

3.2. Mean number of cercariae (± standard error) recovered from tanks containing different potential diluting organisms in five different experiments. Controls contained no diluting organism. Also shown are the P values from Dunnett's family error post-hoc tests, which compared controls with the respective treatments and the overall ANOVA results for each experiment; N = 8 replicates per treatment. Degrees of freedom for all F values = 23. SE, standard error; P, significance level of test.

Experiment	Treatment	Mean No. of cercariae			SE	P
1	Control	115.75			20.56	–
	S. muticum	15.25			1.89	<0.001
	F. vesiculosus	114.13			8.77	0.944
			ANOVA:	F = 19.75		<0.001
2	Control	31.13			2.75	–
	C. crangon	2.25			1.24	<0.001
	H. takanoi	14.25			3.27	0.002
			ANOVA:	F = 31.91		<0.001
3	Control	21.38			1.03	–
	C. gigas shells	12.00			0.84	0.005
	Mixed shells (*C. edule, M. edulis, E. americanus* and *L. littorea*)	20.75			1.93	0.647
			ANOVA:	F = 15.02		<0.001
4	Control	14.00			1.39	–
	C. gigas	4.50			1.05	<0.001
	S. balanoides	4.13			0.72	<0.001
			ANOVA:	F = 26.46		<0.001
5	Control	10.63			1.85	–
	G. marinus	0.00			0.00	<0.001
	I. balthica	8.13			1.38	<0.001
			ANOVA:	F = 17.35		<0.001

Discussion

The experiments showed that most of the potential diluters tested significantly reduced the number of cercariae, with all significant diluters resulting in a >24% reduction. Only two of the 10 tested organisms did not show any dilution effect. This suggests that dilution effects on the trematode *H. elongata* are widespread, with the potential to cause strong effects on the parasite's population dynamics.

The results shown here are in line with previous findings from a study conducted in the brackish Limfjord in Denmark, where the shrimp *Crangon crangon* and the bivalve *Crassotrea gigas* also strongly reduced the numbers of cercariae of *H. elongata* (Thieltges et al., 2008a; Table 3.3). This suggests that dilution effects on this trematode species may be more widespread than shown previously and are not confined to specific habitats or ecosystems. The Limfjord study also found additional diluters not tested in this study (Table 3.3).

Table 3.3. Organisms shown to reduce free living Himasthla elongata cercariae in the Limfjord, Denmark (Thieltges et al., 2008a, 2009) and in the Wadden Sea (this study); ns, no significant effect.

Taxon	Species	Reduction (%) of infections or cercariae	
		Limfjord	Wadden Sea (this study)
Amphipod	*Gammarus marinus*	Not tested	100%
Barnacle	*Semibalanus balanoides*	Not tested	71%
Bivalve	*Crassostrea gigas*	95–99%	67%
Bivalve	*Macoma balthica*	ns	Not tested
Bivalve	*Mya arenaria*	64%	Not tested
Decapod	*Carcinus maenas*	65%	Not tested
Decapod	*Crangon crangon*	78%	93%
Decapod	*Hemigrapsus takanoi*	Not tested	54%
Gastropod	*Crepidula fornicata*	93–99%	Not tested
Isopod	*Idothea balthica*	Not tested	24%
Macroalgae	*Fucus vesiculosus*	Not tested	ns
Macroalgae	*Sargassum muticum*	Not tested	87%
Shells	Mixed (non-oyster) shells	Not tested	ns
Shells	Oyster shells	Not tested	44%

Our study now adds several new diluting species, including filter feeders and dead and live biotic obstacles, to the list of known diluters of *H. elongata*. While filter feeder ingest cercariae via their filtration current, the effect of biotic obstacles is different. The debris from organisms such as shells probably act as a physical barrier: cercariae become entangled in the structure preventing the free-living parasite stages from getting to their hosts within their short lifespan. The convoluted structure of oyster

shells may explain why dilution effects were observed in the oysters shell (44%) but not in the mixed shell treatment (consisting of species with much smoother shell surfaces). Similarly, differences in structural complexity probably also explain the observed differences between the two algae species (see detailed discussion below). However, other mechanisms such as olfactorial cues or other exudates cannot completely be ruled out and deserve further study.

The resulting inventory of diluters shows that a wide range of organisms interferes with the transmission of *H. elongata* cercariae (Table 3.3). In general, we observed relatively high dilution rates and it can be questioned whether this reflects actual dilution rates under natural conditions in the field. In addition to the biotic dilution effect there are also various abiotic factors (e.g. temperature and salinity) known to affect the transmission stages of trematodes (Pietrock and Marcogliese, 2003; Thieltges and Rick, 2006; Studer and Poulin, 2013). This suggests that the observed dilution effects may actually be simply compensatory rather than additive, since mortality of cercariae is very high in the field anyway. However, there is evidence from a field experiment that dilution effects can be additive and comparably high in more natural settings: treatments with Pacific oysters showed a more than three-fold reduction in infection levels of target hosts with *H. elongata* compared to controls without oysters, despite the presumed high natural background mortalities of cercariae (Thieltges et al., 2009).

The interference caused by diluters on cercarial transmission may potentially have strong effects on trematode population dynamics, and thus, have consequences for the hosts. Metacercarial infections (resulting from penetrating cercariae) of trematodes have been shown to have a range of detrimental effects on their invertebrate intermediate hosts, including reducing survival and growth (Jensen et al., 1998; Desclaux et al., 2004; Fredensborg et al., 2005; Thieltges, 2006). Since such effects are usually density-dependent (Fredensborg et al., 2005; Thieltges, 2006), any reductions of parasite loads will relieve the hosts from the negative effects of infections. Here we looked at single species effects on dilution rates. However, in natural settings, cercariae will encounter many different identities and densities of diluters with the potential for a multitude of compensatory, additive or synergistic effects on dilution rates resulting in an interesting avenue for further research.

In general, the strength of the dilution effect seems to depend more on the diluter identity than on the respective dilution mechanism. Direct comparison among different diluters and mechanisms is difficult because the strength of the dilution effect is related to the density of diluters (Thieltges et al., 2009). However, for two diluters a direct comparison is possible, since the same weight was used for the two algae

species, *S. muticum* and *F. vesiculosus*. While *S. muticum* significantly reduced cercariae by 87%, *F. vesiculosus* did not. The difference between the two species probably results from the fact that *S. muticum* has a very fine branching habitus, leading to the entanglement of cercariae, while *F. vesiculosus* has broad blades which probably do not trap cercariae. Interestingly, *S. muticum* is classed as an invasive species in the Wadden Sea, and this draws attention to potential 'positive' impacts that non-native organisms may have on their new habitat (Buschbaum et al., 2006; Thieltges et al., 2006). Here, it was shown that *S. muticum* may alleviate native host organisms from parasites more than the native *F. vesiculosus* which is found to grow in the same locality. Dilution effects on cercariae of *H. elongata* were also caused by other invasive species, e.g. the Pacific oyster *Crassostrea gigas* and the American slipper limpet *Crepidula fornicata* (this study; Thieltges et al., 2009). Similar effects have also been observed in other trematode species. For example, the invasive barnacle *Austrominius modestus* interferes with the transmission of cercariae of *Echinostephilla patellae* and *Parorchis acanthus* (Prinz et al., 2009). Likewise, the invasive algae *Caulerpa taxifolia* interfere with the transmission of various trematode species, presumably due to toxic exudates (Bartoli and Boudouresque, 1997). These examples point to the potential importance of invasive species in mediating parasite–host interactions with potential 'positive' effects on native hosts in contrast to the usually perceived 'negative' effects (Harvell et al., 2004; Kopp and Jokela, 2007; Kelly et al., 2009; Keesing et al., 2010).

In conclusion, the experiments in this study have produced an extended inventory of dilutors, and indicate that there are many non-host species that interfere with the transmission of cercarial stages of *H. elongata*. Together with published data from other marine parasites and systems this suggests that trematode transmission can be interfered with by a multitude of organisms. However, despite increasing evidence of biotic factors interfering with parasite transmission pathways, there is still much that is unknown about how and under what conditions organisms remove the most parasites. Therefore, the future challenge is to determine the mechanisms which result in a reduction in disease risk caused by diluting organisms and to understand the effects of whole communities on pathogen transmission pathways.

Acknowledgements

We would like to thank Hans Witte, Siem Gieles, Rob Dekker, Anouk Goedknegt and Andreas Wasser for their assistance with collecting the organisms. We also thank the two referees for their comments.


Snails with
„Renicula roscovita“
Snails with
„Himasthia elongata“
Snails with
„Cryptocotyle lingua“

Consumer and host body size effects on the removal of trematode cercariae by ambient communities

Jennifer E. Welsh, Anke Hempel, Mirjana Markovic, Jaap van der Meer and David W. Thieltges

Abstract

Parasite transmission can be altered via the removal of parasites by the ambient communities in which parasite–host interactions take place. However, the mechanisms driving parasite removal remain poorly understood. Using marine trematode cercariae as a model system, we investigated the effects of consumer and host body size on parasite removal rates. Laboratory experiments revealed that consumer or host body size significantly affected cercarial removal rates in crabs, oysters and cockles but not in shrimps. In general, cercarial removal rates increased with consumer (crabs and oysters) and host (cockles) body size. For the filter feeding oysters and cockles, the effects probably relate to their feeding activity which is known to correlate with bivalve size. Low infection levels found in cockle hosts suggest that parasite removal by hosts also leads to significant mortality of infective stages. The size effects of crab and shrimp predators on cercarial removal rates were more complex and did not show an expected size match-mismatch between predators and their cercarial prey, suggesting that parasite removal rates in predators are species-specific. We conclude that to have a comprehensive understanding of parasite removal by ambient communities, more research into the various mechanisms of cercarial removal is required.

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Introduction

Parasite transmission between hosts can be significantly altered by the ambient communities in which parasite–host interactions take place. The associated change in disease risk for hosts in a given environment can result from indirect mechanisms, e.g. via competitors or predators affecting host densities (Keesing et al., 2006; Johnson and Thieltges, 2010), but can also stem from direct mechanisms in form of the removal of free-living infectious stages by other organisms (Thieltges et al., 2008a; Johnson and Thieltges, 2010). In the latter case, consumption of parasites by non-hosts living in the vicinity of target hosts has been identified to lead to significant reduction in infectious stages which subsequently leads to lower infection levels and disease risk for hosts (Johnson et al., 2010). For example, consumption of cercarial stages of trematodes by various aquatic invertebrates and juvenile fish has repeatedly shown to reduce cercarial density and lower infection levels in target hosts (e.g. Thieltges et al., 2008b; Kaplan et al., 2009; Orlofske et al., 2012; Welsh et al., 2014; Mironova et al., 2019). The removal of cercariae and other infective stages of parasites is not limited to specific feeding types but occurs in pursuit and ambush predators as well as in filter and deposit feeders (Thieltges et al., 2008a; Johnson et al., 2010). Similarly, parasite removal can also be caused by hosts, either in form of conspecifics or by susceptible alternative host species. In both cases, additional hosts can become infected by infectious stages and thereby remove infective stages from the local infection pool and reduce average infection intensity of individual target hosts (Thieltges and Reise, 2007; Orlofske et al., 2012; Magalhães et al., 2017). It is likely that both forms of removal of infectious stages are very common in natural systems and thus understanding the mechanisms driving the magnitude of parasite removal are important for our understanding of the multiple effects of ambient communities on disease risk.

One of the factors likely to affect parasite removal rates is the body size of both consumers and hosts of infective stages. The importance of consumer size for resource consumption is well known from predator–prey interactions where it is strongly linked to both prey and predator population dynamics (Caswell, 1989; Fryxell and Lunberg, 1998; Beaugrand et al., 2003). Predators usually target prey of specific sizes with larger predators generally consuming larger prey and smaller predators consuming smaller prey (Brose et al., 2006; Costa, 2009). The preference for specific prey size classes can result in a match-mismatch between a predator and its prey if prey items are either too small or too large (e.g. Neill, 1975; Nilsson and Bronmark, 2000; Strasser, 2002). Such a match-mismatch can also be expected to occur in the case of predation upon infective stages of parasites. Indeed, a negative relationship

between predator body size and parasite removal has been observed in invertebrate predators (damselfly nymphs and dragonfly larvae) and vertebrate (juvenile vs. adult mosquitofish) of trematode cercarial stages in freshwater systems (Orlofkse et al., 2015; Catania et al., 2016). Further studies, also from different ecosystems, would be helpful to evaluate the generality of the effects of predator body size on parasite removal. Secondary to the size match-mismatch phenomenon, consumer-size may also determine the per capita removal rates of parasites, with larger consumers removing more infective stages than smaller ones. This may be particularly true for some known parasite consumers such as filter feeders. For example, the filtration rate of bivalve filter feeders increases as filter feeders grow (Møhlenberg and Riisgård, 1979; Jones et al., 1992; Gosling, 2003). Consequently, the number of parasites of a given size range removed (the clearance rate) can potentially increase with an increase in the size of the filter feeder. Finally, similar to consumers, the size of alternative hosts is likely to affect parasite removal rates as host body size is generally positively correlated with parasite infection levels (Poulin, 2011). Hence, larger hosts may remove more infective stages than smaller hosts. To date, neither the effects of filter feeder or alternative host body size on parasite removal rates have been investigated.

In this study, we investigated the effects of consumer and host body size on parasite removal using cercariae of a marine trematode as a model system. The echinostome trematode *Himasthla elongata* (body length: 605–665 μm; tail length: 535–605 μm; Werding, 1969) is found in marine intertidal systems around Europe and has a complex life cycle with birds serving as definitive hosts and periwinkles (*Littorina littorea*) as first intermediate hosts (de Montaudouin et al., 2009). The cercarial stages released from the periwinkles infect bivalves such as the common cockle *Cerastoderma edule* as second intermediate host (Thieltges and Reise, 2007; de Montaudouin et al., 2009). Several intertidal non-host organisms (organisms which are not infected by *H. elongata*) have been shown to remove the cercariae of *H. elongata* and subsequently reduce infection levels in bivalve target hosts (Thieltges et al., 2008a; Welsh et al., 2014). Among those are predatory brown shrimps *Crangon crangon* and shore crabs *Carcinas maenas* and filter feeding Pacific oysters *Magallana gigas* (also known as *Crassostrea gigas*) which do not become infected by the parasite. We used mesocosm experiments to investigate the removal rates of these consumers (crabs, shrimps, oysters) and hosts (cockles) depending on their body size to identify potential size match-mismatches and to quantify whether larger individuals remove more cercariae than smaller individuals. With this experimental approach investigating several different parasite

removal mechanisms, we aim to advance our understanding of the phenomenon of parasite removal and its effects on disease risk.

Materials and methods

Source of consumers and hosts

Shore crabs (*Carcinus maenas*), brown shrimps (*Crangon crangon*), common cockles (*Cerastoderma edule*) and Pacific oysters (*Magallana gigas*) of various sizes were collected from the intertidal area along the eastern coast of the island of Texel (Wadden Sea, The Netherlands). Cockles (known to serve as intermediate hosts for *Himasthla elongata*) were collected from an intertidal sand flat north of Texel where *H. elongata* infections are known to be low (confirmed by dissecting 50 cockles: 3 infected individuals with <2 metacercariae per host). The other three species do not serve as hosts for *H. elongata* (Thieltges et al., 2006) and were therefore not dissected. After collection, all epibionts, if present, were gently removed and all four species were kept in tanks containing filtered and aerated seawater within a climate-controlled room (15 °C) at the NIOZ Royal Netherlands Institute for Sea Research (Texel, The Netherlands).

Source of cercariae

Periwinkles (*Littorina littorea*) collected from the intertidal area around the island of Texel were screened for the presence of *H. elongata* infections by checking for the release of cercariae under increased temperature treatments (for details see Welsh et al., 2014). Infected periwinkles were stored in flow through aquaria at 15 °C and regularly fed sea lettuce (*Ulva lactuca*). To obtain cercariae for the experiments, 150–200 infected snails were incubated in 1800 mL of filtered seawater at 27 °C under light for 3 h to encourage the release of cercariae. From this concentrated cercariae solution, 50 *H. elongata* cercariae (40 in the crab experiment) were pipetted under a stereo microscope into small 100 mL plastic containers within 1.5 h and then added to the experimental units (thus a maximum cercariae age of 4.5 h).

Experimental setup

The effect of consumer and host body size on removal rates of cercariae was investigated in laboratory experiments by determining removal rates of five size

categories of each species (Table 4.1). In addition, a sixth treatment without consumers or hosts served as a control to account for potential losses of cercariae due to other factors (knowing the number of added cercariae was 40 or 50) and to test for the general presence of a cercarial reduction effect (control vs. species addition treatments). The experiments were conducted in four separate runs, with each run testing removal rates of a single species (one individual per replicate, 6 replicates per treatment level, Table 4.1). Each replicate consisted of a 2 L aquarium filled with 1500 mL of filtered seawater and randomly placed in a single climate-controlled room (18.5 °C ± 0.2 °C).

Table 4.1. Size ranges (cm) of the five size categories (A–E) used in the experiments to determine the effect of a consumer or host body size on the removal of cercariae of *Himasthla elongata*. Size measurements based on carapace width for shore crabs (*Carcinus maenas*), carapace length for brown shrimps (*Crangon crangon*), shell length for common cockles (*Cerastoderma edule*) and maximum shell diameter (accounting for the usually irregular shape) for Pacific oysters (*Magallana gigas*; also known as *Crassostrea gigas*).

Species	**Size categories (cm)**				
	A	**B**	**C**	**D**	**E**
Crab	0.4–0.9	1.6–2.2	3.2–4.0	4.3–5.4	6.1–6.5
Shrimp	1.5–1.9	3.2–3.5	4.1–4.5	5.1–5.5	6.0–6.5
Oyster	4.0–5.0	7.1–8.0	10.0–11.0	13.0–14.0	16.0–17.0
Cockle	1.6–1.8	1.6–1.8	1.6–1.8	1.6–1.8	1.6–1.8

All consumers and hosts were starved and kept in the experimental aquaria for 24 h prior to the experiments to allow for acclimation. After this acclimation period, 40 or 50 (for crab treatments and all other species, respectively) cercariae were added to each replicate aquaria and left undisturbed for 3 h. This time period ensured full swimming ability of cercariae for the whole experimental period, which is known to slowly decrease after about 8 h (Thieltges and Rick, 2006; Studer and Poulin, 2013). At termination, the test organisms were quickly removed from the aquaria using long forceps and the water from the aquaria was sieved using a 25 μm sieve. The retained cercariae were backwashed into individual 100 mL pots which contained 10 mL of 99% ethanol for fixation and 0.5 mL Rose Bengal for staining. Cercariae were later enumerated under a light stereomicroscope. In addition, 24 h after the experiment ended all cockles were dissected under a light microscope and metacercariae counted to determine infection intensity. This allowed for the determination of actual cercarial removal from cercarial loss due to infections.

Statistical analyses

The effect of presence/absence and of the size of cercarial consumers and hosts was analysed using three binomial Generalized Linear Models with log-links. Including a log-link assumed a linear pure death process, i.e. all predatory incidences were considered to be independent events (see Liddell et al., 2017 for further details).

The first model (1) included all factor levels for each consumer or host body size class, the second model (2) included only two levels, the control vs. consumer or host presence and the third model (3) only included a constant, thus assuming that the control and all size class treatments show the same removal rate. Comparing model 1 and 2 allowed to test whether consumer or host body size had a significant effect on the removal of cercariae. Comparing models 2 and 3 allowed to test whether there was an overall effect of consumer or host presence. Model comparisons were done using analysis of deviance. The difference in deviance between two models (Δ Dev) was divided by the dispersion factor (ϕ) from the most complete model and then compared with the delta degree of freedom $\chi 2$ at 0.05. Calculations of ϕ were derived by dividing the residual deviance for the most complex model by the degrees of freedom. When two models significantly differed, this indicated that the most complex model had the better fit. From the best fitting models, the clearance rate (L h−1) of each consumer or host was calculated by dividing the instantaneous cercarial removal rates retrieved from the model outputs by the volume of the experimental units (2 L). This was done in an effort to allow for comparisons with literature data on clearance rates.

All analyses were carried out using R (R Development Core Team, 2013) version 3.0.2 in R Studio (version 0.98.1103; R Studio Team, 2014).

Results

Consumer or host body size significantly affected cercarial removal in crabs, oysters and cockles, but it did not affect removal by shrimps (Fig. 4.1; Table 4.2). Similarly, while these three species lead to a significant removal compared with the control, the presence of shrimps had no significant effect on the number of remaining cercariae (Table 4.2). The removal of cercariae by crabs showed an overall increase with an increase in crab size, i.e. the number of cercariae remaining decreased with an increase in crab size class (Fig. 4.1). As such, the clearance rate of crabs increased with crab size (Table 4.3). A similar pattern was seen in the filter-feeding oysters and cockles (the

latter also serving as host for the parasite). In both cases the number of cercariae remaining decreased with an increase in shell length (Fig. 4.1, Table 4.2), hence the clearance rates increased with an increase in oyster and cockle size (Table 4.3).

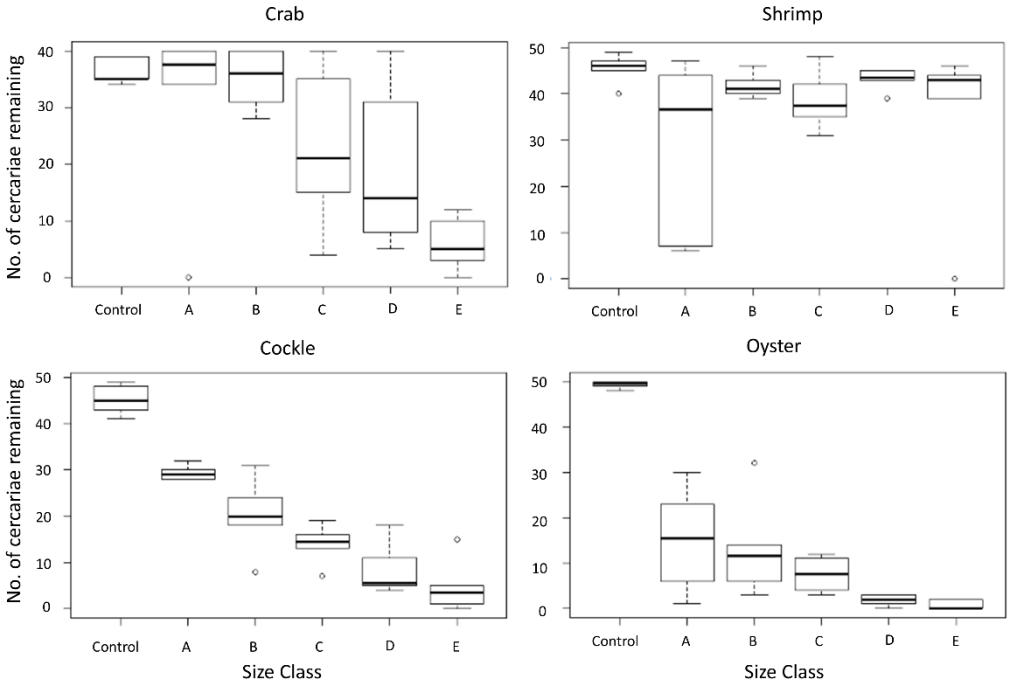

Fig. 4.1. Number of cercariae remaining after 3 h in the presence of consumers (crabs, shrimps, oysters) and hosts (cockles) of different body size categories (A–E) and in absence of any organisms (control). For size classes see Table 4.1, for raw data see Welsh et al. (2018).

Table 4.2. Results of the binomial GLMs for the different consumer and host species showing model deviance for model 1 (including consumer or host body size), model 2 (including only presence-absence of consumer or host) and model 3 (only the constant), the dispersion factor (ϕ) and results of significance tests for effects of predator or host body size (model 1 vs. 2) and predator or host presence (model 2 vs. 3) on cercarial removal.

	Model deviance			Dispersion factor	Significance	
Species	**1**	**2**	**3**	**ϕ**	**Body size (1 vs. 2)**	**Presence (2 vs.3)**
Crab	488.82	844.66	957.85	16.29	***	**
Shrimp	356.93	431.4	472.76	11.9	n.s.	n.s.
Oyster	146.86	326.32	1175	4.9	***	***

Table 4.3. Clearance rates (L h−1) for each consumer or host size category in the three experiments (crabs, oysters, cockles) that showed a significant effect of body size on cercarial removal. Clearance rates were calculated by dividing the instantaneous cercarial removal rates retrieved from the respective model outputs by the volume of the experimental units (2 L).

Species	Size class	Clearance rate (L h^{-1})
Crab	A	0.120
	B	0.065
	C	0.285
	D	0.380
	E	0.965
Oyster	A	0.595
	B	0.675
	C	0.950
	D	1.655
	E	2.16
Cockle	A	0.265
	B	0.455
	C	0.635
	D	0.905
	E	1.185

Table 4.4. Number of infected cockles from a total of 30, prevalence (%) and mean intensity (± S.D.) of cercariae recovered as metacercariae in cockles in each of the five cockle size categories.

Size	Number of infected cockles	Prevalence (%)	Mean intensity
A	3	50.0	2.7 (0.7)
B	4	66.7	1.5 (0.5)
C	1	16.7	1.0
D	1	16.7	2.0
E	0		

Although cockles are known to serve as intermediate host for Himasthla elongata, overall infection levels were unexpectedly low. Prevalence varied between 16.7 and 66.7% among the cockle size classes but infected cockles harboured, on average, only between 1 and 2.7 metacercarial stages (Table 4.4), suggesting that the observed

cercarial loss in the experimental units only marginally resulted from cercariae infecting the cockles.

Discussion

Our series of experiments revealed that consumer and host body size significantly affected cercarial removal in crabs, oysters and cockles but not in shrimps. In general, cercarial removal rates increased with consumer or host body size.

In the two filter feeders, oysters and cockles, this increase in parasite removal rates with oyster or cockle body size relates to the general feeding ecology of the species. Bivalves constantly filter water via their gills and the filtration rate is a function of gill area which is positively correlated with bivalve body size (Møhlenberg and Riisgård, 1979; Jones et al., 1992; Gosling, 2003). However, as the filter feeding mesh of the bivalves' gills is largely independent of body size (Gosling, 2003), prey size selection in bivalve filter feeders is much less affected by organism size compared with predators. Hence, particles that are captured by the filter-feeding mesh of the gills will be filtered at an increasing rate with increasing body size. As cercarial stages of trematodes fall within the size range of particles filtered by bivalves (Gosling, 2003), cercarial removal can also be expected to increase with bivalve body size as observed in our experiments. As the positive relationship between body size and filtration rates are universal in bivalves and possibly also in other filter feeders, measures of filtration capacity such as clearance rates can probably, as long as the size range of the particles captured overlaps with the size of the respective infective stages of parasites, be used as a proxy for the parasite removal capacity of any filter feeding organism. As filtration is often relatively unspecific within the range of particles filtered, a large range of filter feeding organisms such as bivalves may be able to remove infective stages of parasites and may thus play an important role in altering parasite transmission in aquatic ecosystems (Burge et al., 2016).

Although both oysters and cockles significantly removed cercariae from the water, the subsequent fate of the removed cercariae is likely to differ between oysters and cockles. In the case of oysters, the uptake of cercariae of *Himasthla elongata* will not lead to infections as Pacific oysters are not infected with metacercarial stages of the species (Krakau et al., 2006). The cercarial removal capacity of oysters has previously been recognized and has been shown to lead to reduced infection levels in the parasite's target hosts (Thieltges et al., 2009). However, when those studies were conducted, the dependency of cercarial removal on oyster body size was, and

remained, unknown until now. In contrast to the oysters, common cockles do serve as hosts to metacercarial stages of *H. elongata* (Thieltges et al., 2006; de Montaudouin et al., 2009). Hence, in this case, the uptake of cercarial stages by cockles via their filtration can lead to infections. As filtration rates increase with body size, larger cockle hosts will be exposed to larger numbers of infective stages and most likely have higher infection levels. While there is some evidence for a positive relationship between cockle size (or age) and metacercarial infection levels in the literature (de Montaudouin et al., 1998; Jensen et al., 1999; Thieltges, 2008; but see de Montaudouin et al., 2012), cercarial removal only lead to very low infections of cockles in our experiment, with a mean intensity of 1–2.7 metacercariae and no relationship with cockle size. This suggests that the uptake of cercariae by cockles does not necessarily lead to infections but that a large number of cercariae may rather be lost in the course of the filtration, possibly by immobilizing cercariae on the cockles' gills and subsequent digestion. However, the larger infection success reported from previous experiments on cerariae of *H. elongata* suggests that the effect may vary depending on conditions. Wegeberg et al. (1999) reported an infection success of *H. elongata* cercariae of about 60% in cockles ranging from 0.6 to 1.4 cm which is much higher than the 5.4% observed in the smallest size class (1.6 cm) in our experiment. Whether the difference in infection success relates to differences in cockle size classes or ambient conditions (Petri-dishes in Wegeberg et al. (1999) vs. 1.5 L aquaria in our experiments), remains to be studied.

Like oysters and cockles, crabs also showed a positive relation between body size and cercarial removal but this pattern differed from our expectation. We had assumed that a size match – mismatch would occur in crabs, whereby the infective parasite stages would be too large for smaller crabs and too small for larger crabs to remove. However, cercarial stages were removed by all size classes of crab with larger crabs removing more cercariae than smaller crabs. As the range in crab sizes used in our study not only covered the most common sizes found in our study area but also included very small and very large crabs, it is not expected that a size mismatch has been missed. The observed increase in cercarial removal rates with crab size suggests that cercarial removal is possibly not a result of direct predation by crabs (using their claws) but rather a different mechanism. Various crab species have been shown to use their mouth parts to catch small particles, similar to filter feeding in bivalves (Gerlach et al., 1976; Watts, 2014). In addition, small 10 μm polystyrene microspheres have been shown to be taken up by *C. maenas* crabs and retained by their gills which are normally used only for oxygen uptake and not particle filtration (Watts, 2014). It is thus likely, that cercarial removal in shore crabs is based on mechanisms similar to filter feeding in bivalves and indeed we have observed cercariae stained with fluorescent dye in the

digestive tract and on the gills of shore crabs (pers. obs.). Such alternative mechanisms of cercarial removal may explain the different findings in other aquatic predators where a negative relationship between predator body size and parasite removal due to prey size mismatches has been observed (e.g. damselfly nymphs, dragonfly larvae, mosquitofish; Orlofkse et al., 2015; Catania et al., 2016). Our findings thus suggest that predator effects on cercarial removal rates may be more diverse and may also include mechanisms such as removal via mouth parts or gills similar to the filter feeding in bivalves and other filter feeders.

In contrast to shore crabs, the second predator investigated, the brown shrimp, did not significantly remove cercariae. This contradicts with previous studies which have reported cercarial removal by brown shrimps (Thieltges et al., 2008a; Welsh et al., 2014). However, these differences in findings are probably related to differences in the experimental designs used among the studies. Welsh et al. (2014) used 6 shrimps with a length of 3 cm per replicate and found a significant reduction in the number of cercariae by 93%. In contrast, our study only used a single shrimp per replicate and we observed about 20% fewer cercariae in a comparable size class (3.2–3.5 cm). Although cercarial removal was not statistically significant in this study, the results from Welsh et al. (2014) suggest that higher densities of shrimps would have probably led to higher removal rates. In a different previous study, brown shrimps of 1.5–2.5 cm length lead to a reduction in infection levels of cockle hosts by 78% (Thieltges et al., 2008a). However, this study used 10 shrimps per replicate and the observed effect was most likely not only due to cercarial removal by shrimps but also due to interactions of shrimps and cockles leading to disturbances in cockle filtration and subsequently to lower infection levels (Thieltges et al., 2008a). These comparisons show that brown shrimps have the ability to remove cercariae but only at higher shrimp densities. The absence of an effect of shrimp body size shown in this study may further suggest that cercarial removal may be independent of body size in shrimps in general, again differing from the expectation that there should be a sizedependent match-mismatch as observed in other cercarial predators (Orlofkse et al., 2015; Catania et al., 2016). Hence, the effect of predators on cercarial removal may be less predictable than in filter feeders and probably depends strongly on the mechanisms of cercarial removal in a respective predator.

In conclusion, our study shows that consumer and host body size can significantly affect cercarial removal rates and that removal rates generally increased with the body size of consumers (crabs and oysters) and hosts (cockles). In the case of filter feeders

(oysters and cockles), the observed effects probably directly relate to the filter feeding activity and suggest that general measures of filtration capacities, such as clearance rates, may be used as proxies of cercarial removal capabilities. In contrast, size effects on cercarial removal rates by predators were more complex and did not show a consistent size match-mismatch, suggesting cercarial removal rates depend on species-specific mechanisms. Future studies on other host and nonhost organisms would be informative to confirm the generality of our findings. In addition, research on other species of cercariae would be useful to investigate the potentially mediating effects of cercarial size and behaviour. Our results indicate that more research into the various mechanisms of cercarial removal is needed to arrive at a comprehensive understanding of the mechanisms underlying parasite removal in communities.

