Parasitism in a changing world:
The intertidal trematode
Maritrema novaezealandensis and its hosts

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This thesis is dedicated to my godson

Noe Sol Achermann-Murpf

Abstract

There is growing concern about increases in diseases and parasitism with on-going and predicted global climate changes in many ecosystems, including marine ecosystems. Understanding and predicting the possible consequences of these changes on a particular host-parasite system, however, requires a sound knowledge of various aspects of the life cycle components, including their interactions with the environment.

This thesis investigated the ecology of the intertidal microphallid trematode *Maritrema novaezealandensis* and the hosts associated with its complex life cycle: first intermediate snail hosts *Zeacumantus subcarinatus*, second intermediate amphipod hosts *Paracalliope novizealandiae* and definitive bird hosts. An integrative approach was adopted to examine current temporal patterns on a high prevalence mudflat (field study), study the sensitivity of individual steps of the parasite's transmission process from first to second intermediate host (both ectotherms) to abiotic (temperature, salinity and ultraviolet radiation) and biotic environmental factors (laboratory studies), and subsequently use that information to model the life cycle of the parasite and assess the consequences of predicted global warming on the study system, in particular on amphipod hosts.

The seasonal field monitoring showed not only that most of the parasite transmission from first to second intermediate invertebrate hosts takes place during warm summer months, but also that probably the entire life cycle of this parasite is accelerated during that time. This is due to temperature directly, but also due to the availability of hosts.

All environmental factors investigated in the laboratory studies emerged as potentially strong modulators of the transmission of *M. novaezealandensis*, with temperature having the most pronounced effects overall. The two steps of the transmission process identified as the most sensitive were the survival of the free-living parasitic transmission stage (affected by all factors, also interactively) and the survival of amphipods. Results indicated that conditions considered optimal for transmission (low tide, warming water in tide pools) benefited the successful transmission of the parasite to amphipod hosts up to an optimum temperature. Furthermore, the presence of a non-host community member that preys upon the parasitic transmission stages was not strong enough to counteract an increased transmission success of the parasite under conditions of warmer temperatures.

In contrast, ultraviolet radiation may account for substantial mortality of the free-living parasite stages in nature, due to the absence of protective mechanisms. This effect may, however, be compensated by an increased susceptibility of amphipods to infection.

The mathematical model simulating the dynamics of the life cycle of *M. novaezealandensis* under different temperature increases was based on information from the field, laboratory experiments or the literature. The simulations indicated that the predicted temperature increases capable of driving the modelled amphipod host population to collapse mostly fell within the range of temperatures predicted to prevail in the study area over the next 80 years.

Overall, *M. novaezealandensis* and its impacts on hosts were not only shown to be influenced by environmental conditions intrinsic to intertidal ecosystems, but are also predicted to be affected by the changing conditions due to global change.

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CHAPTER ONE

General introduction

1.1 Ecological consequences of climate change

The world is constantly changing - it always has, and it always will. However, recent anthropogenic impacts have considerably altered the extent and nature of climatic and other environmental changes, and these changes are occurring at unprecedented rates (IPCC, 2007). As a consequence, climate change has been identified as one of the biggest challenges that humanity and natural systems face (e.g. Halpern *et al.*, 2008; Sala *et al.*, 2000). Natural systems are expected to alter in terms of their structure, function and species composition, and the impacts of climate change are considered extremely likely to exacerbate existing stresses, increase the probability of species extinctions, degrade natural systems and reduce ecosystem services (IPCC, 2007).

Climate and weather naturally affect many ecological processes; large-scale climatic fluctuations, for example, have been linked to changes in ecosystem properties and ecosystem functioning (e.g. Holmgren *et al.*, 2001; Mysterud *et al.*, 2001; Ottersen & Stenseth, 2001; Stenseth *et al.*, 2002; Stenseth *et al.*, 2003). Hence, any anthropogenically induced environmental changes are bound to have significant ecological consequences. Indeed, ecological and biological consequences of climate change have already been reported from all continents and most oceans (e.g. reviews by IPCC, 2007; McCarty, 2001; Parmesan, 2006; Parmesan & Yohe, 2003; Root *et al.*, 2003; Rosenzweig *et al.*, 2008; Walther, 2010; Walther *et al.*, 2005; Walther *et al.*, 2002), despite the fact that, for example warming, has only been relatively minor compared to what is expected to occur during the current century (Walther, 2010). Most studies have focussed on the effects of climate change on individuals or species, and on shifts in phenology, physiology or changes in the distribution and range of species (Root *et al.*, 2003; Walther, 2010). However, it has become increasingly acknowledged that, because individual species represent interconnected components of multilevel ecological networks, considering biotic interactions is highly crucial when studying and attempting to predict the consequences of environmental changes (e.g. Gilman *et al.*, 2010; Harmon *et al.*, 2009; Kiers *et al.*, 2010; Kordas *et al.*, 2011; Sutherst *et al.*, 2007; Traill *et al.*, 2010; Tylianakis *et al.*, 2008; van der Putten *et al.*, 2010; Walther, 2010).

Species interactions are among the most important forces structuring ecological communities, and they are commonly climate dependent (Gilman *et al.*, 2010 and references therein; Tylianakis *et al.*, 2008). The differential response or susceptibility of an individual

species to a particular change (or changes) may disrupt the interaction with others and thus may alter the outcome of an interaction (Walther, 2010; Walther *et al.*, 2002). For example, increased sea temperatures that result from the relaxation of upwelling mediated by El Niño Southern Oscillation events have caused changes in the interaction strength between a keystone predator and its prey (Sanford, 1999). A more recent study by Helland *et al.* (2011) provides another excellent example of how the outcome of the competitive interaction between two fish species is modified by the duration of ice cover during winter. In addition to the above described effects, the way that species interact may also alter the probability of local extinctions, and there is empirical evidence that climate driven changes in interacting species can drive such extinctions (Gilman *et al.*, 2010). These have not only been linked to altered phenology or behaviour of competitors and mutualists (e.g. Memmott *et al.*, 2007), predator or competitor efficiency (Huey *et al.*, 2009), and changes in the body size of prey (Anderson *et al.*, 2001), but also to pathogen prevalence (Pounds *et al.*, 2006).

Understanding effects of environmental factors on interaction strengths remains a critically important, as yet to be realised, step towards predicting the consequences of climate change, such as global warming, for natural ecosystems (e.g. Abrahams *et al.*, 2007). This is of great relevance in particular in the case of host-parasite interactions. Importantly, the consequences of global changes on diseases and parasitism have been widely acknowledged as being a major concern and great challenge (e.g. Jones *et al.*, 2008; Rohr *et al.*, 2011; Thomas *et al.*, 2004). Moreover, there is evidence that climate change is also already affecting a range of important diseases of humans, livestock and wildlife, although this evidence often remains anecdotal (e.g. Epstein, 2001; Harvell *et al.*, 1999; Harvell *et al.*, 2002; Johnson & Paull, 2011; Lafferty *et al.*, 2004; Marcogliese, 2001, 2008; Mas-Coma *et al.*, 2008; Patz *et al.*, 2000; Polley, 2005; Rohr *et al.*, 2011 and references therein; Ward & Lafferty, 2004).

1.2 Climate change, parasitism and diseases

Parasites and pathogens are ubiquitous components in natural systems. Despite the fact that they are often presented as potential threats for the viability of populations (de Castro & Bolker, 2005; Holmes, 1996; McCallum & Dobson, 1995), they are also important mediators and components of biodiversity (e.g. Dobson *et al.*, 2008; Hudson *et al.*, 2006b; Mouritsen & Poulin, 2002b). Parasites play crucial ecological roles (e.g. Marcogliese, 2004, 2005); they

can regulate host populations (e.g. Albon *et al.*, 2002; Cattadori *et al.*, 2005; Hudson *et al.*, 1998; Scott & Dobson, 1989), influence the composition and structure of communities and food webs (e.g. Combes, 1996; Lafferty *et al.*, 2008; Lafferty *et al.*, 2006; Minchella & Scott, 1991; Mouritsen & Poulin, 2002b, 2010; Thompson *et al.*, 2005), and affect the functioning of entire ecosystems (e.g. Thomas *et al.*, 2005). Parasites can also account for a highly underestimated amount of biomass in natural systems compared to their free-living counterparts (Kuris *et al.*, 2008).

The extent and intensity of parasitism can be modulated by climatic or environmental conditions (e.g. Cattadori *et al.*, 2005; Mouritsen & Poulin, 2002a; Poulin & Mouritsen, 2006). Therefore, changes in climate or in the local environment are bound to affect levels of parasitism with potentially important repercussions for host individuals, populations, communities and ecosystems (see e.g. Kutz *et al.*, 2005). Generally, the more complex the life cycle of a parasite, the more likely it is that it will be influenced by changes in environmental parameters (Koh *et al.*, 2004; Overstreet, 1993; Poulin & Morand, 2004).

Climate change will affect parasites species directly, but also through changes in the condition, distribution and abundance of their hosts (Fig. 1.1) (Harvell *et al.*, 2009; Marcogliese, 2001, 2008). Marcogliese (2001) provides a review of the effects of a range of environmental factors on parasites in aquatic environments and as a consequence, discusses how climate change may impact those systems. His synthesis shows that among the environmental factors that influence host-parasite interactions, temperature is often considered the most important abiotic parameter. Temperature has been shown to strongly affect parasites at all life-cycle stages (Chubb, 1979). Temperature, however, also affects hosts as well as the interaction between parasites and their hosts. For example, any form of stress such as thermal stress, can increase parasite-induced mortality or lower the performance of infected hosts (Esch *et al.*, 1975; Lee & Cheng, 1971; Lutterschmidt *et al.*, 2007; McDaniel, 1969; Tallmark & Norrgren, 1976; Vernberg & Vernberg, 1963) (but see Sousa & Gleason, 1989).

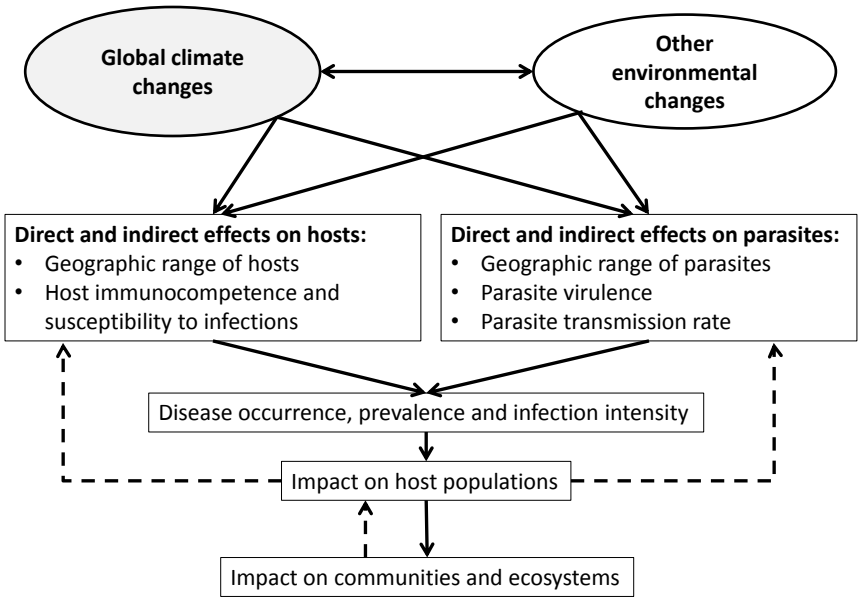


Figure 1.1 Schematic representation of the effects of global climate changes and other environmental changes on parasites and hosts. Environmental factors have cascading effects on hosts, populations, communities and ecosystems (solid lines). These effects will also have feedback effects onto hosts and their parasites (dashed lines). All effects will be influenced and modified by interactions with any other environmental variable or stressor. This figure is a modified version of Figure One in Marcogliese, (2008).

Parasites and pathogens with complex life cycles and/or those with ectothermic hosts may be disproportionally affected by changes such as global warming (Harvell *et al.*, 2002; Marcogliese, 2008). As a consequence of their intimate relationship with hosts, parasites are also particularly sensitive to secondary extinctions (de Castro & Bolker, 2005; Dunn *et al.*, 2009; Lafferty & Kuris, 2009a; Marcogliese, 2001). For example, many parasites have adapted their life cycles to the brief seasonal overlap between definitive and intermediate hosts. Breakdown in synchronicity between the presence of infected definitive hosts, available intermediate hosts and parasite reproduction will lead to the disruption of the parasite's life cycle and the reduction or extirpation of its population (Marcogliese, 2001). Also, the spatial distribution of a parasite with a complex life cycle is dependent on that of all hosts; a modified host distribution will determine where parasites persist and where they colonise (Dobson & Carper, 1992). This will not only depend on the relative sensitivity of hosts and

parasites to environmental conditions, but also on the interplay between the parasite and its hosts.

It needs to be highlighted that hosts and parasites are affected by the interactions of multiple abiotic and biotic factors (e.g. Marcogliese, 2008; Thieltges *et al.*, 2008b), which may impact the interactions and dynamics between them. Such multiple factor effects have been increasingly taken into consideration in many ecological studies, including in marine intertidal systems and in relation to the context of global climate change (e.g. Przeslawski *et al.*, 2005). However, parasite-specific processes, such as transmission dynamics, have only received little attention with regard to interactions of environmental factors, despite the fact that interactive effects can conceivably diminish or facilitate parasite transmission in response to multiple stressors (Pietrock & Marcogliese, 2003). Thus, several authors have highlighted the need to better incorporate ecological complexity into climate change-disease studies (e.g. Lafferty, 2009; Rohr *et al.*, 2011). This includes the importance of biodiversity in disease transmission, i.e. the presence of host and non-host organisms. This is of great importance for the mediation of disease patterns (e.g. Johnson & Thieltges, 2010; Keesing *et al.*, 2010). Climate change has and is expected to further reduce (host and non-host) biodiversity (e.g. Thomas *et al.*, 2004; Thuiller *et al.*, 2011), which may also affect transmission dynamics of parasites and therefore impacts on their hosts. Thus, the inclusion of not only parasites and their hosts, but also non-host organisms, is considered imperative when forecasting the effects of climate change on host-parasite interactions.

Because parasites are influenced by the abiotic factors of an environment, as well as the distribution, abundance and condition of their hosts, predicting the implications of climate change for parasites and their hosts becomes highly complex and context-dependent (Marcogliese, 2001). In this thesis, the focus was on understanding the effects of environmental factors on an intertidal parasite and the hosts associated with its complex life cycle. Particularly, the focus was on the transmission dynamics of the parasite and the influences of abiotic factors as well as a biotic factor. Despite the fact that studies and reports about climate change as well as parasitism and diseases in the marine environment lag behind those in other ecosystems, climate change has also already been linked to the increased occurrence of diseases in the oceans (e.g. Harvell *et al.*, 1999; Ward & Lafferty, 2004). In the absence of long-term baseline data in marine systems and due to the lack of information on

many host-parasite systems, our ability to forecast the potential consequences for diseases and parasitism in marine systems is very limited.

1.3 Climate change, marine and intertidal soft-sediment ecosystems

Marine ecosystems are undergoing impacts from multiple stressors, which not only include global warming, but also increased ultraviolet radiation exposure, overfishing, changes in circulation and stratifications, salinity and acidification (e.g. Brierley & Kingsford, 2009; Halpern *et al.*, 2008; Harley *et al.*, 2006; Hoegh-Guldberg & Bruno, 2010). Reports of the consequences of climate change in marine ecosystems are mounting, including changes in abundance and shifts in geographic ranges of species, as well as changes in phenology and trophic mismatch (e.g. Beaugrand *et al.*, 2002; Edwards & Richardson, 2004; Firth & Hawkins, 2011 and references therein; Hawkins *et al.*, 2008; Hays *et al.*, 2005; Rosenzweig *et al.*, 2008; Southward *et al.*, 1995). The mostly distributional shifts of marine organisms have occurred even faster than most recorded shifts of terrestrial species (Helmuth *et al.*, 2006b). As in other ecosystems, the importance of species interactions is increasingly acknowledged (Kordas *et al.*, 2011), as impacts of changes on habitat-forming or other key ecological species in the marine environment can also result in community reorganisation and thus affect ecosystem functioning (Hoegh-Guldberg & Bruno, 2010; Walther, 2010).

Intertidal ecosystems are at the interface between marine and terrestrial habitats. They are characterised by extreme natural fluctuations in abiotic factors which are influenced by changes in oceanic, atmospheric as well as terrestrial conditions (Nybakken & Bertness, 2005). In contrast to rocky shores, soft-sediment ecosystems are restricted to areas which are sheltered from strong wave actions and thus only occur in bays, harbours, lagoons or estuaries. Due to the accumulation of organic matter, these ecosystems often sustain a high density of organisms and are important feeding (e.g. migratory shorebirds) and nursery grounds. At low tide, mudflats are a mosaic of tide pools and patches of sediment that are exposed to ambient conditions. Similar to tide pools on rocky shores, conditions in soft-sediment tide pools can change markedly compared to times of immersion, especially if low tides occur around noon during warm summer months. These conditions may become more extreme with on-going and predicted climate change and thus pose additional physiological challenges for their inhabitants.

Thermal conditions and the associated risk of climate change for intertidal organisms have received considerable attention in the literature, especially in the case of rocky shores (e.g. Helmuth *et al.*, 2006a; Mislan *et al.*, 2009). This is due to the fact that ectothermic organisms have a limited ability to regulate their internal body temperature, and thus many intertidal organisms are expected to display strong responses and a high vulnerability to changes in climatic conditions, such as extreme temperatures (Fields *et al.*, 1993; Hofmann & Todgham, 2010; Somero, 2002). Even in temperate regions with greater selection for tolerance of environmental variability, intertidal species are, due to their limited potential for adaptation and acclimatisation, expected to be already close to their thermal tolerance limits (Hofmann & Todgham, 2010; Somero, 2002; Stillman & Somero, 2000). Because extreme temperature events have already been linked to mortality in intertidal organisms (Harley *et al.*, 2006), intertidal systems have been proposed as early warning systems for the detection of signals of a changing climate (Helmuth *et al.*, 2006b).

For the area where the present research was carried out (i.e. New Zealand), warming of about 0.9°C already has occurred over the past 100 years (NIWA, 2008), including an increase in surface seawater temperature of around 0.7°C (Folland & Salinger, 1995). Furthermore, forecasts indicate a continuous warming in the range of 0.2 - 2.0°C until 2040 and 0.7 - 5.1°C by 2090 (NIWA, 2008).

However, a range of environmental factors simultaneously fluctuate in intertidal systems in both space and time. For example, during low tide, the relatively small volumes of water in pools can warm up rapidly, which may be either accompanied by an increase in salinity due to evaporation, or, during periods of rainfall, by a reduced salinity. Salinity is a major fluctuating parameter in coastal systems, but predictions regarding large-scale changes in ocean salinity are less straightforward than those for temperature (see above). On the scale of an intertidal mudflat, we might expect increasing stressful osmotic conditions during warmer and more arid weather in summer, whereas in areas with increased precipitation, organisms may be exposed to lower than normal salinities more often.

On the other hand, conditions at low tide also entail the exposure to ambient levels of solar irradiation and thus ultraviolet radiation (UVR). Current levels of UVR are still in their maximum range and significant recovery from the anthropogenically induced ozone depletion is not predicted for at least another several decades (McKenzie *et al.*, 2007). Moreover, UVR

is expected to be especially problematic in clear, shallow aquatic systems and penetration of UVR is expected to increase as a result of warming, acidification and the consequent reduction of dissolved organic matter (Haeder *et al.*, 2011; Schindler *et al.*, 1996). Both, salinity and UVR have been shown to have significant biological and ecological effects in aquatic ecosystems (e.g. Crain *et al.*, 2004; Haeder *et al.*, 2007).

Hence, temperature, salinity and UVR were the abiotic factors considered in the present thesis as they may also be of particular relevance for parasite transmission and host-parasite interactions in intertidal systems, especially for parasites with complex life cycles. The relative importance of these factors and their interactive effects in affecting species interactions in the intertidal zone has rarely been considered, particularly in the context of parasite transmission and particularly for UVR.

1.4 Intertidal trematodes

Among metazoan parasites, trematodes (phylum Plathyhelminths) are the most common parasites in intertidal soft-sediment habitats, and they occur in all major taxa found in these ecosystems (Lauckner, 1984; Mouritsen & Poulin, 2002b; Poulin & Mouritsen, 2006; Sousa, 1991). The majority of trematode species belong to the sub-class Digenea (Gibson, 2001), which typically have a complex life cycle involving three hosts, each used by a different developmental stage of the parasite. Of these hosts, the first and the second intermediate hosts are often ectothermic invertebrates and the transmission of the parasite between them depends on free-living larval transmission stages known as cercariae, which are highly sensitive to environmental conditions (e.g. Pietrock & Marcogliese, 2003). Definitive hosts, in which sexual reproduction of adult worms takes place, are usually vertebrates such as birds or fish. An example of a typical complex trematode life cycle will be discussed and illustrated in detail below, when introducing the model system studied in this thesis.

Trematodes play major roles in intertidal ecosystems affecting the growth, reproduction, behaviour and survival of intertidal organisms (e.g. Lauckner, 1987; Mouritsen & Poulin, 2002b; Sousa, 1991). The impact of trematodes on individuals can be substantial. For example, infected snail first intermediate hosts can get castrated by the parasite whose tissue replaces the snail's gonads (Probst & Kube, 1999). On the other hand, cercarial transmission

stages of the parasite penetrate a host and the impact of infection has been associated with the induction of intensity-dependent mortality in organisms such as amphipods; a small number of parasites does not affect the survival of amphipod hosts, whereas a moderate or high number of parasites highly decreases host survival (Fredensborg *et al.*, 2004b; Jensen *et al.*, 1998; Meissner & Bick, 1999a, b). These effects can translate into effects on the population or even higher levels including the structure and functioning of entire intertidal communities (e.g. Mouritsen *et al.*, 1998; Mouritsen & Poulin, 2002b).

Processes related to the transmission of trematodes and their induced pathology are expected to be altered by climate change (Paull & Johnson, 2011; Poulin & Mouritsen, 2006). In particular, global warming will almost certainly result in enhanced proliferation of trematode infective stages (Poulin, 2006). Hence, warmer temperatures are expected, in general, to lead to increased transmission efficiency and higher infection levels in hosts and may also accelerate parasite development (Marcogliese, 2008; Poulin & Mouritsen, 2006).

However, predictions based on temperature effects on cercarial transmission stages only are highly simplistic and ignore the complexity of natural systems in which host-parasite interactions take place. For example, non-host community members that prey on cercariae have been shown to interfere with the transmission process of parasites (e.g. Hopper *et al.*, 2008; Prinz *et al.*, 2009; Thieltges *et al.*, 2008a) and under increasing temperatures, the increased metabolic rate of non-host organisms may be of substantial importance in buffering an increased transmission pressure of trematodes. Also, environmental factors such as UVR are usually associated with negative effects on organisms, particularly on infective stages of parasites and pathogens (Pietrock & Marcogliese, 2003; Ruelas *et al.*, 2007), and thus this effect may also alter the predicted transmission dynamics based solely on temperature effects. Furthermore, interactive effects of environmental conditions and infections by trematodes on a host may, under exceptional circumstances, cause substantial mortality in these hosts. This has been inferred for mollusc as well as amphipod hosts, even in the field (Jensen & Mouritsen, 1992).

Due to the high sensitivity of trematodes to environmental conditions, the important ecological impact of parasites and the fact that intertidal ecosystems are naturally exposed to extreme fluctuations in environmental conditions, intertidal trematodes and their hosts provide an excellent opportunity to study the effects of the abiotic and biotic environment on

parasite transmission dynamics in order to anticipate the possible impacts of climate change. Parasites have been considered potential indicators of environmental change, especially in the context of pollution and other anthropogenic habitat alterations (Lafferty, 1997). The sensitivity of trematodes has also led to the notion that they may provide a useful tool for monitoring ecological impacts of climate change (Poulin & Mouritsen, 2006). This may be of particular relevance for intertidal ecosystems: responses by parasites (e.g. infection intensity and/or prevalence) can be expected to be, at least to some extent, even more indicative than responses by free-living organisms.

1.5 The model system: *Maritrema novaezealandensis* and its hosts

As a model system, the life cycle of the New Zealand endemic intertidal trematode parasite *Maritrema novaezealandensis* Martorelli *et al.* 2004 (Digenea, Microphallidae) was chosen. Like most trematodes, *Maritrema novaezealandensis* has a complex life cycle, consisting of several members of an intertidal community (Martorelli *et al.*, 2004) (Fig. 1.2).

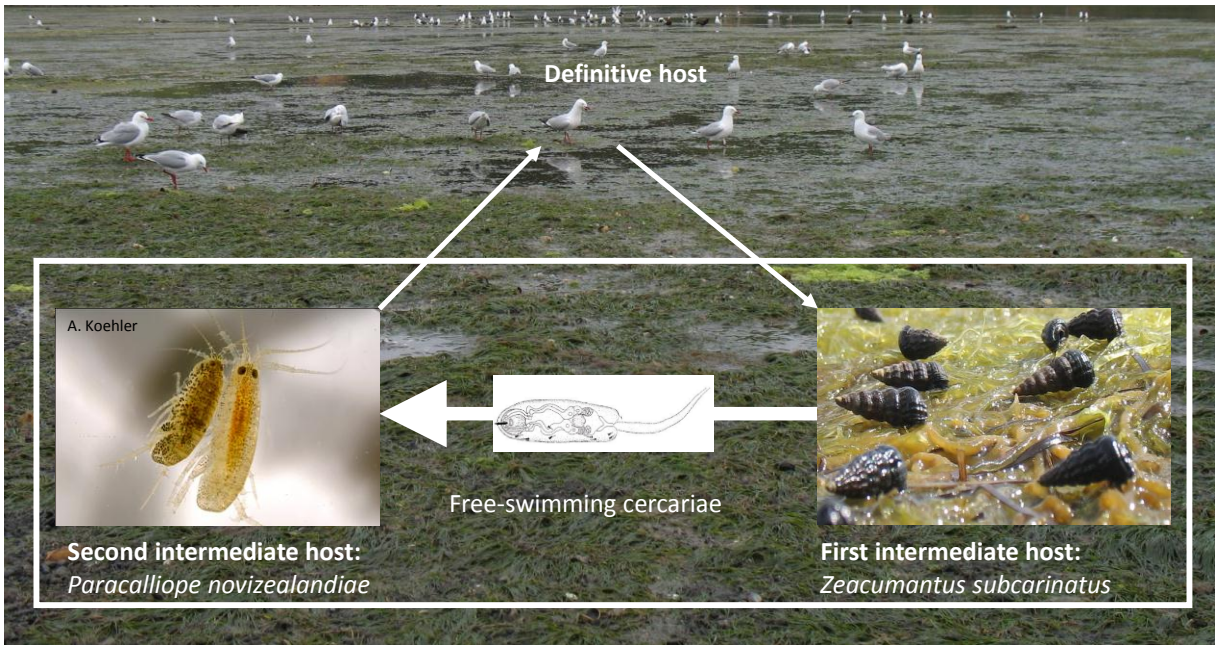


Figure 1.2 Life cycle of *Maritrema novaezealandensis*, involving definitive bird hosts (infected by adult worms), first intermediate snails hosts *Zeacumantus subcarinatus* (infected by sporocysts), and second intermediate crustacean hosts such as the amphipod *Paracalliope novizealandiae* (infected by metacercariae). The focus of this thesis was on the transmission process from first intermediate snail host to the second intermediate amphipod host via free-swimming cercarial transmission stages.

The first intermediate snail host of *M. novaezealandensis* is the mud snail *Zeacumantus subcarinatus* Sowerby 1855 (Prosobranchia, Batillaridae). The geographical distribution of *Z. subcarinatus* is mainly along the East coast of the South Island of New Zealand and the Southern end of the North Island. The snail is an abundant grazer of microalgae, with densities reaching up to 18,000 per m^2 in some areas (Jones & Marsden, 2005). The snails reproduce during austral spring and summer and lay egg strings containing crawl-away larvae. These snails thus lack a planktonic phase, and populations seem to rely mostly on self-recruitment (Fredensborg & Poulin, 2006). *Zeacumantus subcarinatus* snails get infected when accidentally ingesting eggs of *M. novaezealandensis* (defecated by birds) while foraging on the mudflat. Inside the snail, a miracidium hatches from an egg and migrates to the gonads where it develops into a sporocyst. After asexual proliferation, numerous sporocysts eventually replace the entire gonadal tissue of a snail, leading to its castration. In some areas, snail populations show infection prevalences of up to 86.8% (all trematodes) and 61.5% (only *M. novaezealandensis*) (Fredensborg *et al.*, 2005), implying a substantial impact of this parasite on snail populations (e.g. selection for early maturation and rapid growth, as well as reduced biomass and density where prevalence is high; Fredensborg *et al.*, 2005; Fredensborg & Poulin, 2006). While *Z. subcarinatus* is host to at least five trematode species, *M. novaezealandensis* is specific to *Z. subcarinatus*.

Within the sporocysts, the parasite asexually produces larval transmission stages called cercariae. The emergence of cercariae from infected snail hosts is known to be temperature dependent (Chapter Three, Fredensborg *et al.*, 2005). Cercariae of *M. novaezealandensis* are small (approx. 170 - 200 µm body length including tail, see Koehler *et al.*, 2011; Martorelli *et al.*, 2004), short-lived (< 24 hours depending on conditions), non-feeding and translucent. They emerge into the environment in order to infect a second intermediate host. Optimal conditions for the transmission of *M. novaezealandensis* are thought to be during low tides on warm, sunny days when water in the tide pools warms up (Chapters Two and Three, Bates *et al.*, 2010; Fredensborg *et al.*, 2004b; Studer *et al.*, 2010). These conditions often coincide with elevated salinity and high levels of UVR, but it is not known how these factors may affect, separately and interactively, the transmission process of this parasite. The transmission from first to second intermediate host (both ectothermic) is a crucial step in the completion of the life cycle of this parasite: this is the only stage of the entire life cycle where the parasite is directly exposed to ambient conditions while actively seeking its next host. Hence, this process and its responsiveness to environmental factors were a main focus of this thesis.

Successful transmission to a second intermediate host is achieved when a cercaria penetrates the cuticle of (or enters via the gills or joints), and establishes within the body cavity, of a second intermediate host. *Maritrema novaezealandensis* uses a range of benthic and epi-benthic crustaceans as hosts (Koehler & Poulin, 2010). In this thesis, the focus was on the amphipod *Paracalliope novizealandiae* Dana 1853 (Amphipoda, Paracalliopiidae). These amphipods (max. body length of 3.5 mm for females and ca. 5 mm for males) mostly graze on epiphytic diatoms or feed on detritus. Due to their high abundance on mudflats in New Zealand (Barnard, 1972), *P. novizealandiae* amphipods are believed to be an important part of intertidal food webs (Fredensborg *et al.*, 2004b). These amphipods have only ever been found to be infected with *M. novaezealandensis* (Fredensborg *et al.*, 2004b; this thesis; Koehler & Poulin, 2010). Furthermore, these amphipods are affected by intensity-dependent, parasite-induced mortality (Fredensborg *et al.*, 2004b), making them an ideal host species to consider in the context of climate change research. Furthermore, protocols for experimental infections of these amphipods have been repeatedly and successfully applied (e.g. Bryan-Walker *et al.*, 2007; Keeney *et al.*, 2009a).

Within the body cavity of a crustacean host such as *P. novizealandiae*, the parasite undergoes development from an immature to a mature metacercaria, the latter encased within a cyst (see Keeney *et al.*, 2007b). This development is accompanied by an almost 200-fold increase in body volume of the parasite (Fredensborg *et al.*, 2004b). As a fully mature and thus infective metacercaria in a second intermediate host, the parasite awaits ingestion by a definitive host. The definitive bird hosts are the main dispersal agents for this parasite. So far, only red-billed gulls *Chroicocephalus scopulinus*, formerly *Larus novaehollandiae scopulinus*, have been confirmed as definitive hosts of *M. novaezealandensis* (Fredensborg *et al.*, 2004a). However, host specificity of trematodes in definitve hosts is often low compared to first and second intermediate hosts (Combes, 2001), and therefore, a range of other bird species that feed on crustaceans are probably also suitable hosts. Within the intestine of birds, the parasite excysts and attaches itself to the inner gastrointestinal wall by means of its anterior and ventral suckers and starts to reproduce. Although *M. novaezealandensis* is hermaphroditic like most trematodes, reproduction in definitive hosts is likely to be mainly sexual (Galaktionov & Dobrovolskij, 2003). Parasite eggs pass into the environment with the bird's faeces, where the life cycle is completed when a first intermediate snail host ingests a parasite egg.

In this thesis, a specific spatial focus was on the life cycle dynamics of *M. novaezealandensis* occurring on the Lower Portobello Bay (LPB) mudflat (Otago Harbour, 45° 50'S, 170° 40'E). Lower Portobello Bay has been identified as a high prevalence mudflat with by far the highest proportion of *Z. subcarinatus* snails infected when compared to other local mudflats (Fredensborg & Poulin, 2006). This has been related to the relatively high number of birds visiting this mudflat (Fredensborg *et al.*, 2006). Due to the high prevalence in first intermediate snail hosts, infection levels in second intermediate hosts are, as a consequence, expected to also be particularly high. Based on this, the LPB mudflat seems to be the best candidate locality for studying the effect of *M. novaezealandensis* on its host populations. Moreover, under exceptional circumstances, an area with a high prevalence should be considered a "hotspot" for parasite-induced mortality in second intermediate hosts - a major concern with regards to on-going and predicted global climatic changes, in particular global warming.

1.6 Objectives

Climate change is a global problem with ultimately global consequences. However, immediate effects occur on the local scale where global, regional and local factors interact (e.g. Reise & van Beusekom, 2008). The overall aim of this thesis was to investigate transmission processes in the model system chosen, i.e. the intertidal trematode parasite *M. novaezealandensis* and the hosts associated with its life cycle, in order to better forecast how host-parasite systems may be influenced by on-going and predicted global changes on the local scale. This requires a sound understanding of mechanisms by which organisms are impacted by their ambient environment, both directly, but also indirectly through mediation of biotic interactions (Helmuth *et al.*, 2006b).

An integrative approach was chosen combining a field study, laboratory experiments and modelling. Firstly, a seasonal assessment of the dynamics of *M. novaezealandensis* and the hosts associated with its complex life cycle was conducted on LPB. This assessment provided the basis for a better understanding of how this system is currently functioning "in the real world". Secondly, experimental studies were conducted in laboratory settings in order to quantify how temperature, salinity and UVR affect the transmission dynamics of *M. novaezealandensis* from its first intermediate snail host to its second intermediate amphipod host, as this is likely to be the most vulnerable process of the entire life cycle. These studies on single abiotic factors allowed an assessment of the importance of each environmental factor for the various steps of the transmission process, and provided important information in terms of parasite transmission in "a simplified world". Acknowledging that parasite transmission is, however, embedded in "a complex world", interactive effects between temperature, salinity and UVR on the survival of cercariae were investigated in a separate experiment, as was the potential of a non-host predator to interfere with the transmission process under increased temperature. Finally, based on data generated for the study system, the entire life cycle of *M. novaezealandensis* and in particular its amphipod host were modelled, in order to forecast how this system may respond to global warming in "the future world".

The most novel aspects of this research thesis are its inclusive approaches. In terms of the consideration of all hosts of the complex life cycle in the field study, the inclusion of all transmission steps from first to second intermediate hosts in the single factor studies (also

including long-term acclimatisation of organisms used in the experiments), the acknowledgement of natural complexity of abiotic and biotic factors and their relevance for parasite transmission, and also in terms of the environmental factors considered, particularly in the case of UVR and the mechanisms by which this factor influences the transmission of the parasite.

This thesis had the following specific objectives:

1. To assess the seasonal pattern of parasitism in a high prevalence site using the model system chosen for this study and all hosts associated with its complex life cycle (Chapter Two).

2. To investigate the effects of temperature on the transmission steps of *M. novaezealandensis* from first to second intermediate host (Chapter Three).

3. To investigate the effects of salinity on the transmission steps of *M. novaezealandensis* from first to second intermediate host (Chapter Four).

4. To investigate the effect of UVR on the transmission steps of *M. novaezealandensis* from first to second intermediate host (Chapter Five).

5. To investigate potential mechanisms by which UVR influences the transmission of *M. novaezealandensis* from first to second intermediate host (Chapter Six).

6. To identify relevant factor interactions between temperature, salinity and UVR influencing the highly sensitive step of cercarial survival (Chapter Seven).

7. To quantify the potential for a non-host community member to interfere with parasite transmission from first to second intermediate host under different temperature regimes (Chapter Eight).

8. To model the life cycle of *M. novaezealandensis* and in particular an amphipod host population in a high prevalence site, exploring the potential consequences of predicted global warming on the model system (Chapter Nine).

1.7 Structure of the thesis

Chapters Two to Nine were written in manuscript style for submission and publication in scientific journals. For this reason, there is some element of repetition among them. I am the first author of all of these chapters, which means that I have carried out the research, analysed the data and written the text with technical help and/or constructive criticism from co-authors (see Table 1.1). The only part of the research in which I was not directly involved were the oxidative stress and DNA damage assays (Chapter Six), which were carried out by my co-author D.J. Burritt.

Table 1.1 Structure of the thesis.