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Parasites as prey: the effect of cercarial density and alternative prey on consumption of cercariae by four non-host species

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Abstract

In parasites with complex life cycles the transmission of free-living infective stages can be influenced by ambient community diversity, in particular via predation. Here, we experimentally investigated whether parasite density and the presence of alternative prey can alter predation rates on free-living cercarial stages of a marine trematode by several non-host predators. All four predator species consumed increasing numbers of cercariae with an increase in cercarial density, indicating that the removal of cercariae by predators is effective over a range of natural densities as well as in the presence of alternative prey for a number of predators typical of marine ecosystems. However, the relative removal rates and the effects of cercarial density and alternative prey differed among predator species. In barnacles and shrimps, significant interactive effects of cercarial density and alternative prey on cercarial predation occurred while in oysters and crabs cercarial removal rates were unaffected by both factors. As changes in cercarial densities directly translate into changes in infection levels in down-stream hosts in this parasite–host system, the observed predator-specific responses suggest that cercarial predation effects on disease risks will depend on the specific species composition of ambient communities and not on non-host biodiversity per se.

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Introduction

Across the globe biodiversity is being lost at a high rate. In general, decreased biodiversity is believed to reduce ecosystem functioning and service provision (Hooper et al. 2005; Worm et al. 2006; Keesing et al. 2010). An important and increasingly studied additional consequence of biodiversity loss is the potential increase in the transmission of infectious diseases. The relationship between biodiversity and reduced disease transmission has been shown across a variety of ecosystems involving various pathogens, hosts and transmission pathways (Keesing et al. 2006; Johnson et al. 2015). This apparent mediation of disease risk and reduction of infection levels by ecological community diversity is explained by the so called 'dilution effect'. The term has been widely applied as a concept in terrestrial disease ecology, notably in studies on Lyme's disease and other vector-borne diseases (Keesing et al. 2006). Here, an increase in species diversity is said to reduce disease risk by altering the abundance of competent disease reservoirs relative to non-competent reservoir species. This in turn reduces the encounter rate between disease vectors and competent hosts, thereby reducing the number of vectors and their infection prevalence in the system (Ostfeld and Keesing, 2000; Schmidt and Ostfeld, 2001; Keesing et al. 2006). However, whether this effect is universal or whether the actual amplification or dilution of disease risk in a system depends on the specific species composition of reservoir hosts and vectors of that system and not on biodiversity per se is hotly debated (Randolph and Dobson, 2012; Salkeld et al. 2013; Lafferty and Wood, 2013; Wood and Lafferty, 2013; Johnson et al. 2015).

A similar 'dilution effect' as that observed in vector-borne diseases occurs in parasites with complex life cycles where the transmission of free-living infective parasite stages can be strongly influenced by changes in ambient community diversity and composition (Thieltges et al. 2008a; 2008b Johnson and Thieltges, 2010). Changes in species richness can interfere with the transmission of infectious stages to their suitable hosts through a wider variety of mechanisms than simply changing the relative abundance of competent to non-competent hosts (Orlofske et al. 2012). These include predation and hyperparasitism, physical disturbances or barriers, chemical disruption in the form of toxic exudates and interference by decoy and alternative host organisms (Thieltges et al. 2008a; 2008b Johnson and Thieltges, 2010). Of these mechanisms, predation on free-living stages has been particularly well studied, indicating that predators often interfere with parasite transmission by removing substantial numbers of parasitic free-living infectious stages from their environment, thereby reducing encounters between hosts and parasites and ultimately lowering

infection levels in down-stream hosts (Thieltges et al. 2008a; Johnson et al. 2010; Orlofske et al. 2012). However, these removal rates are typically obtained from experiments using specific densities of parasites (i.e. number of infectious stages) and not for a range of different densities. Given that there tends to be a relationship between the consumption rate of a predator and the abundance of its prey (functional response, Oaten and Murdoch, 1975) it may be that the strength of the observed transmission interference differs across a range of parasite densities. Hence, it remains to be determined whether organisms removing parasites reach a saturation point thereby impairing the transmission interference. If predators were to reach saturation at high parasite densities or even reduce their consumption rate due, for example, to swarming effects [i.e. where a high abundance of prey diminish consumption rate through a variety of mechanisms, such as clogging of filters (Jeschke et al. 2004)] this would have important implications for the generality of observed effects of transmission inference. In addition, the consumption rate of predators is also known to be affected by the presence of alternative prey (Oaten and Murdoch, 1975; van Baalen et al. 2001). Under natural conditions predators have access to a range of prey species, while experimental setups typically involve a simple one predator – one prey design. The recorded consumption rate of predators may therefore merely be a phenomenon observed in the lab in the absence of any alternatives. Unfortunately, to date, studies on the density of infective stages and the presence/absence of alternative prey mediating the rate of parasite removal by predators are limited to a single system, cercarial stages of the trematode *Ribeiroia ondatrae* infecting freshwater amphibians (Schotthoefer et al. 2007; Orlofske et al. 2012, 2015). This clearly hinders our understanding of the generality and magnitude of the effect of predator interference with parasite transmission.

In this study, we experimentally investigated the effect of parasite density and alternative prey on the consumption of free-living cercarial stages of a marine trematode (*Himasthla elongata*) by several non-host predators. Previous work had shown that cercariae of this species are frequently consumed by a variety of predators (Welsh et al. 2014). The trematode species uses the gastropod *Littorina littorea* as first intermediate and some bivalves (mainly mussels and cockles) as second intermediate hosts and bivalve-eating birds as definitive hosts (Thieltges et al. 2006). By exposing shrimps (*Crangon crangon*), crabs (*Hemigrapsus takanoi*), oysters (*Crassostrea gigas*) and barnacles (*Semibalanus balanoides*), which either actively prey upon motile, free-living cercarial stages or passively filter them out of the water column, to several ecologically

relevant densities of cercariae (based on calculations from literature data) in presence or absence of alternative prey we aimed to quantify the effect of both factors on parasite removal rates by predators. As cercarial densities directly translate into metacercarial infection levels in down-stream hosts in this system (Liddell et al. 2017), any changes in cercarial densities due to cercarial predation can be expected to ultimately affect disease risk in down-stream hosts. Hence, our experiments contribute to our still limited understanding of the presence and magnitude of the effects of ambient community diversity on parasite transmission interference.

Materials and methods

Experimental organisms and alternative prey

Cercariae of *H. elongata* were used for the experiments. After emergence from the hosts, the relatively large cercariae (body length: 605–665 μm; tail length: 535–605 μm; Werding, 1969), which are visible to the naked eye, swarm actively through the water column. For the experiments, cercariae were obtained from common periwinkles (*L. littorea*) collected in the vicinity of the NIOZ Royal Netherlands Institute for Sea Research on Texel (Wadden Sea, The Netherlands). 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 the necessary numbers of cercariae were pipetted within 1 h (thus the maximum age of cercariae was 4 h) into pots to be administered to the appropriate containers of the experiment.

Four species with different feeding mechanisms or hunting strategies and which do not serve as hosts for the trematode species were used in this study: shrimps and crabs as motile active predators and oysters and barnacles as sessile filter feeders. Shrimps (*C. crangon*; mean ± S.D.: 34.4 ± 1.9 mm length), crabs (*H. takanoi*; 18.8 ± 1.5 mm carapace width), barnacles (*S. balanoides*, attached to empty mussel shells; 34.5 ± 8.2 barnacles of 2–3 mm diameter per shell) and oysters (*C. gigas*; 48.6 ± 4.1 mm diameter) were collected in the vicinity of the NIOZ in the south east of Texel (The Netherlands). Collected organisms were housed in aerated containers or flow through aquaria in the same climate chamber at 15 °C and fed regularly. Crabs were fed on a diet of oysters, mussels, fish (herring) and shrimp. Shrimps were fed fish (herring) and consumed

conspecifics. Oysters were fed algal bivalve feed (*Isochrysis galbana*). Barnacles were collected shortly before the experiment and thus did not require feeding.

The type of alternative prey items offered to predators was chosen based on knowledge on the natural diets of the predators used in the experiments. The alternative prey for the crabs and shrimps consisted of frozen fish (herring) which was defrosted the night before administration and cut into small portions (approx. 0.96 g per crab, 0.72 g per shrimp) at a size that predators could easily handle. The alternative prey for the oysters and barnacles consisted of highly concentrated *I. galbana* algal bivalve feed (Instant Algae by Reed Mariculture Inc. USA; 4.1 billion cells mL^{-1}), administered as 3–4 drops of algal feed per oyster and per unit of barnacles, resulting in algal concentration inducing feeding activity in oysters and barnacles based on observations in preliminary experiments. In all four predator experiments, the alternative prey items added were of a significantly larger volume or quantity than the potential cercarial prey to ensure that predators were offered attractive alternative choices to cercariae at all cercarial densities.

Experimental set-up

Plastic containers (25 ×11×9.5 cm^3) were filled with 500 mL of seawater, constantly aerated and placed on a bench in a completely randomized block design with two temporal blocks. The room temperature was maintained at 18 °C (the average summer water temperature in the study area; van Aken, 2008). In the case of crabs, shrimps and oysters, a single individual was placed in each container and the assigned treatment administered. Barnacles were added attached to a single mussel valve (34.5 ± 8.2 barnacles per container). The four species were tested in four separate experiments, each using the same two-factorial block design, with cercarial density (20, 60, 100 or 300 cercariae) and alternative prey (present or absent) as main factors and two temporal blocks (days 1 and 2). Each treatment combination was replicated four times in each block, i.e. eight replicates for each treatment combination in total.

Cercarial density selection was based on literature data on cercarial shedding rates of *H. elongata* from their first intermediate host, the common periwinkle *L. littorea*, and on literature data on the average abundance of periwinkles (for details see Liddell et al. 2017). These calculations suggested a realistic maximum shedding of about 300 cercariae in the vicinity of an infected snail per tide and we thus used this as the maximum cercarial density administered. As this maximum cercarial concentration is likely to be diluted in the field in the water column and by intra-specific dilution in form of up-take by down-stream hosts such as mussels and cockles (Mouritsen et al.

2003; Thieltges and Reise, 2006; Magalhães et al. 2017) we used several lower cercarial densities (100, 60 and 20 cercariae) to mimic various levels of cercarial dilution.

Crabs, shrimps, oysters and barnacles were placed in their containers a day before the experiment to acclimatize. Treatments were then administered and the experiments run for 3 h. After that the organisms were removed and the contents of the containers sieved through a 20 μM mesh and dyed using Rose Bengal stain (test runs had proven this method to retrieve 100% of cercariae). The number of parasites remaining in the sieved contents was recorded using a light microscope.

Statistical analyses

The relationship between parasite density (20, 60, 100 or 300 cercariae), the presence of alternative prey (absent vs present), and a block factor on the number of remaining parasites 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 removals are independent events, the number of free-living cercarial stages remaining at the end of the experiment follows a binomial distribution. 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:

$$P=e^{-\theta}$$

where θ is the removal rate per unit of experimental time. It is further assumed that this removal rate is a function of parasite density, the presence of alternative prey, their interaction, and a block effect. So:

$$\theta=\mu + \alpha_i + \beta_j + \gamma_{ij} + \delta_k$$

where μ is the intercept, α is the effect of cercarial density, β of the presence of alternative prey, γ is their interaction, and δ is the block effect. The model used the absolute number of remaining parasites after the 3 h experimental time period.

A series of GLM models from the most complex to the least complex were fitted (see Fig. S 5.1). The most complex model included all explanatory variables (cercarial density, alternative prey, their interaction, and a block effect) 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 the Analysis of Deviance. For example, model 1 which included all terms was tested against model 2

in which the interaction was left out. The delta deviance (the difference in deviance between the two models) was subsequently divided by the dispersion factor (φ) from the most complete model (Δ Dev/φ) and compared to the delta degree of freedom χ^2 at 0·05. The dispersion factor was calculated by dividing the residual deviance for the most complex model by the degrees of freedom. A significant difference between two models reveals that the most complex model of the two is the better fit.

From the best fitting models, cercarial removal rates (per experimental runs) and cercarial survival (%) were calculated. Removal rates were calculated for the 3 h experimental period and based on the estimates of the intercept for each significant factor included in the best fitting model output. Cercarial survival was calculated from the estimates of the intercept for each significant factor included in the best fitting model output. From these cercarial survival data, the proportion cercariae removed (%) can be calculated (proportion cercariae removed = 100−cercarial survival).

All analyses were carried out using R (R Development Core Team, 2013) version 3.0.2 in R Studio (version 0.98.1103; R Studio, 2015).

Results

All four predators consumed more cercariae when higher densities of cercariae were offered, both when alternative prey was absent and present, i.e. the absolute removal in terms of numbers of cercariae generally consumed increased with cercarial density (Fig. 5.1). However, the relative cercarial removal rates (i.e. consumption per unit time) differed among the four predators depending on cercarial density and alternative prey (Table 5.1; Fig. S 5.2; for raw data see Welsh et al. 2017).

For the barnacles, the best fitting model included the interaction between cercarial density and presence/absence of alternative prey (model 1; Table 5.1). This probably resulted from the fact that cercarial removal rates were higher at presence than at absence of alternative prey at intermediate cercarial densities while they were lower at high densities (Fig. 5.2). In addition, the best fitting model also included a temporal block effect, which resulted from overall higher removal rates during the second run of the experiment (Fig. 5.2; Table S 5.1).

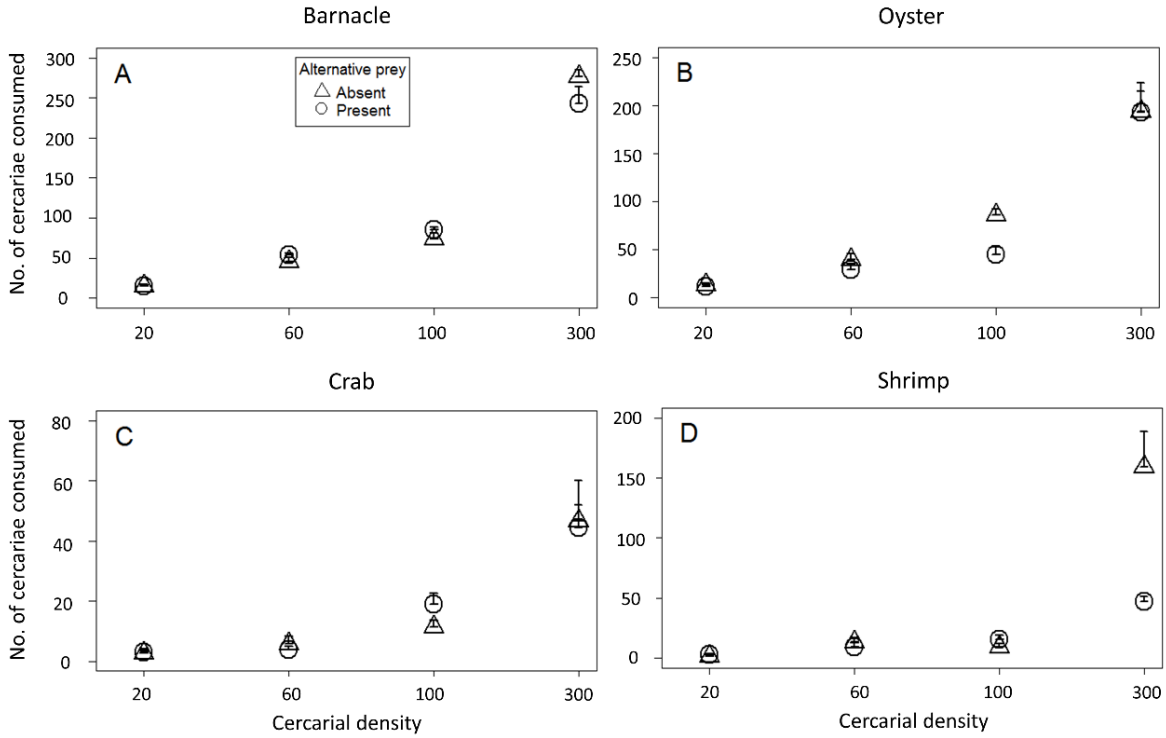

Fig. 5.1. Number of cercariae consumed by (A) barnacles; (B) oysters; (C) crabs; and (D) shrimps across a range of cercarial densities when an alternative food source was either absent or present. Note the different y-axes.

Table 5.1. Results of model selection procedures. From the most complete (model code 1) to the least complete model (10) the degrees of freedom (D.F.) and model deviance are given for each model. Model 1 included the factors cercarial density (α), presence/absence of alternative prey (β), their interaction (γ), and a block effect(δ). Model deviances of the best fitting model for each species/experiment are shown in bold. The dispersion factor (φ) is given for the best fitting model only.

			Deviance			
Model code	**Model**	**D.F.**	**Barnacle**	**Oyster**	**Crab**	**Shrimp**
1	$\alpha+\beta+\gamma+\delta$	55	**864·9**	1968·9	460·7	1067·8
2	$\alpha+\beta+\delta$	58	1049·1	2213·3	485·7	1627·0
3	$\alpha+\beta+\gamma$	56	1051·3	2019·6	500·3	**1085·9**
4	$\alpha+\beta$	59	1235·6	2267·4	523·0	1629·2
5	$\alpha+\delta$	59	1060·6	2325·3	485·9	1890·2
6	$\beta+\delta$	61	1111·2	2234·3	521·2	1768·9
7	δ	62	1125·8	2346·1	**521·2**	2289·8
8	α	60	1245·5	2378·4	523·1	1892·5
9	β	62	1299·6	2286·8	562·1	1779·3
10	1	63	1312·4	**2396·2**	562·9	2295·8
φ best fitting model			15·7	43·6	9·48	19·4

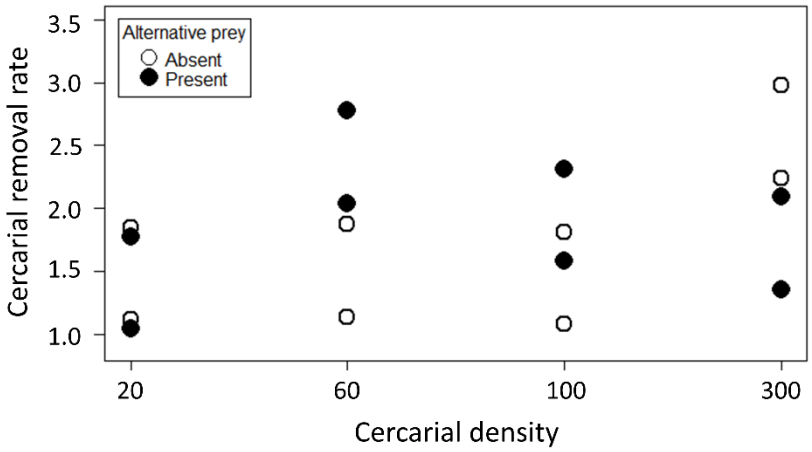

Fig. 5.2. Relative cercarial removal rates (per experimental run of 3 h) of barnacles across a range of cercarial densities and when an alternative food source was either absent or present. Plot based on model output and the factors contributing to the best fitting model (see Table 5.1).

Overall, the survival of cercariae after removal by barnacles was between 5 and 35% (Table S 5.1). In contrast to barnacles, none of the factors tested affected cercarial removal rates by oysters (Table 5.1), i.e. oysters were removing cercariae at a constant rate, independent of the cercarial density or the presence/absence of alternative prey. The cercarial removal rate of oysters was 1.01 and 36% of cercariae survived.

For crabs, the best fitting model only included the block effect (model 7; Table 5.1). Cercarial removal rates by crabs were slightly higher in the first (0.21) than in the second (0.14) experimental run. Accordingly, cercarial survival was slightly lower in the first compared with the second run (81% and 87% respectively). Finally, for shrimps the best fitting model included an interaction between cercarial density and the presence/absence of alternative prey (Table 5.1). This interaction was based on an almost 5-fold increase in searching rates of shrimps at the highest cercarial density when alternative prey was absent (Fig. 5.3). Here, cercarial survival was relatively low with 47%, while in all other cases cercarial survival ranged between 77 and 91% (Table S 5.2).

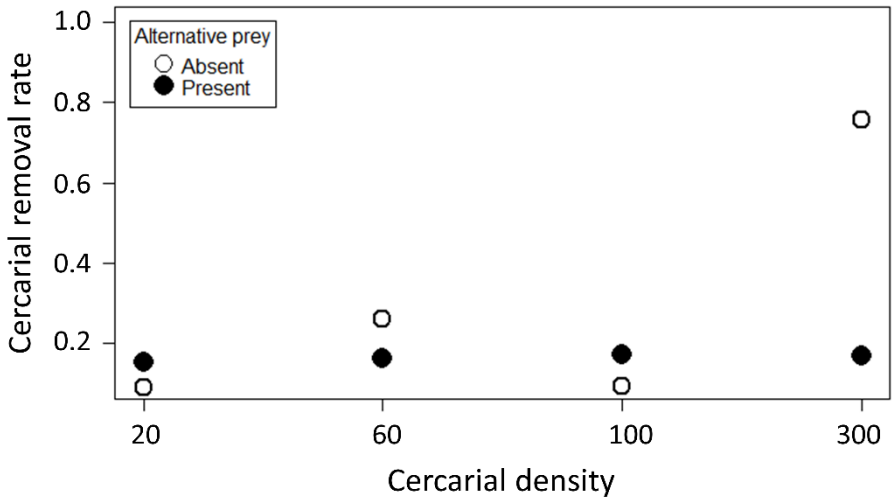

Fig. 5.3. Relative cercarial removal rates (per experimental run of 3 h) of shrimps in the presence of different cercarial densities and in the presence or absence of alternative prey. Plot based on model output and the factors contributing to the best fitting model (see Table 5.1).

Discussion

All four predator species consumed increasing numbers of cercariae with an increase in cercarial density, i.e. the absolute cercarial removal increased with cercarial density. However, the relative cercarial removal rates (i.e. per unit time) and the effect of cercarial density and alternative prey differed among predator species. In barnacles and shrimps, significant interactive effects of cercarial density and alternative prey on cercarial consumption were present, while in oysters and crabs neither cercarial density nor the presence/absence of alternative prey had a significant effect on cercarial removal rates by the predators.

The increase in the numbers of cercariae consumed by all four predator species with increasing cercarial density can be explained in terms of the mass action principle, which assumes that predators encounter their prey randomly and that the number of encounters a predator makes is proportional to the density of its prey (Arditi and Ginzburg, 1989). Interestingly, none of the predators reached saturation across the range of parasite densities tested in this experiment. As the parasite densities administered in this study were selected based on natural shedding rates of cercariae from their host snails and therefore represent abundances of infective stages that a predator is likely to encounter under natural conditions (see 'Materials and Methods' section), the experiments suggest that swarming effects, e.g. by clogging of filters, do not seem to occur at realistic parasite densities in the predators tested. However, while

the absolute numbers of cercariae consumed generally increased with increasing cercarial density in all four predator species, the relative removal rates showed different responses to cercarial density and presence/absence of alternative prey in the four predator species. The fact that species sharing the same feeding mechanism (active predation: crabs and shrimps vs passive filtration: barnacles and oysters) showed different patterns suggests that the responses are not universal or linked to specific feeding traits but rather species-specific.

In barnacles and shrimps, the best fitting models included an interaction between cercarial density and presence/absence of alternative prey. This resulted from cercarial removal rates at low and intermediate cercarial densities being similar or higher at presence compared to absence of alternative prey, while at the highest cercarial density removal rates they were highest in absence of alternative prey. This was particularly the case for shrimps which showed an almost 5-fold increase in searching rate at the highest cercarial density when alternative prey was absent. In contrast, removal rates did not differ much between presence and absence of alternative prey at lower cercarial densities. This may indicate the phenomenon of prey switching (Murdoch, 1969; Cornell, 1976) whereby a predator initially focuses on the most abundant or easily accessible prey type in its environment (in this case the alternative prey, i.e. the piece of fish or algae) and then switches to a new prey type as this becomes more abundant (in this case the parasites). However, whether such prey-switching really underlies the observed pattern in our experiments deserves further studies. Other work on trematodes from freshwater ecosystems also found more complex relationships between cercarial consumption and cercarial density, depending on both the identity of the predator (mosquitofish or damselfly nymphs) as well as of the parasite species (*Echinostoma trivolvis* or *R. ondatrae*; Orlofske et al. 2015). Together with our study, these results suggest that the effect of cercarial density on cercarial removal rates by predators actually depends on the particular parasite and predator species and may be further mediated by the presence or absence of alternative prey.

In the other two cercarial predators investigated in our experiment, oysters and crabs, neither cercarial density nor the presence/absence of alternative prey affected the rates with which they removed cercariae. Relative removal rates were similar over the range of cercarial densities administered within the two predator species and generally higher in oysters than in crabs (36 and 87% cercarial survival, respectively). Oysters have previously been reported as very effective predators of cercariae without serving as hosts to *H. elongata* (Thieltges et al. 2008a, 2009). They are very efficient filter

feeders with high pumping rates (Ren et al. 2000; Ropert and Goulletquer, 2000) and bivalves, including oysters, have generally been shown to selectively consume particles of comparable size to cercariae of *H. elongata* from algae mixtures (Barillé et al. 1997; Cognie et al. 2003). Bivalves can generally show food density dependent filtering activity (Gosling, 2003) but within the realistic food levels and parasite densities administered in our experiments this does not seem to occur as removal rates were not affected by cercarial density or presence/absence of alternative prey. Crabs in turn remove cercariae either by active predation or by uptake via their gills (without becoming infected themselves; pers. observation). Given the lower removal rates in crabs, these mechanisms do not seem to be as effective as in oysters, leading to lower overall cercarial removal rates by crabs. However, in both cases removal rates did not differ in absence or presence of alternative prey, suggesting that parasite removal is often likely to be maintained even in complex communities with multiple prey species under more natural settings. Similar conclusions were made by two studies on predators of the cercariae of *R. ondatrae* in freshwater systems where dragonfly and damselfly larvae, cyclopoid copepods, hydroid polyps and mosquitofish continued to prey on cercariae when alternative prey was present (Schotthoefer et al. 2007; Orlofske et al. 2012). Our study expands on these findings with results from additional taxonomic groups (shrimps, crabs, barnacles, oysters) and mechanisms (e.g. filter feeding bivalves) and suggests that many predator species will maintain their parasite removal capabilities under more realistic multiple prey situations.

In two of the predator species investigated in our experiments, crabs and barnacles, the best fitting model also included a (temporal) block effect. This resulted in significant differences in the cercarial removal rates of predators between the two runs of the experiments. While every effort was made to ensure that conditions remained constant in each experiment, conditions may still have been experienced differently by the predators. For instance, the batch of administered cercariae came from different groups of snails each day and may have been of different quality in terms of motility or life span. In addition, the behaviour of predators may have been affected by slight differences in ambient conditions between the different runs. However, the general patterns observed were consistent between runs and by incorporating a temporal block factor into the statistical models we ensured that these temporal differences were taken into account when investigating the main effects.

In conclusion, the removal of cercariae by predators has been shown to be effective over a range of natural cercarial densities as well as in the presence of alternative prey

for a number of predators typical of marine ecosystems. However, the response of removal rates of predators to different cercarial densities and presence/absence of alternative prey differed among the four predator species without an obvious link to specific predator traits. As changes in cercarial densities directly translate into changes in infection levels in down-stream hosts in this system (Liddell et al. 2017), the predator-specific responses observed suggest that cercarial predation effects on disease risks will depend more on the specific species composition of ambient communities than on biodiversity per se. These results mirror the recent discussion about the generality of dilution and related effects which suggest that the actual amplification or reduction of disease risk in a system may depend more on the specific species composition of ambient communities and not on biodiversity per se (Randolph and Dobson, 2012; Salkeld et al. 2013; Lafferty and Wood, 2013; Wood and Lafferty, 2013; Johnson et al. 2015). Our results suggest that predator specific responses to parasite density and presence/absence of alternative prey add a further layer of complexity to the general interference potential of predators on parasite transmission.

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Supplementary material

Table S 5.1. Model results for cercarial removal rates (searching rates) of barnacles per experimental runs of 3 hours and % survival of cercariae for each treatment combination (cercarial density and presence/absence of alternative prey) and the block effect which were all included in the best fitting model (model 1; see Table 5.1).

Cercarial density	Alternative prey	Block	Cercarial removal rate	Cercarial survival
20	Present	1	1.04	35.2%
20	Present	2	1.78	16.9%
20	Absent	1	1.12	32.7%
20	Absent	2	1.85	15.7%
60	Present	1	2.04	13.0%
60	Present	2	2.77	6.2%
60	Absent	1	1.14	32.0%
60	Absent	2	1.87	15.3%
100	Present	1	1.58	20.6%
100	Present	2	2.31	9.9%
100	Absent	1	1.08	33.9%
100	Absent	2	1.81	16.3%
300	Present	1	1.36	25.7%
300	Present	2	2.09	12.3%
300	Absent	1	2.24	10.6%
300	Absent	2	2.98	5.1%

Table S 5.2. Model results for cercarial removal rates (searching rates) of shrimps per experimental runs of 3 hours and % survival of cercariae for each treatment combination (cercarial density and presence/absence of alternative prey) which was included in the best fitting model (model 3; see Table 5.1).

Cercarial density	Alternative prey	Cercarial removal rate	Cercarial survival
20	Present	0.16	85.6%
20	Absent	0.09	91.3%
60	Present	0.16	84.8%
60	Absent	0.26	77.1%
100	Present	0.17	84.1%
100	Absent	0.10	90.9%
300	Present	0.17	84.4%
300	Absent	0.76	46.9%

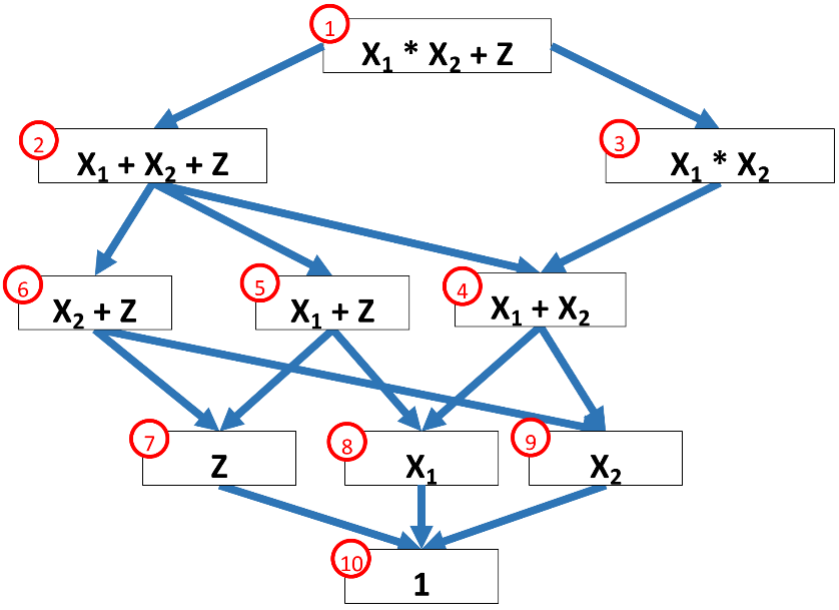

Fig. S 5.1: A) Schematic representation of the model selection procedure based on the best fitting model. Numbers in red circles represent model number from the most complex (1) to the least complex (5) model. X indicates explanatory variables with X_1 representing cercarial density and X_2 representing presence/absence of alternative prey. Blue arrows indicate which model was tested with which. The testing procedure started with testing the most complex model to the next, less complex model, and so on (i.e., model 1 was tested against model 2, model 2 against model 3 and 4, model 3 against model 5 and model 4 against model 5). When a significant difference between two models occurred it was not necessary to continue (as indicated by the red arrows in Figures S 5.2) with a reversed direction).