Chapter	Content
One	General introduction
Two	Seasonal fluctuations in a high prevalence intertidal mudflat: a look at all steps of a complex trematode parasite life cycle. *To be submitted as: A. Studer and R. Poulin*
Three	Parasites and global warming: net effects of temperature on an intertidal host-parasite system. *Published as: A. Studer, D.W. Thieltges and R. Poulin, 2010. Marine Ecology Progress Series.*
Four	Effects of salinity on an intertidal host-parasite system: is the parasite more sensitive than its host? *To be submitted as: A. Studer and R. Poulin*
Five	Effects of ultraviolet radiation on the transmission of an intertidal trematode parasite. *To be submitted as A. Studer, M.D. Lamare and R. Poulin*
Six	Effects of ultraviolet radiation on an intertidal trematode parasite: an assessment of damage and protection. *To be submitted as: A. Studer, V. Cubillos, M.D. Lamare, R. Poulin and D.J. Burritt*
Seven	Survival of an intertidal trematode cercaria: a multifactorial experiment with temperature, salinity and ultraviolet radiation. *To be submitted as: A. Studer and R. Poulin*
Eight	Biotic interference in parasite transmission: can the feeding of anemones compensate an increased risk of parasitism in amphipods at higher temperature? *To be submitted as: A. Studer, L. Kremer, J. Nelles, R. Poulin and D.W. Thieltges*
Nine	Local effects of a global problem: modelling the risk of parasite-induced mortality in an intertidal trematode-amphipod system. *To be submitted as: A. Studer, R. Poulin and D.M. Tompkins*
Ten	General discussion

CHAPTER TWO

Seasonal fluctuations in a high prevalence intertidal mudflat: a look at all steps of a complex trematode life cycle

2.1 Abstract

Seasonal fluctuations of host densities and environmental factors are common in many ecosystems and have consequences for biotic interactions such as the transmission processes of parasites and pathogens. Here, we investigated seasonal patterns in all host stages associated with the complex life cycle of the intertidal trematode *Maritrema novaezealandensis* on a high prevalence mudflat (Lower Portobello Bay, Otago Harbour, New Zealand). The first intermediate snail host, *Zeacumantus subcarinatus*, a key second intermediate crustacean host, the amphipod *Paracalliope novizealandiae*, and definitive bird hosts were included in the study. Density (snails, amphipods), abundance (birds), and prevalence, i.e. percentage of infected individuals, as well as infection intensity (snails, amphipods) were assessed. Furthermore, temperature was recorded continuously in tide pools, where transmission mainly occurs, over a one year period. Overall, trematode prevalence in snail hosts was 64.5%, with 88.4% of infected snails harbouring *M. novaezealandensis*. There was a strong seasonal signal in prevalence and infection intensity in second intermediate amphipod hosts, with peaks for both parameters in summer (over 90% infected; infection intensity 1 - 202 parasites/amphipod). This peak coincided with the highest abundance of bird definitive hosts present on the mudflat. These observations support that conditions for transmission for all transmission steps are optimal during warmer months when the water in shallow tide pools warms up during low tides. However, mudflats where prevalence in first intermediate snail hosts is high must be considered high risk areas for intensity-dependent mortality of second intermediate hosts such as amphipods - a particular concern with regards to on-going global warming.

2.2 Introduction

Climatic and seasonal fluctuations in environmental factors govern many processes in nature, including patterns of diseases and parasitism (e.g. Altizer *et al.*, 2006; Cattadori *et al.*, 2005; Kim *et al.*, 2005; Mouritsen & Poulin, 2002a). The role of seasonality is comparatively well explored in terms of the dynamics of infectious diseases, with a multitude of environmental drivers capable of generating periodic changes including temperature and host availability (e.g. Altizer *et al.*, 2006). However, environmental changes caused by climate change as well as natural climate oscillations (e.g. El Niño Southern Oscillation) are bound to alter seasonality in ways that will also influence the transmission of parasites and, as a consequence, impact host populations (e.g. Harvell *et al.*, 2002; Kutz *et al.*, 2005). In particular, the predicted increase in extreme weather events such as more intense and more frequent heat waves (IPCC, 2007) may have serious consequences for temperature-sensitive host-parasite systems.

The extent of environmental fluctuations is particularly pronounced in intertidal ecosystems, where conditions vary considerably in space and time. Trematode parasites are very common and highly influential components of these ecosystems. These parasites have been shown to play crucial roles in host population dynamics as well as community and food web structures (e.g. Fredensborg *et al.*, 2005; Lauckner, 1984; Mouritsen & Poulin, 2002b, 2010; Sousa, 1991; Thompson *et al.*, 2005). Moreover, parasites and especially trematodes have also been shown to constitute a considerable and highly underestimated amount of biomass in natural systems (Kuris *et al.*, 2008).

The impact of parasites on their hosts can be through a parasite-induced increase in mortality and/or a decrease in fecundity, which translates into a reduced mean rate of host population increase (e.g. Anderson & May, 1979, 1981). Therefore, effects on individual hosts can translate into effects at the population level. For instance, the snail host used in the present study, like many gastropod hosts of trematodes (Lafferty & Kuris, 2009b), are castrated by the parasite thus lowering the reproductive potential of an affected population (Fredensborg *et al.*, 2005). In addition, infection intensity-dependent host mortality has been shown for a number of species including the trematode parasite and its amphipod host studied here (Bates *et al.*, 2010; Fredensborg *et al.*, 2004b). Intensity-dependent mortality effectively removes reproductive hosts from an affected system thus directly reducing the number of

reproducing hosts present and lowering the birth rate in a population. Since parasites critically depend on successful transmission from one host to the next in their life cycle, death of a host, e.g. the intermediate host, before transmission can occur, will also negatively impact the parasite. Despite the fact that parasites and their impact on population dynamics and community structure have been increasingly acknowledged also in marine ecosystems, few studies have investigated seasonal dynamics of parasites and their hosts in this environment and therefore, little is known in qualitative terms regarding seasonal fluctuations in the magnitude of parasite impact.

The dynamics between parasites, especially parasitic transmission stages, and their hosts are modulated by environmental conditions affecting both the host and parasite. For example, temperature is highly influential, both directly and indirectly. The production and output of transmission stages of trematodes from first intermediate snail hosts (cercariae), as well as their survival and infectivity are all influenced by temperature (e.g. Poulin, 2006). However, temperature also affects hosts (e.g. their activity and survival), or may indirectly influence host availability (e.g. for seasonally migrating birds). Higher parasite transmission to susceptible hosts under elevated temperatures may subsequently translate into increased infection levels. As a consequence, parasites and hosts which are sensitive to temperature can be expected to show strong seasonal patterns in infection levels (prevalence and infection intensity). Under exceptional circumstances, and thus of particular relevance when considering on-going and predicted global warming, higher temperatures may increase the risk of parasite-induced intensity dependent mortality of hosts. For instance, in areas where prevalence in first intermediate snail hosts is high, increased transmission under high temperatures may lead to a die-off of second intermediate hosts, as observed with *Corophium volutator* amphipods in the Danish Wadden Sea (Jensen & Mouritsen, 1992). Such die-off events can have large scale, ecosystem-wide consequences (Mouritsen *et al.*, 1998). Thus, a better understanding of seasonal dynamics under current conditions may allow more realistic assessments of potential consequences of predicted changes in a given host-parasite system.

We used the intertidal microphallid trematode *Maritrema novaezealandensis* (Digenea, Microphallidae) and the hosts associated with its complex life cycle as a model system. The first intermediate host of this parasite, the mudsnail *Zeacumantus subcarinatus* (Prosobranchia, Batillariidae), acquires infections by ingesting parasite eggs while foraging on the mudflats. Within a snail, miracidia hatching from eggs replace the snail's gonads by

developing into sporocysts. Within the sporocysts, large numbers of cercariae are produced asexually. These cercarial transmission stages emerge from the snail under optimal conditions and are directly exposed to environmental conditions during transmission to a second intermediate crustacean host, such as the amphipod *Paracalliope novizealandiae* (Amphipoda, Paracalliopiidae). After penetrating a crustacean host, the parasite develops into a mature cyst stage (metacercaria). The final transmission event occurs when an infected crustacean gets ingested by a definitive host, i.e. birds attracted to mudflats such as red-billed gulls *Chroicocephalus scopulinus* (Fredensborg *et al.*, 2004a; Martorelli *et al.*, 2004). Within the intestine of a bird, adult worms reproduce sexually. Eggs pass into the environment with the bird's faeces, where they may get ingested by the snail host, thereby completing the life cycle.

The overall aim of the present study was to investigate seasonal patterns related to all transmission steps in the life cycle of *M. novaezealandensis* on a mudflat where prevalence in first intermediate snail hosts is high. Although little seasonal variation in trematode prevalence is expected in *Z. subcarinatus* snails (Fredensborg *et al.*, 2005), based on the effects of temperature on the transmission process from snails to amphipods (Studer *et al.*, 2010) and the fact that the survival of infected organisms, especially amphipods, is generally negatively affected by a parasite (e.g. Bates *et al.*, 2010; Fredensborg *et al.*, 2004b, 2005; Meissner & Bick, 1999b; Mouritsen & Jensen, 1997), we hypothesised a strong seasonal pattern in prevalence and infection intensity in second intermediate amphipod hosts. We also expected seasonal patterns with regard to host densities (snails and amphipods) or abundance (birds) and thus host availability, complementing our current understanding of the optimal transmission period for the life cycle of this parasite.

Our approach comprised of three main parts. 1.) The concomitant assessment of snail and amphipod density, prevalence (% of infected individuals) and infection intensity (for snails as the proportion of parasite tissue within infected snails, for amphipods as the number of metacercariae per infected individual) across all seasons. 2.) An assessment of birds present on the mudflat at low tide over a one year period. And 3.), a year-long temperature recording in tide pools in order to provide more detailed information on the thermal conditions experienced by organisms in the actual microhabitat where transmission takes place. The results presented here contribute important knowledge about temporal dynamics of trematode parasitism in intertidal soft-sediment ecosystems.

2.3 Materials and Methods

Study site. Lower Portobello Bay (LPB) is an intertidal soft-sediment mudflat in the Otago Harbour (45°50'S, 170°40'E; South Island of New Zealand), which has been identified as a high prevalence area for trematodes infections in *Z. subcarinatus* snails. Fredensborg *et al*. (2005) reported that 86.6% of the snails in this locality were infected with trematodes, of which 61.5% were parasitized by *M. novaezealandensis*. The mudflat (approx. 180 x 200 m; tidal range approx. 2 m) is a mosaic of shallow indentations (referred to as tide pools). Most of the bay is covered lightly, but some areas are densely vegetated with eelgrass (*Zostera novaezelandensis*) and/or other algae. The crustacean community in this locality has been well investigated with regard to its parasite fauna (see Koehler & Poulin, 2010). Due to their abundance, *Paracalliope novizealandiae* amphipods are considered an important component of the local food webs (Fredensborg *et al.*, 2004b; Thompson *et al.*, 2005). These amphipods have never been found infected with any other species of parasite and thus *M. novaezealandensis* is the sole agent affecting the LPB population (Fredensborg *et al.*, 2004b; Koehler & Poulin, 2010, this study). Fredensborg *et al.*(2006) also provided a list of confirmed and potential definitive bird hosts for *M. novaezealandensis* visiting LPB and neighbouring mudflats. The presence of these birds has been linked to the particularly high prevalence in snail hosts on LPB (Fredensborg *et al.*, 2006).

Sampling of snail and amphipod hosts. Samples were collected on two sampling trips per season (within a maximum of three weeks of each other, July/August 2009 (winter), October/November 2009 (spring), Januray 2010 (summer), April/May 2010 (fall); three replicate samples per trip) during low tide in haphazardly selected tide pools in the mid-upper shore area (exposure time per low tide approx. 4 - 6 h; pools > 0.5 m^2 and > 3 cm deep) using a 0.5 m^2 rectangular aluminium box. Amphipods were sampled within the enclosed area using a dip-net with a 250 µm mesh size. Subsequently, snails were collected from within the enclosure by finger-dredging. Both amphipods and snails were sampled as thoroughly as possible and stored in separate 3 l plastic containers for transport back to the laboratory. For all statistical analyses described in detail below, replicates from the two sampling trips were pooled to increase the sample size per season and for the sake of simplicity in presenting the results.

1st intermediate snail hosts. For density estimates, live snails from each sample were counted and shell lengths measured using calipers. For assessment of prevalence, snails were screened for trematode infections by placing them individually in wells (20 x 23 mm) of a 12-well plate containing 3 ml of seawater and incubating them under constant illumination at 25°C for several hours. Snails not shedding parasitic transmission stages (cercariae) during a first incubation were screened again after one week. This conservative method only detects mature infections and thus results are an underestimation of the true prevalence. Cercariae were identified according to species descriptions of local parasites (Martorelli *et al.*, 2008; Martorelli *et al.*, 2004; Martorelli *et al.*, 2006). Due to the high morphological similarity between the cercariae of *M. novaezealandensis* and the rare *Microphallus* sp. (prevalence of *Microphallus* sp. < 2%; Martorelli *et al.*, 2008), the two species were not distinguished and the proportion of each later calculated based on the prevalence of *Microphallus* sp. of 1% for LPB (determined from the dissection of 100 "*Maritrema*"-infected snails). After the screenings, snails were returned to the mudflat. Arbitrarily, only snails ≥ 8 mm were considered in the analyses (see below) as the reliability of sampling smaller snails with the method employed is limited.

As a measure of infection intensity, a minimum of 30 snails of similar size infected with *M. novaezealandensis* (average size ± standard error 14.28 mm ± 0.12) was used each season to assess the proportion of the overall wet weight of the snails actually consisting of parasite tissue (sporocysts). For this, snails were dried on a paper towel for approx. 15 min and snail shell length was measured before weighing them (all weights recorded to the nearest 0.0001 g). Snails were cracked open and then dissected in a drop of seawater. The parasite tissue was isolated and the excess liquid absorbed using a paper towel before weighing. For a subset of snails (n = 32), the soft tissue of the snail was also weighed in order to calculate the proportion of infected tissue from the total snail weight including and excluding shell weight.

For the statistical analysis, differences in density of snails between seasons were analysed using a Generalised Linear Model (GLM) fitted with a quasi-Poisson error structure. A GLM fitted with a binomial error structure was used to assess the effects of season and size of snails on the infection status of snails. The effect of season on the proportion of parasite tissue from the total snail weight was analysed using a GLM fitted with a quasi-binomial error structure.

2nd intermediate amphipod hosts. For density estimates, amphipods were either counted or numbers estimated (counts in 5, 10 or 50 ml subsamples) in samples with many small individuals. When not enough amphipods ≥ 2.25 mm (minimum size limit for sex determination) were collected during the sampling, additional amphipods were obtained by random sampling in various tide pools to ensure a large enough number of amphipods was dissected per season. Amphipods were measured (body length; size classes: 2.5, 3.0, 3.5, 4.0, ≥ 4.5 ± 0.25 mm) and sexed prior to dissection. Upon dissection, the number and developmental stages of the metacercariae of *M. novaezealandensis* within the amphipods were assessed. The developmental stages distinguished were early immature, late immature, early cyst and mature cyst stage (according to Keeney *et al.*, 2007b).

Differences in amphipod density between seasons were analysed using a GLM fitted with a quasi-Poisson error structure. Using the entire dataset for all amphipods ≥ 2.25 mm, the effects of season, sex and size of amphipods on their infection status were analysed using a GLM fitted with a binomial error structure. For all infected amphipods ≥ 2.25 mm, the effects of season, sex and size of amphipods on the number of infecting parasites (i.e. infection intensity) was analysed with a GLM fitted with a quasi-Poisson error structure.

Definitive bird hosts. The mudflat was visited 12 - 14 times per season during low tide and all birds present on the entire mudflat including birds wading along the waterline were counted (one count per visit upon arrival). To date, only red-billed gulls have been confirmed as final hosts of *M. novaezealandensis* (Fredensborg *et al.*, 2004a). However, trematodes often show little specificity in definitive hosts and most trematodes present within *Z. subcarinatus* snails probably use birds as final hosts (Leung *et al.*, 2009). Therefore, all birds present were counted regardless of species. Mean bird abundance per season was calculated and the months when most birds were present specified.

***In situ* temperature logging**. To describe the thermal conditions occurring in tide pools in which essentially all transmission steps take place, five temperature loggers (DS 1921 Thermochron iButtons; ± 0.5°C) were installed in haphazardly selected pools in the mid-upper shore area of the mudflat (pools varying in size and degree of vegetation). The loggers, wrapped in a strip of parafilm, were placed in white submersible housings (HOBO SUBC2-WH, 10 x 6.5 cm), which were attached with cable ties to a 60 cm stainless steel pole anchored in the sediment. The main bodies of the housings were constantly submerged in

water. Temperature was recorded in 30 min intervals. Logger data was downloaded monthly during the logging period (November 2009 - November 2010). Average data from four loggers are presented; readings from the fifth logger were omitted as its pool was covered with a thick layer of sludge for extended periods. Additional temperature measurements were taken on several occasions in the tide pools with a digital thermometer. Furthermore, daily temperature data of the main water body in the harbour measured at the nearby Portobello Marine Laboratory were obtained for comparison.

2.4 Results

1st intermediate snail hosts

Density. Snail density did not differ among seasons at the 5% significance level (GLM, quasi-Poisson; $F_{3, 20} = 2.94$, $p = 0.058$; deviance explained by the model = 0.30). Density was, however, higher in summer and fall than in winter and spring (Table 2.1, Figure 2.1), with an overall mean density of 161.3 snails (range: 1 - 1053) 0.5 m^{-2}. Mean shell length of snails (≥ 8 mm) was 13.67 ± 0.04 mm (n = 3043) (winter: 14.02 ± 0.11 (n = 257), spring: 14.47 ± 0.11 (n = 356), summer: 14.11 ± 0.06 (n = 1197), fall: 12.95 ± 0.08 mm (n = 1233); ± standard error as in all following results).

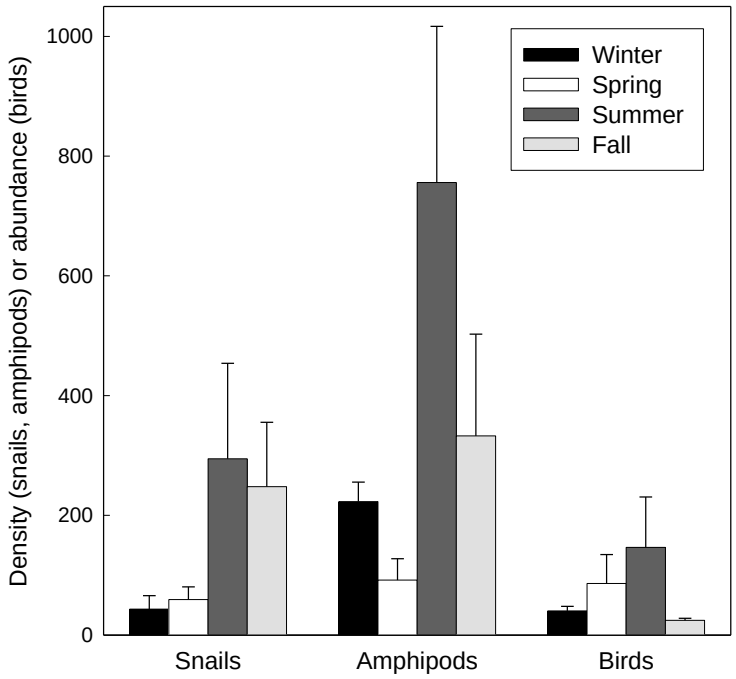


Figure 2.1 Density of snails and amphipods (per 0.5 m^2) and abundance of birds on the Lower Portobello Bay mudflat across four seasons (mean ± standard error).

Table 2.1 Prevalence and infection intensity of snail (*Zeacumantus subcarinatus*) and amphipod (*Paracalliope novizealandiae*) hosts of *Maritrema novaezealandensis* from the Lower Portobello Bay mudflat across all four seasons. Prevalence (percentage of hosts infected; for snails: overall trematode prevalence and prevalence of *M. novaezealandensis* in brackets) and infection intensity (snails: percentage of snail weight being parasite biomass; amphipods: number of metacercariae per host; all ± standard error) are reported. Results are based on six replicate samples per season (pooled from two sampling trips).

		Snails		**Amphipods**	
			n		*n*
Prevalence	*Winter*	71.6 ± 0.03 (61.1)	257	3.3 ± 0.01	540
	Spring	78.1 ± 0.02 (63.8)	356	60.6 ± 0.03	274
	Summer	64.4 ± 0.01 (59.3)	1577	90.6 ± 0.02	320
	Fall	59.2 ± 0.01 (51.2)	1233	68.4 ± 0.02	389
Infection intensity	*Winter*	8.1 ± 0.5	32	1.1 ± 0.1	18
	Spring	8.6 ± 0.4	30	6.7 ± 1.4	178
	Summer	9.1 ± 0.5	31	11.4 ± 1.0	339
	Fall	8.1 ± 0.4	32	5.2 ± 0.5	287

Prevalence. Overall trematode prevalence in snails was 64.5% (n = 3423) for snails ≥ 8 mm. 88.4% of these infections consisted of *M. novaezealandensis*. Besides infections with *M. novaezealandensis*, infections with the following trematodes were found: *Philophthalmus* sp. (prevalence: 4.7%), *Acanthoparyphium* sp. (3.0%), *Galactosomum* sp. (0.8%), *Microphallus* sp. (0.1%); double infections of *M. novaezealandensis* and *Philophthalmus* sp. (1.3%), *M. novaezealandensis* and *Galactosomum* sp. (0.1%). Highest prevalences of *M. novaezealandensis* in snails were found in winter (71.6%) and spring (78.1%) (Table 2.1) and infection status of snails was significantly influenced by season and size of snails (GLM, binomial; season: $\chi^2 = 10.02$, df = 3, p = 0.018; size: $\chi^2 = 289.12$, df = 1, p < 0.001). In fall, the high number of small snails collected lead to the lowest prevalence observed (Fig. 2.2, Table 2.1). Infected snails (of all snails ≥ 8 mm) were generally larger than uninfected snails (average 14.21 ± 0.05mm (max. 24 mm; n = 2028) and 12.59 ± 0.08 mm (max. 21 mm; n = 1015) for infected and uninfected snails, respectively). The proportion of infected snails was lowest for small snails, showed a hump-shaped curve for snails 11 - 21 mm and was 100% for the largest snails collected (22 - 24 mm) (Fig. 2.3).

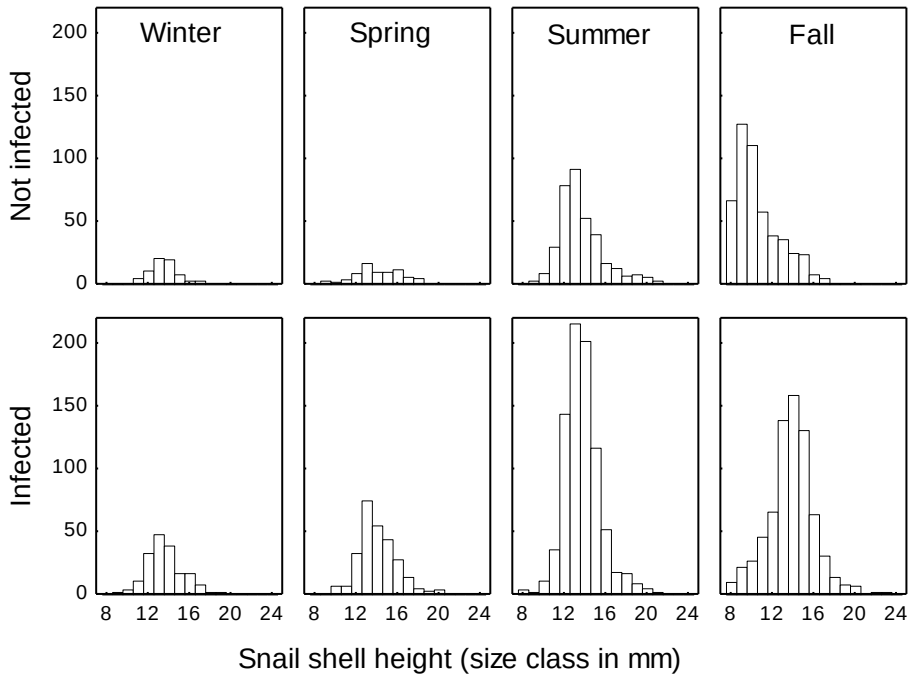


Figure 2.2 Size frequency distributions (shell height in mm, grouped into size classes) of *Zeacumantus subcarinatus* snails infected and uninfected with *Maritrema novaezealandensis* across four seasons.

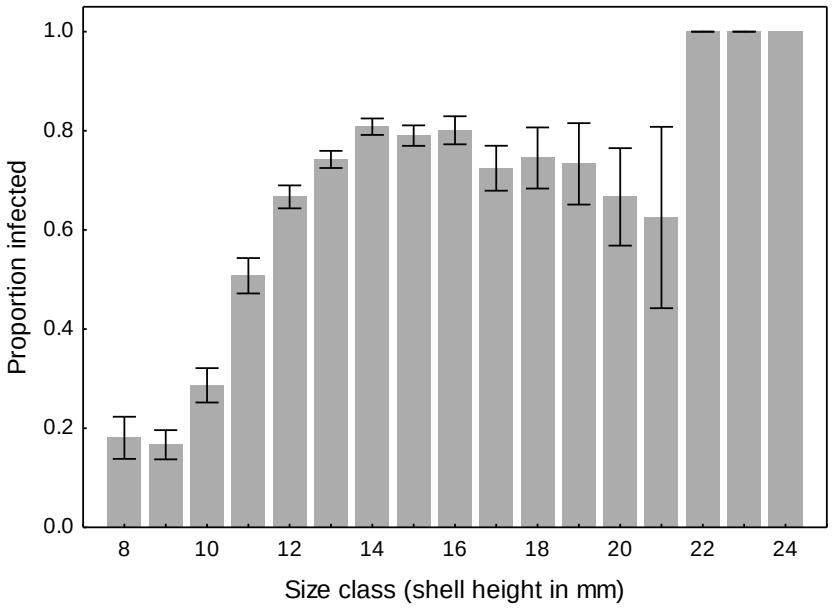


Figure 2.3 Proportion of *Zeacumantus subcarinatus* snail hosts infected with *Maritrema novaezealandensis* per size class (shell height in mm). Note that sample sizes for the three largest size classes is only very small (n = 3, 2 and 1, for size class 22, 23 and 24, respectively).

Infection intensity. On average, 8.5 ± 0.22% (n = 125) of the total *Z. subcarinatus* snail weight (including shell) and 36.8 ± 0.98% of the soft tissue weight (excluding shell) was *M. novaezealandensis* tissue. Mean weight of the parasite tissue in a snail was 0.017 ± 0.001 g. Although there was a seasonal trend with a peak in summer (Table 2.1), the effect of season on the proportion of parasite tissue was not significant (GLM, quasi-binomial; $F_{3, 121} = 1.16$, $p = 0.329$).

2nd intermediate amphipod hosts

Density. Amphipod density varied significantly between seasons (GLM, quasi-Poisson; $F_{3, 20} = 4.62$, $p = 0.013$), being by far the highest in summer and lowest during spring (Fig. 2.1). Overall, the average amphipod density was 350.0 (range 4 - 1926) 0.5 m^{-2}. For amphipods ≥ 2.25 mm, size class distributions were relatively similar across seasons, except that amphipods ≥ 4.25 mm (i.e. males) were absent and only few were present from the second largest size class (4.0 ± 0.25mm) in summer (Fig. 2.4). Juvenile amphipods (< 2.25 mm) were mostly collected in summer and fall. There was a weak positive correlation between amphipod and snail density within sampled areas (Spearman's $\rho = 0.46$, $p = 0.002$), indicating that in enclosures where many snails were counted, comparatively many amphipods were also present.

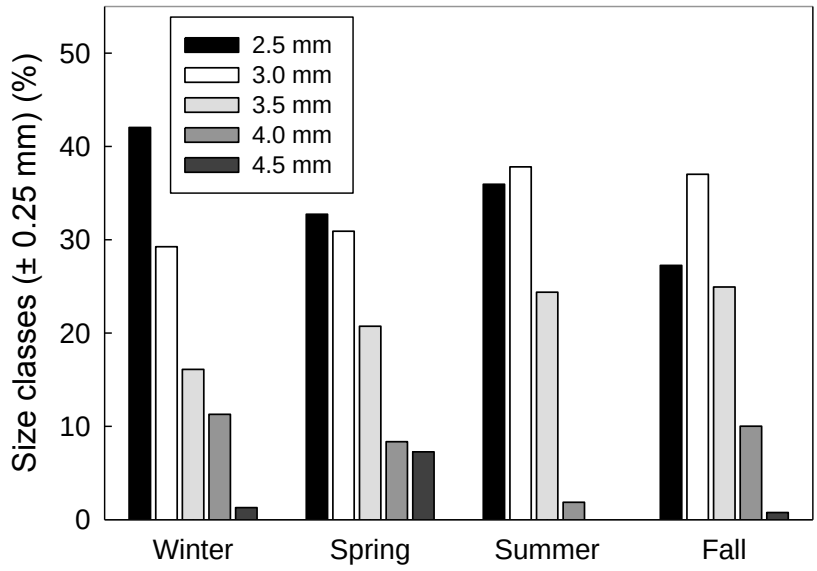


Figure 2.4 Percentage of size classes of *Paracalliope novizealandiae* amphipods (body length 2.5, 3.0, 3.5, 4.0 and ≥ 4.5 ± 0.25 mm) collected across seasons.

Prevalence. The percentage of infected amphipods showed a strong seasonal pattern with the highest prevalence (over 90%) in summer (Table 2.1). Season, sex and size of amphipods all had a significant effect on amphipod infection status (GLM, binomial; season: $\chi^2 = 925.92$, df = 3; sex: $\chi^2 = 15.04$, df = 1; size: $\chi^2 = 131.11$, df = 4, $p < 0.001$ for all; Fig. 2.5) with larger amphipods and males having a higher prevalence (adult males are larger than females with maximum body length for females of 3.5 mm and 5.0 mm for males). Within the same size class (comparing 2.5, 3.0 and 3.5 ± 0.25 mm separately), the proportion of infected amphipods did not differ between sexes (Mann-Whitney U, $p > 0.05$ for all comparisons).

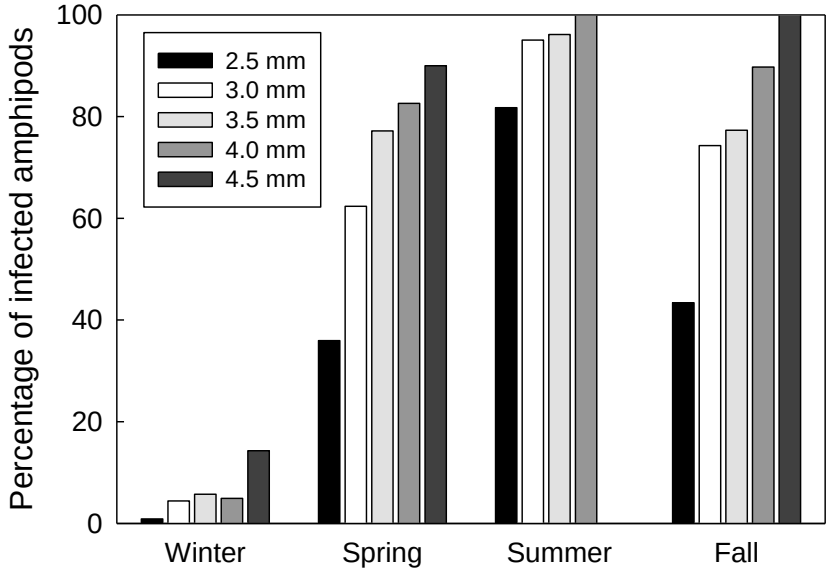


Figure 2.5 Percentage of *Paracalliope novizealandiae* amphipods infected with *Maritrema novaezealandensis* per size class (body length 2.5, 3.0, 3.5, 4.0 and ≥ 4.5 ± 0.25 mm) across all seasons.

Infection intensity. There was also a seasonal pattern with regard to the number of metacercariae per infected amphipod. Mean infection intensity was lowest in winter (range 1 - 19) and highest in summer (range 1 - 202) (Table 2.1). The effects of season, sex and size of amphipods on the number of parasites per amphipod were significant (GLM, quasi-Poisson; season: $F_{3, 733} = 24.27$; sex: $F_{1, 733} = 43.53$; size: $F_{4, 733} = 30.81$, $p < 0.001$ for all). Males were on average more infected than females and larger amphipods more than smaller ones. Mean infection intensities varied from 6.1 ± 0.4 (females) to 11.3 ± 1.1 (males), and from 4.6 ± 0.5 (2.5 ± 0.25 mm size class) to 30.1 ± 10.1 metacercariae/amphipod (≥ 4.5 ± 0.25 mm size class). When comparing infection intensities in the same size class separately, there was a

significant effect of sex with males being more infected than females in the 3.0 and 3.5 mm size classes (Mann Whitney U, 3.0 mm: $Z = -3.39$, $p < 0.001$, 3.5 mm: $Z = -2.64$, $p = 0.01$), but not in the 2.5 mm size class (see also additional information in Box 1 and 4 at the end of this chapter, p. 38-39).

Definitive bird hosts

A seasonal trend was also observed for the abundance of birds on the mudflat, with highest numbers counted from October to January (austral spring and summer) (see also Fig. 2.1). The most common species observed were red-billed and black-backed gulls (*C. scopulinus* and *Larus dominicanus*), oystercatchers (pied and variable; *Haematopus ostralegus finschi* and *H. unicolor*) and ducks (especially paradise shell ducks, *Tadorna variegate* and mallards *Anas platyrhynchos platyrhynchos*). The large flocks visiting the mudflat during summer were mostly gulls (red-billed and black-backed gulls), whereas the most consistent birds present on the mudflat throughout the year were ducks.

Temperature

The mean temperature measured by the loggers between November 2009 and November 2010 was 11.7°C. Highest temperatures were recorded from the end of November 2009 through to March 2010, whereas the coldest period was between June and July 2010 (Fig. 2.6). Temperatures measured during summer occasionally reached above 25°C (maximum: 26.5°C) and dropped below 5°C during winter (minimum: 3.0°C). The magnitude of daily fluctuation of temperature was also greatest in summer (above 10°C compared to 0.5°C in winter). Data measured daily from the main water body of the harbour at the nearby Portobello Marine Laboratory for the same period showed a mean temperature of 11.6°C, minimum of 6.5°C and maximum of 18.1°C. During warmer months, temperatures measured by the loggers on the mudflat were generally higher than those measured from the main water body in the harbour whereas during the colder months they were lower (Fig. 2.6). Additional temperature measurements taken with a digital thermometer indicated that especially during warm periods, loggers in the housings underestimated the actual water temperature on average by about 2.4°C. With a digital thermometer, temperatures above 30°C were measured in several tide pools on one occasion (2. Feb. 2010).

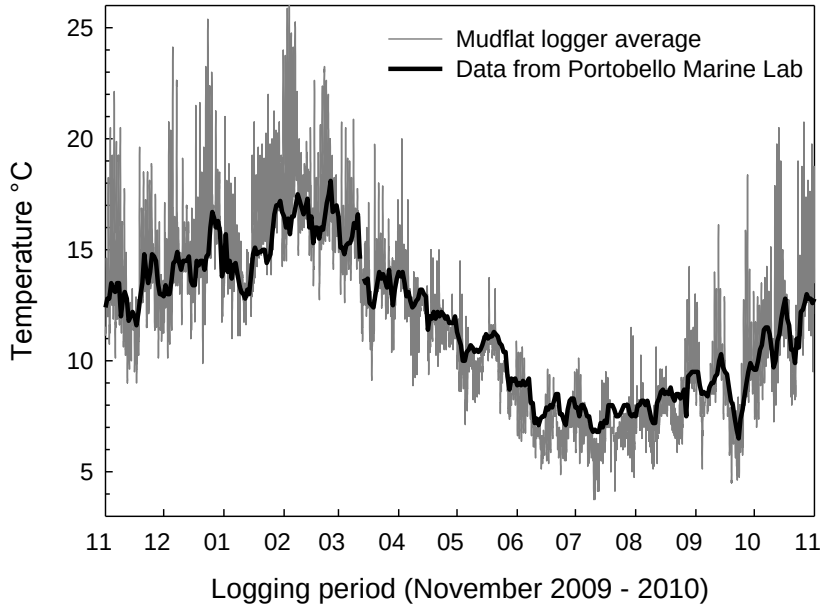


Figure 2.6 Average temperature profile from four loggers deployed in tide pools on the Lower Portobello Bay mudflat from November 2009 - 2010 (grey line) and additional daily measurements obtained at the nearby Portobello Marine Lab from the main water body of the harbour during the same period (black line).

2.5 Discussion

Seasonal fluctuations in the hosts involved in all stages of the complex life cycle of the intertidal trematode parasite *M. novaezealandensis* were investigated to reveal patterns of density (snails and amphipods) and abundance (birds), as well as prevalence and intensity of infections (snails and amphipods). While parasite related parameters in snail hosts remained relatively stable across seasons, temperature-mediated parasite transmission from first to second intermediate hosts was the likeliest main cause of the strong seasonal pattern observed for infections in amphipod hosts. The peaks in density, prevalence and infection intensity in these amphipods during warmer months further coincided with the high abundance of bird hosts present, indicating that all steps in the life cycle of this parasite are completed mostly during warmer months.

1st intermediate snail hosts

Although the density of *Z. subcarinatus* snails was not significantly different between seasons due to substantial variability, the higher mean densities in summer and fall were mainly due to small snails growing to sizes that were detectable by the sampling method used. The higher numbers of small and still uninfected snails consequently affected prevalence, which was significantly lower especially during fall when compared to other seasons. Snail reproduction is thought to take place from November to March (austral spring and summer, Fredensborg *et al.*, 2005), thus the new snail cohort will lead to an overall reduction of prevalence even in populations where prevalence of castrating trematodes is high. This, however, is unlikely to affect the transmission potential of the parasite during that time, as the number of infected medium to large snails remains relatively constant across all seasons and thus supports the expectation of a highly dynamic, yet overall relatively stable prevalence across seasons (Lauckner, 1984).

Fredensborg *et al.* (2005) inferred parasite-induced mortality of snails from a reduction in prevalence among larger size classes and demonstrated that the prevalence of castrating trematodes negatively affected the population density and biomass of *Z. subcarinatus* snails. Our data do not completely support parasite-induced mortality among larger size classes, as the proportion of infected snails remained high even in the largest size class found (Fig. 2.3). Moreover, the reduction in the proportion of *Maritrema* - infected snails in the size classes 17 to 21 mm is most likely due to infections being replaced by another trematode species (i.e. *Philophthalmus* sp.) (see Keeney *et al.* 2008), rather than a reduction in infections. In addition, no indication of mortality was found based on the collection of empty but intact snail shells (data not shown, see Box 2). However, due to the limited dispersal ability of *Z. subcarinatus* and based on evidence of a lower tolerance of infected snails to physical, thermal and/or osmotic stress (e.g. Fredensborg *et al.*, 2005; McDaniel, 1969; Tallmark & Norrgren, 1976) (but see Sousa & Gleason, 1989), the highly infected snail population of LPB may still be at an increased risk for a population crash under extreme conditions such as a heat wave (see Jensen & Mouritsen, 1992).