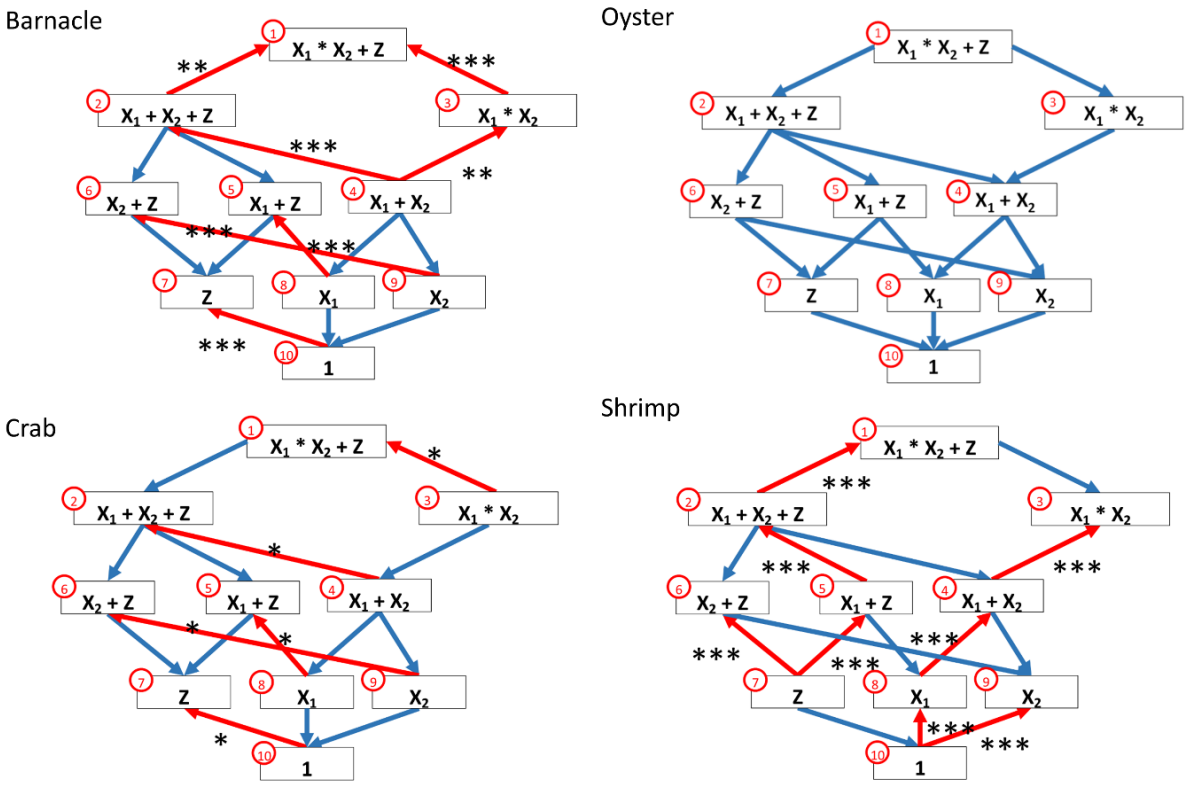

Fig. S 5.2. Actual model selection procedure for the effect of cercarial density and presence/absence of alternative prey on cercarial removal by all test species. Significant differences between models are indicated by red arrows (* denoting a significance level of 0.05).

Climate change and parasite transmission: how temperature affects parasite infectivity via predation on infective stages

M. Anouk Goedknegt, Jennifer E. Welsh, Jan Drent, and David W. Thieltges

Abstract

Climate change is expected to affect disease risk in many parasite-host systems, e.g., via an effect of temperature on infectivity (temperature effects). However, recent studies indicate that ambient communities can lower disease risk for hosts, for instance via predation on free-living stages of parasites (predation effect). Since general physiological theory suggests predation effects to be temperature dependent, we hypothesized that increases in temperature may lead to reduced parasite infectivity via elevated consumption rates of free-living parasite stages (temperature-predation interaction). We experimentally investigated such interactions in three marine predators of infective parasite stages. Two species (the oyster *Crassostrea gigas*, and the barnacle *Austrominius modestus*) significantly reduced cercarial stages of the trematode *Renicola roscovita* in mussel hosts (*Mytilus edulis*), while the third (the crab *Hemigrapsus takanoi*) did not show a reduction of infective stages at all. In barnacles, cercarial consumption significantly interacted with temperature, with lowest infectivity at highest temperatures. Since these patterns reflected the known thermal responses of the three cercarial predators' feeding rates, parasite consumption rates may be predictable from temperature dependent feeding rates. Our results suggest that integrating temperature-predation interactions into studies on parasite transmission and on climate change effects is essential and that predators of free-living stages of parasites may play an important role in indirectly mediating disease risk under climate change.

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Introduction

Various studies have suggested that climate change may lead to elevated disease risk in wildlife and humans with wide-ranging ecological and economic effects (Harvell et al. 2002, Patz et al. 2005). However, the actual relationship between temperature and disease is often complex, with varying underlying mechanisms that are better understood for some disease agents than for others (Lafferty 2009, Rohr et al. 2011). A particularly clear and strong effect of temperature on disease dynamics is known from trematodes (flukes) in which it directly affects crucial steps in their transmission between life cycle stages. The production and emergence of their free-living infective stages (cercariae) in the first intermediate hosts (mollusks) is strongly positively correlated with temperature (see review by Poulin 2006). At the same time, the infectivity of cercariae in the down-stream second intermediate hosts (invertebrates or fish, depending on the species) is also positively correlated with temperature (e.g., Evans 1985, Thieltges and Rick 2006, Studer et al. 2010). Since cercarial transmission is a crucial step in the trematode life cycle, it has been proposed that global warming might dramatically increase future infection levels in hosts (Marcogliese 2001, Poulin 2006, Poulin and Mouritsen 2006).

Parasite-host dynamics are not only influenced by abiotic conditions like temperature but also by interactions with other species in the environment. For example, some organisms of the ecological community from which a parasite-host system is part of can reduce the risk of certain diseases via a process called the dilution effect. This effect has mostly been related to varying host compatibility, with the presence of low competency hosts leading to a reduction in disease risk for competent hosts (Keesing et al. 2006). However, the initial concept has recently been broadened to include effects of species which do not serve as hosts at all (non-host; Johnson and Thieltges 2010, Johnson et al. 2010). When such non-hosts prey upon free-living infectious stages of parasites, they can interfere with transmission (equivalent to the 'encounter reduction' mechanism of Keesing et al. 2006) and lead to reduced infection levels in the target host (Johnson and Thieltges 2010). Experimental and observational studies, both from the lab and the field, indicate that such predation effects can occur in many parasites with free-living stages (Thieltges et al. 2008b), including trematodes (e.g., Thieltges et al. 2009, Orlofske et al. 2012, Welsh et al. 2014), and they are increasingly proposed as important regulatory mechanisms for many diseases (Keesing et al. 2010, Ostfeld and Keesing 2012).

Given the general positive relationship between metabolic rates of ectothermic organisms and ambient temperature (Q_{10}-rule; Schmidt-Nielsen 1997), non-host predation effects are, like trematode emergence and infectivity, also expected to be mediated by temperature. An increase in metabolism translates into an increase in feeding rates and feeding rates of organisms generally scale positively with temperature up to a maximum, after which they decrease due to temperature stress (e.g., Newell 1970, Englund et al. 2011). This suggests a potential interaction between temperature effects and predation effects: an increase in parasite production and infectivity at elevated temperatures might actually be compensated by increased feeding rates of predators of free-living parasite stages. Although an interaction between temperature and predation effects is very likely, to the best of our knowledge, to date no experimental studies have detected their existence (but see Studer et al. 2013 for a first attempt in this direction).

This study aimed to investigate whether predation effects caused by non-hosts can interact with temperature effects so that increasing infection levels due to elevated temperatures may be counterbalanced by predation on free-living stages of parasites. We used controlled lab experiments and a marine parasite-host system widespread in the eastern North Atlantic (cercariae of the trematode *Renicola roscovita*, its second intermediate host, the blue mussel *Mytilus edulis*) and three common cercarial predators (Pacific oysters *Crassostrea gigas*, Asian shore crabs *Hemigrapsus takanoi* and Australasian barnacles *Austrominius modestus*), to investigate the following specific questions: (1) Does the strength of the predation effect depend on temperature? (2) If so, could this predation effect compensate for increased infection levels with rising temperatures? (3) Do predation effects vary between cercarial predators and can this be explained by their known temperature responses in feeding rates? Identifying potential interactions of temperature and predation effects has important implications for our understanding of parasite transmission in general and the role of non-hosts in mediating effects of climate change in particular.

Materials and methods

Organisms

To obtain a source of *Renicola roscovita* cercariae, its first intermediate host, the periwinkle *Littorina littorea*, was collected from different mussel beds and dykes on the east coast of the island of Texel (Wadden Sea, The Netherlands). After an acclimation period of at least 24 h at 15°C, the periwinkles were divided over 6-well plates, filled with seawater (16 mL per well) and placed in an incubator at 25–29°C. After 3 h, the wells were screened for cercariae of *R. roscovita* under a dissection microscope. Infected snails were kept in groups of 20 in aerated aquaria (3.6 L) and fed ad libitum with *Ulva spp.* Uninfected blue mussels (*Mytulis edulis*; 30–35 mm) were used as target hosts and collected from groins along the west coast of Texel. Here the first intermediate host of the parasite does not occur and the mussels are therefore uninfected (verified by dissecting 30 mussels—no infections found). The three cercarial predators were collected as follows: Pacific oysters (*Crassostrea gigas*; average volume of 38.3 ± 8.5 mL) were collected from a small oyster bank in the Mokbaai inlet at the southeast end of the island of Texel (53°00′21″ N; 4°46′10″ E). At the same location, Asian shore crabs (*Hemigrapsus takanoi*; average carapace size of 1.0–1.5 cm) and empty mussel shells covered with the Australasian barnacle (*Austrominius modestus*) were collected between the oysters. All organisms were kept in aerated sand filtered seawater at 15°C and fed ad libitum with blue mussel flesh (crabs) or with the unicellar algae *Isochrysis galbana* (mussels, barnacles, and oysters).

Experimental design

The experiment was carried out as a partly nested two-factorial split-plot design (Quinn and Keough 2002), with temperature (between plots: 12.5°, 18° and 25°C) and cercarial predator presence (within plots: yes or no) as fixed factors and water bath (plots) as random factor, nested in temperature (see details below). The temperatures used were within the normal physiological temperature range of the three cercarial predators (Pacific oyster: Bougrier et al. 1995, Ren et al. 2000; Asian shore crab: Dehnel 1960; Australasian barnacle: Southward 1955) and reflect the average spring temperature (12.5°C) in the study area (Western Wadden Sea) over the last 140 years (van Aken 2008) and temperatures that are commonly (18°C) or occasionally observed during extreme warm days during summer (25°C) in tidal pools and shallow waters on the tidal flats (the habitat of the parasite-host system and cercarial predators; Kühl 1952, van Aken 2008, Onken et al. 2010). Moreover, the highest temperature level (25°C) is predicted to occur more frequently and for prolonged periods under future

climate change scenarios (Philippart and Epping 2009). The standardized amount of cercarial predators was within the range observed in the field (see Discussion for details) and was as follows: one oyster, two crabs and empty mussel shells with a total of approximately 7 cm^2 of barnacle cover (≈ 140 individuals). To all experimental units three individuals of uninfected blue mussels were added as the target hosts for cercariae.

For each of the three temperature levels (12.5°, 18° and 25°C), we used four individually heated and temperature controlled (to avoid the common pseudo-replication present in temperature experiments; Rohr et al. 2011) water baths (functioning as plots; 40 x 30 x 25 cm) that were distributed randomly within a single climate chamber (10°C). Within each water bath we placed two sets of two plastic containers (12 x 10 x 22 cm, filled with 1.5 L of seawater) that served as the experimental units to apply the within-plot factor cercarial predator presence. Two of the four containers included cercarial predators, while the other two served as controls, containing hosts only. During the experiments and the acclimation periods, the organisms experienced full light exposure. Cercariae only emerge at daylight and their infectivity ceases after 10–15 hours (Greve 1997, Thieltges and Rick 2006), thus for our experiment (cercariae already about 3 hours old when added) a day-night cycle was considered irrelevant. No food was supplied during the experiment as cercarial predators are known to maintain cercarial consumption under starvation (Orlofske et al. 2012, Liddell 2014).

Experimental procedure

Due to logistical constraints, three separate experiments (one for each cercarial predator) were conducted. All organisms (cercarial predators and hosts) were acclimated for 24 hours in the experimental set-up as described above. After 24 h, 50 mL of cercariae stock (seawater with cercariae) was added to all containers. The stock was obtained by incubating 112 infected snails in 3.1 L seawater in an incubator at 28–29°C. After 3 h, the snails were removed and the stock was transferred into a glass beaker and processed immediately (thus the cercariae had a maximum age of ~3 h at the start of the experiment). The stock was carefully stirred (3 times clockwise and 3 times counter-clockwise) each time before 50 mL was removed, to avoid aggregations of cercariae on the bottom of the beaker. A pre-study showed that this method was appropriate to provide consistently similar numbers of cercariae. To estimate the added dose, two subsamples of 50 mL were taken before, four in between, and two after adding of cercariae stock (i.e., 8 subsamples in total). They were fixed with 10 mL of 96% ethanol, stained with 1 mL Rose Bengal and immediately counted under a

dissection microscope (mean number of cercariae (± SE) added to the containers: oysters: 299 ± 10; crabs: 51 ± 7 and 246 ± 8; barnacles: 45 ± 2 and 55 ± 7). In the oyster experiment only a single dose was administered as the first dose was already above our target of at least 100 cercariae (considered to be a realistic dose) while in the other two experiments a second round of infection was necessary due to low numbers of cercariae shed by the snails in the first infection round. The second infection round was done on the next day in the same way as the first. The resulting different total doses were considered unproblematic as cercarial dose does not affect infectivity and does not lead to satiation of cercarial predators at the levels used in our experiment (Liddell 2014). After 24 h in the oyster and 48 hours in the other two experiments, all mussels were placed into seawater and incubated for an additional 48 h to ensure full encystment of metacercariae. Finally, the mussels were dissected, the complete tissue squeezed between two large glass slides, and the numbers of metacercariae determined under a stereomicroscope.

Statistical analyses

Infectivity of cercariae was calculated as the ratio of the mean number of metacercariae encysted per mussel host divided by the number of cercariae added to the experimental unit. The analysis of the partly nested two-factorial splitplot design followed Quinn and Keough (2002), using R (R Development Core Team 2014).

Temperature (A) and cercarial predator presence (C) were used as fixed factors. The water baths (B) functioned as plots and were nested within temperature (B(A)) and analyzed as a random factor. Between plots, we tested for the effects of temperature (A). Within plots, we tested for the effects of cercarial predator presence (C) as well as the interaction between temperature and cercarial predator presence (A 3 C). For all analyses we averaged the two replicates of cercarial predator presence within each bath as an independent replicate (Quinn and Keough 2002). To check for potential differences in mussel size within or among the three experiments, Welch two sample t-tests were used for within trials (diluter vs control) and one-way ANOVA for among trials. For all analyses, the assumptions of normality and homogeneity of variances were checked by inspecting residual plots. To meet the assumptions we applied an arcsine square-root transformation to the infectivity data of all experiments.

Results

The three cercarial predators showed different responses resulting in different cercarial infectivity with increasing temperature (Fig. 6.1). While temperature had a significant effect on cercarial infectivity in the experiments using crabs as cercarial predators, the presence of crabs had no effect (Table 6.1): cercarial infectivity followed a similar pattern as in the absence of crabs (Fig. 6.1). In contrast, in the other two experiments (oysters and barnacles) the presence of cercarial predators had a significant effect on infectivity with a decrease in infectivity compared to the controls (Fig. 6.1, Table 6.1). In the presence of oysters, all cercarial predator treatments showed lower infection levels in mussels compared to the controls but no significant temperature-predation interaction (Fig. 6.1, Table 6.1).

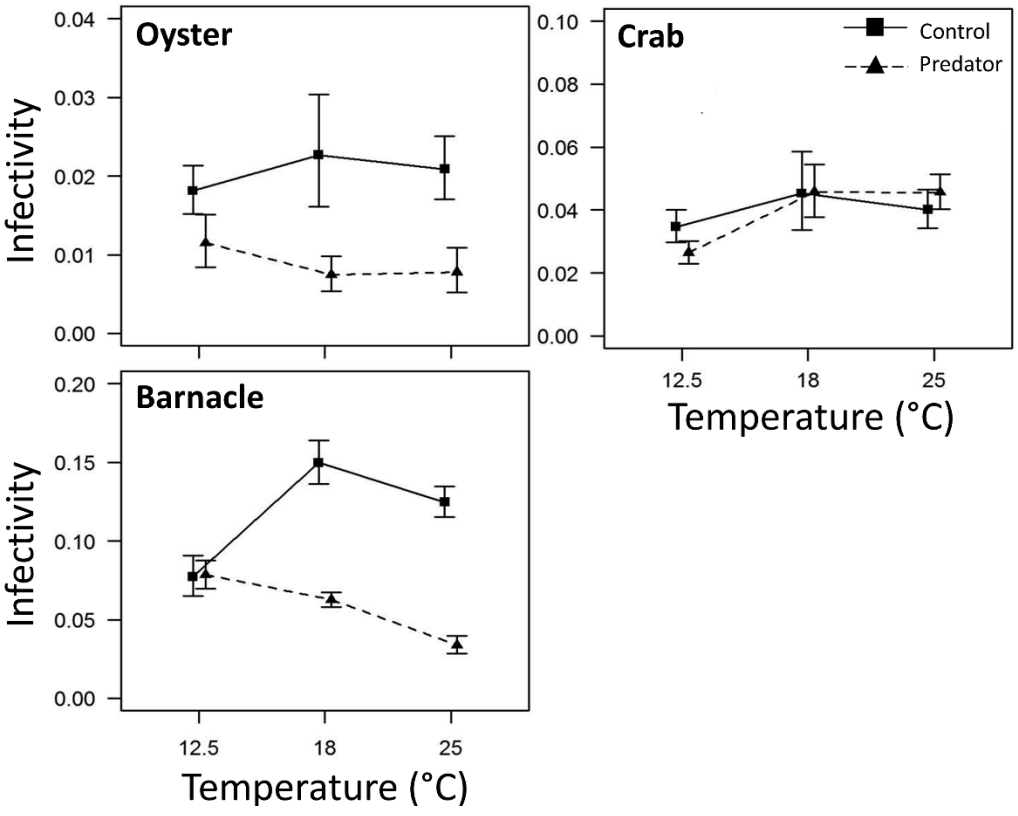

Fig. 6.1. Infectivity of *Renicola roscovita* cercariae in the mussel *Mytilus edulis*, depending on temperature and the presence of cercarial predators: Pacific oysters (*Crassostrea gigas*l), Asian shore crabs (*Hemigrapsus takanoi*) and Australasian barnacles (*Austrominius modestus*). The infectivity is the average number of cercariae found per mussel divided by the amount of cercariae added to each container. Infectivity values are back transformed asin(sqrt()) averages. The bars denote ± SE.

Table 6.1. Results of the partly nested two-factorial splitplot ANOVA for the three separate experiments (oysters, crabs, barnacles). We tested for the effects of temperature (T) and water bath (nested in temperature) between plots and the effects of cercarial predator presence and an interaction between temperature and predator presence within plots. Temperature and predator presence were fixed factors, while water bath (nested in temperature) was a random factor. The analyses and presentation of results follow Quinn and Keough (2002).

Source of variation	df	MS	F	p
Oyster				
Between plots				
Temperature	2	0.0000	0.048	0.954
Water bath (T)	9	0.0009		
Within plots				
Predator	1	0.0148	12.068	0.007
Temperature x Predator	2	0.0008	0.622	0.558
Residual	9	0.0012		
Crab				
Between plots				
Temperature	2	0.0036	4.337	0.048
Water bath (T)	9	0.0008		
Within plots Predator	1	0.0001	0.032	0.863
Temperature x Predator	2	0.0007	0.381	0.694
Residual	9	0.0019		
Barnacle				
Between plots				
Temperature	2	0.0066	3.449	0.077
Water bath (T)	9	0.0019		
Within plots				
Predator	1	0.0721	110.363	< 0.001
Temperature x Predator	2	0.0192	29.462	< 0.001
Residual	9	0.0007		

In contrast, in the barnacle experiment, the observed predation effect showed a significant interaction with a temperature effect: infectivity in the presence of the predators decreased with an increase in temperature, suggesting an increase in the strength of the predation effect with rising temperatures. The mean infectivity of cercariae per individual mussel differed among the three experiments: it was lowest in the oyster experiment (0.7–2.3%), intermediate in the crab (2.6–4.4%) and highest in the barnacle experiment (3.6–15.9%). Sizes of mussels did not differ among treatments within and among the three trials (all $p < 0.3$).

Discussion

Our experiments demonstrated a strong interaction between temperature and predation effects on cercarial infectivity in one of the cercarial predators tested (barnacles). While infectivity of cercariae generally increased with elevated temperatures in the absence of cercarial predators, it decreased when barnacles where present. This suggests that temperature-mediated increases in infectivity and host infection levels can be counteracted by predation on free-living parasite stages. This has several important implications.

First, an increase in the strength of predation effects with rising temperatures suggests that predicted effects of climate change (Poulin 2006, Poulin and Mouritsen 2006) may not be so severe or even non-existing because temperature induced increases in predation on free-living parasite stages could counterbalance increases in parasite infectivity. Our experiments focused on identifying potential temperature-predation interactions on cercarial infectivity by keeping cercarial doses constant, i.e., they did not integrate elevated doses due to increases in cercarial production at higher temperatures. However, given the known temperature response of cercarial production of Renicola roscovita, a potential compensation of increased cercarial production by cercarial consumption of barnacles at elevated temperatures under climate change seems likely. While snails infected with *R. roscovita* shed 3.4 times more cercariae at 20°C compared to 15°C, production is 2.3 times lower at 25°C compared to 20°C (Thieltges and Rick 2006). This suggests that the observed increased cercarial consumption by barnacles will not keep up with the increase in cercarial production from low to intermediate temperatures (3.43 higher production but only a 2.43 decrease in infectivity when barnacles are present; see Fig. 6.1) but could easily override the production difference between 20° and 25°C (2.3 times lower cercarial

production but also a 4.23 decrease in infectivity when barnacles are present; see Fig. 6.1). Since the last step is the one relevant for climate change scenarios in our region (Philippart and Epping 2009), the net effect is likely to be a lowered transmission due to increased cercarial consumption. Field experiments and observations in our (Thieltges et al. 2009) and other ecosystems (e.g., Upatham and Sturrock 1973, Mouritsen and Poulin 2003, Kaplan et al. 2009, Venesky et al. 2014) suggest that laboratory derived indications for predation effects of a variety of predators of free-living parasite stages hold true under field conditions. Hence, the observed temperature-predation interaction is likely to translate into a compensation of elevated parasite production under climate change.

However, these calculations are based on the specific densities used in our experimental set-up. The experimental densities of all three cercarial predators fall within the range observed in the field, but this range actually spans over several orders of magnitude, depending on the habitat where mussel hosts occur (mussel dominated beds, mixed mussel-oysters beds, oyster dominated beds, and hard substrates like the extensive Dutch dykes and harbors) and on local factors (e.g., tidal height, exposure). For example, the barnacle used in our experiments occurs at densities ranging from a few individuals to mean densities of about 70,000 individuals m^2 on mussel/oyster beds in the Wadden Sea ecosystem (Witte et al. 2010). Hence, the actual strength of predation effects will probably be strongly mediated by the density of cercarial predators and may be locally much stronger than observed in our experiments (about 12,000 barnacles m^2). In addition, predation effects of different cercarial predators may also be additive when they co-occur, resulting in further reductions of infection levels (Thieltges et al. 2009). With all three cercarial predators co-occurring locally in the Wadden Sea (Troost 2010, Witte et al. 2010, Landschoff et al. 2013), the actual total cercarial consumption will thus depend on the species composition around target mussels. Interestingly, the mussels themselves will also act as sentinels, i.e., increasing mussel densities will lead to reduced infection levels per individual host (Thieltges et al. 2009). However, experiments with trematode-mussel (e.g., Thieltges et al. 2009) and other parasite-host systems (see e.g., review by Thieltges et al. 2008b) have shown that predation effects by cercarial predators exist in the presence of target hosts, suggesting that they actually cause an additional dilution of infective stages. Finally, there are also other factors besides cercarial predators and sentinel hosts that mediate infectivity. For example, host condition and also abiotic factors are likely to affect transmission of cercariae to mussels (Pietrock and Marcogliese 2003) and the observed differences in infectivity between the three experiments may have resulted from differences of some of these factors among trials. In conclusion, the exact net effect of temperature increases

on infectivity will depend on the interplay of cercarial production, cercarial predator and host densities as well as other infectivity mediating factors. More detailed studies focusing on entangling the relative contributions of these factors will be a promising avenue for future research. Moreover, studies on the effects of climate change on parasitism would generally benefit from integrating effects of predation on parasite free-living stages given the potential for temperature-predation interactions.

Second, a significant interaction between temperature and predation effects has also important implications for our understanding of the role of non-hosts in mediating parasite transmission in general (dilution effects sensu lato). Many organisms have been identified to act as predators for free-living infectious stages of parasites, not just for trematodes but also for many other macroparasites as well as microparasites (Thieltges et al. 2008, Johnson and Thieltges 2010, Ostfeld and Keesing 2012). The activity levels and feeding rates of most predators of parasite free-living stages will be temperature-dependent, following basic physiological rules (Newell 1970, Englund et al. 2011). Hence, the strength of their respective predation effects can also be expected to be strongly temperature-dependent. This suggests that parasite consumption rates of predators may vary greatly under different temperature regimes, strongly calling for an integration of temperature as an additional factor in experimental studies on the role of non-host diversity in mediating parasite transmission.

Finally, our results point to a potentially important role of invasive species in mediating the effects of climate change on host infection levels. Many invasive species are generally expected to extend their ranges poleward (Hellmann et al. 2008) and increase in population size (Dukes and Mooney 1999, Walther et al. 2009). This is also true for the three cercarial predators used in our study which are all invasive in the study area and expected to benefit from climate change by increasing in abundance (Diederich et al. 2005, Witte et al. 2010, van den Brink et al. 2012). Such an increase in abundance together with temperature-mediated increases in predation rates on parasite stages may have profound effects on future disease risks. Negative effects on the transmission of parasite free-living stages have also been reported from other invasive species (e.g., Bartoli and Boudouresque 1997, Kopp and Jokela 2007) and may be more common than currently known. Hence, invasive species may play an important role in mediating disease risk due to climate change thus integrating the indirect effects of invaders on disease risk would be highly relevant for impact assessments of invasive species.

Although two of the cercarial predators we tested showed a significant predation effect, the type of their response differed: while oysters showed similar predation effects at intermediate and high temperatures, the predation effect by barnacles increased with rising temperatures showing highest predation effects at 25°C. This pattern exactly mirrors the known temperature response of both species regarding their feeding rates. In the Pacific oyster, the thermal response curve is hump-shaped, with clearance rates increasing from 10° to about 20°C and then levelling off at higher temperatures (Bougrier et al. 1995, Ren et al. 2000). In contrast, the feeding activity of Australasian barnacles increases from 4° to 25°C (Southward 1955). This close match of the temperature response of predation effects with the general feeding activity of the respective predators suggests that predation rates can be predicted from temperature response curves of feeding rates. However, this does not mean that there will always be predation effects. Asian shore crabs have a similarly shaped activity rate response curve as the barnacles, with increasing activity rates from 5° to 20°C (Dehnel 1960), but they did not show any predation effect. This was probably due to a size-selective mismatch between predator and prey, with the cercariae of *R. roscovita* (129–330 μm; Werding 1969) being too small to serve as prey for the crabs. In contrast, the size of *R. roscovita* cercariae are within the prey size range of oysters (Möhlenberg and Riisgård 1979) and barnacles (Crisp and Southward 1961), allowing predation effects to occur. Since size-dependent match-mismatches between predator and cercarial sizes have also been observed in juvenile fish preying on cercariae (Kaplan et al. 2009), a match of cercarial size and prey size range of a predator is crucial in determining which species has the potential to act as a cercarial predator. This in turn suggests that the capacity to prey on free-living stages of parasites is, to a certain extent, predictable from the general prey size spectrum of predators.

In conclusion, our experiments revealed that there can be strong interactions between temperature and predation effects. Such interactions can potentially offset predicted increases in disease risk under climate change and have important implications for our general understanding of the effects of non-hosts on parasite transmission, because consumption rates of most predators will be temperature-dependent. In addition, our results suggest that invasive species may play an important role in mediating disease risk in the course of climate change. All this calls for the integration of temperature in future studies on the role of non-hosts in parasite transmission as well as in studies on the effects of climate change on disease risk and on the impact of invasive species.

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Idiosyncratic effects of non-host diversity on the removal of infective stages of parasites

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Abstract

Transmission pathways of parasites and pathogens can be altered by surrounding communities but whether biodiversity leads to a decrease in infections (dilution effect) is still a matter of debate. Previous studies focussed on vector-borne diseases or compared hosts within the same ecological guild but with different competence. However, for the removal of infective free-living parasite stages by non-host organisms, experimental studies on diversity effects on disease risk are limited, partly due to difficulties in comparing non-hosts from different taxa. Here, we used a response surface experimental design to overcome such issues and studied the effects of non-host diversity on the removal of cercarial stages of a marine trematode. Non-hosts exhibiting diverse removal mechanisms: predatory crabs, filter feeding oysters and a physical trap in form of seaweed, were used in laboratory experiments. Combinations of two non-hosts at four density levels were tested and the number of remaining infective stages at the end of the experiment were recorded. In all three combinations of non-host species, the effect of a specific non-host species on cercarial removal depended on the presence and density of the other non-host species. Thus, the addition of a second non-host species either neutralised, amplified or reduced the parasite removal effects exerted by the first non-host species. Our experiments unanimously revealed non-host diversity effects on parasite removal. However, diversity effects did not generally result in a dilution effect but the alteration direction of disease risk depended on the specific non-host species combination. Given the likelihood of complex species interactions in diverse communities, diversity effects on parasite removal are probably generally idiosyncratic and response surface experimental designs are a promising approach to unravel the underlying mechanisms.

Manuscript in preparation

Introduction

Transmission pathways of parasites and pathogens can be altered by surrounding ecological communities, ultimately affecting disease dynamics. Recent research into such effects of communities on parasite transmission has identified several mechanisms of how surrounding ecological communities can affect disease risk in focal hosts. These mechanisms relate to a phenomenon called *dilution effect*, which is the idea that increased biodiversity results in a reduction in disease risk (Keesing et al. 2006, Ostfeld and Keesing 2012). While there are several mechanisms that can lead to a biodiversity-mediated alteration of disease risk (Keesing et al. 2006), one of the mechanisms receiving most attention has been *encounter reduction,* which is a reduction in encounters occurring between susceptible and infectious hosts or infective stages caused by surrounding communities. The most prominent examples for this mechanism come from vector-borne diseases with frequency–dependent transmission such as Lyme disease in which hosts of lower competence can act as decoys for vectors and pathogens and thereby dilute the pathogen pool and lead to reduced prevalence in focal hosts (Ostfeld and Keesing 2000, 2012). A second line of research expanded on this by assessing how changes in the diversity of hosts with different competence affect non-vector borne diseases with density-dependent transmission. Most of this work has been conducted with free-living trematode cercarial stages infecting tadpoles and has indicated that less competent hosts can act as decoys for infective stages, thereby lowering infection levels in the main competent host (Johnson et al. 2008, Johnson et al. 2013). Finally, a third line of research has been focussing on how non-hosts (i.e. organisms which do not serve as competent host or less competent decoy and thus do not become infected) can interfere with the transmission of free-living infective stages (Thieltges et al. 2008, Johnson and Thieltges 2010). This interference can, for example, occur in the form of non-hosts acting as physical traps for or preying on free-living infective stages and in doing so, lower the pool of infective stages and reduce infection levels in focal hosts (Johnson and Thieltges 2010, Johnson et al. 2010). This transmission interference is probably widespread and does not only occur in free-living infective stages of macroparasites but also during the transmission from one host to the next of microparasites such as viruses (Welsh et al. submitted).

While there is no doubt that many organisms can affect disease transmission and dynamics via the mechanisms discussed above, there has been a debate about whether a reduction of disease risk with an increase in diversity (dilution effect) is a

universal phenomenon or whether diversity effects are rather idiosyncratic (Randolph and Dobson 2012, Salkeld et al. 2013, Ostfeld and Keesing 2013, Lafferty and Wood 2013, Wood and Lafferty 2013, Civitello et al. 2015, Johnson et al. 2015). In the case of vector-borne diseases such as Lyme disease, dilution effects have been reported from several disease systems (Ostfeld and Keesing 2012) but the mechanisms linking biodiversity and disease risk for focal hosts may often be more complex and include habitat changes, host densities and spatial scales, resulting in reductions as well as in amplification of disease risk depending on the specific circumstances (Wood and Lafferty 2013). Studies on the effects of differential host competence on disease risk in parasites with density-dependent transmission have capitalised on the fact that in this case experimental manipulations are much easier to realise. In addition, the experiments also allow the separation of real diversity effects from density effects (simply increasing the density of a host species may have the same effect as increasing diversity). For example, experimental manipulations of amphibian host diversity and density found a decrease in the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) at increased richness of hosts with differential competence, independent of host density (Searle et al. 2011). Similarly, experiments with the trematode *Ribeiroia ondatrae* infecting amphibians showed a decline in tadpole infection levels in the focal host in the presence (and at the same total host density levels) of another amphibian species with lower host competence (Johnson et al. 2008). For the third type of encounter reduction, the removal of infective stages by non-hosts, experimental evidence of diversity effects is, to date, very limited. In mesocosm experiments with trematode cercariae infecting tadpoles, diversity effects of odonate larvae preying on cercarial stages were observed but depended on odonate density, however, they were probably not only the result of parasite removal but also of predation on focal hosts and non-consumptive predator effects (Rohr et al. 2015). Many other studies have shown that a multitude of non-host species can remove infective stages of a large range of parasite groups (for a review see Thieltges et al. 2008b) and also that this removal by non-hosts is density-dependent (Thieltges et al. 2009, Rohr et al. 2015). However, whether the addition of other non-hosts results in real diversity-mediated effects and not just density effects is little studied. In part, this is probably related to the methodological difficulties in conducting meaningful comparisons of non-hosts of very different morphologies, sizes and removal mechanisms (Johnson and Thieltges 2010). This scarcity of studies hampers our understanding of the mechanisms underlying the general relationship between biodiversity and disease (Johnson et al. 2015).