There was also no significant seasonal pattern regarding infection intensity in snails, despite a slight trend showing a higher proportion of parasite tissue compared to the overall snail weight during summer. This is in contrast to the seasonal difference described by Hechinger *et al.* (2009) for trematode biomass in *Cerithidea californica* snails. Parasite

biomass in natural systems has only recently been recognised as substantial, in particular in the case of trophically transmitted parasites and parasitic castrators (Hechinger *et al.*, 2009; Kuris *et al.*, 2008). The trematode biomass for the area of the LPB mudflat from *Z. subcarinatus* snails alone was estimated to be about 35.3 kg/ha (see additional information in Box 3). This is comparable to results shown for trematodes in Kuris *et al.* (2008), but clearly exceeds the 1 - 10 kg/ha specified for parasitic castrators and trophically transmitted parasite stages. Moreover, the percentage of soft-tissue weight of individual infected snails in the case of *M. novaezealandensis* in *Z. subcarinatus* (36.8%) matches the upper range of what has been described for various trematode species infecting *C. californica* (6 - 49%) and is well above the 20 - 28% described in earlier studies on other castrating trematodes (Hechinger *et al.*, 2009 and references therein).

2nd intermediate amphipod hosts

In contrast to the relatively stable parameters in first intermediate snail hosts across seasons, second intermediate amphipod hosts showed strong and consistent seasonal variations with respect to density, prevalence and infection intensity. The density of amphipods on LPB was highest in summer and fall, mainly due to the high number of juveniles in those samples. Low density of amphipods in spring has also been reported in other studies and has been mostly attributed to mortality of the overwintering generation after reproduction in spring and/or mortality and drifting of juveniles (e.g. Meissner & Bick, 1997).

The seasonal pattern for amphipod density was accompanied by seasonal patterns in prevalence and infection intensities with peaks for both infection parameters during the warm seasons. Because density of snails and infection levels in snails remain relatively constant across seasons (see above), this finding supports the assumption that transmission from first to second intermediate host is highly influenced by prevailing thermal conditions (i.e. triggered by temperature) and thus mostly takes place during low tide when water in tide pools warms up. The surprisingly high infection intensities in amphipods (well above 100 parasites per amphipod of the same developmental stage) in summer confirm that “bursts” of cercarial emergence are frequent (Fredensborg *et al.*, 2004b; Keeney *et al.*, 2007b). Some of these high infection intensities were captured because sampling took place during optimal conditions for transmission. Amphipods infected with a certain number of metacercariae beyond an unknown lethal threshold are, however, unlikely to survive for long, as mortality tends to peak within the first two days after infection (Fredensborg *et al.*, 2004b).

This raises the issue of an increased risk of parasite-induced mortality for these amphipods under conditions where repeated massive releases of cercariae from first intermediate snail hosts are occurring. The potential of *M. novaezealandensis* to induce intensity-dependent mortality in these amphipods has been shown experimentally and has been inferred from field data (Bates *et al.*, 2010; Fredensborg *et al.*, 2004b). Consistent with Fredensborg *et al.* (2004b), prevalence and infection intensity both increased with increasing amphipod size and in particular larger size classes and thus reproductively active amphipods were absent from summer samples. They are therefore considered to be most at risk of parasite-induced mortality; as they accumulate parasites throughout their lifetime, such repeated bursts of cercariae are most likely to cause mortality in these hosts because they surpass the tolerable infection threshold (see also Bates *et al.*, 2010). Despite these high infection levels, density of amphipod hosts was still highest during summer and hence did not indicate any substantial impact of the parasite on the population. This is in accordance with Fredensborg *et al.* (2004b), stating that under normal conditions *M. novaezealandensis* is not expected to exert a strong influence on amphipod population dynamics. Induced mortality is thus considered compensatory, i.e. amphipods would die regardless of the increased impact of the parasite (Fredensborg *et al.*, 2004b). However, massive infection events under exceptional circumstances are likely and are expected to lead to extensive mortality especially among larger and thus reproductively active size classes.

Definitive bird hosts

Definitive bird hosts are the most mobile of all hosts in the life cycle of *M. novaezealandensis* and therefore, are the main dispersal agent for this parasite (Fredensborg *et al.*, 2006; Keeney *et al.*, 2009b). The peak in abundance of bird hosts in the present study was consistent with the main breeding season of gulls in the area. In accordance with Fredensborg *et al.* (2006), the large bird flocks observed during the late spring and summer were mainly red-billed and black-backed gulls (see also McClatchie *et al.*, 1991). The presence of large numbers of birds in the present study not only coincided with the highest density and infection levels in amphipods, but also with the highest density of snails (especially small and still uninfected snails). Due to the temperature dependence of snail activity and thus foraging, it is likely that most snails acquire new infections during this time. This indicates that the continuation of the entire *M. novaezealandensis* life cycle via the bird hosts is accelerated during warmer months and slowed if not halted during colder months. However, besides the various components of the life cycle studied, linking the presence of definitive hosts, effects

of the parasite on the bird hosts, the fate of the parasite eggs expelled by the birds, and parasite recruitment into the first intermediate snail population, remain a major challenge for future investigations.

Temperature

In situ temperature logging provided important details of the thermal fluctuations occurring in the actual habitat where the transmission processes of *M. novaezealandensis* take place. While it was not surprising to identify strong seasonal patterns with regards to temperature, detailed conditions in a microhabitat such as soft-sediment tide pools over long periods of time have rarely been quantified. The measurements taken by the loggers within the housings, however, did not optimally reflect the actual conditions in the pools, as there were considerable discrepancies with additional measurements taken with a thermometer directly in the tide pools. The use of directly submersible temperature loggers is therefore strongly recommended for future research of temporal or small scale spatial thermal conditions. Nonetheless, the temperature profile complemented the overall patterns of densities and parasite related aspects described for the model system on this mudflat and provides an important data set for further research (see Chapter Nine).

Temperature probably affects most aspects of the complex life cycle of *M. novaezealandensis* and as a consequence, the optimal transmission window for this parasite occurs during summer months, especially during low tide when water in tide pools warms up. Other environmental factors may also be influential but have not been investigated. Albeit at very low levels, it is possible that transmission to definitive hosts takes place even during colder months, as amphipods harbouring mature cysts were found and some birds visited the mudflat also in winter. This may be of particular relevance regarding the increasing winter temperatures expected from global warming (IPCC, 2007), which may contribute to an increase in baseline levels of infections especially in second intermediate host populations. Furthermore, as most transmission events, especially transmission from first to second intermediate hosts, probably occur in waves, it can be expected that these would increase in direct relation to the expected increase in frequency and intensity of heat waves (IPCC, 2007). Despite the potential for accelerated reproduction and development of amphipod hosts at increased temperatures, such transmission waves (possibly in combination with direct negative effects of temperature, Studer *et al.*, 2010) may be strong enough to cause significant mortality in amphipods, especially in locations where prevalence of first intermediate hosts is

high, such as on LPB. Since mortality particularly affects the reproductively most active amphipods, this may considerably lower the potential of an affected population to recolonize or rebound to sustainable densities, thus increasing the risk of local extinction. Further spatial investigations are needed to identify areas of high prevalence, which may face an increased risk of parasite-induced mortality events.

In conclusion, seasonal patterns exist for all hosts involved in the complex life cycle of the intertidal trematode parasite *M. novaezealandensis*. These patterns were consistent, suggesting that all transmission processes of *M. novaezealandensis* take place mainly during warmer months and that this is especially true for transmission from first to second intermediate host. The results described here provide important insights into temporal variation of parasitism in a natural host community, providing data on first intermediate, second intermediate and definitive hosts as well as on thermal conditions experienced in the actual microhabitat where transmission occurs. It must be emphasised that areas experiencing a high prevalence in first intermediate snail hosts should be considered high risk areas of intensity-dependent mortality in second intermediate hosts. This is of great concern in the context of on-going and predicted global warming.

Box 1. Population parameters of *Paracalliope novizealandiae* amphipods

The present study allowed the quantification of seasonal changes in population parameters of *P. novizealandiae* amphipods on the Lower Portobello Bay (LPB) mudflat, including seasonal changes in the sex ratio and a better description of the breeding season of these amphipods. The male:female ratio showed a proportionally higher number of females present in winter (0.60) and summer (0.88), almost equal numbers of males and females in fall (1.01), and more males than females were present in spring (1.25) (overall sex ratio 0.86; n = 1524 for amphipods ≥ 2.25 mm; i.e. 53% females). The proportion of gravid females showed a peak in summer (79.0%), intermediate levels in spring and fall (54.5 and 64.2%, respectively), and was considerably lower in winter (14.2%). Gravid females had the highest number of eggs in spring (average of 10.5 ± 0.5; overall average brood size: 7.7 ± 0.2 eggs per female). During summer, both the body length of a female (categorical predictor) as well as the number of parasites infecting a female (continuous predictor) were significantly related to the number of eggs present (ANCOVA; size class: $F_{2, 137} = 11.27$, $p < 0.001$; infection intensity: $F_{1, 137} = 5.87$, $p = 0.017$). These results indicate that, at least at low levels, amphipod reproduction occurs all year round. The main breeding season, however, is from spring to fall. These results also indicate that larger females, the ones with the highest reproductive output, also have the highest number of parasites and thus face an increased risk of parasite-induced mortality.

Box 2. Empty snail shells

The number and average size of empty and intact shells collected from within the sampled 0.5 m^2 areas during this study was lowest in winter (n = 83; 13.0 ± 0.29 mm), but similar in spring, summer and fall (n = 228, 246 and 225, respectively; spring 13.71 ± 0.19; summer 13.73 ± 0.12 mm; fall: 14.1 mm ± 0.18). Based on these empty but intact snail shells, no evidence was found for an increased mortality of especially larger snails or for seasonal differences in snail mortality.

Box 3. Trematode biomass estimation

The average weight of *M. novaezealandensis* tissue per snail was used to estimate the total biomass of trematodes from *Z. subcarinatus* snails present on the LPB mudflat. This was based on the following assumptions: 1) an overall homogeneous snail density of 104 infected snails per 0.5 m^2 (= 64.5% of an average 161 snail density per 0.5 m^2; snails of average size; see Results); 2) an average weight of the parasite tissue per snail of 0.017 g (as measured for *M. novaezealandensis*); and 3) a total area for the mudflat (mid-upper shore area as a rectangle) of approx. 180 x 200 m (i.e. 3.6 ha). The estimated total trematode biomass from infected *Z. subcarinatus* snails for this mudflat was 127 kg (i.e. 35.28 kg ha^{-1}).

Box 4. Developmental stages of the metacercariae

Developmental stages of the parasite's metacercariae within amphipod hosts also showed seasonal variation. Overall, 6556 metacercariae were counted from dissected amphipods, of which 78.9% were at an early immature, 17.6% late immature, 2.6% early cyst and 0.9% mature cyst stage. In spring and summer, the vast majority of metacercariae were at the early immature stage (91.9 and 83.0%; n = 1193 and 3862, respectively), whereas in winter and fall, metacercariae at more advanced stages were found relatively more frequently (e.g. in fall: 38.4% late immature stage (n = 569), 1.9% early cyst (n = 28) and 1.2% mature cysts (n = 18)). This result does not reflect that parasite development within amphipods is greatly accelerated at higher temperatures(Studer *et al.*, 2010), but indicates that new infections during warmer months occur in addition to existing infections especially in larger amphipods, which may reach the lethal threshold of infection intensity and thus have a higher risk of mortality (see also Bates *et al.*, 2010). According to Fredensborg *et al.* (2004b), this accumulation of parasites throughout the amphipod's life time and the reaching of lethal levels in larger amphipods indicated that parasite-induced mortality is more compensatory than additive and that *M. novaezealandensis* is thus not expected to exert a strong regulatory influence on amphipod populations under normal circumstances.

CHAPTER THREE

Parasites and global warming: net effects of temperature on an intertidal host-parasite system

3.1 Abstract

Climate changes, particularly global warming, are likely to impact host-parasite interactions. However, our understanding of the effects of environmental factors on marine host-parasite systems is limited. We conducted a series of laboratory experiments on the effects of temperature on all transmission steps of the intertidal trematode *Maritrema novaezealandensis* from its first intermediate snail host *Zeacumantus subcarinatus* to the second intermediate amphipod host *Paracalliope novizealandiae*. By measuring output of cercarial transmission stages from snails, cercarial survival and infectivity, susceptibility of amphipods to infections, amphipod survival and parasite development within amphipods, we evaluated overall net effects of temperature. At low temperatures (< 20°C), transmission was low and amphipod survival unaffected. At intermediate temperatures (20 to 25°C), output and infectivity of cercarial transmission stages was at an optimum, which may increase the risk of intensity-dependent mortality of amphipods. Also, temperature directly increased amphipod mortality, but accelerated parasite development within amphipods. At high temperatures (≥ 30°C), transmission of the parasite was reduced (few cercariae, low infectivity), but temperature-induced mortality of amphipods was most pronounced. Our approach revealed that temperature strongly, but differentially, affects the various steps of the transmission process, pointing to the amphipod as the most vulnerable component. An increased impact of parasites on amphipod populations with global warming is predicted and the possible disruption of the host-parasite system seems realistic under unusual future circumstances such as prolonged heat waves. We suggest that more holistic studies of host-parasite interactions are essential for a better understanding of potential responses of host-parasite systems to global changes.

3.2 Introduction

Parasites are ubiquitous components of the biosphere and constitute an important part of biodiversity (Marcogliese, 2004; Poulin & Morand, 2000). Parasites affect not only host individuals, populations and communities, but can play an important role in ecosystem functioning and food web dynamics (e.g. Lafferty *et al.*, 2008; Sousa, 1991; Thomas *et al.*, 2005). This is the case not only for terrestrial and freshwater ecosystems, but also for the marine environment where parasitism is equally important, but comparatively little studied. A climate-mediated increase in the frequency and severity of disease outbreaks in marine environments has been reported over the last decade (Harvell *et al.*, 1999; Lafferty *et al.*, 2004). This has directly affected vertebrates, invertebrates and plants, with the potential to alter the structure and function of marine ecosystems (Ward & Lafferty, 2004). Given the pivotal role of parasites and pathogens, understanding the potential ramifications of climate change, particularly global warming, on parasitism should be of major concern.

Probably the best studied marine ecosystems with respect to parasites are intertidal ecosystems, in which digenean trematodes comprise the dominant macroparasite group (Lauckner, 1987; Mouritsen, 2002; Sousa, 1991). Typically, they have a complex life cycle involving three different hosts. In the definitive host (a vertebrate), adult worms reproduce sexually and eggs are expelled into the environment. First intermediate hosts (molluscs) either ingest the eggs or are actively infected by miracidia that hatch from these eggs. Within the first intermediate host the parasite reproduces asexually, generating large numbers of cercariae, which emerge from their host to infect second intermediate hosts (invertebrates or vertebrates, depending on the trematode species). Cercariae are free-living, short-lived (generally < 24 h), non-feeding transmission stages, that are directly exposed to ambient conditions (Pietrock & Marcogliese, 2003). After successful infection of a second intermediate host, the cercariae mature into metacercariae. The life cycle is completed when an infected second intermediate host is consumed by a definitive host.

All of these stages of a trematode life cycle are directly affected by temperature (Chubb, 1979), in particular the transmission process of the parasite (Poulin, 2006). Usually, with increasing temperature, there is an increase in production within, and emergence of cercariae from, the first intermediate host (e.g. Mouritsen, 2002; Thieltges & Rick, 2006). Infectivity of cercariae (leading to successful transmission to the second intermediate host) increases up to

an optimum temperature, whereas survival of cercariae decreases with increasing temperature (Evans, 1985; McCarthy, 1999). These observations have led to the prediction that global warming might enhance the impact of trematodes through the increased number of infective stages present in a system (Poulin, 2006; Poulin & Mouritsen, 2006).

However, this prediction may be too simplistic, as it is not only the parasites and their free-living stages that are sensitive to temperature changes but also their hosts (Marcogliese, 2001). To anticipate the net effect of an increase in temperature on a host-parasite system, both, parasites and hosts have to be considered. Temperature effects on hosts range from a weakening of the host defence and an increase in susceptibility to disease to a boost in immune defences (e.g. Harvell *et al.*, 2009; Lafferty *et al.*, 2004). Temperature can also act as a stressor on hosts and may lead to an increase in parasite-induced mortality (Esch *et al.*, 1975). The impact on individual hosts may then also translate into effects at the population level, which in turn, may negatively affect the parasite's success at completing its life cycle.

To date, most studies on the effects of temperature on trematodes have only investigated a few aspects of the transmission and infection processes. In addition, most studies have only looked at the short-term responses of parasites to different temperatures (i.e. direct exposure without, or with only very short, previous acclimatisation) and/or focussed on first intermediate hosts. However, responses of parasites to long-term increases in temperature might be different from responses to short-term temperature changes. Also, it is necessary to consider differential effects on hosts and parasites as well as the possible interaction between temperature and parasitism to be able to make more realistic predictions.

In the present study, we used a host-parasite system from the intertidal soft sediment ecosystems of Otago Harbour and surrounding bays (South Island, New Zealand; higher latitude [colder] end of the parasite's geographical distribution) to investigate the net effect of temperature on the different steps of the transmission and infection processes of the trematode *Maritrema novaezealandensis*, from its first intermediate snail host *Zeacumantus subcarinatus*, to its second intermediate amphipod host *Paracalliope novizealandiae.* The complex life cycle of *M. novaezealandensis* includes birds such as the red-billed gull *Chroicocephalus scopulinus* as definitive hosts (Martorelli *et al.*, 2004). The mudsnail *Z. subcarinatus* acts as the first intermediate host, from which cercariae (mean body length, including the tail approx. 170 µm, Martorelli *et al.*, 2004) emerge under optimal conditions in

order to infect a range of second intermediate crustacean hosts including *P. novizealandiae*. Second intermediate hosts have been identified as crucial components of a trematode life cycle (Mouritsen *et al.*, 2005), as mortality of these hosts is highly sensitive to infection intensities (Fredensborg *et al.*, 2004b; Meissner & Bick, 1999a, b; Mouritsen & Jensen, 1997). For the completion of a trematode life cycle and the overall assessment of the local impact of a parasite, the second intermediate host might thus be a key component. Due to its high abundance (mean: 350 amphipods 0.5 m^{-2}, range: 4 - 1926 amphipods 0.5 m^{-2}; Chapter Two), *P. novizealandiae* is considered an important component of the food web of these intertidal mudflats. Temperature-dependent cercarial output from snails and infection intensity-dependent mortality of amphipod hosts have previously been shown (Fredensborg *et al.*, 2004b, 2005), making this an ideal model system to investigate the net effect of temperature on host-parasite interactions and formulate predictions of the potential outcome of this interaction with regard to global warming.

We focussed on understanding the effects of temperature on this trematode-amphipod system by conducting a series of laboratory experiments that address each step of the transmission and infection processes from first to second intermediate hosts (see Fig. 3.1): (1) long-term cercarial output from first intermediate snail hosts; (2) cercarial survival; (3) cercarial infectivity; (4) susceptibility of second intermediate amphipod hosts to infection; (5) survival of infected and uninfected amphipod hosts; and (6) development of the parasite within the amphipod hosts. Our overall objective was to examine the net effects of temperature and to predict how this system is likely to respond to global warming. Although we could not consider the complete life cycle (steps related to the definitive bird host were not included), the investigation of the entire transmission process from first to second intermediate hosts (both ectotherm), as well as the survival of second intermediate hosts and the assessment of long-term responses, allowed more realistic predictions to be made regarding the consequences of global warming on the impact of trematode parasitism in second intermediate host populations.

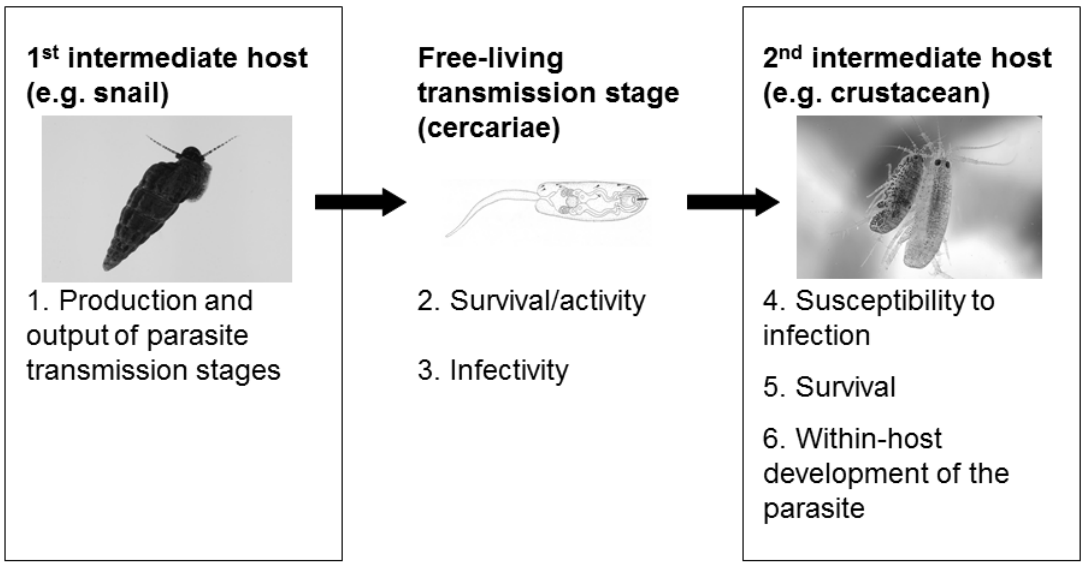


Figure 3.1 Conceptual model of the different steps of the transmission process studied. Note that first intermediate snail host *Zeacumantus subcarinatus*, cercaria of the trematode *Maritrema novaezealandensis* and second intermediate amphipod hosts *Paracalliope novizealandiae* are not to scale. Photos of snail and amphipods courtesy of A.V. Koehler.

3.3 Materials and Methods

Parasite and host material. First intermediate snail hosts *Z. subcarinatus* infected with *M. novaezealandenis* were collected from the upper intertidal zone of a high prevalence site (Lower Portobello Bay, Otago Harbour, 45°50'S, 170°40'E; Fredensborg & Poulin, 2006) on a single occasion in October 2008. Snails were screened for infections by incubating them individually in wells with approx. 3ml seawater (12-well plate; 23 x 20 mm) at 25°C under constant illumination for 2 - 4 h, and then checking for the presence of emerged cercariae. All snails used in the temperature experiments (see below) were of intermediate size (range 11 - 16 mm shell length). For experimental infections of second intermediate amphipod hosts, uninfected *P. novizealandiae* were collected from Hooper's Inlet (Otago Peninsula; 45°52'S, 170°42'E) on several occasions 2 - 3 d before the start of an experiment to allow for adequate acclimatisation to laboratory conditions. As the first intermediate snail host is absent, amphipods from Hooper's Inlet have never been found naturally infected (neither by *M. novaezealandensis* nor by any other metazoan parasite; Chapter Two; Bryan-Walker *et al.*, 2007; Fredensborg *et al.*, 2004b).

General remarks on the experiments. Tidal flats are subject to extreme fluctuations in abiotic factors, particularly temperature (Chapter Two, de Wilde & Berghuis, 1978). Whereas for the main water body in the harbour temperatures are around 7 to 16°C (long-term mean minimum and maximum water temperature measured at the Portobello Marine Laboratory), water temperatures > 30°C have been measured in tide pools on the local mudflats during summer (Chapter Two). Climate projections for New Zealand suggest an increase of about 0.9°C in mean air temperature by 2040 (Mullan *et al.*, 2008). Although these general large-scale predictions are expected to lead to a similar increase in coastal water temperatures, there are no data available for small-scale localities like the local tidal flats and tide pools used in the present study. Extreme events such as heat waves are also expected to become more frequent. Hence, we decided to base the temperature range used in the experiments on the current temperature range experienced on the mudflats, including the summer extremes, as well as a temperature level beyond that. Experiments therefore included an acclimatisation temperature (16, 20, 25, and 30 ± 0.5°C), and an incubation temperature (16, 20, 25, 30 and 34 ± 0.5°C; the lowest temperature for the cercariae and amphipod survival experiments was 15 ± 0.5°C due to logistical reasons). In these experiments, we focussed on temperature, acknowledging that temperature effects may be due to a combination of heat and levels of dissolved oxygen.

For temperature experiments employing wells, including all experimental infections of amphipods, 96-well plates (wells 7 x 10 mm wells) were used. In all experiments (except the cercarial output time series), the cercariae used were pooled from 40 snails (either randomly selected snails from stock aquaria kept under variable conditions or acclimatised snails). These snails were incubated in eight replicate Petri dishes containing 8 ml of aerated seawater at the next higher temperature from their acclimatisation temperature (16 at 20, 20 at 25°C etc., or at 25°C for the snails from the stock aquaria) for 1 h under constant illumination. After removal of the snails, seawater containing the emerged cercariae was combined and carefully mixed (see below). This allowed a genetically mixed array of cercariae to be used in the experiments. To assess the number of cercariae added per volume of each mixture, cercariae in 10 aliquots were counted. All amphipods used were measured, grouped into size classes (2.5, 3.0, 3.5, 4.0, 4.5 ± 0.25 and ≥ 4.75 mm) and sexed prior to their dissection under a dissecting microscope. General husbandry of snails and amphipods included weekly changes of water (long-term shedding experiment) and feeding with *Ulva* sp. algae, *ad libitum*. Prior to all statistical analyses (see details under each section below) we tested for assumptions

regarding parametric tests and for differences between replicates. Data were transformed or non-parametric tests were used where assumptions were violated. Data are presented as means ± standard error unless otherwise stated.

Output of *M. novaezealandensis* cercariae from first intermediate snail hosts. Long-term cercarial output (Fig. 3.1, Step 1) was assessed by counting the number of cercariae released by snails acclimatised to different temperatures during weekly incubations over a period of eight weeks. Snails were marked individually using numbered plastic tags (The Bee Works) to allow repeated counts of the number of cercariae released by individual snails over time. In an attempt to clear the parasite tissue within these snails of fully developed cercariae, snails were first incubated at 25°C for 24 h under constant illumination, after which cercarial output has been found to be much reduced for several days when continuously kept under these conditions (A. Studer unpubl. data). Snails were then distributed into two replicate aquaria at each of 16, 20, 25, and 30°C (temperatures ± 0.5°C; n = 28 per temperature level). After one week at the respective acclimatisation temperatures, half of the snails were incubated for 24 h at this same temperature (16 at 16°C, 20 at 20°C, 25 at 25°C and 30 at 30°C; equal temperature treatment), while the other half were incubated at one temperature level higher (16 at 20°C, 20 at 25°C, 25 at 30°C and 30 at 34°C; temperature boost treatment; all incubations under constant illumination). The temperature boost was meant to simulate the higher temperatures that are experienced by snails at low tide on hot sunny days, occasions thought to trigger emergence of readily developed cercariae from snails (Fredensborg *et al.*, 2004b).

For the incubations, snails were individually placed in 1.5 ml Eppendorf tubes filled with 1 ml aerated seawater at the respective temperature. To preserve the emerged cercariae after removal of the snails, tubes were centrifuged (5 min, 20 817 x g), and 900 µl of the seawater was replaced with 70% ethanol. The discarded water was checked for the presence of cercariae. This procedure was repeated weekly for seven weeks, except that subsequent incubations were shortened to 6 h to prevent any potential decay of cercariae at high temperatures and to provide a realistic time frame of potential shedding in relation to the tides. Samples containing preserved cercariae were counted under a dissecting microscope. A General Linear Model (GLM) was used to determine the effect of the acclimatisation and incubation temperatures (incubation equal or not equal to acclimatisation temperature) on the log-transformed average output of cercariae from each snail per shedding event.

Survival of cercariae. This experiment monitored survival and activity of cercariae (Fig. 3.1, Step 2) at different temperatures, using cercariae from infected snails that were acclimatised to different temperature levels (15 - 16, 20, 25 and 30 ± 0.5°C) for several weeks. A cercarial mixture was obtained for the different acclimatisation temperatures (see above). Snails acclimatised to 15 and 30°C did not shed enough cercariae to be included in the experiment. For the 20 and 25°C acclimatisation levels, groups of approx. 20 to 25 cercariae (corresponding to 40 µl [20°C] and 25 µl [25°C] of the cercarial mixtures) were then transferred into wells of a 96-well plate and incubated under constant illumination at 15, 20, 25, 30 and 34°C (12 wells in two replicate well plates per temperature level; volume of water in wells standardised to 50 µl). The survival of the cercariae was checked 2, 4, 7, 12, 22 and 26 h post-emergence by assessing the number of cercariae that were fully active, sluggishly motile, or immotile/dead. The data were analysed using a repeated measures ANOVA to determine the effect of the acclimatisation and incubation temperatures on the proportions of fully active cercariae (arcsine-square root transformed) 2, 4, 7 and 12 h post-emergence.

Infectivity of cercariae. Infectivity (Fig. 3.1, Step 3) of cercariae was assessed by comparing their success at infecting amphipod hosts. For these experimental infections, 40 uninfected amphipods were put individually in wells of two replicate well plates filled with 75 µl of seawater at the respective temperature. A cercarial mixture was obtained from acclimatised snails (see above). To each well, 50 µl of the cercarial mixture from snails acclimatised at 16, 20 or 30°C (corresponding to an addition of an average of 3 ± 0.45 (± SE), 25 ± 1.02 and 20 ± 0.91 cercariae per amphipod), or 25 µl (plus 25 µl of seawater to standardise the volumes) of the cercarial mixture from snails acclimatised at 25°C (corresponding to an addition of 35 ± 1.19 cercariae per amphipod) was added. Two different approaches were used to investigate infectivity of cercariae. First, for all acclimatisation temperatures, experimental infections were conducted to assess infectivity of the cercariae at the acclimatisation temperature, compared with the infectivity of these cercariae one temperature level higher (16 at 16 and 20°C, 20 at 20 and 25°C, etc.). Second, the infectivity of cercariae from snails acclimatised to 20°C was assessed at all experimental temperature levels (16, 20, 25, 30 and 34°C). The two approaches were conducted in order to assess the importance of long-term acclimatisation versus short-term exposure on the infectivity of the cercariae. The incubation period was 2 h under constant illumination. Unlike previous studies (Bryan-Walker *et al.*, 2007; Fredensborg *et al.*, 2004b), the shorter incubation period for the

experimental infections was chosen in order to reduce amphipod mortality at high temperatures. After incubation, amphipods were placed in two replicate groups per treatment in small containers (approx. 300 ml aerated seawater) and left for 1 to 2 d at 16°C. Amphipods were then dissected to assess the proportions of cercariae that successfully infected the amphipods. A GLM was used to assess the effect of acclimatisation and incubation temperatures on the proportion of cercariae (arcsine-square root transformed) that successfully infected amphipods.

Susceptibility of amphipods to infections. Susceptibility of second intermediate hosts to infection (Fig. 3.1, Step 4) was investigated by exposing amphipods to different temperatures, prior to adding non-acclimatised cercariae, and then comparing the infection success of the cercariae in these hosts. Groups of 17 amphipods (two replicate groups per temperature) were put through an acclimatisation series at 1 h steps until reaching the final temperature level (16, 20, 25, 30 and 34°C, all amphipods handled similarly). Amphipods were then distributed into wells containing 75 µl aerated seawater at the respective temperature. From the cercarial mixture generated (from snails kept in a stock aquarium), 30 µl was added to each well that contained an amphipod (20 ± 0.86 cercariae per amphipod). Well plates were then incubated under constant illumination at the different temperatures for 2 h and amphipods were subsequently dealt with as described for the infectivity experiment (Step 3). A GLM was used to determine the effect of the incubation temperature on the proportion of cercariae (arcsine-square root transformed) that successfully infected the amphipods.

Survival of infected and uninfected amphipods and development of parasites within amphipod hosts. Infected and uninfected amphipods were exposed to different temperatures and their survival (Fig. 3.1, Step 5) was monitored over a 12 d period. Amphipods (n = 400) were placed individually in wells filled with 75 µl aerated seawater (40 amphipods in each of two replicate well plates per temperature level). A cercarial mixture was prepared from snails kept in stock aquaria. Of this mixture, 50 µl was added to half of the wells (corresponding to an addition of 25 to 30 cercariae per amphipod), and the same volume of pure seawater was added to the controls. Well plates were incubated for 2 h at 25°C under constant illumination to allow the cercariae to infect the amphipods. Amphipods were then transferred into containers filled with 300 ml aerated seawater and stored overnight at 15°C to allow the cercariae to fully penetrate the hosts. The following day, infected and uninfected

control amphipods were put through an acclimatisation series (1 h at every temperature level; all amphipods handled similarly) and subsequently were placed into two replicate aquaria (6.5 l; half filled) at the final experimental temperature level (15, 20, 25, 30 and 34 ± 0.5°C). Survival was subsequently monitored two to three times a day. Dead amphipods were dissected to assess the infection status, infection intensity and the developmental stage of the parasites (Fig. 3.1, Step 6); developmental stages of metacercariae were classified as early immature, late immature, early cyst and mature cyst, according to Keeney *et al.* (2007b). After 12 d, all remaining amphipods were sacrificed.

For the statistical analyses, Kruskal-Wallis tests were used to compare the survival times of the amphipods (only those that died during the experiment) between temperature levels and between amphipod size classes and Mann-Whitney U-tests were used to determine the significance of the effect of infection status and sex of the amphipods (data not normally distributed). A Spearman's rank correlation was used to assess the relationship between infection intensity (number of metacercariae per amphipod) and survival time in the experiment. A survival analysis was carried out comparing the survival of all amphipods in the experiment at different temperatures (χ^2). Further survival analyses were run to assess the survival of infected and uninfected amphipods separately at 20 and 25°C (Cox's F-Test).

3.4 Results

Cercarial output from snails

The total average output of cercariae per snail over the entire eight weeks at each acclimatisation/incubation level varied substantially, with a mean of 8.1 ± 3.9 cercariae per snail at 16/16°C, 107.1 ± 34.2 at 16/20°C, 170.1 ± 68.6 at 20/20°C, 615.9 ± 181.4 at 20/25°C, 592.8 ± 200.2 at 25/25°C, 1483.2 ± 339.9 at 25/30°C, 127.4 ± 56.0 at 30/30°C and 531.5 ± 142.5 at 30/34°C (± SE; n = 14, range: 0 - 1177 cercariae per snail per incubation period). These differences were significant among acclimatisation levels (Table 3.1) and cercarial production over the whole period was highest for the snails kept at 25°C (Fig. 3.2). Cercarial production was similar at 20 and 30°C, whereas at 16°C, it was consistently low (Fig. 3.2). The temperature boost during snail incubation (acclimatisation < incubation level) also had a significant effect on the average and total output of cercariae per snail (Table 3.1) at all temperature levels (GLM, temperature boost: $F_{1, 104}$= 28.37 and 27.43, respectively, $p < 0.001$,

for both total and average output; Fig. 3.3). There was no significant difference in shell length among the snails used in the different replicates and treatments (n = 112, size range 11.0 - 15.7 mm; one-way ANOVA, $F_{15, 96} = 1.23$, p = 0.220).

Table 3.1 Effect of acclimatisation temperature (16, 20, 25 and 30°C) and temperature boost (incubation temperature > acclimatisation temperature, compared with incubation temperature = acclimatisation temperature) on the average number of *Maritrema novaezealandensis* cercariae released from infected snail hosts over the entire period of the experiment (one 24 h incubation, followed by seven weekly 6 h incubations; log-transformed; n = 28 per acclimatisation temperature). Results of the General Linear Model.

Factor	**df**	**MS**	**F**	**p**
Acclimatisation temperature	3	1.43	13.54	< 0.001
Temperature boost	1	1.67	15.75	< 0.001
Acclimatisation x boost	3	0.06	0.55	0.649
Error	56	0.11		

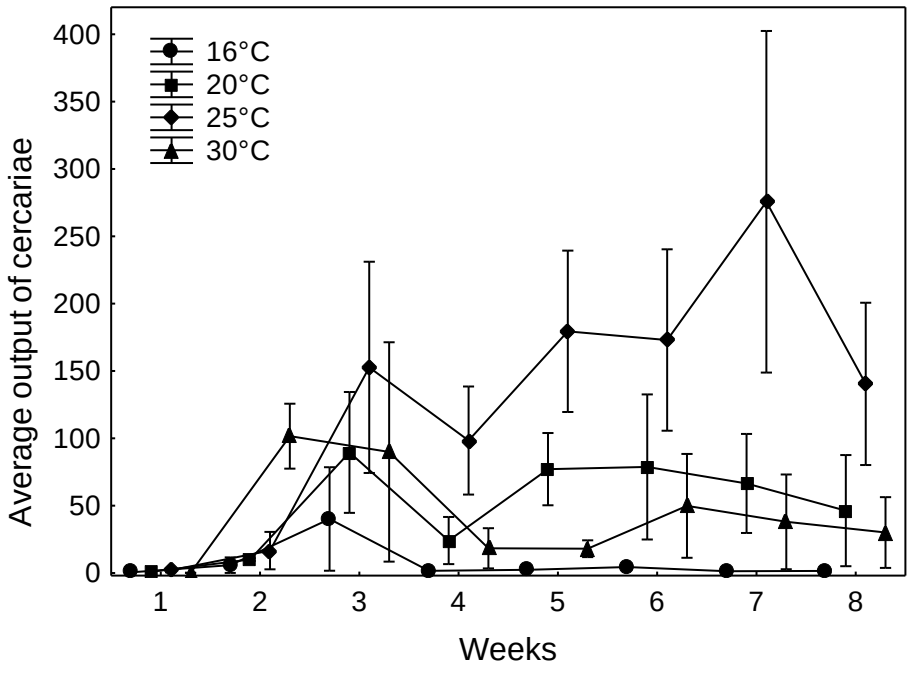


Figure 3.2 Mean ± SE output of *Maritrema novaezealandensis* cercariae from infected *Zeacumantus subcarinatus* snail hosts over eight weekly incubations for four acclimatisation temperatures (n = 28 per temperature).