In this study we borrowed an experimental approach , named response surface design, from general community ecology to overcome methodological issues in studying diversity effects of non-hosts of different taxa with different parasite removal mechanisms. Response surface design experiments incorporate two different competitive species at various densities (Inouye 2001). The design enables inter and intra-species interactions to be statistically tested and thus the effects of species density can be disentangled from species diversity. For our laboratory experiments, we used cercariae of a common marine trematode species (*Himasthla elongata*) as our focal parasite. This species uses periwinkles (*Littorina littorea*) as first intermediate host from which cercariae are released into the water column and then infect a second intermediate host as metacercarial cysts (second intermediate hosts are bivalves such as the blue mussel *Mytilus edulis;* definitive hosts are birds; Werding 1969). Infection levels in the second intermediate are dose-dependent, i.e. the number of infective stages a host is exposed to is positively correlated with the resulting infection level (Liddell et al. 2017). Metacercarial infections in the second intermediate host result in reduced fitness in form of, among others, lower condition, filtration rates and growth and these negative effects are generally considered to be density-dependent (i.e. the more metacercariae the stronger the effect; Thieltges 2006, Stier et al. 2015). Hence, in this system any increase or reduction in the number of infective stages by non-hosts directly translates into higher or lower infection levels and associated disease risk for the down-stream host. To study the effect of non-host diversity on parasite removal we used three non-host species from widely different taxa common in coastal waters which have been shown to interfere with cercarial transmission via different removal mechanisms: the predatory shore crab *Carcinus maenas*; the filter feeding Pacific oyster *Crassostrea gigas* and a physical trap in form of the seaweed *Sargassum muticum*. As response, instead of using the infection levels in down-stream hosts to identify diversity effects related to parasite removal, we determined the number of remaining free-living parasite stages for the different non-host combinations. and thus the results were not confounded by predation on hosts or non-consumptive effects such as behavioural changes of parasites and down-stream hosts in presence of non-hosts. Our study illustrates a promising experimental approach to study diversity effects on parasite removal by non-hosts from divergent taxa with different morphologies and parasite removal mechanisms.

Materials and methods

Experimental organisms

To obtain sources of cercariae, we collected periwinkles (*Littorina littorea*) in the intertidal in the vicinity of the NIOZ Royal Netherlands Institute for Sea Research on Texel (Wadden Sea, The Netherlands). Snails infected with *Himasthla elongata* were identified by shedding trials (release of cercariae under light and heat), kept in aerated flow-through aquaria and fed with sea lettuce (*Ulva lactuca*). For the experiments, we obtained cercariae from infected snails by incubating approximately 30 snails under light at 27°C in 3 L of seawater for 3 hours. The required amount for cercariae was then pipetted within one hour and administered to the experimental units (i.e. cercarial age was no more than 4 h at the start of the experiment).

Three non-host species which all coexist in the same habitats in the study area were used: oysters which are sessile filter feeders, crabs which are motile active predators and seaweed, which forms a physical barrier or maze-like structure throughout the water column. The sizes of non-hosts reflected common size ranges observed in the field: Pacific oysters (*Magallana* (previously *Crassostrea*) *gigas;* 10.51 ±1.2 cm diameter), shore crabs (*Carcinus maenas;* 3.1 ± 0.3 cm carapace width), and seaweed (*Sargassum muticum;* branches of individual plants). All three species were previously identified as interfering with *H. elongata* transmission (Welsh et al. 2014) and were collected from the intertidal area along the eastern coast of the island of Texel (Wadden Sea, Netherlands). Immediately after collection, any epibionts were carefully removed and all test organisms were housed in aerated flow through aquaria in the same climate chamber at 15°C. Crabs were fed on a diet of mussels while oysters were fed algal bivalve feed (*Isochrysis galbana*, Instant Algae by Reed Mariculture Inc. USA; 4.1 billion cells ml^{-1}; administered as 4 drops of algal feed per oyster, as recommended by Reed Mariculture).

Experimental design

To test for the effects of non-host diversity on the removal of cercariae we used a two-factorial response surface design, with two different non-host species and four density levels. This design combines both additive (varying non-host diversity but also density at the same time) and substitutive (varying diversity but keeping density constant)

designs and allowed us to separate diversity from density effects as well as identify potential interactive effects between both factors (Inoye 2001; Fig. 7.1). Density levels of the three non-host species reflected realistic densities observed by us in the field and were as follows: oysters (0, 1, 2, 6 ind.), crabs (0, 1, 2, 3 ind.), and seaweed (0, 5, 15, 30 g fresh weight after drying on a paper towel). The treatment with zero densities of both non-host species served as a control for potential background losses of cercariae.

All experiments were carried out in a temperature- and light-controlled room (18.0°C ± 0.2°C; 10:14 hour light/dark cycle) in three successive runs. Each experimental run tested two different non-host species at four density levels and each treatment was replicated four times (i.e. 64 replicate units in total per run; Fig. 7.1). Each of the replicate units consisted of a 2 L aquarium with 1500 ml of filtered seawater. To allow for acclimation, all test organisms were starved and kept in the experimental containers for 24 hours. At the start of the experiment, 100 cercariae were added to each experimental unit and the aquaria were left undisturbed for the following three hours. After three hours, the experiment was terminated by quickly removing all non-hosts with forceps. The water from each experimental unit was filtered through a 25μm sieve to retain any remaining cercariae, the units were then flushed with filtered seawater and sieved a further two times to reduce chances of cercarial adhesion to the walls of the units. Subsequently, the cercariae were washed from the sieve and fixed using 10 ml of 96% ethanol and stained using Rose Bengal. After a minimum of 24 hours to allow sufficient staining, all cercariae were counted in Petri dishes under a stereo microscope.

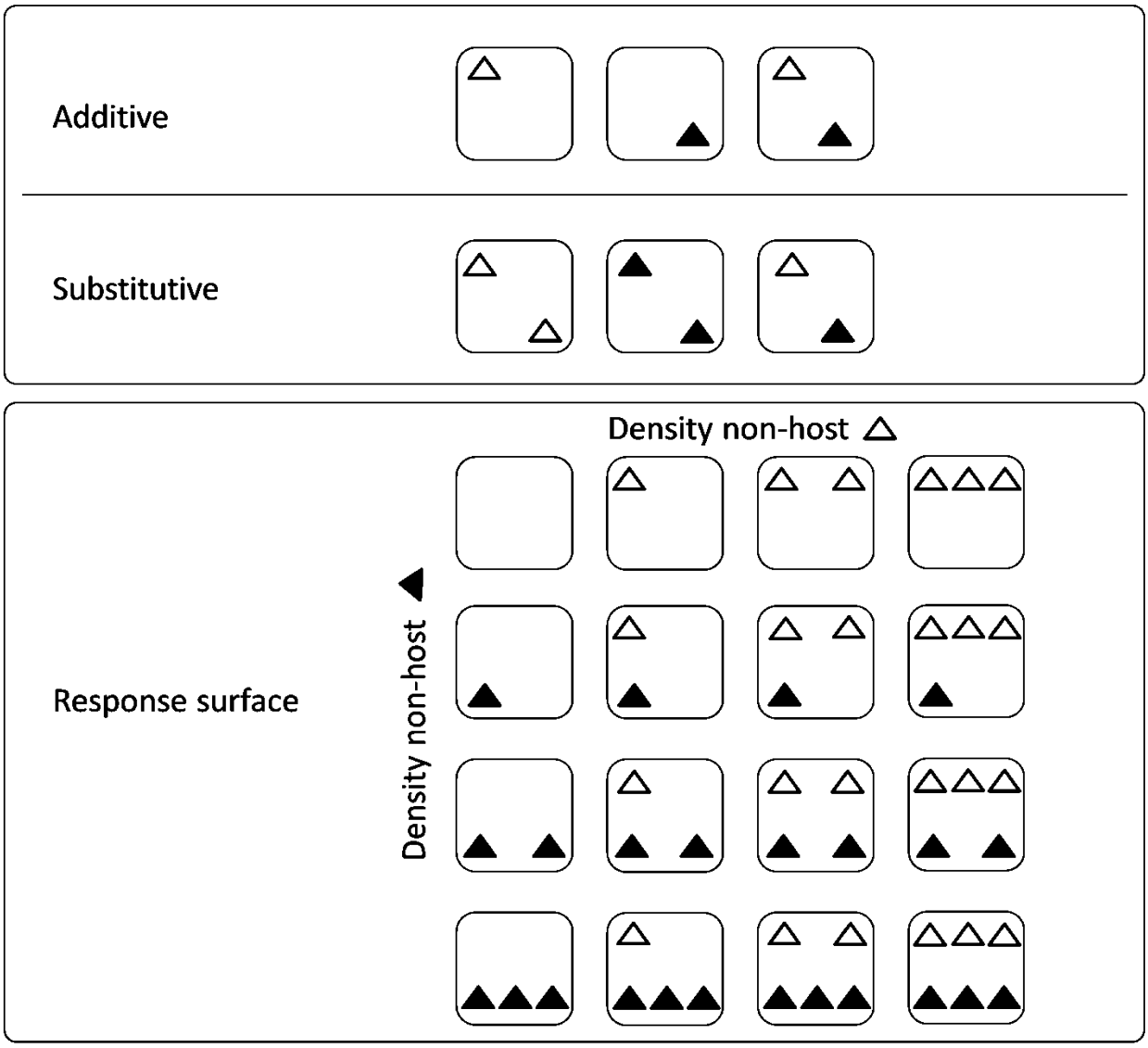

Fig. 7.1 Differences between additive and substitutive experimental designs (above) and the response surface experimental design used in this study (below). The white and grey symbols indicate two different non-host species, the small blue rectangles individual experimental units. In our experiments, we used a two-factorial response surface design, with two non-host species and four density levels each.

Statistical analyses

To visualise the results, we plotted the mean absolute numbers of cercariae remaining per treatment combination by placing the different density levels of one of the two non-host species on the x-axis and separating the different densities of the second non-host species into four different line series. Although combining data points with lines in a categorical design is usually not appropriate, this graphical depiction allowed for an easy visualisation of potential interactions between the two main factors. Parallel lines would suggest additive effects of a second non-host species while crossing or diverging lines would indicate interactive effects of the two non-host species on cercarial removal.

The effects of non-host species on cercarial removal was investigated using binomial Generalized Linear Models (GLMs) with a log-link. We assumed a *linear pure death process* (i.e. each cercarial removal by non-hosts is an independent event) so that the number of cercarial stages remaining at the end of the experiment is binomially distributed, with a probability of cercariae surviving until the end being equal top=$e^{-\theta t}$

where θ is the rate at which cercariae are removed per unit of experimental time, and hence t=1. This cercarial removal rate was assumed to be a function of non-host diversity, density and the interaction between both, hence

$$\theta=\mu+\alpha_i+\beta_j+\gamma_{ij}$$

where α_i represents the effect of the first non-host at the i^{th} density, β of the second non-host, and γ_{ij} their interaction.

We then fitted a series of GLMs from the most complex to the least complex model (for an illustration of the model selection procedure see Fig. S 7.1). In the most complex model all explanatory variables were included (including the interaction) while the simplest model only contained the intercept (null model). We identified the best fitting model by testing for significant differences between models of decreasing complexity using Analysis of Deviance. To illustrate the procedure, the most complex model was tested against the next less complex model (including the effects of both species but not their interaction). The difference in deviance between the two models was then divided by the dispersion factor (φ) and compared to the delta degree of freedom χ^2 at 0.05 to identify statistical significance. The dispersion factor was calculated by dividing the residual deviance for the most complex model by the degrees of freedom (Δ Dev/φ). A significant difference between two models indicated a better fit of the

more complex model. Using the model coefficients and unique estimates of intercepts for each of the factors included in the best fitting model, we calculated cercarial removal rates and parasite survival (%). All analyses were carried out using R (R Development Core Team, 2019) version 3.0.2 in R Studio (version 1.2.1335; RStudio, 2018).

Results

For all three combinations of non-host species, the model fitting the data best included the interaction term, thus the effect of a specific non-host species on cercarial removal depended on the presence and density of the other non-host species (Table 7.1 and Fig. 7.2; Fig. S 7.1). When plotting the number of remaining infective stages for both non-host species (Fig. 7.2), diverging, converging and crossing of the lines denoting the different densities of the second non-host species occurred. Hence, depending on the non-host species combination, the presence of a second non-host species resulted in a neutralisation, amplification or reduction of the parasite removal effects exerted by the first non-host species.

In the experiment using crabs and seaweed, the number of infective stages remaining at the end of the experiment decreased with increasing crab and seaweed density (Fig. 7.2 A). At low densities of crabs, the addition of seaweed to the experimental units had an additive effect (suggested by the roughly parallel lines), while at higher crab densities survival of cercariae strongly decreased with increasing seaweed densities (diverging lines; Fig. 7.2 A). The survival of cercariae was lowest (28 %) and thus the total removal rate by the non-hosts highest (1.26) in the treatment with the highest crab and seaweed density levels (Fig. 7.2 A; Table S 7.1). In absence of seaweed, increasing crab densities lead to a decrease in the number of cercariae remaining, however at the highest crab density level (3 crabs), cercarial survival increased again, leading to a trough-shaped curve of the numbers of cercariae remaining (Fig. 7.2 A; Table S 7.1). In absence of crabs, cercarial survival decreased with increasing seaweed density, however, at the highest seaweed density level (30 g) cercarial survival was relatively similar to the one observed at the second highest density level (73% and 76%, respectively; Fig. 7.2 A; Table S 7.1).

In the experiment investigating the effects of seaweed and oysters, the number of infective stages remaining at the end of the experiment decreased with increasing seaweed density in absence of oysters (Fig. 7.2 B). Likewise, when seaweed was absent, cercarial survival decreased with increasing oyster densities (Fig. 7.2 B). However, when oysters were added to experimental units where seaweed was present, total removal rates were lower than in oyster only treatments. In addition, the difference in total cercarial removal between seaweed only and mixed treatments decreased with increasing seaweed densities (converging lines; Fig. 7.2 B). At the highest seaweed density, the three treatments with oysters showed similar cercarial survival, of approximately 60%, as the seaweed only treatment (Fig. 7.2 B; Table S 7.2).

Finally, in the experiment investigating oysters and crabs we observed a traversing of the different lines (Fig. 7.2 C). In absence of crabs cercarial survival decreased with increasing oyster density, however, the addition of crabs to the experimental units resulted in a much lower effect of oyster density (Fig. 7.2 C). While cercarial survival decreased with increasing crab density in absence of oysters, this patterns was fully reversed at the highest oyster density (6 ind.), with cercarial survival increasing with increased crab density and thus the highest cercarial survival was observed at the highest crab density (Fig. 7.2 C; Table S 7.3). Alike the experiment with crabs and seaweed, cercarial survival in absence of the second non-host species decreased with crab density but then, albeit less strongly, increased again at the highest crab density (63%) compared to the survival at the intermediate crab density (60%; Fig. 7.2 C; Table S 7.3). Such slight differences of cercarial removal rates/survival at the same density levels in the treatments without the second non-host species were observed in all three experiments, however, the general removal patterns were similar among the different runs (Fig. S 7.2).

Table 7.1 Model selection results, showing the degrees of freedom (df) and deviances for each model from the most complex (model 1) to the simplest model (model 5) for each non-host species combination. The best model in all three cases was the most complex model which included the first non-host species (X_1), the second non-host species (X_2) and an interaction term (X_1:X_2). The dispersion factor (φ) for the best fitting model for each non-host species combination is shown. For details of model selection procedures see text and Fig. S 7.2.

Model code	**Model**	**df**	**Deviance**		
			Crabs and seaweed	**Seaweed and oysters**	**Oyster and crab**
1	$X_1+X_2+X_1:X_2$	48	130.7	840.1	183.1
2	X_1+X_2	57	306	1034.7	638.8
3	X_1	60	615.3	1873.3	683.4
4	X_2	60	1090.3	1232.3	1302
5	1	63	1373.9	1930.5	1355.5
φ from best fitting model			2.72	17.5	3.81

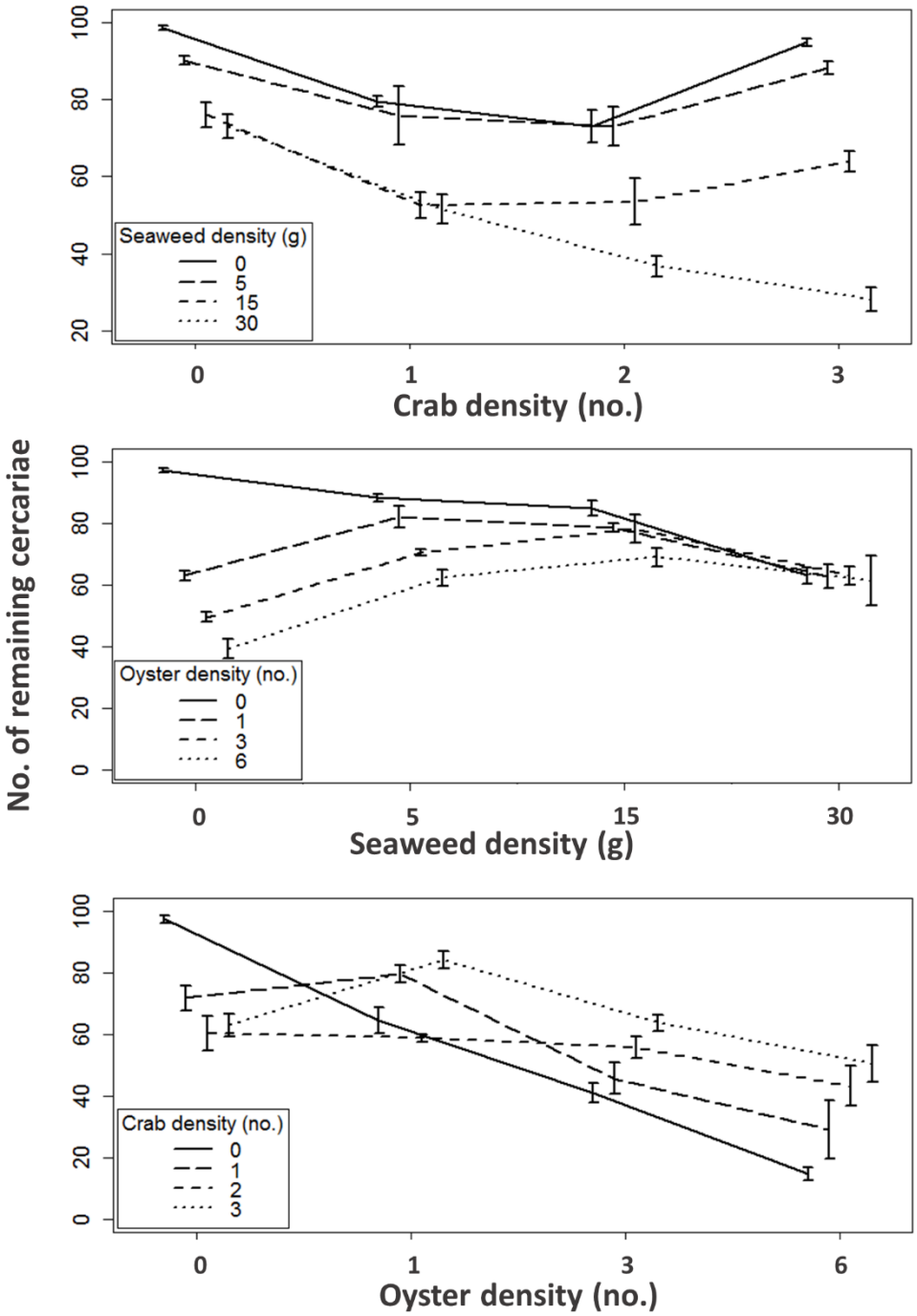

Fig. 7.2. Number of infective stages remaining (± SD) after exposure to combinations of two non-host species at different densities in response surface design experiments with A) crabs and seaweed, B) seaweed and oysters, and C) oysters and crabs. Each treatment combination was replicated four times, i.e. total N=64 per experiment.

Discussion

In all three experiments, total parasite removal by the non-host community was a function of the interactive effects between the two non-host species, providing evidence for diversity effects of non-hosts on parasite removal. However, adding a second non-host species to the experimental units did not result in a universal increase of parasite removal rates with non-host diversity, thus contradicting the as expected reduction in disease risk suggested by dilution effects. Instead, increasing non-host diversity led to a neutralisation, amplification or reduction of the parasite removal effects exerted by the first non-host species, depending on the non-host species combination. This suggests that diversity effects in respect to parasite removal by non-hosts are idiosyncratic and strongly conditional on the respective non-host combinations.

These idiosyncratic effects of non-host diversity on parasite removal probably resulted from differences in the interactions among non-host species arising in the different non-host combinations. Adding a second non-host species to experimental units is likely to affect the behaviour of one or both non-host species, with potential effects on their parasite removal rates. In the case of the crab-seaweed combination, it is likely that the addition of seaweed to the experimental units allowed the crabs to move through the seaweed matrix so that they could access cercarial stages, which were swirling up and down through the entire water column, that would otherwise be unreachable if no seaweed was present (crabs remove cercariae via their mouth parts and gills; Welsh et al. 2019). As higher densities of seaweed provided a denser matrix for the crabs, which themselves removed cercariae in a density-dependent fashion, removal rates in the combined treatments increased with increasing crab and seaweed densities and community removal rates were highest in the treatments with the highest crab and seaweed densities. Hence, the addition of seaweed strongly amplified the parasite removal effect exerted by crabs alone. In contrast, in the oyster-seaweed combination, the matrix created by the seaweed did not result in increased parasite removal by oysters, instead with an increase in seaweed density the seaweed probably trapped more and more cercariae so that fewer cercariae made it to the bottom of the experimental units where the oysters were positioned (oysters remove cercariae by filtration; Welsh et al. 2019). Hence, in this case, the addition of seaweed lead to a neutralisation of the parasite removal effects exerted by oysters alone. Finally, in the case of the oyster-crab combination, the addition of crabs, a known predator of Pacific oysters (Mascaro and Seed, 2000; Mascaro and Seed, 2001), lead to a reduction of the parasite removal effects exerted by oysters alone, possibly because the movements of

crabs throughout the experimental units disturbed the oysters inducing valve closure and thus reduced their filtration activity. This interference by crabs is likely to increase with crab density and this matches the observation that at the highest seaweed density cercarial survival was highest at the highest crab density, thus reversing the pattern of highest cercarial survival at lowest crab densities when seaweed was absent. We acknowledge that further experiments will be needed to verify the suggested interactions, however, it is plausible that different behavioural changes initiated by the addition of a second non-host species underlie the idiosyncratic diversity effects observed in our experiments. In addition, to interspecific interactions, intraspecific interactions may have further modified parasite removal rates of non-hosts. For example, crabs showed slightly lower parasite removal rates at high compared to intermediate crab densities (albeit less pronounced in one of the two experiments with crabs). This may have resulted from intra-specific interactions among crabs which are known to show aggressive display and fighting behaviour in presence of conspecifics which can lead to reduced predation rates due to interference competition (Smallegange et al. 2006). Similar interference interactions may have occurred in our experiments leading to a reduction in parasite removal rates at high crab densities. Such intra-specific interactions may also have an individual component as individual crabs can exhibit different competition strength (Sneddon et al. 2000) which may explain the slight variation in removal rates at the highest crab densities between the two experiments involving crabs. Whatever the exact mechanisms, the experiments clearly revealed that diversity effects of different directions were present in the three non-host combinations.

The different diversity effects on parasite removal translate into different consequences for the down-stream hosts of the parasite. Infection levels in the second intermediate hosts of the parasite species used in our experiments are dose-dependent (Liddell et al. 2017), thus any alteration of the number of infective stages in the locality of a down-stream host will affect infection level and, as metacercarial infections are intensity-dependent (Thieltges 2006), also alter disease risk. Hence, the amplification of parasite removal rates observed in the crab-seaweed combination can be expected to lead to a reduction in infection levels and the associated disease risk in down-stream hosts. In contrast, in the seaweed-oyster combination, total parasite removal rates of oysters were only increased at low seaweed densities but neutralised at high seaweed densities. This would suggest a reduced infection level at lower seaweed density levels but a similar risk at high density levels. Finally, in the case of the oyster-crab combination, the reduced cercarial removal rates in combinations of crabs and oysters compared to oysters only treatments suggest that diverse communities would lead to

increases in infection levels in down-stream hosts as more infective stages remain in the surroundings. Hence, depending on the non-host species combination, down-stream hosts are likely to experience very different parasite pressures and the different diversity scenarios will either increase or decrease the disease risk for the down-stream hosts. This conclusion seems to contradict with findings from meta-analyses based on published studies on diversity effects on disease risk which found evidence for the generality of dilution effects among diverse host and disease systems (Civitello et al. 2015; Huang et al. 2017). However, the database underlying these analyses included a diversity of studies, many of which only contrasted the addition of a diluting species with a control but did not really investigate diversity effects of more than one single diluter. In addition, most of these studies were not designed to disentangle diversity from density effects. The few studies that did include measures of diversity versus density effects are surprisingly rare and paint a more diverse picture. Studies that investigated diversity effects of hosts of differential competence found diversity effects for both fungal pathogens and trematodes infecting amphibian hosts (Johnson et al. 2008, Searle et al. 2011). In contrast, substitutive designed experiments (varying diversity while keeping density constant; Rohr et al. 2015) on cercarial predation by three larval odonate species did not reveal diversity effects . However, in a mescosom experiment of the same study which included snails as sources for cercariae, the odonate cercararial predators and the down-stream hosts, diversity effects were observed, albeit depending on odonate density (Rohr et al. 2015). In this case, the resulting dilution effect was not only the result of parasite removal but also of predation on focal hosts by some of the odonate species and non-consumptive predator effect on odonate and host behaviour (Rohr et al. 2015). This suggests that the addition of hosts to experimental units adds yet another layer of diversity-mediated effects, further suggesting that diversity-disease relationships are probably highly conditional on the disease system at hand.

With such a complexity of factors already observed modifying the relationships between diversity and disease in relatively simple experimental settings, the question arises to what extentcan diversity effects also be observed in the field. Ensuing the parasite-host system investigated in our experiments, large-scale investigations of the correlates of infection levels in blue mussels (*Mytilus edulis*) in our study area did not reveal evidence for dilution effects of oysters on infections of *Himasthla elongata* in mussel hosts living on mixed mussel and oysters beds (Goedknegt et al. 2019). However, this does not mean that disease-mediated effects do not occur in this system

in the wild as field experiments have shown a decrease in infections in mussels in the presence of oysters (Thieltges et al. 2009). Instead it is more likely that the complexity of species interactions with direct and indirect effects on parasite transmission hampers the detection of specific diversity effects. This may be exacerbated in marine ecosystem where high parasite dispersal occurs which may lead to additional mediating effects such as those caused by tides, ocean currents and other physical dynamics . Studies in much more closed ecosystems such as freshwater lakes and wetlands have been more successful in finding some evidence for diversity-mediated reductions in parasite infections levels (Lagrue and Poulin 2015, Rohr et al. 2015). In contrast to these findings, a meta-analysis of field studies on the relationship between diversity and zoonotic diseases in terrestrial ecosystems only found weak and idiosyncratic diversity effects (Salkeld et al. 2013). However, whether there really are differences in the relevance and strength of diversity-disease relationships among major realms remains to be investigated. In any case, more studies from different hosts and disease systems, ideally combining experimental and correlative field approaches, are needed to identify general patterns in the direction and strength of diversity effects on disease rick in different ecosystems.

Conclusions

Our experiments unanimously revealed non-host diversity effects on parasite removal. However, diversity effects did not generally result in a dilution effect but the alteration direction of disease risk depended on the non-host species combination. This conditionality of diversity effects probably resulted from behavioural changes in non-hosts initiated by the presence of another individual or another species. Given the likelihood of complex species interactions in diverse communities, diversity effects on parasite removal are probably generally idiosyncratic and thus response surface experimental designs are a promising approach to unravel the underlying mechanisms.

Acknowledgements

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Supplementary material

Table S 7.1. Removal rates and cercarial survival (%) for each diversity and density combination of seaweed and crabs as extracted from the best fitting model (model 1; see Table 7.1). X_1 and X_2 refer to the factor coding used in the model selection procedure (see Fig. S 7.2). N= 4 per treatment combination.

Non-host density			
Seaweed (X_1)	**Crabs (X_2)**	**Removal rate**	**Cercarial survival (%)**
0	0	0.02	98.5%
0	1	0.23	79.5%
0	2	0.31	73.0%
0	3	0.05	94.8%
5	0	0.11	90.0%
5	1	0.28	75.8%
5	2	0.31	73.0%
5	3	0.13	88.0%
15	0	0.27	76.0%
15	1	0.64	52.5%
15	2	0.63	53.5%
15	3	0.45	64.0%
30	0	0.31	73.0%
30	1	0.66	51.5%
30	2	1.00	36.8%
30	3	1.26	28.3%

Table S 7.2. Removal rates and cercarial survival (%) for each diversity and density combination of oysters and seaweed as extracted from the best fitting model (model 1; see Table 7.1). X_1 and X_2 refer to the factor coding used in the model selection procedure (see Fig. S 7.2). N= 4 per treatment combination.

Non-host density			
Oysters (X_1)	**Seaweed (X_2)**	**Removal rate**	**Cercarial survival (%)**
0	0	0.03	97.0%
1	0	0.46	63.0%
2	0	0.70	49.5%
6	0	0.94	39.3%
0	5	0.12	88.3%
1	5	0.20	82.0%
2	5	0.35	70.5%
6	5	0.47	62.3%
0	15	0.17	84.8%
1	15	0.24	78.5%
2	15	0.25	78.3%
6	15	0.37	69.0%
0	30	0.46	63.0%
1	30	0.47	62.8%
2	30	0.46	63.0%
6	30	0.49	61.3%

Table S 7.3. Removal rates and cercarial survival (%) for each diversity and density combination of oysters and crabs as extracted from the best fitting model (model 1; see Table 7.1). X_1 and X_2 refer to the factor coding used in the model selection procedure (see Fig. S 7.2). N= 4 per treatment combination.

Non-host density			
Oysters (X_1)	**Crabs (X_2)**	**Removal rate**	**Cercarial survival (%)**
0	0	-0.03	97.3%
0	1	-0.33	71.8%
0	2	-0.51	60.3%
0	3	-0.46	63.0%
1	0	-0.44	64.5%
1	1	-0.23	79.5%
1	2	-0.53	58.8%
1	3	-0.17	84.0%
2	0	-0.89	41.0%
2	1	-0.78	45.8%
2	2	-0.58	55.8%
2	3	-0.45	63.8%
6	0	-1.91	14.8%
6	1	-1.23	29.3%
6	2	-0.84	43.2%
6	3	-0.68	50.5%

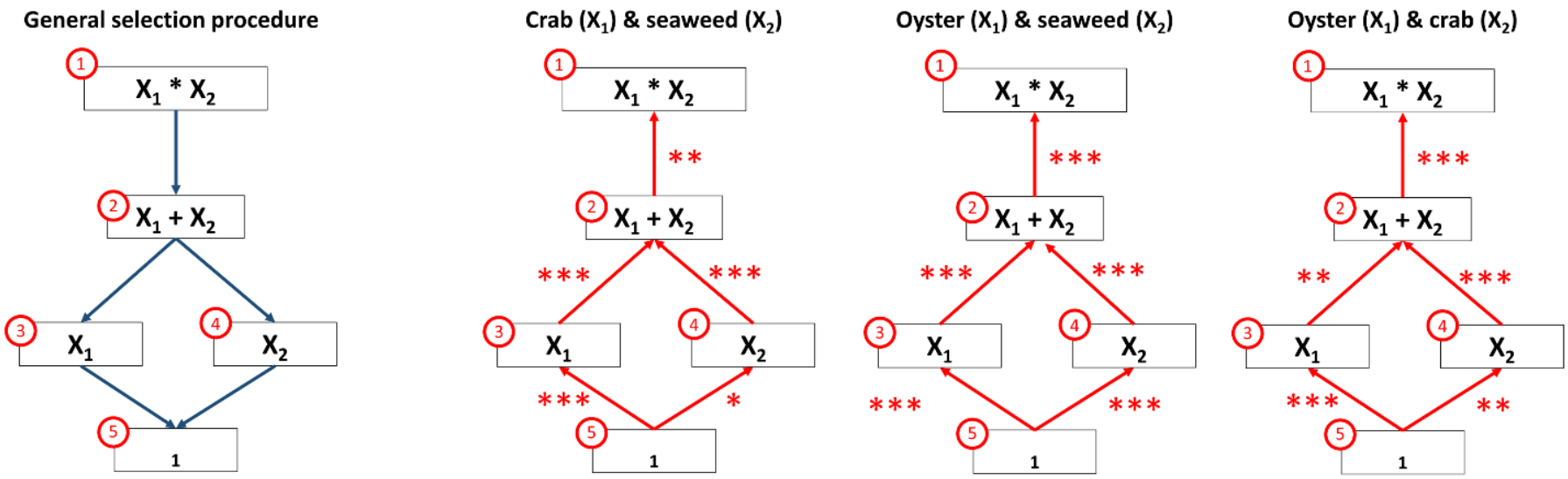

Fig. S 7.1. A) General model selection procedure followed for testing for significant differences between the different models relating the two non-host species (X_1 and X_2), starting with the most complex model (1) down to the simplest model (5), and the respective pathways and significance results for each of the three non-host combinations: crabs and seaweed, seaweed and oysters, and oysters and crabs. Asterisks denote significance levels: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1; ' ' 1. For details see main text.