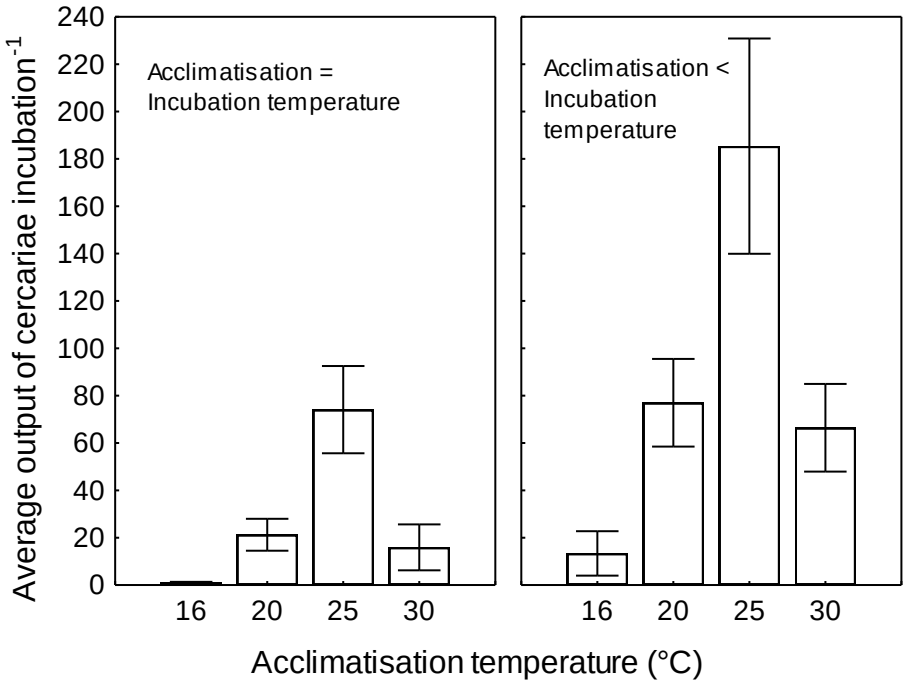


Figure 3.3 Effect of the temperature boost (acclimatisation < incubation temperature) on the mean ± SE output of *Maritrema novaezealandensis* cercariae per weekly incubation (n = 14 per treatment).

Cercarial survival

Full activity decreased steadily with increasing temperature. Incubation temperature, time and their interaction (but not acclimatisation temperature or any associated interaction) had a significant effect on the proportions of fully active cercariae (Table 3.2). Full activity of cercariae ceased within 12 h post-emergence except for cercariae incubated at 15°C (Fig. 3.4).

Table 3.2 Effect of acclimatisation (15/16, 20, 25 and 30°C) and incubation (15/16, 20, 25, 30 and 34°C) temperatures on the activity of *Maritrema novaezealandensis* cercariae (proportion of fully active; arcsine-square root transformed; n = 12). Results of the repeated measures ANOVA (with multivariate within-subjects results).

Factor	**df**	**MS**	**F**	**p**
Between subjects				
Acclimatisation temperature	1	0.02	0.03	0.854
Incubation temperature	4	3.07	5.23	0.001
Acclimatisation x incubation	4	0.34	0.58	0.675
Error	110	0.59		
Within subjects				
Time	3	10.62	66.56	<0.001
Time x acclimatisation	3	0.04	1.39	0.249
Time x incubation	12	0.44	7.84	<0.001
Time x acclimatisation x incubation	12	0.03	0.82	0.627
Error (Time)	330	0.07		

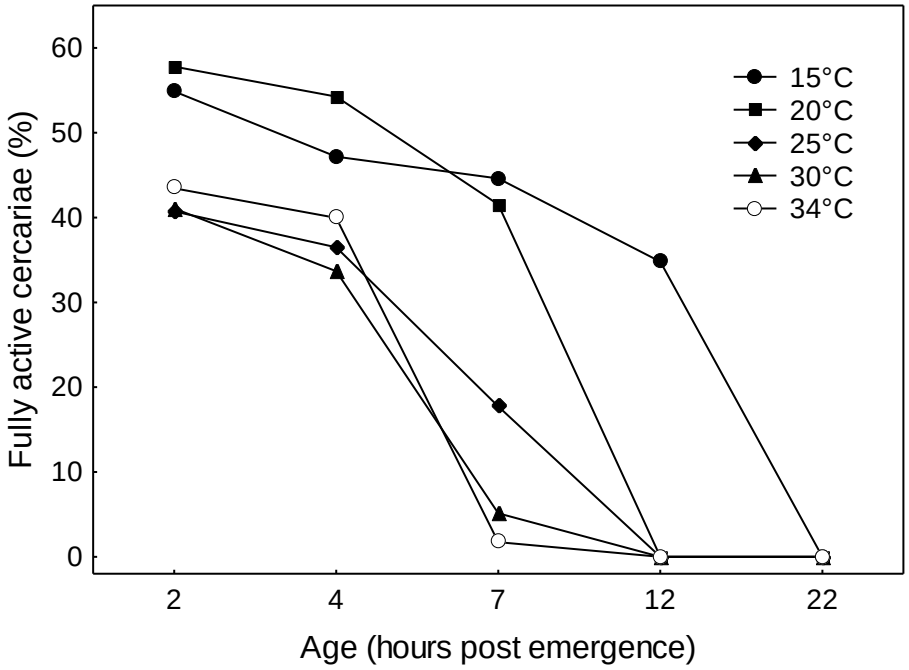


Figure 3.4 Percentage of fully active *Maritrema novaezealandensis* cercariae at 15, 20, 25, 30 and 34°C measured 2, 4, 7, 12 and 22 h post-emergence (n = 12 per temperature).

Infectivity of cercariae

For cercariae from snails acclimatised to the range of experimental temperatures, infectivity significantly differed between acclimatisation levels (GLM, $F_{3, 312} = 34.36$, $p < 0.001$). The optimum temperature for successful infection was 25°C, with 30 and 16°C showing the lowest proportions of cercariae successfully infecting an amphipod host (Fig. 3.5). The percentage of infected amphipods was 38, 100, 100, and 99% at 16, 20, 25, 30°C (n = 40 for each acclimatisation temperature). Mean infection intensities varied from 0.5 ± 0.1 (16°C), to 6.0 ± 0.4 (20°C), 14.2 ± 0.7 (25°C) and 4.2 ± 0.3 (30°C) parasites per amphipod. No significant difference was found in the infectivity of cercariae that were incubated at their respective acclimatisation level compared to the cercariae that were incubated at one temperature level higher (GLM, $F_{1, 312} = 2.69$, $p = 0.102$).

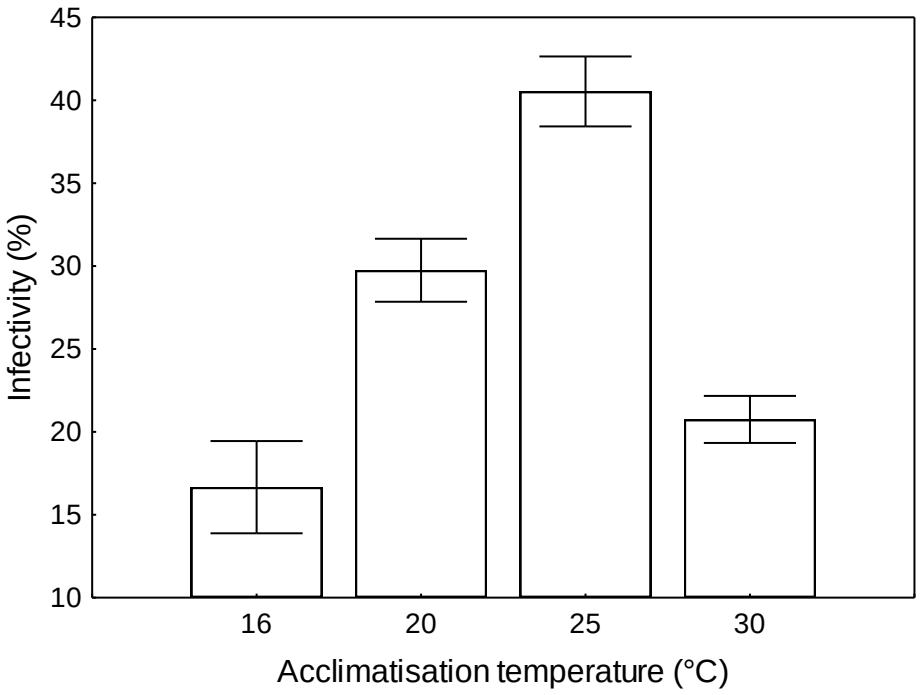


Figure 3.5 Infectivity of *Maritrema novaezealandensis* cercariae: Mean ± SE percentage of cercariae from snails acclimatised to four different temperatures successfully infecting a *Paracalliope novizealandiae* amphipod host (n = 40 for each temperature).

In contrast to the results described above, infectivity of cercariae from snails acclimatised at 20°C and then exposed to all experimental temperatures did not vary significantly between incubation temperatures (GLM: $F_{4, 195} = 0.91$, $p = 0.462$). Infection status of the amphipods varied only slightly between incubation temperatures (98, 100, 100, 93 and 100% infected amphipods at 16, 20, 25, 30 and 34°C; n = 40 for each temperature).

The mean proportion of cercariae successfully infecting an amphipod was 0.29 ± 0.01 and mean infection intensity was 5.8 ± 0.3 parasites per amphipod (n = 200). For both experiments, size and sex of the amphipods had no significant effect on infectivity of cercariae (ANOVA's).

Susceptibility of amphipods

There was no significant difference in the susceptibility of amphipods regarding either the number of parasites within the hosts or the proportion of cercariae successfully infecting amphipods (mean: 0.27 ± 0.01; n = 170) when exposed to different temperatures prior to and during the experimental infection (GLM, log number of parasites: $F_{4, 165} = 1.61$, $p = 0.173$; arcsine-square root proportion of successful cercariae: $F_{4, 165} = 2.10$, $p = 0.084$). Of all amphipods in the experiment (n = 170), 99% were infected by at least one parasite (100% at 16, 20 and 25°C, 97% at 30 and 34°C; n = 34 for each temperature), and the overall mean infection intensity was 5.3 ± 0.3 parasites per amphipod. Size and sex of amphipods did not differ between treatments and had no significant effect on the proportion of cercariae that successfully infected an amphipod host (one-way ANOVA, sex: $F_{1, 168} = 0.03$, $p = 0.872$; size classes: $F_{4, 165} = 2.16$, $p = 0.075$).

Survival of infected and uninfected amphipods

The average time of the amphipods in the experiment until death varied significantly between temperatures, with time until death decreasing with increasing temperature (Kruskal-Wallis, $H_{3, n = 252} = 216.24$, $p < 0.001$). Infection status had no significant effect on the average time until death (Mann-Whitney U-test, $Z = 0.29$, $p = 0.770$), nor did amphipod sex or size (sex: Mann-Whitney U-test, $Z = 0.49$, $p = 0.623$; size classes: Kruskal-Wallis, $H_{4, n = 244} = 1.83$, $p = 0.768$). There was a weak, positive correlation between infection intensity and survival time in the experiment (Spearman's $\rho = 0.12$), with highly infected individuals surviving for slightly longer (mean survival time of amphipods in the experiment: amphipods with no parasites, 3.3 ± 0.3 d; with 1 parasite, 3.7 ± 0.9 d; with > 1 parasite, 3.9 ± 0.5 d). A total of 376 amphipods were recovered during the experiment (203 females, 169 males, 4 unsexed). Overall, 48% of the amphipods were infected (54% at 16°C, n = 76; 43% at 20°C, n = 76; 49% at 25°C, n = 70; 41% at 30°C, n = 78; 53% at 34°C, n = 76). Mean infection intensities did not vary significantly between temperatures (overall mean infection intensity: 5.3 ± 0.3; GLM, log number of parasites: $F_{4, 371} = 1.66$, $p = 0.159$).

Survival of amphipods at various temperatures differed significantly (χ^2 = 226.72, df = 4, p < 0.001). All amphipods at 34°C were dead within 2 h, whereas the survival of amphipods at 30°C was approximately 2 d, regardless of their infection status (Fig. 3.6). Survival at 20 and 25°C was similar, with infected amphipods surviving slightly longer; however, this was only significant at 20°C (survival analysis, Cox's F-test, infection status at 20°C: $F_{26, 50}$ = 2.01, p = 0.017; 25°C: $F_{58, 62}$ = 1.14, p = 0.307). There was almost no mortality in infected or uninfected amphipods kept at 15°C.

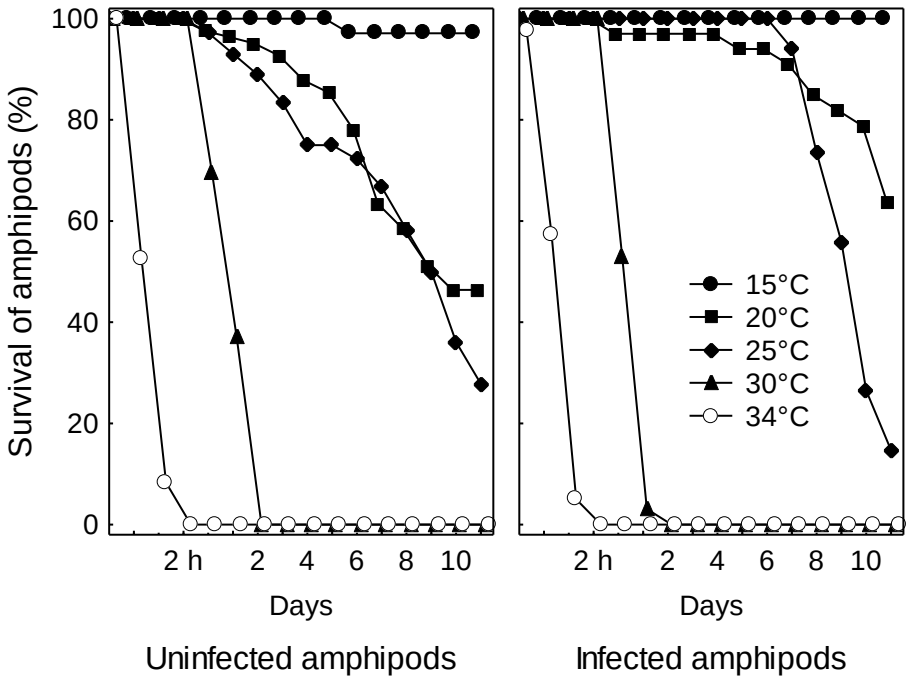


Figure 3.6 Survival (%) of infected and uninfected *Paracalliope novizealandiae* amphipod hosts at 15, 20, 25, 30 and 34°C over a 12 d period. Note that the x-axis is not to scale: the first intervals represent 30, 90, 120 and 150 min, whereas all following intervals represent days.

Parasite development within the amphipod host

The temperature at which amphipods were kept had a strong effect on the development of the parasites. Metacercariae from amphipods kept at 20°C (n = 259) were mostly at late immature (59%) and early cyst stages (39%) (those recovered from dead amphipods both during and at the end of the experiment). When kept at 25°C, 29% of the metacercariae completed development during the course of the 12 d experiment (n = 264). The metacercariae recovered from amphipods incubated at 30 and 34°C (maximum survival of 2 d and 2 h, respectively), were still in the early immature stage (100% for both; n = 125 and 221,

respectively). Similarly, metacercariae recovered from amphipods kept at 15°C and sacrificed after 12 d, were between the early immature and late immature stages (n = 225).

3.5 Discussion

Parasites are strongly influenced by environmental conditions and rely on their hosts for completion of the life cycle. A change in these conditions, particularly temperature, is very likely to differentially affect the parasite, the host and/or their interactions. The outcome of such changes on host-parasite relationships in marine systems has rarely been assessed. The present study experimentally investigated the effects of temperature on an intertidal trematode-amphipod system, incorporating aspects of the parasite and the host in order to make more realistic predictions about the potential impact of global warming on trematode parasitism.

Effects on the parasite

With increasing temperatures, the production and output of *M. novaezealandensis* transmission stages (cercariae) from first intermediate snail hosts *Z. subcarinatus* increased up to an optimum temperature. Temperature has been shown to trigger the emergence of cercariae and to accelerate the production of new cercariae within first intermediate hosts (e.g. Ataev, 1991). Previous studies have also reported similar hump-shaped production patterns (e.g. Thieltges & Rick, 2006). However, the present study is one of very few assessing long term cercarial production and output. Our results suggests that raising temperatures could cause a considerable increase in cercarial production and emergence, which should be of particular relevance for the ongoing and predicted increase in winter temperatures, but also for more frequent heat waves in summer which would, as long as temperatures lie within the optimal range for the parasite, result in more infective stages being present in an ecosystem.

Survival of cercariae decreased steadily with increasing temperature, as described in other studies (e.g. Evans, 1985; McCarthy, 1999; Mouritsen, 2002). Increasing temperatures seem to directly affect the activity of cercariae, leading to a faster depletion of their finite energy reserves (Pechenik & Fried, 1995). Infectivity increased with temperature until dropping off at high temperatures. It has been suggested that as cercarial activity increases, so does the number of contacts with a potential host per unit time (Evans, 1985). At high

temperatures, the decrease in infectivity results from the rapid depletion of the limited energy reserves, so that reserves might not be sufficient to allow successful penetration and subsequent establishment in the host. However, in the present study, infectivity was different for cercariae that were produced in snails acclimatised to different temperatures, but not when comparing cercariae from one acclimatisation temperature that were exposed to the range of experimental temperatures. The most likely explanation for this discrepancy is that cercarial density within snails affects the per capita infectivity rate of cercariae. As shown previously (Evans & Gordon 1983), per capita infectivity increases with increasing cercarial density up to a threshold before dropping off. Therefore, temperature has not only a direct effect on cercarial production, but also an indirect effect on the success of cercariae at infecting second intermediate hosts. This notwithstanding, it should be noted that the long-term acclimatisation in our experiment led to different numbers of cercariae being used for the different treatments, which may account for some of the pattern observed, especially in the case of the coldest treatment. It is acknowledged that the most appropriate approach would have been to use the same number of cercariae in each treatment.

The overall transmission success or efficiency is a measure that combines cercarial survival and infectivity. Previous studies have shown that transmission efficiency can be relatively high over a wide range of temperatures; at low temperatures, low infectivity is offset by low mortality, whereas at high temperatures, higher infectivity compensates for higher mortality (Evans, 1985; McCarthy, 1999). The results from the present study indicate a similar pattern, except that at very high temperatures, both survival and infectivity are negatively affected. Assuming that cercarial survival and infectivity are counterbalanced up to a certain threshold (based on our results estimated to lie between 25 and 30°C for *M. novaezealandensis*), rising temperatures up to that threshold would nevertheless lead to an increase in the number of cercariae present in a system (through cercarial production and emergence). High infection intensities with *M. novaezealandensis* induce mortality in amphipod hosts (Fredensborg *et al.*, 2004b) and increasing temperatures are likely to cause more frequent exposures of amphipods to large numbers of cercariae, thereby increasing the risk of parasite-induced host mortality. Rising temperatures beyond this threshold would negatively affect cercarial emergence, survival, and infectivity and, therefore, the transmission success of this parasite.