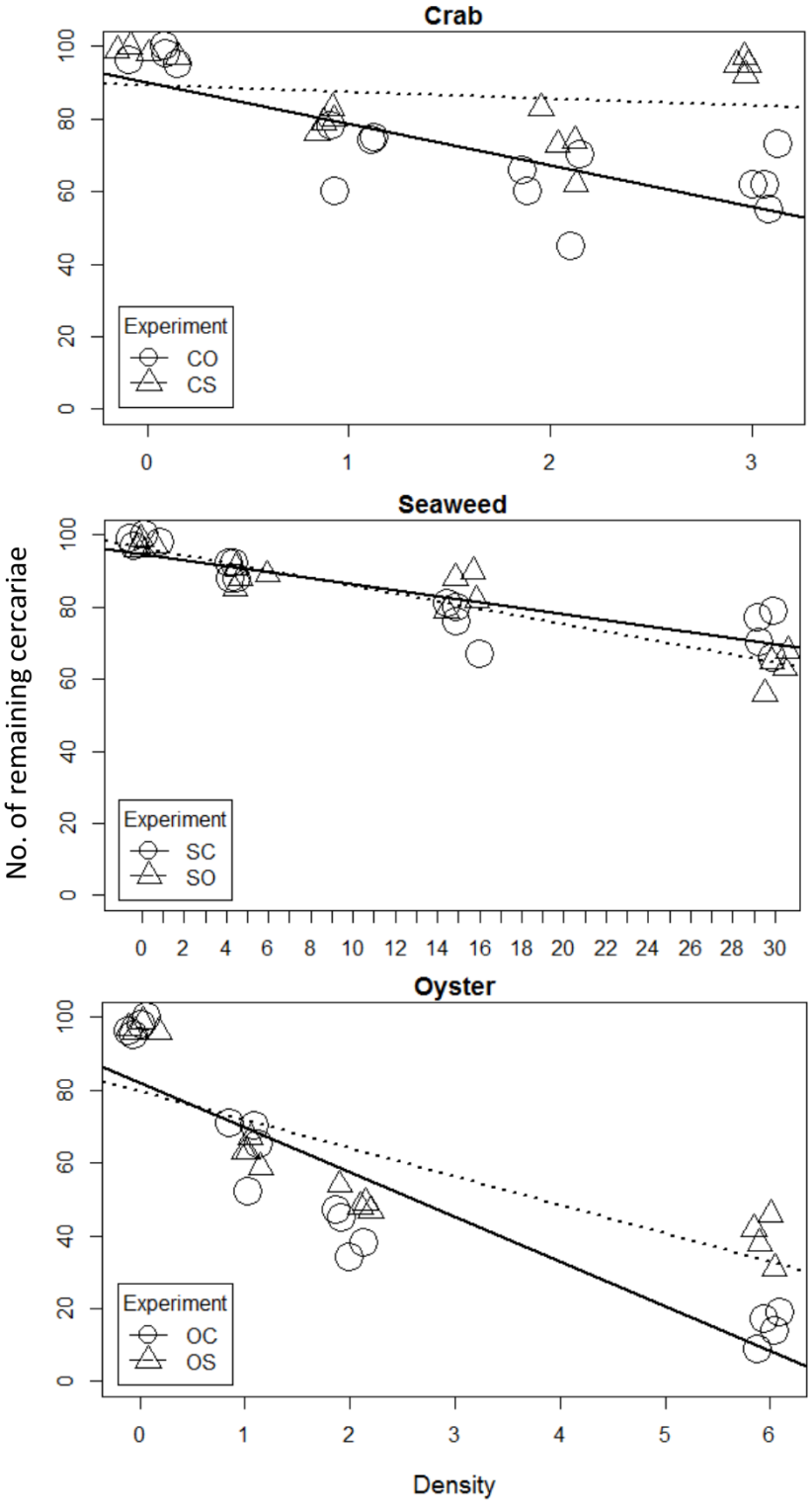

Figure S 7.2. Number of remaining cercariae at different densities of the three non-host species in absence of the second non-host in the two different experiments conducted per non-host species. Shown are the individual replicates per non-host density level and best fit regression lines for the means of each combination.

Marine virus predation by non-host organisms

Jennifer E. Welsh, Peter Steenhuis, Karlos Ribeiro de Moraes, Jaap van der Meer, David W. Thieltges and Corina P. D. Brussaard

Abstract

Viruses are the most abundant biological entities in marine environments, however, despite its potential ecological implications, little is known about virus removal by ambient non-host organisms. Here, we examined the effects of a variety of non-host organisms on the removal of viruses. The marine algal virus PgV-07T (infective to *Phaeocystis globosa*) can be discriminated from bacteriophages using flow cytometry, facilitating its use as a representative model system. Of all the non-host organisms tested, anemones, polychaete larvae, sea squirts, crabs, cockles, oysters and sponges significantly reduced viral abundance. The latter four species reduced viral abundance the most, by 90, 43, 12 and 98% over 24 h, respectively. Breadcrumb sponges instantly removed viruses at high rates (176 mL h^{-1} g tissue dry wt^{-1}) which continued over an extended period of time. The variety of non-host organisms capable of reducing viral abundance highlights that viral loss by ambient organisms is an overlooked avenue of viral ecology. Moreover, our finding that temperate sponges have the huge potential for constant and effective removal of viruses from the water column demonstrates that natural viral loss has, thus far, been underestimated.

Submitted manuscript

Introduction

Viruses are the most numerically abundant entities in the oceans with an estimated abundance up to 10^8 mL^{-1} (Wigington et al. 2016). Via infection and mortality of their microorganism hosts, viruses have the ability to regulate not only host population dynamics but also to drive biogeochemical cycling and carbon sequestration within marine systems (Suttle 2007, Brussaard et al. 2008, Danovaro et al. 2011, Weitz and Wilhelm 2012, Mojica et al. 2015). As lytic virus infections of microbial hosts inevitably result in the death of the host cell, any decay of infectious virus particles is likely to have important repercussions for hosts as it results in lower encounter rates and thus reduced infection levels. Despite marine viruses influencing fundamental biological processes (Suttle 2007, Brussaard et al. 2008), research investigating virus decay (e.g. loss of infectivity) and particle loss has primarily focused on abiotic factors such as UV radiation and temperature (loss of infectivity) and adhesion to clay particles and aggregates (loss of viral particles) (Lipson and Stotzky 1985, Wommack and Colwell 2000, Brussaard et al. 2005, Syngouna and Chrysikopoulos 2010, Mojica and Brussaard 2014). However, biological factors resulting in removal of virus particles from the water column is still understudied (Fig. 8.1). Marine heterotrophic nanoflagellates have been reported to graze on viruses, albeit at a relatively low rate of 0.1% of the viral population day $^{-1}$, or a clearance rate of around 4% (Suttle and Chen 1992, González and Suttle 1993). Furthermore, the Red Sea sponge *Negombata magnifica* was reported to filter viruses with an average efficiency of 23% (Hadas et al. 2006). And recently the appendicularian stage of the pelagic *Oikopleura dioica* tunicate was reported to remove viruses at significant rates(Lawrence et al. 2018).

While such reductive effects of non-host organisms on marine viral abundance are still understudied, similar removal effects have been well demonstrated in other aquatic host-pathogen systems. For instance, infective stages of helminths are removed by a wide range of non-host organisms via predation and other mechanisms (Thieltges et al. 2008, 2009, Kaplan et al. 2009, Prinz et al. 2009, Welsh et al. 2014), with reductions in free-living infective stages reducing infection levels in downstream hosts (Liddell et al. 2017). The variety of non-host organisms causing the reductions is not only limited to active predators but also includes passive predators such as filter feeders and organisms creating physical barriers between the parasite and its host (Johnson et al. 2009, Welsh et al. 2014). Thus, non-host organisms are known to affect parasite transmission in macro parasite-host systems, a phenomenon known as transmission interference (Keesing et al. 2006, Thieltges et al. 2008, Goedknegt 2017, Welsh et al. 2017). In this study, we used various ecologically relevant non-host organisms to assess their effects on transmission interference on a microparasite-host system.

Specifically, we investigated the potential of a variety of pelagic and benthic marine non-host species, including bivalve filter feeders and decapod predators, to remove a marine algal virus from the surrounding seawater. We used the marine algal virus PgV, which is host specific and known to infect the bloom forming algae, *Phaeocystis globosa* (Baudoux and Brussaard 2005), and a selection of non-host organisms that are found in coastal areas where the algal host, virus, and non-host organisms coincide. In addition, the organism most proficient in reducing viral abundance was studied in more detail to determine if efficient removal of viruses could be sustained for a prolonged time. Understanding which organisms and to what extent non-host organisms regulate marine viral abundances is unreservedly important for more accurate predictions of the ecological impact viruses have on host population dynamics in the seas and oceans.

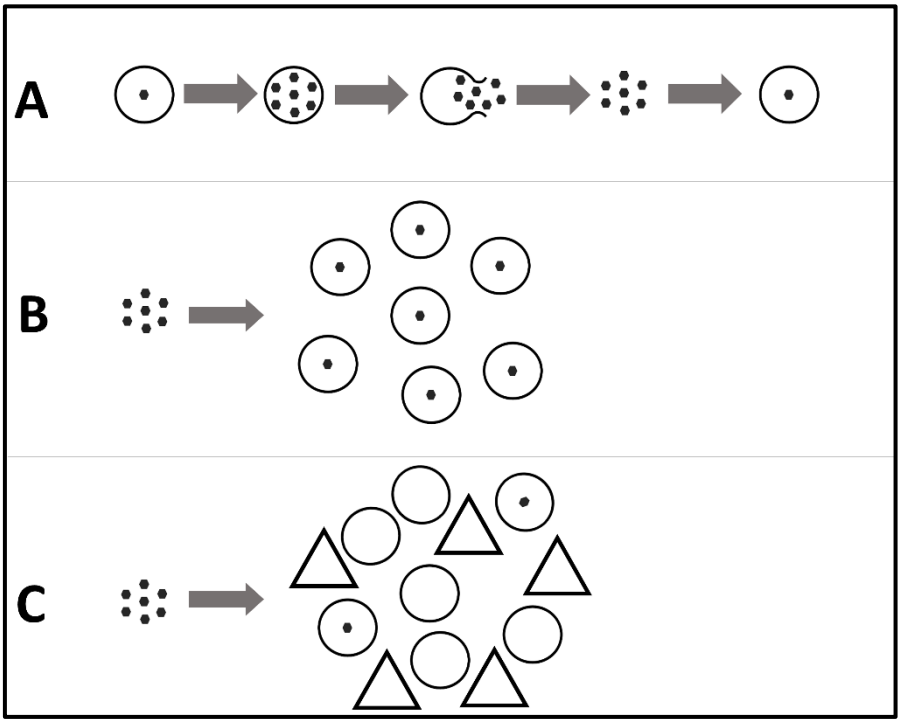

Fig. 8.1. Lytic viruses (⬣) infect and replicate inside their susceptible host (○) until the host lyses, releasing virus progeny into the surrounding environment where they can proceed to infect a new host (A). In a simple system these newly produced viruses are available to infect the available succeeding hosts (B). In complex systems, typically found in nature, viruses may be lost due to interactions with non-host organisms (△), resulting in reduced encounter rates and disease prevalence within the ecosystem (C).

Materials and methods

Viruses and non-host organisms

The marine phytoplankton virus PgV-07T (NIOZ culture collection (Baudoux and Brussaard 2005) was chosen as a model system because it can be clearly discriminated (and enumerated) from co-occurring bacteriophages using flow cytometry, based on fluorescence after staining with the nucleic acid-specific dye SYBR Green I (Brussaard 2004b). *Phaeocystis globosa* strain G (A) (culture collection of the University of Groningen, the Netherlands) is the host of the double stranded DNA virus PgV-07T (hereafter known as PgV; around 150 nm diameter and 470 nm kbp genome size (Baudoux and Brussaard 2005) and is an ecologically important primary producer with a wide distribution in temperate (coastal) seas during spring and summer (Lancelot and Billen 1984, Cadée and Hegeman 1986, 2002, Brussaard 2004a, Brussaard et al. 2005, Ruardij et al. 2005, Baudoux et al. 2006). Algal cultures were grown in Mix-TX medium (Maat and Brussaard 2016) at 15°C and a light:dark cycle of 16:8 h. Cultures were transferred weekly to keep the cells growing exponentially. The virus PgV was produced by infecting exponentially growing hosts and allowing complete lysis to occur during the following week. Prior to experimental use, the lysate was cleared from most of the lysed *P. globosa* cell debris by centrifugation (Eppendorf 5810R, Hamburg, Germany) at 2450 x g for 30 min at 15°C. The supernatant containing the PgV was carefully pipetted off and stored at 15°C until experimental use (max. 2 days). Filtration of the supernatant resulted in reduced PgV abundances in the filtrate and hence we chose to use unfiltered supernatant for our experiments. The infectivity of the PgV batches used for the experiments was determined by most probable number (MPN) endpoint dilution (Suttle 1993). Percentage infectious viruses (obtained by dividing the MPN abundance by the total abundance from flow cytometric analysis (Brussaard 2004b) was near 100%, ensuring that potential removal of PgV by non-host organisms was not a selective process for either infectious or non-infectious virus particles.

PgV was enumerated according to the protocol by Brussaard et al. (Brussaard 2004b) with modification as described by Mojica et al. (2014). In short, stored samples collected during the experiment were thawed and diluted in sterile 0.2 μm filtered TE buffer (10:1 Tris-EDTA, pH 8.2; Minisart high flow Syringe Filter, Sartorius A.G., Göttingen, Germany), stained with the nucleic acid-specific green fluorescent dye SYBR Green I (Invitrogen-Molecular Probes) for 10 min in the dark at 80°C, after which PgV was enumerated using a BD FACSCanto™ flow cytometer (BD Biosciences, USA).

The trigger was set on the green fluorescence for the detection of stained PgVs. Data were processed using FCS Express 4 software (De Novo Software).

Non-host organisms tested for their ability to reduce viral abundance were chosen based on their geographic distribution coinciding with that of the algal host-virus model system and included organisms of varying feeding mechanisms (filter feeders, predators, etc.) as well as range in size (small copepods to large oysters). Bivalves such as oysters, mussels and cockles are found in intertidal-subtidal areas and are considered bioengineers, altering substrate type, filtering vast amounts of water and removing particles <250 μm in size (Gosling 2003). Organisms such sponges, anemones and sea squirts are found on hard surfaces such as harbor walls and are typically subtidal but can also be found in some intertidal areas. Both sponges and sea squirts are capable of filtering large volumes of water (300 L h^{-1} and 200 mL min^{-1} respectively; Petersen and Riisgard 1992, Cebrian et al. 2006) and retaining nano-sized particles (Petersen and Riisgard 1992, Hadas et al. 2006, Petersen 2007, Maldonado et al. 2010). Polychaete larvae and copepods are pelagic, feeding on prey <53μm in size (Hansen et al. 2010). Copepods live in coastal and upwelling regions and switch prey between algae and ciliates under 60 μm in size with clearance rates of < 86 m1 d^{-1} (Jonsson and Tiselius 1990, Kiørboe et al. 1996). The species used, i.e. anemones (*Actinia equina*), barnacles (*Semibalanus balanoides*, attached to one valve of an empty mussel shell), cockles (*Cerastoderma edule*), crabs (*Carcinus maenas*), mussels (*Mytilus edulis*), oysters (*Magallana gigas*), sea squirts (*Styela clava*) and adult copepods (*Acartia tonsa*, >125 μm) and polychaete larvae (a mix of species, >125 μm) were all collected between spring and summer from the coastal area along the island of Texel (the Netherlands). Breadcrumb sponges (*Halichondria panicea*) for the first experiment (Exp. 1) were collected along the same coast, but to prevent impacting the local sponge community too much the sponges for subsequent tests were collected from the Oosterschelde (southern Netherlands) and transported to the NIOZ in cool boxes. After collection, all organisms were gently cleaned to remove any visible epibionts. Before being transferred to sterile 100 mL polystyrene pots for the experiments, the cleaned organisms were starved for 24 h in flow-through aquaria (80 x 40 x 40 cm) at 15°C with a light:dark cycle of 8:16 h.

Experimental set-up

Experiment 1: Removal of viruses when in the presence of non-host organisms

Experiment 1 (Exp. 1) assessed a variety of organisms for their ability to interfere with a marine virus-host transmission pathway by removing infectious virus particles from the water. Sterilized 100 mL polystyrene pots with screw cap (VWR International,

Leuven, Belgium) were used during the experiment as aquaria. Prior to the experiment the pots were sterilized using 6 M HCl for 1 h, followed by rinsing in deionized water and then finally washed in 90°C deionized water to remove any traces of HCl. The experiment consisted of two types of treatment: the first with the non-host organism (typically 1 individual per pot, except for barnacles which were 10 on an empty mussel valve, and copepods and polychaete larvae which had 16 individuals per pot) in 80 mL of the PgV lysate (around 1 x 10^6 mL^{-1}), and the second treatment with only the lysate to assess for adherence to the pots. In addition, an extra control with only the test organism in culture media was used to assess for the introduction of viruses by the test organism. As these controls indicated that no viral particles in the same size range as PgV were introduced, they were left out of further analysis. Each of the two regular treatments was replicated six times. All trials took place in a single climate room kept at 15°C. During the experiment, samples (1 mL) were taken using sterile pipettes (one for each replicate) and placed into 1.5 mL Eppendorf tubes containing 20 µL 25% glutaraldehyde (0.5% final concentration, EM-grade, Sigma-Aldrich, St. Louis, USA) for 30 min at 4°C, flash-frozen in liquid nitrogen and stored at -80°C until further analysis [26]. Samples were taken before the test organisms were added (pre-test or PT), 15 min after the animal were adapted to the pot (T0), after a period of 3 h (T3) and then after 24 h (T24), with the exception of crab, cockle, barnacle and mussel treatments where samples were taken before the test organisms were added (pre-test or PT) and after a period of 3 h (T3).

Experiment 2: Continuous clearance of virus by breadcrumb sponge

Breadcrumb sponges were shown to significantly decrease PgV abundance (Exp. 1) and so Experiment 2 (Exp. 2) was designed to assess the ability of the sponges to continuously remove PgV over a longer time period. The experimental set-up was similar to Exp. 1, but PgV (approx. 2 x10^6 mL^{-1}) was added ('spiked') at 20 min intervals for 6 h to avoid depletion of viruses by the sponges. Samples (1 mL) were taken using individual sterile pipettes prior to and immediately after adding viruses to the system to allow for the calculation of clearance rates. Treatments consisted of test organism spiked with PgV, and two controls (sponge spiked with growth medium to test for disturbance effect due to spiking, and PgV, spiked with PgV to obtain the ultimate PgV abundance without the sponge present). All treatments were replicated four times but due to the mortality of one sponge during the experiment, only three replicates were used for statistical analysis. Samples were processed and stored as in Exp. 1.

Statistical analyses

All statistical tests were carried out using R (R Core Team 2014). For Exp. 1, the effect of the presence of non-host organisms on changes in PgV abundance over time, was statistically tested by comparing changes in PgV abundance from one sampling time to the next, between the treatment where a non-host organism was present and the control treatment. A univariate analysis of variance (ANOVA) was used when samples were only taken at two time points (i.e., when there was only a single change observed between the start and end of the trial), whereas a multivariate analysis of variance (MANOVA) was used when samples were taken at more than two occasions (e.g. at the start, after 15 min, 3 h and then again after 24 h). The univariate test statistic is the F-value, the multivariate test statistic is Pillai's trace. Significances indicate that viral abundances over time differed among treatments. As there are only two treatments (with and without non-host organisms), the tests are in fact equivalent to the t-test and to Hotelling's T^2 test.

For Exp. 2, sponge clearance rates were calculated for 20 min intervals using the viral abundances directly before spiking and the viral abundance immediately after the aquaria were spiked with viruses. Sponge clearance rates were determined using the following formula:

$$C_l = (V/Kt)ln(N_0/N_t)$$

whereby C_l is volume of water cleared of suspended particles per unit of time; V is the volume, K is the number of individual sponges and N_0 and N_t are the virus cell abundance at time 0 and time t, respectively (Riisgård 2001). In this study one piece of sponge (i.e. one individual of 52 mm Ø in size; 1.76 ± 0.99g dry weight; 0.42 ± 0.16 g ash free dry weight) was used per treatment. Clearance rates were calculated for the period after clearance had stabilized, i.e. from 1-5.5 h into the experiment, unless otherwise stated. Linear regressions were subsequently used to test for density dependency by comparing the viral abundances directly before spiking against clearance rates for that specific 20 min period.

Results and discussion

Experiment 1: Removal of viruses by non-host organisms

Ten marine non-host species were tested to assess their ability to reduce abundances of the model virus PgV. As expected, the control treatments containing only PgV

showed no significant decline in PgVs over time and controls containing only the non-host organisms did not display virus enrichment. All non-host organisms, except barnacles, copepods and mussels, significantly affected PgV abundances (Table 8.1, Fig. 8.2), showing that the interference of virus transmission by non-host organism is a common process. Whilst anemones, polychaete larvae and sea squirts tested as significant, the rate of change was very small so that they did not result in an ecologically relevant reduction in viruses by the end of the experiment (Supplement Fig. S 8.1). Conversely, another sessile marine tunicate or 'sea squirt' has been reported to remove up to $7x10^5$ *Emiliania huxleyi* viruses mL^{-1} (by 0.4 animals mL^{-1}), with a clearance rate of 50 mL ind^{-1} day^{-1} (Lawrence et al. 2017) suggesting that removal of viruses by tunicates may be species-specific. The presence of oysters and crabs resulted in significant reduction of PgV abundances over the 24 h experimental period, i.e. <12% and 90% respectively within 3 h. In other studies, after exposure to water containing human enteric viruses, bivalves and crabs showed internal accumulation of the viruses, with recovery of viruses from tissues such as the digestive tract (DiGirolamo et al. 1972, Gerba and Goyal 1978, Hejkal and Gerba 1981, La Bella et al. 2017, Bookelaar et al. 2018). This indicates that both decapods and bivalves have the potential to take up viruses from their surrounding environment and, collectively, significantly contribute to the reduction in viral abundances. Moreover, studies have shown that after uptake by bivalve species viral particles released via fecal matter are inactive and thus suggesting that the digestion process renders the particles non-infective (McLeod et al. 2009, Faust et al. 2009). In other marine macroparasite-host systems crabs and oysters have been shown to effectively reduce the number of free-living trematode parasites (Thieltges et al. 2009, Welsh et al. 2014). While the infective cercarial stages of trematode parasites are considerably larger (body length of several hundred μm (Galaktionov and Dobrovolskij 2003) than virus particles, our study demonstrates the extent of their interactions and their potential to alter a wide range of infectious diseases within a system. Besides the existing studies focusing on the concentration of viruses and their impact on the human food chain we are, to our knowledge, the first to report the removal of non-human viruses by marine shellfish.

Table 8.1. Uni- (ANOVA) and multivariate (MANOVA) analyses results testing for the effect of the presence of a non-host organism on changes in PgV abundance over time, compared to the control treatment where no non-host organisms were present. ANOVA tests used F values and were conducted when samples were only taken at two time points. MANOVA tests, however, were used when samples were taken at more than two time points and used Pillai's trace test. Significance levels indicate whether there was a significant change in viral abundance (over time) between the control and treatments. Significance codes are as follows: ***= 0.001; **= 0.01; *=0.05; and (*) = 0.1.

Test organism	Pillai trace	- F	*P*	Significance
Anemones	0.84	-	<0.001	***
Barnacles	-	0.0	0.970	
Cockles	-	0.0	0.997	
Copepods	0.16	-	0.457	
Crabs	0.99	-	<0.001	***
Mussels	-	4.0	0.073	(*)
Oysters	0.93	-	<0.001	***
Polychaete larvae	0.79	-	<0.001	***
Sea squirts	0.71	-	0.004	**
Sponges	0.87	-	<0.001	***

The strongest removal of PgV was caused by the sponges, not only by removing the most viruses but also because removal of viruses from the system began instantly (Fig. 8.2, Fig. S 8.1). The Breadcrumb sponge reduced PgV abundance by 94% in the first 3h and a minimum of 14-fold by the end of the 24 h experimental period, with an end reduction of 98 %. Sponges are known to be effective at filtering out small particles such as algal and bacterial cells (Reiswig 1975, Sidri 2004, Peterson et al. 2006, Ledda et al. 2014), as well as dissolved organic matter (DOM; De Goeij et al. 2008). According to the standard practical classification and operational definition, particles < 0.2 μm are included in the DOM pool, i.e. the DOM particle size range also includes the nano-meter range of virus particles. In this study we used only algal virus PgV-07T but, given the size range of prey sponges can take up, we would expect comparable removal rates of other viruses (algal viruses and bacteriophages). Natural seawater viruses, dominated by typically smaller-sized bacteriophages (20-60 nm range; Torrella and Morita 1979), have been reported to be removed by a tropical sponge (Hadas et al. 2006). Furthermore, even the very small influenza viruses can be effectively removed by bivalves (Faust et al. 2009).

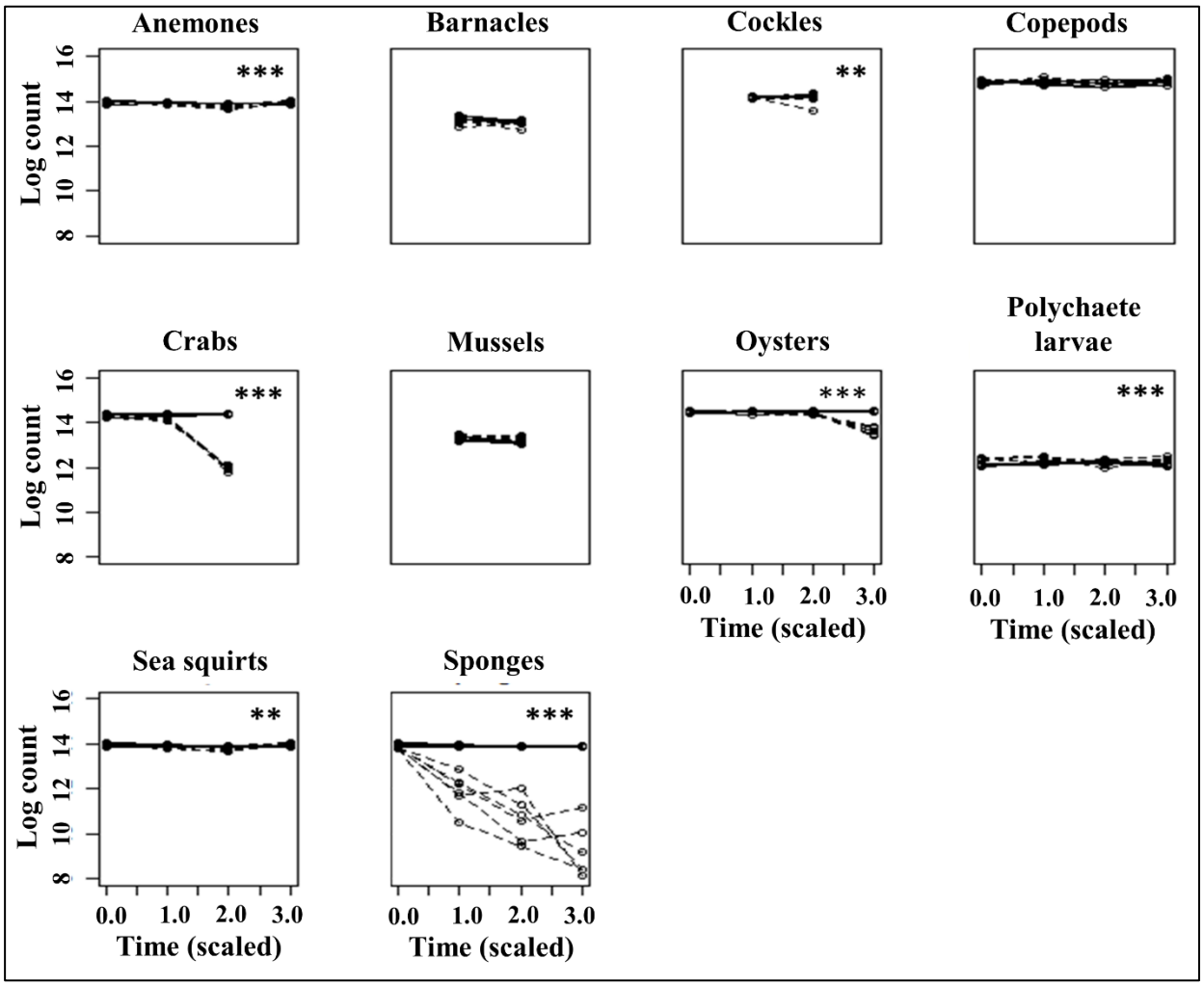

Fig. 8.2. Viral abundance (log PgV mL) over time in the presence (dashed lines) and absence (control; black lines) of a non-host organisms. Asterisks identify non-host organism which had a significant effect on changes in viral abundance ('***' P< 0.001 '**'P< 0.01 '*'P<0.05). Each line represents one replicate. Note that time axis is not continuous, but scaled to four observation times, start of experiment (time 0), approximately 15min (time 1), 3h (time 2), and 24h (time 3) after the start. The y-axis shows the Log of PgV counts, that is the natural log base of the exponential of the virus counts. All virus counts were in the range of x106.

While not the focus of this study, the overall removal of virus particles is likely to have selected consequences for nutrient cycling via the direct loss of viruses (containing and average of 41 C and 16 N and 4.2 P atoms per virus capsid head; potentially accounting for <0.01 to 50% of the total marine DOP, < 7% DON depending on the viral group and other physical parameters (Jover et al. 2014) and the indirect alterations in biogeochemical cycling resulting from host cell lysis (Suttle 2007, Brussaard et al. 2008).

The effective removal of ecologically relevant virus concentrations by the temperate breadcrumb sponge highlights that the filtration activity and the removal of viruses from the surrounding water by non-host organisms is compelling. The virus removal rates shown here are much higher than previously reported for other sponge species. For example the tropical sponge *Negombata magnifica* reduced viruses with an efficiency of 23 ± 3 % (Hadas et al. 2006). Discrepancies in sponge efficiency between their and the present study may be a result of anatomical differences between the sponges used; here we used a temperate sponge from the order Halichondrida, whereas Hadas and co-workers (Hadas et al. 2006) used a tropical sponge from the order 'Poecilosclerida'. Furthermore, the sponge species used in our study has fluctuating removal efficiencies depending on the season. This study was conducted during the period when the sponges exhibit their highest energy demand (April and August; Barthel 1988). Importantly, the differences between the studies illustrate that the contribution of sponges in controlling viral abundance in marine systems is thus far substantially underestimated. Such high reductions in viral abundance are likely to have ecological consequences with knock-on effects for the algal host and virus-host contact rates.

Experiment 2: Continuous clearance of viruses by breadcrumb sponge

To test the sponges' ability to consistently remove viruses over time, we spiked replicates with PgV every 20 min for up to 6 h with approx. 2.5×10^6 PgV mL^{-1} to avoid complete depletion in the surrounding water (Fig. 8.3). Removal rates of the 3 sponges (one sponge died during the experiment) in the first 3 h and over 24 h were comparable to Exp. 1 with a 91% and 94% reduction in viruses. After 1 h the removal of PgV stabilized and the sponge continued to clear PgV at a rate of around 5 mL h^{-1} (Fig. S 8.2). During the stabilized period (1 to 5.5 h into the experiment) the sponges removed a total of about 9.3×10^7 PgVs (Fig. 8.3). Initial PgV abundances varied between the two experiments but all initial abundances were within the natural range found in marine environments (Baudoux 2007). Our results thus demonstrate a constant and very effective virus removal by the breadcrumb sponge. Clearance rates of the viruses by the breadcrumb sponges stabilized after the start of the experiment and remained constant until the end of the testing period. Sponges cleared viruses at a rate of 176 mL h^{-1} g tissue dry wt^{-1} (based on initial 15 min). Given that virus clearance rates reported thus far (e.g. Hadas et al. 2006); 648 mL h^{-1} g tissue wet weight converted to 38 mL h^{-1} g tissue dry wt^{-1} as outlined in Frost (1978) are lower than in our study, the ecological importance of virus loss by sponge activity is most likely extremely underestimated.

Previous studies on viral and DOM loss by sponges have primarily used tropical sponge species (Stuart and Klumpp 1984, Peterson et al. 2006). To our knowledge, we are the first to show that a temperate sponge species is efficient at removing viruses and thus it is likely that other temperate sponge species are also able to reduce viral abundance with equal or better efficiency.

Theoretically there might have been the possibility that sponges filtered the suspension volume (80 mL) more than once, however given that the mean clearance rate was 176 mL h^{-1} g sponge dry weight and the average sponge dry weight per replicate was 1.76 ± 0.99 g, the average clearance rate was 77 mL (range between 34 and 121 mL) within the 15 min sampling period. Although a higher time resolution is recommended it was logistically constrained by the sample processing time required immediately after taking the samples. By utilizing a controlled experimental design and setup such as the one used in this study we are able to show that the loss of viruses was a direct effect of the sponge and not an artefact due to interactions caused by the presence of other organisms within the system. Furthermore, unlike Hadas et al. (2006), by using a controlled experiment and filtered water we can also conclude that there was no other potential 'food' in the water, potentially supporting previous suggestions that viruses act as a nutrient source and a part of the sponge pump (De Goeij et al. 2008, 2013).

Reductions in natural virus abundance by such high degree as shown in our study, have the potential to impact local microbial host population dynamics as the virus-host contact rate drops due to the decline in virus abundance. For PgV specifically, viruses infecting *P. globosa* have been shown to contribute considerably to bloom demise and, depending on the environmental conditions, viral activity may even prevent bloom formation (Brussaard 2004a, Brussaard et al. 2007, Mojica and Brussaard 2014). Thus any reduction in contact rate between PgV and its *P. globosa* host results in reduced virally induced mortality of the algal host, subsequently promoting a longer blooming period or shifting the share of loss factors from viral lysis to grazing (Hallegraeff 1993, Cloern and Dufford 2005). Sponges have a crude ability to remove particles based on size (Ribes et al. 1999, Yahel et al. 2006, Hanson et al. 2009, McMurray et al. 2016) and it is well documented that bacteria and phytoplankton are a major part of sponge prey (Hanson et al. 2009, Riisgård and Larsen 2010). This could influence host population dynamics directly (removal of host algae) and indirectly (removal of viruses). This complex form of transmission interference may thus reduce contact rates between viruses and their hosts even further (due to the dual reduction of host and virus). Therefore, the presence of a non-host organism may be a 'double edged sword' affecting the abundance of both viruses and hosts. We conducted a pilot

experiment to compare simultaneous removal of PgV, *P. globosa* and bacteria, and, indeed, all three types of particles were efficiently cleared (within 24 h) by the breadcrumb sponge. The bacterial clearance rate of 535 mL h^{-1} g tissue dry wt^{-1} falls within the rates published for other sponges (for example, 10-5000 mL h^{-1} g tissue dry wt^{-1}; Frost 1978, Stuart and Klumpp 1984, Riisgård et al. 1993, Ribes et al. 1999, Peterson et al. 2006).

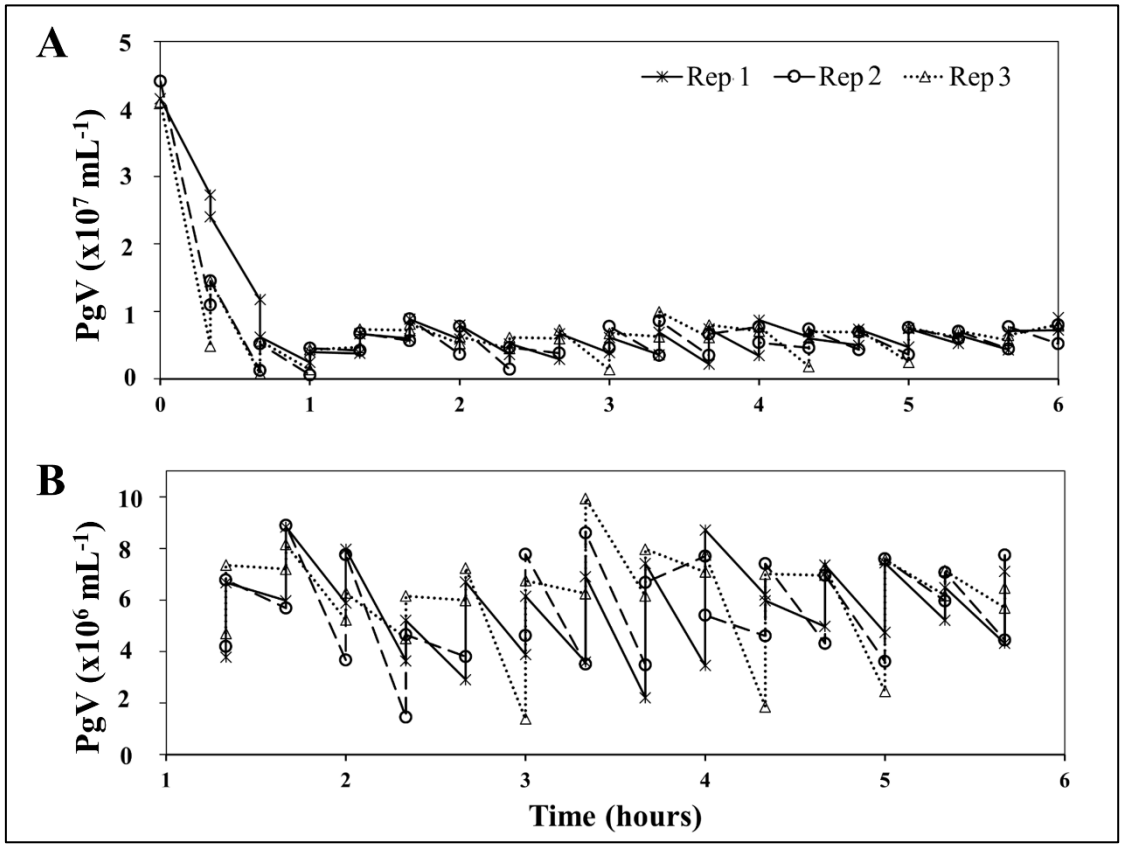

Fig. 8.3. The removal of PgV by breadcrumb sponges over the entire incubation period (A) and in more detail from 1 to 5.5 h into the experiment when PgV was added at 20 min intervals between (B). Experiment was performed in triplicate (Rep 1-3).

The *P. globosa* clearance rate (68.2 mL h^{-1} g tissue dry wt^{-1}) was in the middle range of published rates for phytoplankton (34.4–77 mL min^{-1} g tissue dry wt^{-1}; Riisgård et al. 1993). Substantial variation in clearance rates for both phytoplankton and bacteria do occur which may be caused by factors such as initial particle concentration, time of year, temperature, species and individual sponge behaviour (Stuart and Klumpp 1984, Riisgård et al. 1993, Larsen and Riisgård 1994). Regardless of these differences, our study demonstrates that the virus clearance rates are within the range of the acknowledged natural food particles, bacterial and algal cells.

Given that sponges can be found in high densities in coastal regions, such as harbors (Connell and Glasby 1999), as well as in tropical (Diaz and Rützler 2001) and deep sea reefs (Hogg et al. 2010, Beazley et al. 2013), collectively they are highly likely to continuously interact with the viruses in the water column creating strong localized differences in viral abundance. For example, the overall effects of sponges on viruses here in the Netherlands is likely to have less of an impact than in other regions such as coral reefs or harbors where the majority of surfaces comprise of hard substrate. In such locations sponge coverage can be high, e.g. ranging from 45-70 m^{-2} (Indonesia; Bell and Smith 2004) to >1400 individuals m^{-2} (Ireland; Bell 2002), therefore, in locations such as relatively enclosed bays, the effect of sponges on virus removal is probably vastly underestimated. For example, in a hypothetical hard-substrate bay measuring 10km x 10km x 0.01km, an average sponge density of 1,400 sponges m^{-2}, and a sponge clearance rate of viruses of 5 mL h^{-1} (Exp. 2, this study), an estimated 7 $x10^9$ L h^{-1} is cleared of viruses by the sponges. That equates to 1.7% of the bays water volume every hour. Given stable conditions (e.g. stagnant water), all the viruses in the bay could, hypothetically, be cleared within 3 days. Although such estimates should be approached with caution, the above estimation underlines the enormous potential of sponges as virus loss factor whereby the ultimate ecological impact depends on the local conditions such as tide phase, current strength, local sponge cover, other non-host organisms potentially removing viruses from the water column (e.g. bivalves), as well as host presence and abundance.

In conclusion, our results stress the notion that a wide range of non-host organisms and in particular sponges, have the potential to reduce nano-sized pathogen abundance via transmission interference. It is very likely that there are many more species capable of removing viruses from the water column. It is also likely that there are a variety of factors which facilitate or impede the removal of viruses, such as stratification or mixing of the water column altering contact rates, ambient temperatures affecting non-host organism feeding rates, free-floating clays and aggregates to which viruses may adhere (Gerba 1984, Lipson and Stotzky 1984, Brussaard et al. 2005, Mojica and Brussaard 2014) all of which should be tested for their effects on transmission interference. An interesting follow up experiment would be to expand on the experiments investigating the effect of sponges on the contact rates of host and virus when both are present. Considering that even temperate sponges effectively remove viruses from the water column (this study), the global ecological consequences are expected to be considerable and hence, there is a need for further

investigations to determine how, when and to what extent non-host organisms affect virus-host dynamics.

Acknowledgements

We wish to thank our colleagues for their valued contribution to the research: Anna Noordeloos and Kirsten Kooijman for laboratory assistance, and Douwe Maat, Kristina Mojica and Tristan Biggs for their time given to discussing the research.

Supplementary material.

Experiment 1: Removal of viruses in the presence of non-host organisms.

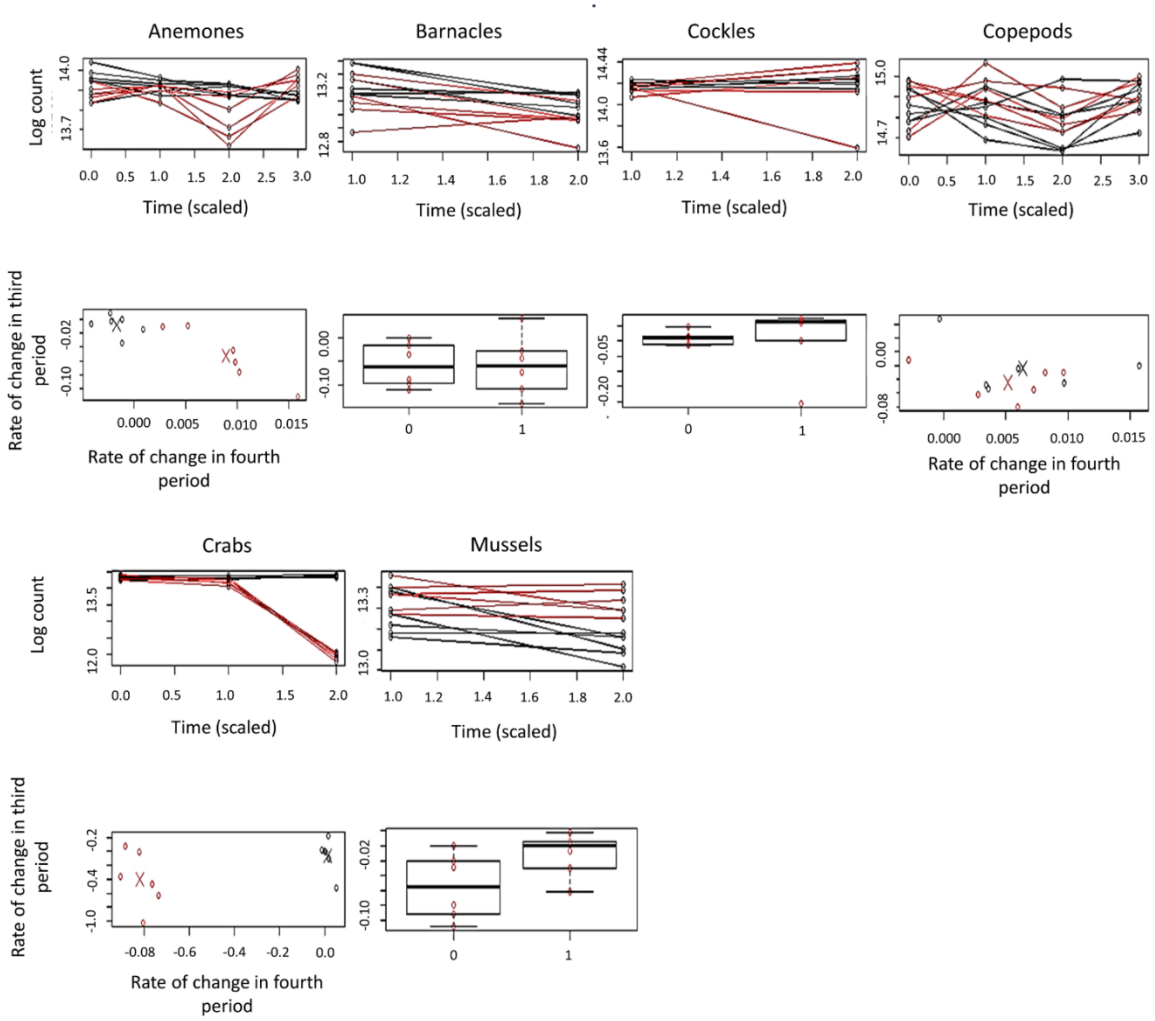

Fig. S 8.1. Viral log counts and rates of change in viral abundance over time for individual replicates (red lines) and for each test organism as well as controls (viruses only, black lines). Viral log counts and rates of change in viral abundance over time for individual replicates (red lines) and for each test organism as well as controls (viruses only, black lines). Rate of change over the fourth period amongst control replicates (black circles) and test organism replicates (red circles) and their means (crosses in associated colour) is shown as negative or a reduction in viral abundance when the rate of change is >0.00. Anemones, crabs, oysters, polychaete larvae, sea squirts and sponges all significantly affected viral abundance (see main text). Whilst anemones, polychaete larvae and sea squirts tested as significant factors in viral abundance, from the above graphs it is evident that they do not have an ecologically relevant influence on overall viral abundance. Crabs, oysters, and sponges, however, clearly significantly reduce viral abundance by the end of the experiment. Sponges reduced viral abundance at a rapid rate from the outset, clearing almost all of the viruses by the end of the experiment.

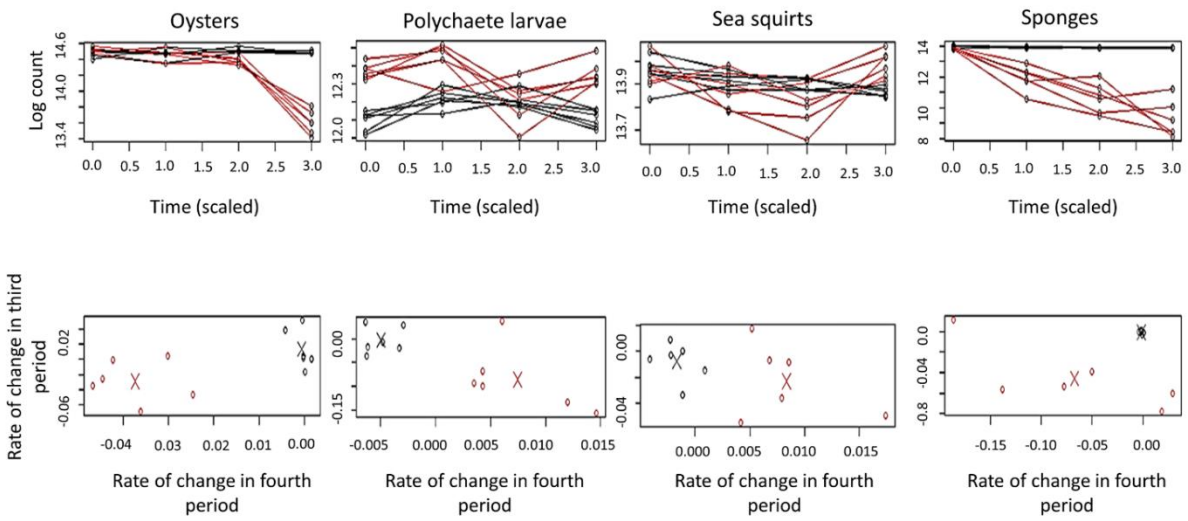

Fig. S 8.1. Continued. Viral log counts and rates of change in viral abundance over time for individual replicates.

Experiment 2: : Continuous clearance of virus by breadcrumb sponge

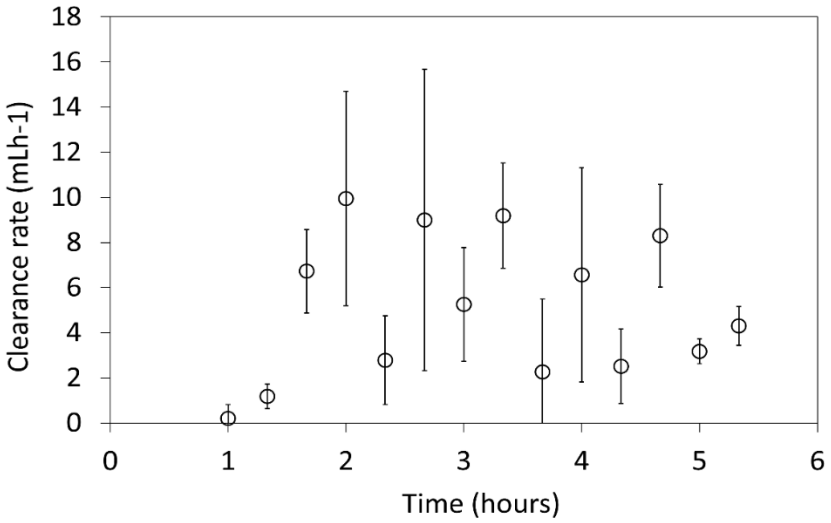

Fig. S 8.2. Mean clearance rates (± standard error) of PgV by breadcrumb sponges during the stable clearance period of Exp. 2.

The Use of Filter-feeders to Manage Disease in a Changing World

Colleen A. Burge, Collin J. Closek, Carolyn S. Friedman, Maya L. Groner, Cody M. Jenkins, Amanda Shore-Maggio, and Jennifer E. Welsh

Abstract

Rapid environmental change is linked to increases in aquatic disease heightening the need to develop strategies to manage disease. Filter-feeding species are effective biofilters and can naturally mitigate disease risk to humans and wildlife. We review the role of filter-feeders, with an emphasis on bivalves, in altering disease outcomes via augmentation and reduction. Filtration can reduce transmission by removing pathogens from the water column via degradation and release of pathogens in pseudofeces. In other cases, filtration can increase pathogen transmission and disease risk. The effect of filtration on pathogen transmission depends on the selectivity of the filter-feeder, the degree of infectivity by the pathogen, the mechanism(s) of pathogen transmission and the ability of the pathogen to resist degradation. For example, some bacteria and viruses can resist degradation and accumulate within a filter-feeder leading to disease transmission to humans and other wildlife upon ingestion. Since bivalves can concentrate microorganisms, they are also useful as sentinels for the presence of pathogenic microorganisms. While somewhat less studied, other invertebrates, including ascidians and sponges may also provide ecosystem services by altering pathogen transmission. In all scenarios, climate change may affect the potential for filter-feeders to mitigate disease risk. We conclude that an assessment including empirical data and modeling of system-wide impacts should be conducted before selection of filter-feeders to mitigate disease. Such studies should consider physiology of the host and microbe and risk factors for negative impacts including augmentation of other pathogens.

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Introduction

Aquatic disease outbreaks can decimate populations, alter community structure, and deplete fisheries, resulting in large economic losses and impacts on fishing communities (Lafferty et al. 2015; Groner et al. 2016). Aquatic environments pose a management challenge as pathogen transmission typically occurs in a 3D water column with complex patterns of water movement, frequently changing water chemistry and temperature, and numerous ecological interactions. In addition to the natural variation in the aquatic environment, climate change impacts both pathogens and their hosts, especially ectothermic species such as invertebrates and fish (Burge et al. 2014). Strategies to manage aquatic diseases and mitigate their impacts are needed and must be able to reduce pathogen loads and or transmission in large bodies of water in a variety of conditions.

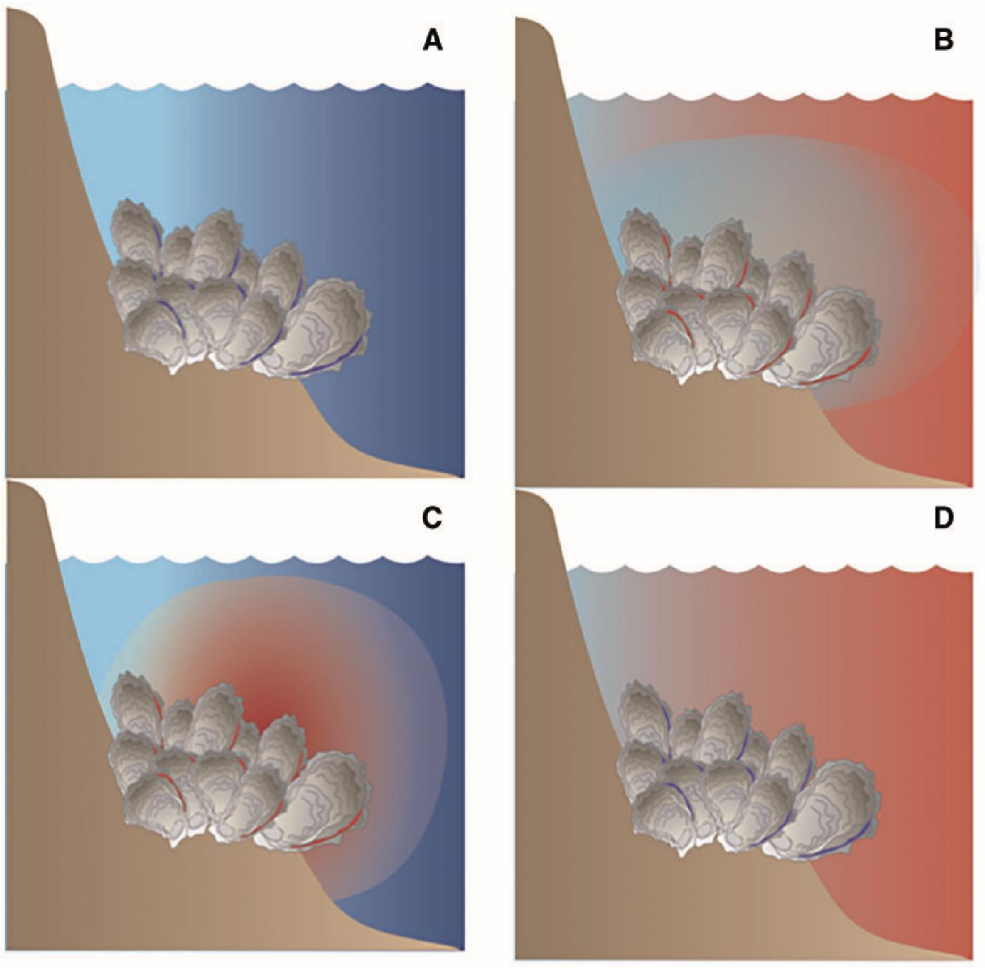

Fig. 9.1. Four scenarios for filtration within the aquatic environment. (A) Neutral; low number of pathogens in both the water column and filter-feeder. (B) Subtractive; filter-feeders are reducing the number of pathogens in the water column. (C) Additive; the filter-feeders are a reservoir for pathogen replication and emitting the pathogens into the environment. (D) No Impact; the filter-feeder does not reduce the pathogen in the water column.

One recent management strategy to alleviate infectious disease capitalizes on naturally occurring filtration services of species, such as bivalves, to alleviate pathogen pressure (Maeda 2004; Defoirdt et al. 2011). Bivalves (Phylum Mollusca) such as mussels, clams, and oysters feed by capturing particles from the water using their gills, where particles are selected by size and density (reviewed by Gosling 2003). Selected particles are ingested and released as feces and rejected particles are expelled as pseudofeces (Beninger et al. 1999; Alexander et al. 2008). Bivalves primarily consume planktonic species, particularly phytoplankton such as diatoms and dinoflagellates (Shumway et al. 1987). Bivalves also consume smaller plankton including bacteria, viruses, and micro-zooplankton, including pathogens, as well as dissolved and particulate organic material (which may contain a rich assortment of microorganisms) (Gosling 2003). As a result, filter-feeding species have the potential to concentrate or remove pathogens.

Individual sessile filter-feeding organisms such as bivalves can clear particles from tens to hundreds of liters of water daily. Filter-feeding species often occur in high population densities, which, when combined with their high filtration capacity, gives these organisms the potential to alter epidemiologic outcomes of pathogens (Ben-Horin et al. 2015). Specific filtration outcomes (Fig. 9.1) may vary and include little or no discernable impact (either at low or high pathogen concentration), reduction (subtractive) of pathogens through either active or passive filtration, or augmentation (additive) of pathogens when the host acts as a reservoir for pathogens either as a passive or active reservoir (Box 3).

Although the use of filter-feeding species for pathogen control is an attractive management option, little is known about specific applications of biofiltration on pathogen abundance and disease. Currently, the majority of literature is focused on bivalves as biofilters. There is no guide to instruct when and under what conditions bivalves or other filter-feeders may be useful for pathogen mitigation.

Given the potential pathogen removal capacity of filter-feeders, we were interested in the following question: How and when can filter-feeders be used to manage disease? In order to answer this question, we considered the following:

(1) What are known impacts of bivalve filtration on marine disease?
(2) Which other high-filtration capacity invertebrates may function as pathogen biofilters?
(3) What makes a pathogen more resilient to filtration or degradation?
(4) What are the potential impacts of ocean or climate change on interactions between filter-feeders and pathogens?
(5) What are management implications of filter-feeders?
(6) How can modeling inform management approaches?

Finally, we provide conclusions and future directions for the use of filter-feeders for disease management.

Box 3. Definitions

Accidental or non-target host: A type of abnormal host in which the parasite is not commonly found, yet is suitable for the parasite's development. In some instances (e.g., cysticercosis), the accidental host becomes a ''dead end'' because even though the parasite develops through its stages, it is unable to be transmitted to the next host and, thus, cannot complete its life cycle.

Active removal: Removal of particles that are targeted and/or selected based on specific parameter (e.g., weight, size, or type).

Clearance rate: Volume of water cleared of suspended particles per unit of time.

Disease mitigation: the act of reducing the impacts of disease (e.g., severity or mortality) in a population. Filtration rate: Flow rate of water moving across the gills (e.g., pumping rate ¼ volume flow rate).

Infectious disease: A disease caused by a transmissible agent (e.g., a virus, bacterium, protist, macroparasite, fungus, alga, or prion) that infects the host tissues, leading to an identifiable illness or syndrome.

Parasite: An organism (often microscopic) that is metabolically dependent on its host and typically gains energy or food from its host, and may or may not cause disease.

Passive removal: Removal of particles as a side effect of filtering water and/or consuming other particles.

Box 3. Definitions (continued)

Pathogen: A causative agent (i.e., virus, bacterium, fungus, protist, etc.) of disease; under certain conditions, metazoan parasites (i.e., helminthes and crustaceans) may also cause disease.

Pathogen source: The species or population from which a pathogen is transmitted to other hosts.

Pathogen sink: A host that may become infected with a pathogen but does not transmit the pathogen to other hosts.

Reservoir host: Hosts (environments or populations) that become infected by a pathogen and maintain infections (with or without disease) and serve to transmit the pathogen to susceptible hosts; often in reference to a defined target population (sensu Haydon et al. 2002).

Sentinel or indicator: A species with known susceptibility to a specific pathogen or toxin that is sampled over time to assess the presence or absence of the target pathogen or toxin.

Spill-over: Transmission of a pathogen (spills-over) from a reservoir or maintenance host, often domesticated, to sympatric wild host species. The reservoir species may be non-native or introduced to a new locale and introduce the pathogen into a native population.

Spill-back: ''Reverse spill-over'' occurs when pathogen transmission occurs from a native host that acts as reservoir for transmission back to maintenance species that introduced the infectious agent. Spill-back may occur when wild, native species transmits the pathogen to back to domesticated animals (sensu Daszak et al. 2000).

What are known impacts of bivalve filtration on marine disease?

Bivalves often exist in dense beds providing necessary structure and habitat for a variety of species, and improving water quality by directly filtering pathogens and suspended matter from the water column (Ward and Shumway 2004; Coen et al. 2007). In Fig. 9.2, we focus on epidemiological outcomes of pathogen filtration in bivalves. In this scenario, pathogens may be amplified through aggregation and replication within reservoir hosts or reduced through direct ingestion. Live pathogens may be released into the environment attached to feces or pseudofeces. We will focus the following section on transmission augmentation (amplification) and transmission reduction.

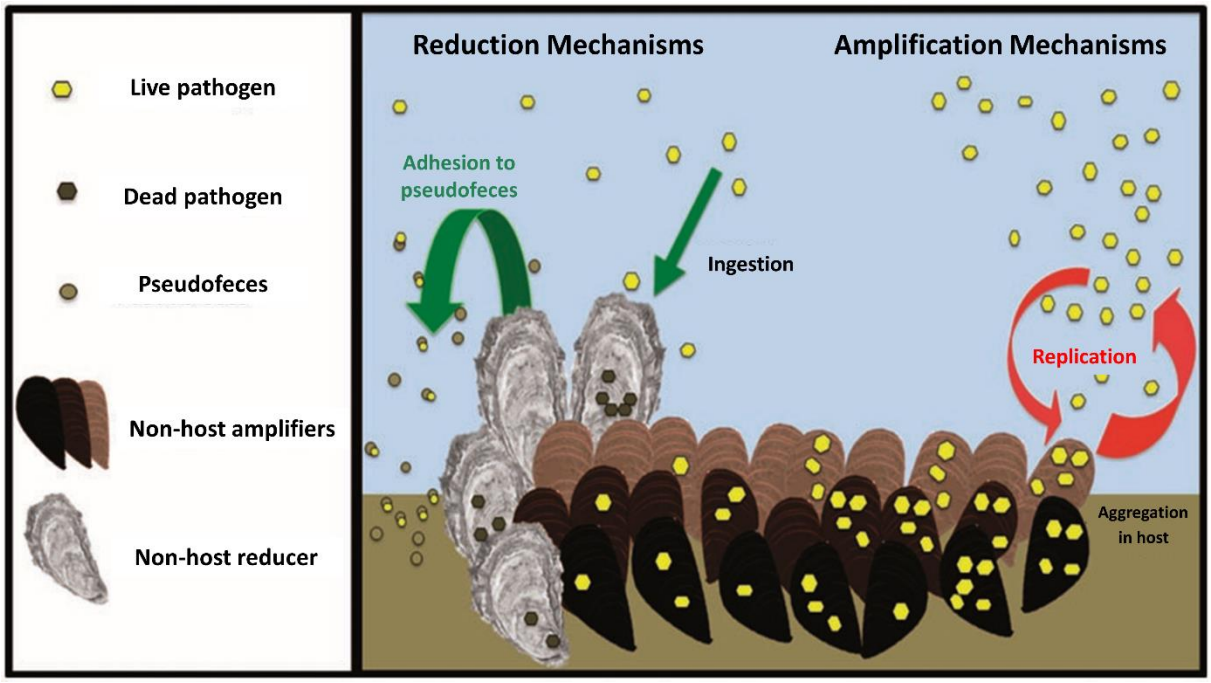

Fig. 9.2. Filter-feeders can alter pathogen transmission through reduction or amplification. Reduction can occur via mechanisms such as ingestion by non-target filter-feeders (dark arrow) or incorporation into pseudofeces made by the filter-feeder that sink out of the water column (curved arrow). Amplification occurs when the pathogen successfully replicates inside or on the filter-feeder (arrows) or if aggregation in the filter-feeder increases the likelihood of transmission

Transmission augmentation

The role of bivalves in the transmission of human pathogens is well documented and their role in transmission of wildlife disease is gaining attention. Bivalves can filter and concentrate human pathogens and serve as a passive reservoir for viruses [e.g., Hepatitis A or Norovirus (Enriquez et al. 1992; Schwab et al. 1998), bacteria (e.g., *Vibrio spp*. and *Escherichia coli*, Ismail et al. 2015)], diatoms and dinoflagellates (e.g., toxin producing *Pseudo-nitzchia spp*., Amzil et al. 2001, *Alexandrium*, and *Gymnodinium spp*., Bricejli and Shumway 1998), and protists (e.g., *Cryptosporidium*, Gómez-Couso et al. 2006). In addition, bivalves can act as a passive reservoir for pathogens of wildlife. For example, mussels (*Mytilus galloprovincialis*), are known intermediate hosts for *Toxoplasmosis gondii*, which commonly infects sea otters (Arkush et al. 2003). Oysters in the genus *Crassostrea* (*C. gigas*, the Pacific oyster, and *C. virginica*, the eastern oyster) have been shown to act as passive reservoirs for pathogenic fish reoviruses (Meyers 1980, 1984). A second type of augmentation occurs when a bivalve is an active reservoir, and pathogen replication occurs within the host leading to transmission within or between species (see Ben-Horin et al. 2015 for an in-depth review of pathogen transmission in bivalves).

Transmission reduction

Consumption and degradation of parasites (referred to as degradation in this review) is an effective means to reduce pathogen loads in aquatic environments (Table 9.1). Filter-feeders that may mitigate disease by pathogen removal, such as mussels and oysters, can specifically select particles based on size (Gosling 2003). Therefore, it is presumed that they ''actively'' remove larger or specific organisms for consumption. For example, in the presence of the non-host Pacific oyster, metacercariae stages of the parasite *Himasthla elongata* in *Cerastoderma edule* (cockles) were reduced by up to 91% (Thieltges et al. 2008a; Welsh et al. 2014). Despite generally selecting for particles of 4–250 mm (Gosling 2003) some species of bivalves, as well as other filter-feeders (e.g., sponges and sabellid worms), are capable of removing microbial pathogens including viruses (Hadas et al. 2006; Faust et al. 2009; Granada et al. 2014). For example, the Asiatic clam (*Corbicula fluminea*) can successfully remove and avian influenza virus from the water column, reducing infection rates in wood ducks (*Aix sponsa*) (Faust et al. 2009).

Pathogens can be removed from the water column by means other than selective filtration. For example, Pacific oyster shells reduced free-living *H. elongate* trematode parasites by 44% despite containing no filter-feeding organism, suggesting that adhesion to the shell may play a role in parasite reduction (Welsh et al. 2014). Bacteria and viruses have also been shown to attach to clay particles, such as those found in sediment and pseudofaeces (Syngouna and Chrysikopoulos 2010). Sediment particles, including clay and pseudofaeces, often sink out of the water column, removing the attached bacteria and viruses from areas where transmission is likely (Haven and Morales-Alamo 1972).

Table 9.1. Examples of freshwater and marine filter-feeders that have caused pathogen reduction from the water column or within a host species. ns: indicates studies where reduced the pathogen loads were not statistically significant.

Habitat	Filter Feeder		Pathogen		
	Taxon	**Species**	**Taxon**	**Species/particle**	**Citation**
Freshwater	Bivalve	*Corbicula fluminea*	Virus	A/Mallard/MN/190/99 (H3N8)	Faust et. al. 2009
		Sphaerium sp.	Trematoda	*Ribeiroia ondatrae*	Orlofske et al. 2012[ns]
		Dreissena polymorpha	Conoidasida	*Cryptosporidium parvum*	Graczyk et al. 2003
Marine	Bivalve	*Crassostrea gigas*	Trematoda	*Himasthla elongata*	Welsh et. al. 2014, Thieltges et al. 2008b, 2009
		Mya arenaria	Trematoda	*H. elongata*	Thieltges et. al. 2008b
		Marcoma balthica	Trematoda	*H. elongata*	Thieltges et al. 2008b[ns]
		Mytilus edulis	Trematoda	*H. elongata*	Thieltges et al. 2008b
	Crustacean	*Semibalanus balanoides*	Trematoda	*H. elongata*	Welsh et al. 2014
		Austrominius modestus	Trematoda	*Echinostephilla patellae*	Prinz et al. 2009
	Gastropod	*Littorina littorea*	Trematoda	*Parorchis acanthus*	Prinz et al. 2009
		Patella vulgata	Trematoda	*P. acanthus*	Prinz et al. 2009
		Crepidula fornicata	Trematoda	*H. elongata*	Thieltges et al. 2008b

Table 9.1. Continued. Examples of freshwater and marine filter-feeders that have caused pathogen reduction from the water column or within a host species. ns: indicates studies where reduced the pathogen loads were not statistically significant.

Habitat	Filter Feeder		Pathogen		
	Taxon	**Species**	**Taxon**	**Species/particle**	**Citation**
Marine	Porifera	*Hymeniacidon perleve*	Bacteria	*Escherichia coli*	Fu et al. 2006
		Chondrilla nucula	Bacteria	*E. coli*	Milanese et al. 2003
		H. perleve	Bacteria	*Vibrio anguillarum II*	Fu et al. 2006
		Negombata magnifica	Virus	Unknown	Hadas et al. 2006
	Polychaeta	*Branchiomma luctuosum*	Bacteria	*V. alginolyticus*	Licciano et al. 2005
		Sabella spallanzanii	Bacteria	*V. alginolyticus*	Licciano et al. 2005

Which other high-filtration capacity invertebrates may function as pathogen biofilters?

While their role in changing water quality may be less well-studied, organisms such as sponges and ascidians are important filter-feeding invertebrates and can also act as bio-filters. Here, we review the known effects of these taxa on pathogen transmission.

Sponges

Sponges (Phylum Porifera) have high filtering and clearance rates of microbes and have been used as remediation tools and biofilters for aquaculture in regions around the world (reviewed by Wilson et al. 2012; Ledda et al. 2014). Sponges filter large amounts of seawater; up to 14l/h/m^2 of tissue (Milanese et al. 2003). As suspension feeders, filtration by sponges is considered non-selective. Nonetheless, this method is effective at removing >25% of the dissolved and particulate total organic carbon (TOC) from the water column, which contributes to sustaining the microbial symbionts that make up more than two thirds of the sponge biomass (Yahel et al. 2003). While it is presumed that pathogen removal is a side effect of filtering pico- and nanoplankton from large volumes of water (Maldonado et al. 2010), selective consumption of specific pathogenic microbes has been measured in controlled laboratory studies with sponges (Maldonado et al. 2010). Pathogens of other organisms, such as the putative causative agent of sea fan aspergillosis, *Aspergillus sydowii*, have been found in sponges with no signs of disease (Ein-Gil et al. 2009; Negandhi et al. 2010). As a result, sponges have been proposed as a bioremediation tool to clear pathogens of wild species from the water column (Milanese et al. 2003; Fu et al. 2006; Stabili et al. 2006a, 2006b; Longo et al. 2010).

Ascidians

Ascidians (Phylum Chordata) are solitary or colonial filter-feeding marine invertebrates and may reduce abundances of potential pathogens in the marine environment. Ascidians pump seawater through their branchial basket and collect particles on a mucus filter that covers the inner wall of the pharynx (Randlov and Riisgård 1979). Although particles that range in size from 0.5 to 100 mm are ingested, those measuring greater than 600 nm have the highest retention rate (e.g., phytoplankton and larger bacteria; Peterson 2007). Filtration rate varies with ascidian size, seawater temperature, and particle concentration (Peterson and Riisgård 1992). Ascidians can filter up to 200ml/min but many filter in the range of 10–100ml/min

(Randlov and Riisgård 1979). In one study, *Ciona intestinalis*, a solitary ascidian that can form dense aggregations, filtered 5–34 ml/min and was estimated to be able to filter the entire volume of the studied cove in Denmark daily (Peterson and Riisgård 1992). The role of ascidians in biofiltration depends upon the pathogens in the water column. The colonial ascidian, *Polyundrocarpa zorritensis*, ingests bacteria and can reduce seawater concentrations of allochthonous bacteria, including pathogens; however, it can also concentrate bacteria and may serve as a reservoir for some pathogen species due to differential digestion of bacterial taxa (e.g., Gram-negative bacteria, Stabili et al. 2015). Similar to bivalves and sponges, ascidians have potential as biofilters for pathogen remediation, but knowledge gaps about whether the target pathogen is filtered, retained, and or digested need to be addressed (Stabili et al. 2015).

What makes a pathogen more resilient to filtration or degradation?

Pathogens have evolved mechanisms to resist degradation from the tissues of filter-feeders. Outbreaks of bacterial and viral diseases in humans associated with bivalve consumption demonstrate that some microorganisms are able to resist degradation and persist in bivalve tissues. Bacterial genera that survive in the tissues of bivalves and resist degradation include Salmonella and Vibrio species (Jones et al. 1991; Wright et al. 1996; Hernroth et al. 2002; Pruzzo et al. 2005). These species are responsible for the most cases of bacterial-caused food poisoning associated with bivalve consumption and are readily isolated from bivalves (Jones et al. 1991; Rippey 1994; Wright et al. 1996; Potasman et al. 2002; Hernroth et al. 2002). Viruses that survive in the tissues of bivalves and resist degradation are frequently non-enveloped viruses, such as noroviruses (Potasman et al. 2002). It is unclear whether this morphological trait influences persistence in bivalve tissues. While components of the envelope often facilitate virus entry and evasion of host immunity (Wyatt and Sodroski 1998; Poranen et al. 2002), non-enveloped viruses are generally more persistent in aquatic environments (Sobsey and Meschke 2003).

Pathogen characteristics that determine resistance or susceptibility to degradation may include mechanical features such as particle size. Many studies have correlated clearance rate as a function of particle size although this varies somewhat with species (Riisgård 1988, 1998; Sprung and Rose 1988; Lei et al. 2001). For example, *C. gigas* and *M. edulis* can filter eukaryotic diatoms (44 mm width) with 100% efficiency, but filtration efficiency decreases with decreasing particle size (Haven and Morales-Alamo 1970; Riisgård 1998). Viruses, which are often less than 200 nm in size, are

generally more resistant to degradation than bacteria (Polo et al. 2015). Human enteric viruses such as Norovirus and Hepatitis A virus are the most common pathogens transmitted by consumption of bivalves (Lipp and Rose 1997; Potasman et al. 2002; Lees 2000). Filtration efficiency of small microorganisms can increase when microbes are attached to organic aggregates, also called marine snow (Lyons et al. 2005; Kach and Ward 2008; Froelich et al. 2013). In particular, pathogenic Vibrio spp. have been shown to accumulate on marine snow (Keyhani and Roseman 1999). Thus, while size affects susceptibility to filtration, this general rule is impacted by particle aggregation.

The bivalve immune system may also influence the resistance of microorganisms to degradation. Persistence of bacteria in bivalve tissues is dependent on their sensitivity to the bactericidal activity of the hemocytes (primary immune cells) and soluble immune factors (Pruzzo et al. 2005). For example, various Vibrio species have a higher capacity to survive in mussel hemolymph than *E. coli* (Prieur et al. 1990; Croci et al. 2002). Specific molecular mechanisms of binding to bivalve tissues can also affect resistance to degradation. For example, Pacific oysters have been shown to differentially concentrate various strains of Norovirus (Le Guyader et al. 2006) via binding to ligands present in different oyster tissues (Maalouf et al. 2010, 2011).

These examples of resistance to bivalve degradation stress the need for more basic research on the interactions between specific microbes and various filter-feeders. This type of research will allow management agencies to match the most effective filter-feeder for a particular environment.

What are the potential impacts of ocean or climate change on interactions between filter-feeders and pathogens?

Current and future climate change conditions in the world's ocean will continue to impact marine species (Doney et al. 2011; Howard et al. 2013; Burge et al. 2014), including disease-causing microbes (Burge et al. 2014) and filter-feeding bivalves (Kroeker et al. 2013). Physical ocean changes (e.g., warming, acidification, circulation, salinity, storms, hypoxia, and additional changes) impact the biology of the organisms inhabiting the ocean, both through physiological changes (linked to temperature, salinity, hypoxia, nutrients, pH, etc.) and population shifts (invasions, biological interactions between species, community composition, and biodiversity), which ultimately can lead to ecosystem changes (Doney et al. 2011; Nagelkerken and Connell 2015). Climate change and its associated processes may, thus, influence how filter-

feeders reduce or augment disease (Fig. 9.3). For example, non-cholera Vibrio disease outbreaks in humans (caused by *V. vulnificus* and *V. parahemaolyticus*), increase with warmer temperatures and extreme storm events (reviewed by Burge et al. 2014). Increased temperatures facilitate bacterial growth as well as increase expression of virulence factors, such as hemolysins (Mahoney et al. 2010). Climate change is expected to alter temperatures at which filter-feeders could be exposed to during emersion and air exposure during low tides (Doney et al. 2011). Both bivalves and particles they filter (i.e., ''microbial'' and ''macroparasite'' pathogens and phytoplankton food sources) have temperature dependent metabolic rates (Burge et al. 2014; Moran 2015; Nagelkerken and Connell 2015). Temperature may act to increase consumption rates by herbivores (including bivalves), and shift abundances and production of available members of the ocean plankton and microbiome (Nagelkerken and Connell 2015). However, we are far from understanding the complex trophic structure of the ocean microbiome under warming conditions (Moran 2015; Sunagawa et al. 2015).

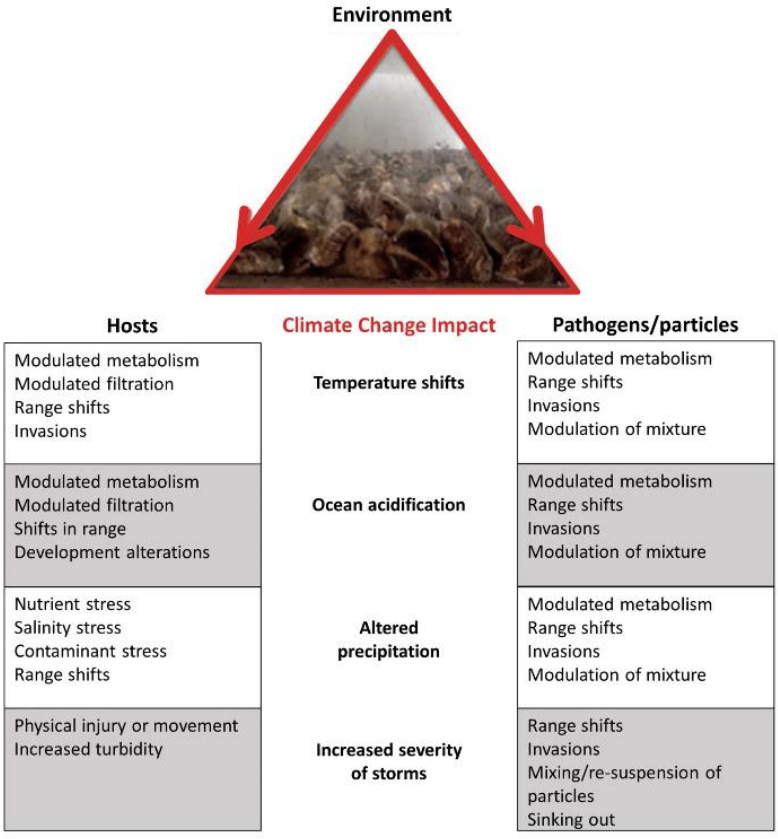

Fig. 9.3. Climate change impacts on pathogen filtration. Shifts in the environment are leading to ocean change, including (1) changes in temperature, (2) ocean acidification (increased C02, decreased pH), (3) altered precipitation (leading to changes in salinity) and (4) increased severity and or number of storms and cyclones. All of these factors are acting to change the interaction between the host and potential pathogens or particles.

For bivalves, elevated water temperature within physiological tolerable ranges typically increases filtration rates. Such increases, in turn, can influence how filter-feeders interact with pathogens. For example, higher filtration rates may clear more pathogens from the water and reduce infection intensity in downstream hosts (Goedknegt et al. 2015). Increases in filtration with increased temperature is not a linear relationship; filter-feeders eventually reach a maximum temperature threshold, after which the filtration rate declines (Sylvester et al. 2005; Goedknegt et al. 2015). Thus, understanding physiological limits is essential when selecting potential species for use as biofilters.

Other ocean conditions and inputs can also be altered as a result of climate change and may affect how filter-feeders interact with pathogens. In marine systems, ocean acidification due to increased atmospheric partial pressure of carbon dioxide (pCO_2), terrestrial run-off, and other biotic and abiotic inputs will result in marine hypercapnia (increased CO_2; Feely et al. 2012). Ocean acidification can affect the soft tissues of filter-feeding organisms by altering metabolism (Lannig et al. 2010; Liu and He 2012; Waldbusser et al. 2015), decreasing body condition (Lannig et al. 2010), and hindering larval development (Gazeau et al. 2010; Barton et al. 2012). In addition, the reduced pH caused by the increase in CO_2 can affect the calcareous shells of bivalve species by reducing their ability to deposit new layers and the dissolution of existing shell material (Barton et al. 2012). Ocean acidification is also predicted to impact the abundance (e.g., by increasing pelagic density) of some potential pathogen species, while having no impact on other species (Nagelkerken and Connell 2015).

Effects of climate change on aspects of filter-feeder development and physiology is species dependent (Liu and He 2012). Extreme weather conditions are expected to occur more frequently with climate change and filter-feeders may not be able to adapt to these predicted extreme and rapid fluctuations (Kayler et al. 2015). The majority of conclusions regarding the effects of climate change on organismal ecology are based on short-term mesocosm or laboratory experiments, and thus the long-term effects of temperature or ocean acidification and adaptations of the filter-feeders and pathogens remain largely unknown. Recent studies have shown positive carryover effects of ocean acidification exposure to adult Sydney rock oysters (*Saccostrea glomerata*) on their progeny and subsequent generation (Parker et al. 2015). Combined with the lack of studies on interactions between filter-feeders and pathogens it is difficult to predict with certainty how the augmentation or mitigation of diseases by filter-feeders will be affected by climate change.

What are management applications and implications of filter-feeders?

Pathogen reduction in aquaculture

Aquaculture is on the rise globally and is an important source of revenue and protein (SOFIA 2014). Losses due to infectious disease have increased over the past decades making disease control a priority in aquaculture development and resource conservation (Krkošek et al. 2007; Groner et al. 2016). Intensive farming often involves the culturing of genetically similar and or high densities of individuals, which can facilitate density-dependent outbreaks of disease (Jansen et al. 2012). At the same time, natural ''controls'' for disease mitigation may be missing from such systems. Control mechanisms include predation of diseased individuals or their pathogens, evolution of disease resistance in hosts, geographical separation of host life stages, and dilution of pathogens among hosts and non-hosts. Pathogen filtration by bivalves has been proposed as a method for reducing disease risk of farmed and nearby wild organisms (Faust et al. 2009; Bartsch et al. 2013) (Table 9.2). The viability of this approach depends upon the pathogen in question, the filtration species chosen, and their interactive effect on other pathogens in the system. For example, lab studies demonstrated that blue mussels (*Mytilus edilus*) and Atlantic sea scallops (*Placopectan megellanicus*) can ingest sea lice (*Lepeoptheirus salmonis*), which are a significant pest of wild and farmed salmon (Molloy et al. 2011; Bartsch et al. 2013). However, lab studies showed that these bivalves may concentrate infectious pancreatic necrosis virus (IPNv), an important pathogen of Atlantic salmon, releasing the virus in their feces for up to 7 days post-exposure (Molloy et al. 2013). The success of bivalve filtration will depend upon the relative risk of these two diseases locally.

Use of bio-filtration in aquaculture needs to consider the influence of environmental conditions (see previous section for more details). For example, low temperature and runoff influenced the uptake and accumulation of F+ Coliphage during the winter months with a concentration up to 99-fold relative to other seasons (Burkhardt and Calci 2000, Hernroth et al. 2002). Intentional manipulation of environmental conditions can be used to facilitate parasite–filter-feeder interactions. Sea lice are attracted to light, thus placement of light near filter-feeders may concentrate sea lice where they can be ingested (Bartsch et al. 2013). This type of application is particularly promising for the development of integrated multi-trophic aquaculture because many filter-feeders have economic value.

Bivalves have also been suggested as a means of reducing pathogen exposure in other molluscs. For example, oysters placed near mussel farms had a lower risk of

exposure to Ostreid herpes virus (OsHV-1) than those placed away from mussel farms (Pernet et al. 2014). Interest in using bivalves to filter out pathogens is also being investigated with land-based farms to reduce the microbial loads in farm effluent. For example, oysters, mussels, and other filter-feeding organisms are being evaluated to reduce the potential for release of a bacterial pathogen from an abalone farm (Friedman et al., unplubl. data). Bivalves are not the only organisms being considered for disease management in aquaculture. Two sabellid worm species from the Mediterranean, *Branchiomma luctuosum Grube* and *Sabella spallanzanii Gmelin*, have been proposed as biofilters in aquaculture for their high-filtration rate of bacterioplankton and reduction of the bacterium *V. alginolyticus* (Licciano et al. 2005). New applications of filter-feeders for pathogen management are likely to emerge as aquaculture continues to increase.

Using bivalves as indicator species

Due to their proclivity to concentrate pathogens, there is considerable value in using bivalves as sentinels to monitor disease risk (Table 9.3). For example, the NOAA mussel watch program monitors bivalves for the presence of human pathogens, toxins, contaminants, and parasites. This program has led to regulatory decisions and follow-up monitoring to evaluate management programs (Kim and Powell 2007). Invasive dresissenid bivalves (*Corbicula fluminea* and *Dresisena spp*) have been used for pathogen monitoring in the Great Lakes system (North America) and in the Shannon River (Ireland), where dresissends are used as sentinels for human and animal pathogens including *Cryptosporidium*, *Giardia*, *Cyclospora*, *Enterocytozoon*, and *Encephalitozoon* (Conn et al. 2013). While highly quantitative comparisons among sites may not be possible using sentinels due to variation in accumulation rates, sampling of bivalves has proven to be an effective method to detect the presence of pathogens, which may be hard to detect in the water column or target hosts.

Table 9.2. Current and proposed uses of filter-feeders to for disease mitigation

	Filter-feeder			
Taxon	**Species**	**Pathogen**	**Pathogen host**	**Citation**
Bivalve	Atlantic sea scallop(*Placopectin megellanicus*)	Sea lice (copepoda)	Atlantic Salmon	Bartsch et al. 2013
	Blue mussels (*Mytilus edulis*)	Sea lice	Atlantic Salmon	Bartsch et al. 2013
	Asiatic clam (*Corbicula fluminea*)	Avian influenza virus	Birds	Faust et al. 2009
	Freshwater mussel (*Anondonta californiensis*)	*E. coli*	Various	Ismail et al. 2015
Porifera	*Hymeniacidon perlevis*	Various bacteria	Mediterranean mussel (*Mytilus galloprovincialis*)	Longo et al. 2010, 2015
Polychaeta	*Sabella spallanzanii*	Various bacteria	Humans	Stabili et al. 2006b,2006a 2010
	Ircinia variabilis	Various bacteria	Various	Ledda et al. 2014
	Agelas oroides	Various bacteria	Various	Ledda et al. 2014
Ascidian	*Polyandrocarpa zorritensis*	Various bacteria	Various	Stabili et al. 2015

Table 9.3. Current and proposed use of bivalves and other filter-feeders as sentinels for pathogens

Filter-feeders				
Taxa	**Species**	**Program or Location**	**Pathogens detected**	Citation
Bivalves	Asiatic clams (*Corbicula fluminea*)	Trials	Avian Influenza	Huyvaert et al. 2012
	Mussels (*Mytilus chilensis*)	Chile	Hepatitis A virus	Enriquez et al. 1992
	Mussels (*M. edulis*)	Skagerrak coast Sweden	Adenoviruses, enteroviruses, Norwalk-like virus	Hernroth et al. 2002
	Mussels (*M. californianus*)	California Coast	*Salmonella spp., C. perfringens, P. shigelloides, Vibrio cholerae, V. parahaemolyticus, V. alginolyticus*	Miller et al. 2006
	Mussels (*M. californianus*)	California Coast	*Cryptosporidium*	Miller et al. 2005
	Mussels and oysters various species	USA (Mussel Watch)	Various	Powell et al. 2015
	Mussels and oysters various species	Spain (Various)	Various	Muniain-Mujika et al. 2003
	Dresseneid mussels (*Corbicula fluminea*, *Dreissena spp*.)	Great Lakes/St. Lawrence River (North America) Shannon River (Ireland)	*Cryptosporidium, Giardia, Cyclospora, Enterocytozoon*	Conn et al. 2013; Ladeiro et al. 2014; Mezzanotte et al. 2016
Polychaeta	*Branchiomma luctuosum*	Mediterranean sea	Various bacteria	Stabili et al. 2006b
	Sabella spallanzanii	Mediterranean sea	Various bacteria	Licciano et al. 2007
Porifera	*Hymeniacidon perlevis*	In trial	Various bacteria	Longo et al. 2010, 2015
	Spongia officinalis	In trial	Various bacteria	Stabili et al. 2008

Impacts of introduced species

The introduction of nonnative and reintroduction of recently extirpated native bivalve species (restoration aquaculture) has been suggested as a viable way to reduce the level of human pathogens in the water column for both saltwater (NAS 2004) and freshwater ecosystems (Ismail et al. 2015). For example, a recent laboratory study indicates the reintroduction of *Anadonta californiensis*, a species of freshwater mussel once found in Mountain Lake, California (once the source of drinking water to the city of San Francisco) may facilitate removal of *E. coli* (Ismail et al. 2015). Other studies have shown that invasive filter-feeders efficiently remove native pathogens from the new location and thus effectively mitigate disease outside of their natural range (Thieltges et al. 2009; Welsh et al. 2014; Goedknegt et al. 2015). For invasive species that thrive in warmer temperatures such relief from pathogens may be welcomed by native hosts, which may already be stressed by increased water temperatures thus making the mitigation effects greater.

Movement of non-native filter-feeders can be risky. *C. gigas* has been cultured outside of its natural range and has subsequently colonized many coastal regions throughout the world (Troost 2010). Introduction of *C. gigas* from Japan to California, USA is linked to the introduction of *Haplosporidium nelsoni* into Tomales Bay, CA. (Burreson et al 2000). Movement of infected *C. gigas* to the US east coast from the Pacific (either directly from Asia or from California) is believed to be the potential vector for transmission of this pathogen to the native oyster, *C. virginica* (spillover; Daszak et al. 2000) (Burreson et al. 2000; NAS 2004). Similarly, native pathogens can infect and be amplified by invasive species and resulting in an increased local pathogen population and spill-back into the native species (Daszak et al. 2000, NAS 2004). For example, the Suminoe oyster, *C. ariakensis*, was considered for introduction to the Chesapeake Bay in order to restore lost ecosystem services (including removal of human pathogens) that were previously provided by oysters (NAS 2004). Ultimately, this oyster was not introduced into the Chesapeake Bay, in part, due to concerns about potential introductions and amplification of oyster diseases by this species (NAS 2004). These observations demonstrate the complex trade-offs that occur when moving filter-feeders.

How can modeling inform management approaches?

The impact of pathogen filtration by non-host organisms on disease dynamics depends on numerous processes occurring within and among filter-feeders (Powell and Hofmann 2015). Much of what we know about the role of filter-feeders on pathogen abundance and persistence comes from laboratory studies and it is unclear how the patterns ''scale up'' in larger, more complex environments. There is potential for non-linear effects of filtration on pathogen dynamics and complex, environmentally dependent mediators of these interactions. In cases where pathogen filtration may be considered as a management approach, modeling is advised for identifying the levels of filtration necessary to alter transmission among host organisms, the environmental conditions when filtration is an appropriate strategy, and potential trade-offs associated with the approach (e.g., unintended pathogen augmentation and non-linear effects). Recent modifications of typical Anderson-May type Susceptible-Infected (SI) models to Susceptible-Infected-Particle (SIP) models may prove valuable for calculating the effects of filtration on transmission in aquatic environments (Murray 2009; Bidegain et al. 2016). Such models were specifically designed to examine the epidemiology of disease in aquatic environments, where transmission is typically density-dependent and occurs from the water column. Pathogens (or particles) are modeled as a concentration in the water column, or an absolute number within host or non-hosts, thus specific effects of the filter-feeder on the pathogen can be modeled directly (e.g., degradation, clearance, replication).

Transmission of pathogens from the water column to receptive hosts is dependent on numerous biological, physical, and chemical properties including environmentally dependent growth and survival of the pathogen, tides, currents, temperature, and pH (Powell and Hofmann 2015). Such factors are often heterogeneous over space and time and may not be amenable to SIP models. An alternative approach is to use gridded hydrodynamic models or FVCOM (Finite Volume Ocean Circulation Models), which model the movement of particles over space and time. Although FVCOM models are computationally expensive and are only practical for simulating dynamics of local or regional areas, they may be useful for identifying source-sink dynamics of pathogens (Salama et al. 2013). In theory, these models could inform strategic placement of filter-feeders to have a maximum effect on disease dynamics. Such environmentally-dependent models can be used to investigate future scenarios (e.g., with climate change).

As with many disease models, parameterization and validation are some of the biggest challenges for the proposed models. Such models require estimation of (1)

filtration and degradation (or replication) rates, (2) contact rates between pathogens, filter-feeders, and hosts, and (3) impacts of infection on host survival, fitness, and production of pathogens. Validation of model outcomes requires field measurements of disease rates, quantification of density, and filtration rates by accidental (non-target) hosts and often requires multidisciplinary collaboration.

Conclusions and future directions

As the necessity for marine disease management increases, so does the need for strategies to reduce pathogen transmission. Bivalves and other species can provide a potent ecosystem service by efficiently filtering some pathogens. Bivalves are also valuable as pathogen sentinels due to their capability to bio-concentrate pathogenic microorganisms. Many knowledge gaps need to be overcome to evaluate the utility of a specific species to filter target pathogens, as well as the potential for the filter-feeder to act an active reservoir and convey pathogenic microorganisms to humans or wildlife. Careful choice of a robust combination of filter-feeder and pathogen is needed for efficient management (for transmission reduction or pathogen monitoring), particularly when the filter-feeder is introduced. Key knowledge gaps to investigate include calculating the optimal density of the filter-feeder's hosts both in the capacity of filtering and evaluating the potential for increased disease risk to both the filter-feeder and possibility of augmentation (active or passive). It is clear that diseases of the filter-feeder themselves may impact ecosystem services provided. In addition to the filter-feeding host, the natural symbionts (metaorganism or holobiont) may be collectively responsible for the filter-feeders ability to reduce pathogen loads, though research in this field is limited. In the future, screening of host symbionts may be an integral part of choosing particular species or cohort of animals for disease management. In addition, paired studies using empirical data and modeling may be useful for understanding how filter-feeders reduce pathogens, and how ocean change may play a role in the capacity of the biofiltration.

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General Discussion

Jennifer E. Welsh

The transmission from one host to another is an essential part of a parasite's life cycle. It enables the parasite to reach a host in which it can reproduce or multiply, but it also necessitates the parasite to leave the safety of its host organism. These free-living infective stages encounter many complex situations, possibly resulting in failure of transmission. For example, an encounter with non-host organisms can prevent them from infecting downstream hosts via one of many mechanisms, one of which is parasite removal by non-hosts (Thieltges et al. 2008a). This can alter the overall abundance of free-living stages of parasites and ultimately affect infection prevalence and intensity in the target hosts. Using marine parasite-host model systems, I explored the fundamental mechanisms driving parasite removal by non-hosts to understand the interactions that occur between non-host organisms and infective stages during transmission. In this final chapter, I discuss the major findings from the experiments performed and place the findings into the broader context of how biodiversity affects disease risk.

Parasite removal by non-hosts is common

In indirect life-cycles, such as those exhibited by the trematodes studied in this thesis, the parasite may have multiple free-living infective stages (Poulin and Cribb 2002, Rauch et al. 2005). The first free-living stage in a trematode life cycle is typically an egg, from which a motile miracidium hatches. The second free-living stage is also motile and has the ability to actively seek its downstream host. These free-living cercarial stages have a glycogen rich tail, which is used up to provide the energy for finding the next host (Lawson et al. 1980, Beers 1995). Due to this limited energy source, cercariae only have a short time frame (usually less than 24 hrs) in which they can infect their downstream host and complete the transmission process (Thieltges and Rick 2006). During this transmission phase, other organisms which do not serve as hosts, can reduce infective stage abundance, e.g. by preying on infective stages. A reduction in the number of infective stages due to parasite removal ultimately leads to lower infection intensity in the target hosts, that is fewer parasites within individual hosts (Chapter 2; Fig. 10.1 A). The second parasite group studied in this thesis are viruses. There the infection of only one parasite can result in the death of the host, and a reduction in parasite dose by non-host organisms leads to a reduction in parasite prevalence (a reduction in the proportion of infected hosts within the host population; Chapter 8; Fig. 10.1 B).

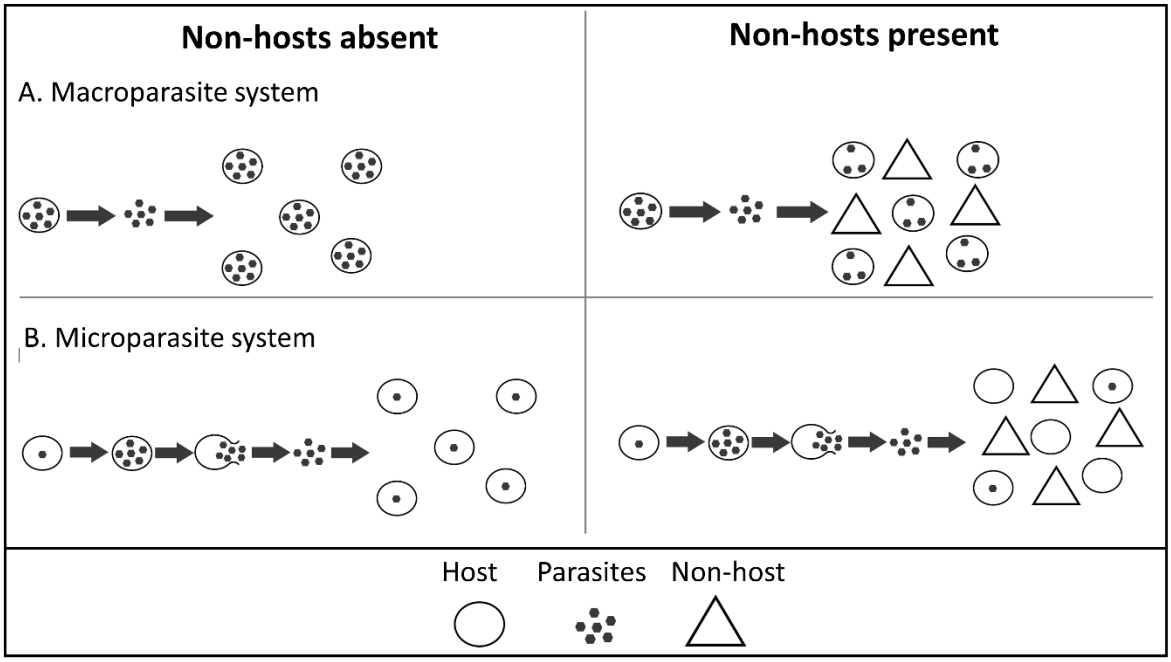


Fig. 10.1. In a simple system, where no organisms other than the parasite's hosts exist, infective stages are able to transmit from one host to another. In more complex systems where non-host organisms are also present, transmission interference can occur leading to lower infection intensity (A). For example, in macroparasite systems such as the trematode system used in this thesis, the presence of non-host organisms may remove free-living infective stages resulting in lower infection intensity, i.e. individual hosts will be infected by fewer parasites; or lower parasite prevalence (B). For example, in microparasite systems such as that of the marine algal virus also used in this thesis, the presence of non-host organisms may remove free-living infective stages resulting in lower parasite prevalence i.e., fewer hosts become infected.

In general, parasite removal by non-hosts as a means of parasite regulation has been largely underestimated. In this thesis I have shown that parasite removal is a common phenomenon occurring in both microparasites (viruses and bacteria) as well as in macroparasites such as helminths (Chapters 2 and 8). Moreover, existing data suggests that there is a large diversity of organisms which use a variety of mechanisms but are, nonetheless, efficient in removing free-living infective stages from their environment (Chapters 2 and 3, Fig. 10.2). The simplest form of transmission interference occurs when an organism acts as a physical barrier. The seaweed *Sargassum muticum* is a good example, reducing cercarial abundance by over 85% in laboratory experiments, by entangling infective stages in the complex structure of the algae (Chapter 3).

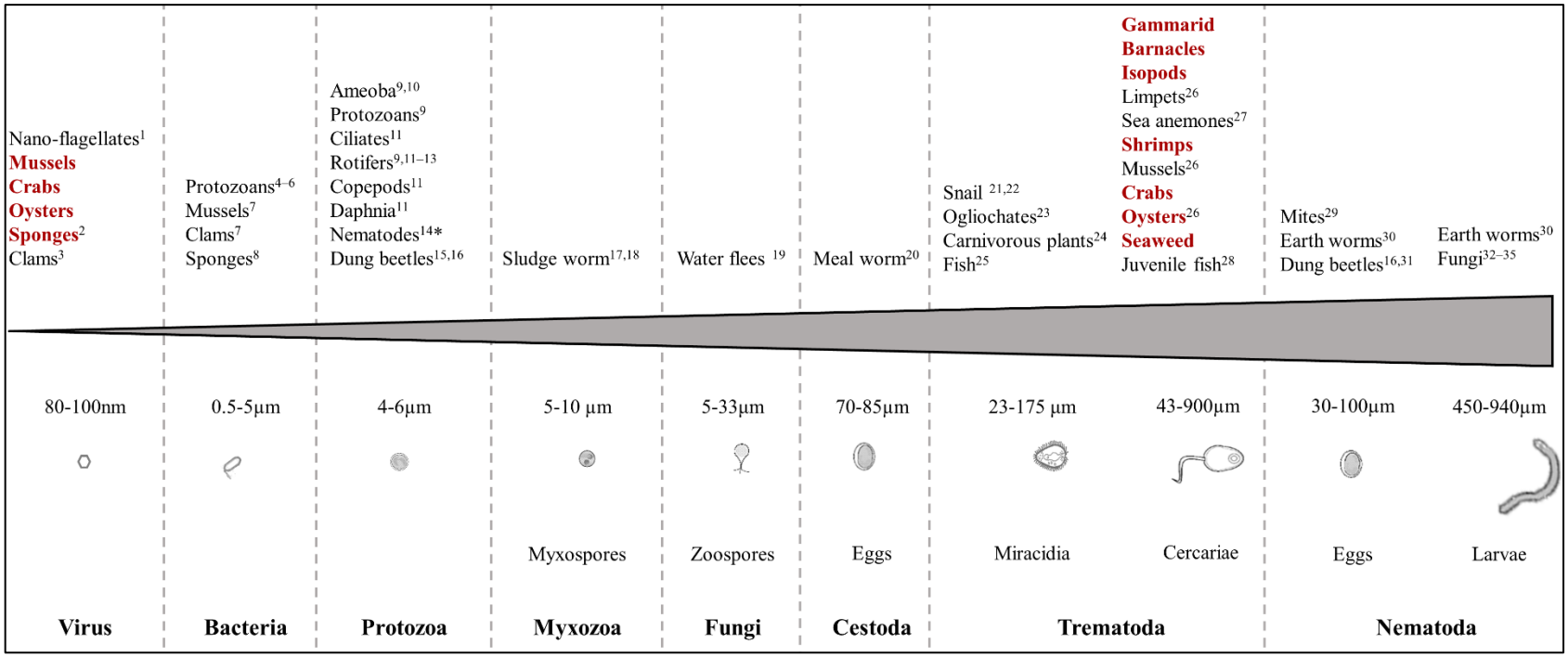


Fig. 10.2. Removal of free-living infective stages by non-host organisms occurs across a variety of parasite systems and encompasses extremely small virus particles to large nematode larvae. The types of non-host organisms causing transmission interference is also varied, with large species reducing the abundance of both small and larger free-living infective stages. In this thesis, previously unknown organisms (shown in red) were identified for their ability to interfere with the transmission process of virus particles and trematode cercariae, highlighting the commonality of transmission interference by non-host organism across both micro- and macroparasite host systems. [1] Bettarel et al. 2005; [2] Pinheiro et al. 2007; [3] Hadas et al. 2006; [4] Faust et al. 2009; [5] Mange et al. 2002; [6] González et al. 1990; [7] Worden et al. 2006; [8] Silverman et al. 1995; [9] Fu et al. 2006; [10] Stott et al. 2003; [11] Siqueira-Castro et al. 2016; [12] Fayer et al. 2000; [13] Trout et al. 2002; [14] Hansen et al. 1993; [15] Connelly et al. 2007; [16] Rønn et al. 2012; [17] Yeates et al. 1993; [18] Mathison and Ditrich, 1999; [19] Salas Iglesias, 2014; [20] Beauchamp et al. 2006; [21] Rácz et al. 2006; [22] Buck et al. 2011; [23] Lethbridge, 1971; [24] Rodgers et al. 2005; [25] Khalil, 1961; [26] Gibson and Warren, 1970; [27] Upatham, 1972; [28] Upatham and Sturrock, 1973; [29] Bunnag et al., 1977; [30] Thieltges et al. 2009; [31] Mouritsen and Poulin, 2003; [32] Hopper et al. 2008; [33] Kaplan et al. 2009; [34] Ismail, 2003; [35] Chen et al. 2013; [36] Waghorn et al. 2002; [37] Grønvold, 1987; [38] Miller et al., 1961; [39] Fincher et al. 1975; [40] Fincher et al. 1973; [41] Bryan and Kerr, 1989; [42] Weber et al. 1952; [43] Barron, 1977; [44] Chandrawathani et al. 1998; [45] Hsueh et al. 2013.

Whilst no study has quantitatively compared the various methods of transmission interference, active and passive predation by non-host organisms have been repeatedly reported to be significant contributors to the demise of free-living infective stages. In marine parasite-host systems there is not only a wide variety of species predating on free-living infective stages, but the extent to which they can reduce parasite abundance can be substantial and can occur rapidly with some non-host species, such as sponges, crabs, shrimps and gammarids, reducing cercarial abundance by over 65% within 3 hours (Chapters 1 and 2) and sponges reducing viral abundance by 98% within 48 hours in experimental settings (Chapter 8).

Mechanisms of parasite removal

Although many organisms haven been shown to remove parasites the studies presented in this thesis show that the removal of parasites from the environment is a complex process modified by various abiotic and biotic factors (Fig. 10.3).

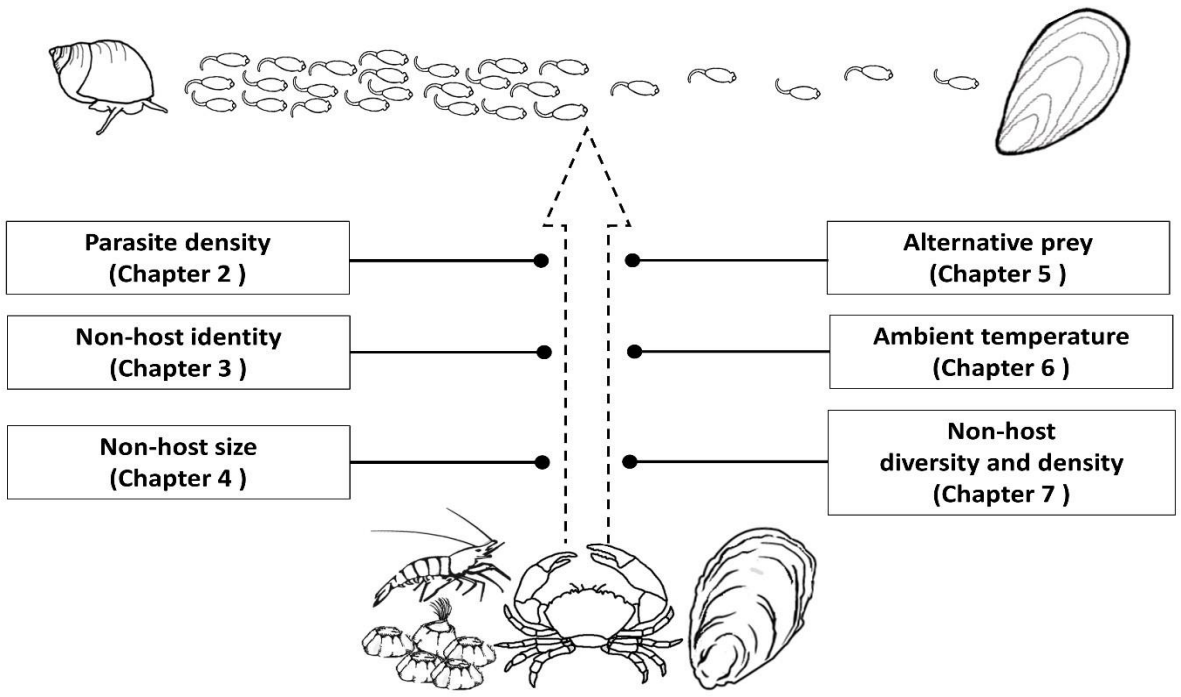


Fig 10.3. Mechanisms mediating the effects of non-host organism removing free-living infective stages in a marine trematode model system. Trematode cercariae ( ) transmission from the first intermediate snail host to the second intermediate mussel host may be altered (dashed arrow) by a variety of non-host organisms such as crabs, oyster, shrimps and barnacles. Mechanisms such as parasite density and non-host organism size, which were tested in this thesis, have been shown to influence transmission interference by non-host organisms, determining when and to what extent they predation upon the free-living cercariae occurs.

Many non-host organisms can remove parasite infective stages (Fig. 10.2). However, the experiments presented in this thesis indicate that the efficiency of removal varies widely and that some non-hosts do not remove trematode cercariae or infective virus particles at all (Chapters 3 and 8). For example, while the feather-like structure of the seaweed *Sargassum muticum* reduced cercarial abundance by over 85%, the broad blades of *Fucus* seaweed were much less efficient (<11%), suggesting that species identity and specific traits play an important role in when transmission interference occurs. Thus, during physical removal of infective stages, structural elements that entangle cercariae are probably the most relevant traits. During predation of infective stages by non-host organisms, the trait expected to be most relevant is that of prey size range. If infective parasite stages fall within this specific size range, they may be consumed and thus removed from the environment. In the studies presented in this thesis, this was a trait commonly shown by filter-feeding bivalves. In particular, Pacific oysters were very efficient in removing infective stages from the water column, this was the case for both macro- and micro-sized free-living stages. This is perhaps not surprising considering their efficient filtering mechanism and their ability to rapidly clear water masses of particles (Chapter 9; Gosling 2003). However, oysters are traditionally known to be selective with the particles which they ingest, selecting for prey of specific shapes and prey within a specific size category, with prey density affecting filtration rate (Bougrier et al. 1997, Cognie et al. 2003, Gosling 2003). While this is not the first time that oysters have been reported to filter free-living trematode cercariae (Thieltges et al. 2008a, 2009), the results in this thesis show that they continue to do so with no sign of density dependency and when another food source is available, suggesting that they may actively select cercariae. Irrespective of the selection process, the life strategy of oysters and their ability to create large beds within in tidal ecosystems and with high filtration rates (approx. 8 l h^{-1}; Riisgård 1988) means that, collectively, oysters are likely to have a huge ecological impact on the free-living parasite abundance.

In contrast to filter feeders, the link between the prey size spectrum of benthic predators such as crabs and shrimps and the sizes of parasites seem to be more complex. Like oysters and cockles, crabs also showed a positive relation between body size and cercarial removal rates but this pattern differed from expectation. It was assumed that a size mismatch would occur in crabs, whereby the infective parasite stages would be too large for smaller crabs and too small for larger crabs to remove. However, cercarial stages were removed by all size classes with larger crabs removing more cercariae than smaller individuals. This suggests that crabs do not use their claws to catch cercariae. Various crab species have been shown to catch small particles in a

manner similar to that seen in filter feeders (Gerlach et al. 1976; Watts 2014). Specifically, small 10μm polystyrene microspheres have been shown to be taken up by *C. maenas* crabs (a species used in this thesis) and retained by their gills which are normally used only for oxygen uptake and not particle filtration (Watts 2014). Furthermore, cercariae stained with fluorescent dye were recovered from the digestive tract and the gills of the crab species (pers. obs. D. W. Thieltges). Such alternative mechanisms of cercarial removal may explain the different findings in other aquatic predators where a negative relationship between predator body size and parasite removal due to prey size mismatches has been observed (e.g. damselfly nymphs, dragonfly larvae, mosquitofish; Orlofkse et al. 2015, Catania et al. 2016). Thus, my findings suggest that predator effects on cercarial removal rates may be more diverse than only relating to direct predation and may also include indirect mechanisms such removal via mouth parts or gills similar to the filter feeding in bivalves.

The effects of parasite density and the presence of alternative prey were also species-specific. Some non-host organisms (for example, oysters) removed parasites across multiple parasite densities and in the presence of an alternative prey source, whereas other non-host organisms such as shrimps did not. The presence of alternative prey and differences is parasite density affected the shrimps' ability to remove free-living parasites, suggesting that prey switching (Murdoch, 1969; Cornell, 1976) occurs within this particular species, a factor not seen with the other non-host organisms investigated. In an ecological context, this shows that while some species can continue to remove free-living parasites under a variety of conditions, other species cannot. Considering that consumption rates also vary between non-host species (Orlofske et al. 2015), the overall effects of non-host species traits on the removal of free-living parasite abundance is likely to be extensive. Consequently, infection intensities in hosts will depend on the specific composition of species within the given ecosystem (Wood et al. 2014) and may be confounded by the specific factors occurring at any given time; a paradox which contradicts the blanket theory that increasing biodiversity reduces disease risk (Keesing et al. 2006).

The actuality that the strength of transmission interference effects depend on the specific composition of species rather than simple species richness per se was also indicated by experiments manipulating the diversity and density of non-host organisms (Chapter 7). In all possible combinations of non-host organisms (seaweed, oysters and crabs), complex interactions and no simple additive effects of diversity occurred. These complex interactions, leading to decreases as well as increases of the disease risk for target hosts, resulted from intra and interspecific interactions between the species involved (Fig. 10.4). This insinuates three essential points: parasite removal

effects of non-hosts are not simply additive, behavioural traits are likely to be a key feature of parasite removal and, perhaps most controversially, an increase in diversity does not always lead to a reduction in infective stages. These findings resemble results on general multiple predator effects, but contrary to theories about the effects of biodiversity on disease risk, due to the intricate behavioural interactions between the predators (Soluk 1993, Sih et al. 1998), the presence of multiple predators can result in fewer parasites being consumed (Chapter 8; Fig. 10.4; Griswold and Lounibos 2006, van Son and Thiel 2006, McCoy et al. 2012, Siddon and Witman 2013).

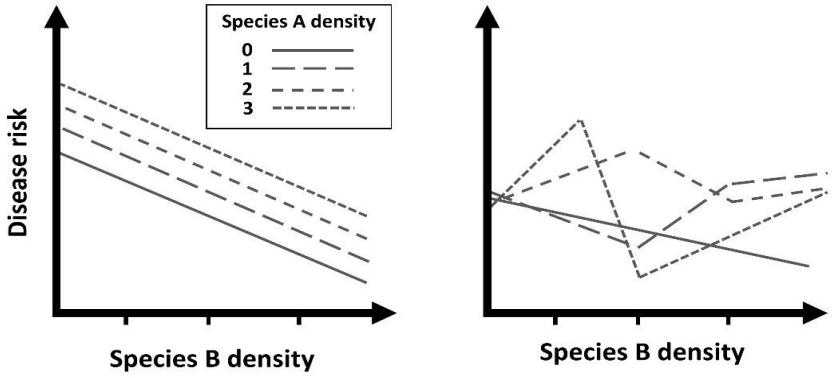


Fig. 10.4. Increasing species diversity and density would, theoretically, result in additive effects on disease risk (left). However, this thesis demonstrates that the complex behavioural interactions which occur between individuals of the same or different species results in very intricate effects on the outcome of disease risk (conceptual graph, right).

In addition to the various biotic factors, affecting the strength of transmission interference, abiotic factors may further modify these processes. In the course of this thesis, ambient temperature was investigated as it is a common driver of environmental processes and is known to affect the production and longevity of free-living infective stages with increased temperatures increasing cercarial production but reducing cercariae longevity (Poulin 2006, Thieltges and Rick 2006, Studer and Poulin 2013). It has been postulated that an increase in global temperatures via climate change, would increase the number of disease incidences with parasites maturing quicker and producing more generations throughout the whole year (for review see Marcogliese 2001). However, in this thesis I show that an increase in ambient temperature can also affect the consumption rates of parasites by non-host organisms (Chapter 6). Higher temperatures increase metabolism, and thus the feeding rate, of non-host organisms resulting in a higher predation rates of cercariae at higher temperatures. Therefore, some predators may be essential in counteracting the increase in free-living infective stages as caused by temperature increases. However, not all non-host species offset the effects of increased temperatures, despite continuing

to reduce overall cercarial abundance. For example the Pacific oyster *Crassostrea gigas* was very efficient at reducing cercarial abundance across all temperatures. This suggests that the studied effects are species specific, a phenomenon which is observed throughout all the studies shown in this thesis.

Finally, although this thesis mainly focused on the removal of macro-parasites using cercarial stages as an experimentally tractable model, similar mechanisms are also likely to influence the removal of micro-parasites (Fig. 10.1 and 10.2). However, more research into this direction is needed to determine the definite consequences of non-host organism size, density and temperature effects as well as the presence of alternative prey on the removal of micro-parasites by non-hosts.

Consequences for target hosts and potential applications

For individual hosts, parasite infections can make the difference between life or death (Brussaard 2004a), for others it can have diverse sub-lethal effects such as reduced condition and increased sensitivity to additional stressors (Marcogliese and Pietrock 2011). I have shown that several non-host organisms are capable of counteracting the effects of both high and low parasite densities (Chapters 2 and 5). Most importantly, any reduction in parasite abundance translates as a reduction in infection intensity in downstream hosts (Chapter 2), a reduced encounter rate between parasite and host or prevents infection altogether (Chapter 3). Thus, parasite removal can affect the disease risk for individual target hosts. However, as parasites can also have far-reaching effects on community and ecosystem levels (Hatcher and Dunn 2011, Thomas et al. 2005), the effects of transmission interference are likely to have wider implications. For example, in systems where timing is key, such as that of viruses infecting bloom forming algae resulting in the termination of the often problematic algal bloom, reductions in infective stages can alter parasite-host encounter rates resulting in lower prevalence. This could lead to the prolonging the algal bloom and have fundamental effects on the whole ecosystem.

The idea of ambient organisms neutralizing the production of parasites through predation is highly appealing, especially regarding disease management (Chapter 9). The manipulation of ambient communities so that they contain more species which are effective at reducing free-living parasites has the potential to help reduce disease incidences. For example, over the last few decades the aquaculture industry has seen an increase in demand for aquatic products but production is hindered and limited by

disease (Ananda Raja and Jithendran 2015, Pérez-Sánchez et al. 2018). The production of aquatic products typically occurs in ponds or cages where the cultured species is kept at high densities and all other species are omitted from the system, allowing specific diseases to thrive (Lotz and Soto 2002, Lafferty et al. 2015). Revaluating these traditional culture methods to introduce additional organisms which are efficient at interfering in parasite transmission may reduce the abundance of parasites such as trematodes, bacteria, viruses and protozoans (Chapter 9, Stabili et al. 2006b, Licciano et al. 2007, Feichtmayer et al. 2017). In addition to their commercial use, ambient organisms have the potential to reduce parasite loads in wild populations, and may be of particular importance where changes in parasite loads and diversity within a given system are a result of anthropological incidences (Scholz 1999, Sepúlveda et al. 2004, Costello 2009) resulting in parasites spilling over into organisms within a new environment (Goedknegt 2017).

Parasite removal by non-hosts in the biodiversity and disease risk debate

It is commonly reported that an increase in biodiversity can reduce the transmission and establishment of a disease within a given ecosystem via the 'dilution effect' (Chapter 1). Despite this, the results of studies testing the effects of biodiversity decline on disease risk have been ambiguous (Keesing et al. 2006, Randolph and Dobson 2012, Lafferty and Wood 2013, Ostfeld and Keesing 2013, Zargar et al. 2015). Whilst it is not disputed that changes in biodiversity have some effect on disease, existing studies did not incorporate several aspects of an ecosystem. Firstly, the majority of studies have focused on the diversity of host species in vector-borne systems and did not include other, equally important, parts of the ecosystem such as non-vector borne diseases (Levi et al. 2012, Wood and Lafferty 2013, Salkeld et al. 2013, Wood et al. 2015). Secondly, those studies which have included non-vector borne diseases used changes in host diversity as a measure of biodiversity (Levi et al. 2012, Johnson et al. 2013, 2015) and only few included non-host organisms (Orlofske et al. 2012). Thus, the full range of the 'net effects of species diversity' (Keesing et al. 2006) driving the dilution effect phenomenon has not yet been explored. In this thesis, I demonstrate how the removal of parasites with free-living infective stages by non-hosts is a valid mechanism contributing to the dilution effect theory (Chapter 8). However, deciphering the effects of only diversity can be problematic as increasing diversity inherently changes the density of the organisms present (Sih et al. 1998, Inouye 2001). Unlike previous

biodiversity and disease risk studies, I used a fully factorial response surface experimental design to identify the difference between diversity and density effects (Chapter 8). In these experiments I show that changes in non-host diversity results in complex behavioural-based interactions, resulting in either no effect, in a significant reduction or increase in the removal of free-living parasites. For example, the presence of a crab (a natural enemy to oysters; Kulp et al. 2011, Grabowski 2012, Pickering et al. 2017) makes the oyster close, a defence mechanism used against the crab but which prevents it from feeding on the free-living parasitic stages. Conversely, when an oyster is combined with seaweed it is postulated that the seaweed acts as an indirect defence barrier, allowing the oyster to feed. The behavioural interactions not only occur between different species as diversity increases but also between individuals of the same species as density increases. For example, crabs of the same species are known to compete with each other for resources such as prey (Smallegange 2007). In Chapter 8 it was seen that in the presence of seaweed, the addition of a second crab to the system resulted in fewer parasite infective stages being removed, but, when increasing crab density the crabs removed more infective stages, ultimately amplifying the parasite removal effects. However, when seaweed and oysters were combined, an increase in seaweed density resulted in fewer parasites being removed compared to oysters alone, resulting in a neutralization in parasite removal effects. The results suggest species specific intra- and inter-species interactions and individual behavioural differences mean that parasite removal is not always consistent and is difficult to predict. Therefore, infection intensities in hosts will depend on the specific composition of species within the given ecosystem; a paradox which contradicts the blanket theory that increasing biodiversity reduces disease risk (Keesing et al. 2006).

Limitations and future propositions

The experiments undertaken during this study show that biodiversity can have an effect on disease risk but the outcomes are often multifaceted and depend heavily on behavioural traits of the non-hosts removing the infective stages. The experiments were conducted in the laboratory so that all factors could be rigorously controlled. This enabled me to give robust conclusions regarding the mechanisms driving transmission interference. However, it is expected that under a natural setting there would be a multitude of other factors interacting with both the hosts and the non-host organisms, further complicating the transmission process. For example, it is expected that predators of both the hosts and non-host organisms would alter their behaviour and

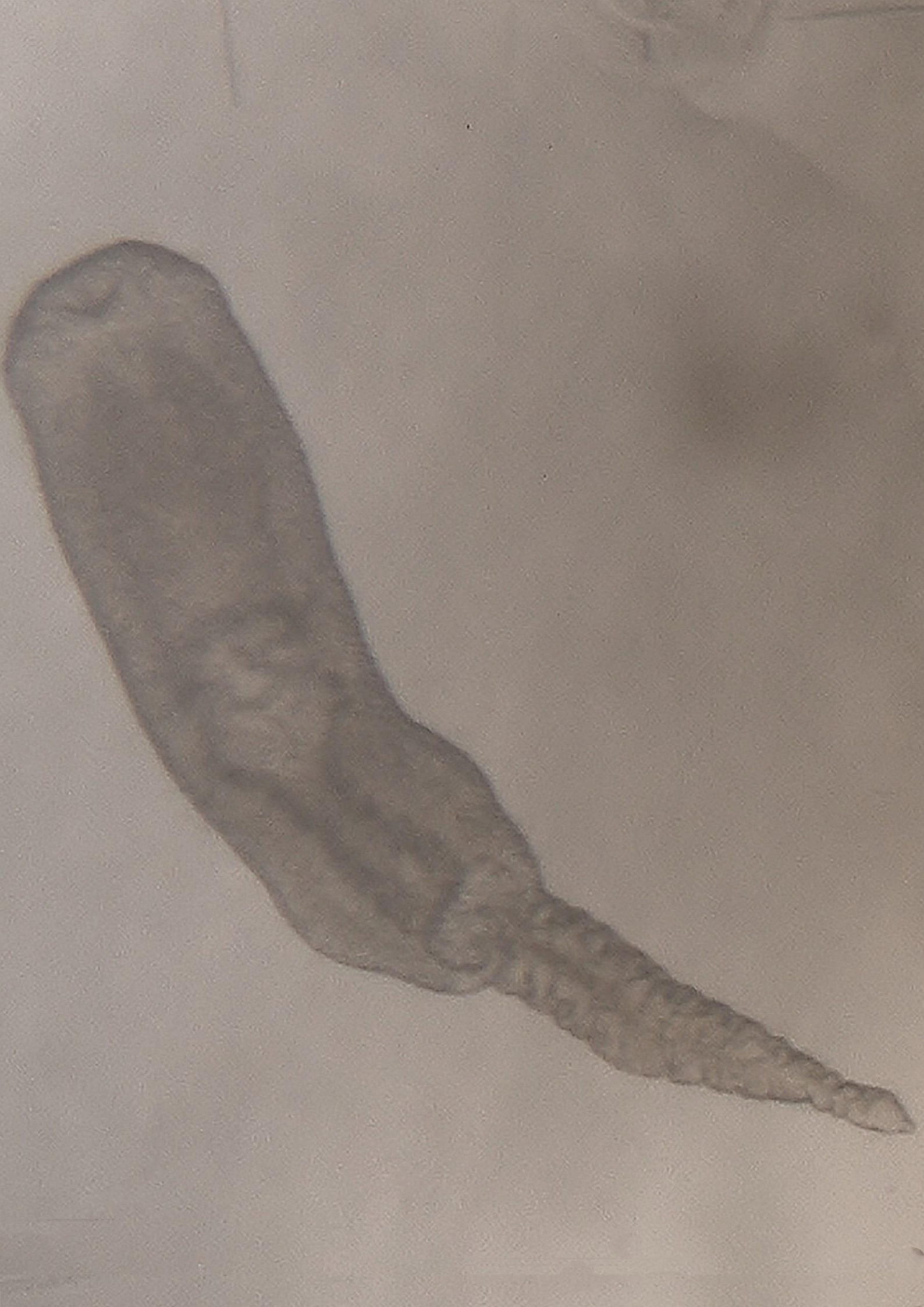
possibly lead to reduced or increased contact rates between the parasite and host. Moreover, in some parasite-host systems, seasonal changes and host migrations may also play a role, not only altering hosts to non-host organisms ratios but also releasing some consumers from their predators, leaving them free to feed (Andresen 2013). Furthermore, non-host organisms may interact with the other free-living stages displayed by indirect lifecycle parasites. For example, the miracidia of intertidal trematodes may be removed from the sediment surface and consumed or buried by benthic organisms such as polychaetes, preventing the parasites from reaching their downstream host. Although these factors can be tested in laboratory experiments it is suggested that they also be applied and compared to field based experiments as other factors such as tides, currents and biofilms may influence the outcome.

This thesis has focused on marine parasite-host systems as an example but the methods used and mechanisms identified serve as a paradigm for other parasite-host systems. Testing the theories shown in this thesis on other systems will extend our understanding of disease interactions. Furthermore, comprehension of the mechanisms driving transmission interference across different systems could aid management decisions and conservation measurements (Garchitorena et al. 2017). Finally, the complex interactions highlighted throughout this thesis warrant an ecological audience and therefore, disease research needs to break away from its traditional clinical setting and be widely considered in an ecological context (Marcogliese 2004, Johnson et al. 2016).

Final remarks

The research in this thesis collectively demonstrates the importance of the interactions which occur between ambient organisms and free-living infective stages during parasite-host transmission. The results reveal that parasite removal caused by non-host organisms is not only omnipresent, occurring in multiple different parasite-host systems, but also an integral part of ecosystems. Through the work I present in this thesis it is evident that many factors such as parasite dose, non-host identity, size, and density, influence how and when non-host organisms affect parasite transmission. It is expected that parasite removal caused by non-host organisms will differ according to each circumstance and, due to the complex interactions involved, may not always lead to an overall reduction in free-living infective stages. Therefore, it is essential to expand on the work conducted in this thesis to fully understand the interplay of

biodiversity and disease risk and to ensure that parasite-host interactions are universally considered a key component of ecosystems.



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