Effects on the host

Susceptibility of amphipods was the only step of the transmission process studied which was not significantly affected by temperature. By contrast, amphipod survival was strongly affected by temperature. Survival of amphipods was dramatically reduced at 34°C and, because amphipod survival was even lower than cercarial survival, amphipods are considered the most vulnerable component of all aspects of the trematode-amphipod system studied here. At high temperatures (30 and 34°C), amphipod hosts died regardless of their infection status. At intermediate temperatures (20 to 25°C), a more subtle effect of the parasite on host survival was apparent. Although only significant at 20°C, infected amphipods had a slightly higher survival than uninfected amphipods. This had already been observed in a previous study, where even amphipods with relatively high infection intensities had a higher survival rate towards the end of the experimental period than individuals from the control group (Fredensborg *et al.*, 2004b). This is rather surprising, given that the parasite's development within the amphipod hosts is accompanied by an approximate 200-fold increase in volume. Infection intensities in our experiments were relatively low, but are representative of the average infection intensity found in some naturally infected populations (Bryan-Walker *et al.*, 2007; Fredensborg *et al.*, 2004b).

Effects on parasite development within the amphipod host

Temperature greatly affected parasite development within the second intermediate host. Martorelli *et al.* (2004) reported the recovery of mature metacercariae from experimentally infected amphipods after four to five weeks (at an unspecified temperature). In the present study, a substantial number of parasites were able to complete development at 25°C within less than 12 d. Reaching the mature metacercarial stage is of vital importance. All immature developmental stages within the second intermediate host that is ingested by a definitive host will not contribute to the adult generation. The dramatic increase in the parasite's development rate at intermediate temperatures boosts the number of fully developed, and thus infective, metacercariae present in a system. An acceleration of the life cycle and a concomitant enhanced impact of parasites could be the consequence, which has already been shown to occur under conditions of increased temperature in some ecosystems (Kutz *et al.*, 2005; Marcogliese, 2001).

Net effects of temperature

Three main net effects of temperature on this trematode-amphipod system can be described, assuming that no other ecological factor changes in a way that influences the inferences made: First, at temperatures below 20°C, the production, emergence and infectivity of cercariae are relatively low, but the survival period of cercariae is prolonged. The survival and, therefore, availability of amphipod hosts is greatest at these temperatures; thus infections in second intermediate hosts are persisting at constant but low levels without having a major impact on the amphipod population. This is currently the prevailing condition.

Second, at intermediate temperatures (20 to 25°C), the production and emergence of cercarial transmission stages are substantially increased, the survival of the cercariae is still relatively long and their infectivity is at a maximum. Amphipod survival is considerably diminished at these temperatures, whereas parasite development within amphipods is greatly accelerated. These conditions seem to be optimal for an acceleration and completion of the life cycle by a greater number of individual parasites. Water temperatures between 20 to 25°C and above are occurring at present during summer days at low tide (Chapter Two). Thus, we predict that the parasite will find such optimal conditions more often and for longer periods of time with global warming. Under such conditions, the impact of the parasite on a host population could be substantial, as massive infection events and, therefore, parasite-induced mortality could lead to rapid reductions in amphipod populations in places where infection prevalence in first intermediate snail hosts is high (Fredensborg *et al.*, 2004b). Such an additive effect of temperature and parasitism has been observed in another intertidal trematode-amphipod system, and the resulting mass mortality of the amphipod hosts caused substantial ecosystem-wide consequences (Jensen & Mouritsen, 1992; Mouritsen *et al.*, 1998).

Third, at high temperatures (~30°C and above), relatively high numbers of cercariae are still being produced but their infectivity is low and their functional lifespan very short. More importantly, the survival of amphipod hosts at these temperatures is even lower. From the parasite's perspective, successful transmission is reduced but might still be possible. The high mortality of amphipods, however, could mean that hosts (individuals and populations) may not be available for infection or not be available long enough for the completion of the parasite's development. A disruption of the host-parasite relationship could result. At present, this is only rarely an issue. However, predicted changes include an increase in the number of

high temperature episodes (Mullan *et al.*, 2008), thus increasing the risk of temperature-induced amphipod mortality and the concomitant disruption of this host-parasite system.

Although temperature is a very important factor, host-parasite systems are influenced by a large and complex network of abiotic and biotic factors that could significantly alter predicted outcomes solely based on responses to constant temperatures in laboratory settings. For example, biotic interference in parasite transmission could reduce the number of cercariae present in a system due to simultaneous increases in the feeding activity of the organisms that prey on them (Thieltges *et al.*, 2008b). Also, temperature variability has been shown to increase parasite development when compared with constant temperature for temperatures below 21°C, and to slow parasite development above 21°C (Paaijmans *et al.*, 2009). In our experiments, the temperature boost to which half of the infected snails were exposed to during incubation (for the other half incubation temperature = acclimatisation temperature) had a significant positive effect on the production and output of cercarial transmission stages at all temperature levels investigated, giving credence to the importance of considering temperature variability.

Furthermore, temperature or other factors such as a rise in sea level or habitat alterations might influence the distribution and behaviour of hosts, especially the definitive bird host, thereby altering parasite recruitment or hindering the completion of the life cycle due to changes in feeding patterns. Finally, since global warming will occur over numerous host and parasite generations, it is possible that natural selection will favour genotypes with certain responses to temperature over other genotypes. Such multi-year evolutionary considerations cannot be addressed within the context of an experiment, but they imply that the characteristics of organisms that might exist in a warmer future environment may differ from those that exist in the present climate.

In conclusion, our results indicate that temperature strongly, but differentially, affects the different steps of the transmission and infection processes of the host-parasite system studied, pointing to the second intermediate amphipod host as the most vulnerable step overall. Based on our results, we predict that the transmission and development of this parasite will be positively affected by increasing temperatures, as long as temperatures lie within the optimal range for the parasite and its hosts. Amphipods are likely to be exposed more often to a greater number of parasites, thereby increasing the risk of parasite-induced

mortality. Under extreme conditions such as heat waves, parasite transmission might be reduced, but the risk of temperature-induced mortality of amphipods is most pronounced, potentially leading to a disruption of this link in the completion of the parasite's life cycle. As studies from another amphipod-trematode system point to a similar temperature sensitivity (e.g. Jensen & Mouritsen, 1992; Mouritsen & Jensen, 1997), intertidal amphipod populations may generally experience increasing parasite pressure under climate change. Given the pivotal role of amphipods as decomposers, prey and ecosystem engineers in many intertidal systems (e.g. Mouritsen *et al.*, 1998), this may have far-reaching effects for these ecosystems.

The inclusion of all stages of the transmission process from first to second intermediate host and the evaluation of net effects are valuable tools for attaining a more realistic understanding of the possible impacts of climate change on parasitism and host-parasite interactions. However, long-term monitoring programs are required to verify the predictions made here and to provide data on the actual interactions between environmental conditions and host-parasite relationships in ecosystems affected by climate change.

CHAPTER FOUR

Effects of salinity on an intertidal host-parasite system: is the parasite more sensitive than its host?

4.1 Abstract

Intertidal habitats are characterised by highly fluctuating environmental conditions including varying salinity regimes. Changes in salinity may be gradual or abrupt; for example, heavy rainfall or evaporation during warm periods can either decrease or increase salinity. Trematodes are the most common parasites in intertidal ecosystems and their transmission is known to be highly influenced by environmental conditions. However, effects of salinity on the transmission of intertidal trematodes are not well studied. Here, we investigated effects of long-term exposure to different salinities (25, 30, 35 and 40 psu) on the transmission of *Maritrema novaezealandensis* from its first intermediate snail host (*Zeacumantus subcarinatus*) to a second intermediate amphipod host (*Paracalliope novizealandiae*), in order to evaluate overall net effects. The following steps were assessed: output of parasite transmission stages (cercariae) from infected snail hosts, survival and infectivity of cercariae, susceptibility of amphipod hosts to infection and survival of amphipod hosts including parasite development within amphipod hosts. Output and survival of cercariae increased with increasing salinity while infectivity of cercariae and susceptibility of amphipods to infection were not clearly affected. Survival of amphipods was significantly longer at lower salinities and parasite development in infected amphipods concomitantly more advanced. Overall, the results suggest that the parasite and the amphipods are differentially affected, and that during the main transmission window of this parasite, i.e. on warm sunny days when water in tide pools warms up and salinities are slightly increased, conditions for the parasite are more favourable than for the amphipod host.

4.2 Introduction

In light of the large-scale environmental changes that are occurring and predicted to occur, understanding the effects of environmental factors on marine species and species interactions, such as those between hosts and parasites, seem not only crucial but urgent. Climate change is expected to have negative consequences particularly for the biota of intertidal and shallow marine areas (e.g. Brierley & Kingsford, 2009; Harley *et al.*, 2006). These ecosystems are naturally subject to extreme environmental fluctuations, including changes in salinity, and therefore impose substantial physiological challenges for many of their inhabitants (e.g. Przeslawski *et al.*, 2005). As an integral part of intertidal ecosystems, trematodes are not only the most common parasite group (e.g. Lauckner, 1984; Mouritsen & Poulin, 2002b; Sousa, 1991), but are also of high ecological importance (e.g. Kuris *et al.*, 2008; Mouritsen & Poulin, 2005, 2010). Despite the fact that parasite transmission is known to be strongly influenced by environmental conditions (e.g. Pietrock & Marcogliese, 2003), little is known about the effects of salinity on the transmission of intertidal trematodes and hence on their host organisms.

Salinity is considered one of the most important environmental factors in marine ecosystems, influencing small and large-scale biotic interactions (Berger & Kharazova, 1997; Ingole & Parulekar, 1998). While most marine systems have on average a relatively stable salinity of approximately 35 practical salinity units (psu), salinity in intertidal zones and especially estuaries can fluctuate gradually (e.g. seasonally) as well as rapidly. Tides, rainfall, freshwater inflow or runoff can cause relatively abrupt decreases in salinity, whereas evaporation in tide pools can raise salinities well above normal levels (e.g. Adam, 1990; Brierley & Kingsford, 2009; Wheatly, 1988). Salinity can affect the distribution (Crain *et al.*, 2004; Kneib, 1984), physiology (Hylleberg, 1975; Pequeux, 1995; Shock *et al.*, 2009) as well as the reproduction (Deschaseaux *et al.*, 2010) of intertidal species, which also possess tolerance mechanisms to cope with changing osmotic conditions (for crustaceans, see Pequeux, 1995).

Salinity has also been recognised as an important environmental factor for parasitism and disease dynamics in estuarine or brackish environments (e.g. Haskin & Ford, 1982; Kesting *et al.*, 1996; Koie, 1999; Messick *et al.*, 1999; Reisser & Forward, 1991; Zander, 1998). However, little is known about the effects of salinity on intertidal trematodes,

especially with regards to long-term effects (but see Lei & Poulin, 2011). Trematode parasites usually have complex life cycles involving several members of a community and transmission processes involve stages of the parasite being directly exposed to environmental conditions. For instance, transmission between first and second intermediate hosts is typically via a free-living, short-lived (< 24 h) transmission stage (cercaria), which is asexually produced within gastropod first intermediate hosts. Like most larval endohelminths, cercariae are strongly affected by environmental factors (e.g. Pietrock & Marcogliese, 2003).

The few studies available on the effects of salinity on marine trematodes are focussed on the emergence and/or the survival of these cercariae as short-term responses and these studies have produced inconsistent results. While some (Lei & Poulin, 2011; Rees, 1948; Sindermann & Farrin, 1962; Sindermann, 1960) reported a general increase in emergence of cercariae from first intermediate hosts with increasing salinity, Mouritsen (2002) found a greater cercarial emergence at higher salinity only at elevated temperatures. In a study by Koprivnikar & Poulin (2009) on two intertidal trematode species using the same first intermediate snail host (same host and parasite as used for this study; see below), cercarial emergence was reported to increase with decreasing salinity. With regards to the survival of cercariae, several studies have reported that survival was generally not affected over a range of salinities (Mouritsen, 2002; Prokofiev, 1999; Rees, 1948; Stunkard & Shaw, 1931).

However, studying just one out of the many steps involved in the transmission process in short-term experiments can provide only limited information about the overall transmission success of a parasite species under certain conditions. To achieve a more comprehensive understanding, several steps, including those relating to the hosts, need to be considered. Here, we used the intertidal microphallid trematode *Maritrema novaezealandensis*, a common and, due to its negative effects on intermediate hosts, important parasite in soft-sediment intertidal areas in New Zealand. The adult worms have been described from red-billed gulls, *Chroicocephalus scopulinus* (Fredensborg *et al.*, 2004a; Martorelli *et al.*, 2004), but probably occur in a range of other definitive bird hosts attracted to intertidal mudflats. Eggs produced by adult worms are expelled with the bird's faeces and are ingested accidentally by first intermediate snail hosts, *Zeacumantus subcarinatus*. Within the snails, the parasite multiplies asexually, producing large numbers of the parasite's free-living cercarial transmission stage. These cercariae (mean body length including tail approx. 170 µm; Martorelli *et al.*, 2004) emerge mostly at low tide when water in tide pools warms up (Chapters Two and Three,

Fredensborg *et al.*, 2004b) to infect and subsequently encyst within second intermediate hosts, consisting of a wide range of crustaceans (Koehler & Poulin, 2010), including the amphipod *Paracalliope novizealandiae* used in this study. Definitive bird hosts acquire infections, thereby completing the life cycle, when feeding on crustaceans harbouring fully developed, mature metacercariae.

The main objective of this study was to assess the effects of salinity on the different steps of the transmission process of the intertidal trematode *M. novaezealandensis* from its first intermediate snail host to its second intermediate amphipod host, in order to evaluate the overall net effects of salinity on this host-parasite system. We investigated: (1) cercarial production and emergence from infected first intermediate snail hosts, (2) cercarial survival, (3) cercarial infectivity, (4) susceptibility of second intermediate amphipod hosts to infection, (5) survival of infected and uninfected amphipods, and (6) development of the parasite within the amphipods (see Fig. 3.1 in Chapter Three). The completion of a trematode life cycle is a multi-step process which may be differentially affected by environmental factors at various steps of the transmission process. Environmental effects on the parasite, on its hosts, as well as on their interactions can occur at any step. Therefore, the goal of the present study was to include the entire transmission process from first to second intermediate host, as well as long-term responses to salinity, in order to develop a better understanding of this environmental component on trematode transmission and hence affected host organisms in intertidal ecosystems.

4.3 Materials and Methods

General remarks. On the local mudflats (Otago Harbour, South Island, New Zealand), there are marked salinity gradients resulting from freshwater inputs (range 0 to ~34 psu). During heavy rainfall at low tide, salinity decreases due to direct mixing with seawater as well as increased run-off, whereas during warm, sunny days, salinity slightly increases due to evaporation (max. ~36 psu; A. Studer, pers. observation). The salinity levels used in the experiments were 25, 30, 35 and 40 psu (salinities ± 1 psu; 20°C). These were chosen to cover a wide range of naturally occurring salinities, including one level (40 psu) beyond what is currently experienced on local mudflats but towards which conditions change during exceptionally warm periods such as heat waves in summer, conditions predicted to occur

more frequently with on-going global climate change (IPCC, 2007). For each salinity level a solution was prepared using artificial sea salt (Red Sea salt®). Solutions were stored at 20°C in 20 l containers and kept aerated. Parasites and hosts were obtained and kept as described in Studer *et al.* (2010) (infected snails were collected from Lower Portobello Bay, Otago Harbour, in July 2009; uninfected amphipods (all > 2.25 mm in body length) from Hooper's Inlet, Otago Peninsula, a few days before the start of an experiment). All amphipods used in the experiments were grouped into size classes (2.5, 3.0, 3.5, 4.0, 4.5 ± 0.25 and ≥ 4.75 mm) and sexed prior to their dissection under a dissecting microscope.

In all experiments (except the cercarial output time series; see details below), the cercariae used were pooled from 25 or 40 snails (either snails randomly selected from stock aquaria kept in natural seawater or from infected snails acclimatised to different salinities for several weeks, depending on the experiment; see below). To obtain cercariae, snails were incubated in five or eight replicate Petri dishes (depending on the number of snails used) containing 7 ml of aerated water of the given salinity or natural seawater, respectively, for 1 h at 25°C under constant illumination. After removal of the snails, water from the different dishes containing the emerged cercariae was pooled to ensure a genetic mixture of parasites was used. To assess the number of cercariae added per volume of each mixture, cercariae in 10 aliquots were counted. For the cercarial survival and all experimental infections of amphipods, 96-well plates (wells 7 x 10 mm; total volume 320 µl) were used. Statistical procedures will be discussed in each of the following subsections. We checked for differences between replicates and assumptions of parametric tests, and data were transformed or other tests used where necessary.

Output of *M. novaezealandensis* cercariae from first intermediate snail hosts. Long-term cercarial output was assessed by counting the number of cercariae emerging from individual *Z. subcarinatus* snails (marked with plastic tags; The Bee Works) during weekly incubations over six weeks. At the beginning of the experiment, 112 snails were incubated at 25°C for 24 h under constant illumination in order to induce emergence of fully developed cercariae. Snails were then distributed to two replicate aquaria at each of 25, 30, 35 and 40 psu (at 20°C). After one week of acclimatisation, half of the snails were incubated for 6 h at 25°C under constant illumination at the same salinity, while the other half were incubated at one salinity level higher (25 at 30, 30 at 35, 35 at 40 psu), to simulate a salinity increase that would be experienced in tide pools at low tide during hot sunny days due to evaporation.

Snails kept at 40 psu were incubated only at 40 psu (n = 16 for each treatment). For the incubations, snails were individually placed in 1.5 ml Eppendorf tubes filled with 1 ml of water of the respective salinity. Emerged cercariae were preserved and counted under a dissecting microscope (see details in Studer *et al.*, 2010). After testing with a General Linear Model (GLM) for the effect of acclimatisation salinity and the salinity increase during incubation in the 25, 30 and 35 psu treatments only, the latter factor was omitted from a subsequent analysis with a GLM assessing the effect of the acclimatisation salinity only on the average number of cercariae per snail per week (square root transformed; including all salinity levels). Due to snail mortality, only snails that survived the entire duration of the experiment were included in the analyses.

Survival of cercariae. Survival and activity of cercariae at different salinities were compared between cercariae from infected snails that were acclimatised to these salinities for approx. four weeks prior to the experiment. A cercarial mixture was obtained for the different salinities using 25 snails per salinity. Approximately 25 cercariae were then transferred into wells of 96-well plates and incubated under constant illumination at 25°C (12 wells in two replicate well plates per salinity; volume of water in wells was standardised to 150 µl). Survival and activity of the cercariae were checked at 3, 5, 7, 9, 12 and 14 h post-emergence. Cercariae were classified as fully active, sluggishly motile or immotile/dead. The data were analysed using a repeated measures ANOVA to determine the effect of salinity on the proportions of fully active cercariae 3, 5, 6, and 9 h post-emergence (arcsine-square root transformed). Due to the assumption of sphericity being violated (Mauchley's test), multivariate results for within-subjects are reported.

Infectivity of cercariae. Infectivity was assessed by comparing the success of cercariae from snails acclimatised to different salinities for approx. six weeks at infecting second intermediate amphipod hosts (*Paracalliope novizealandiae*). For these experimental infections, uninfected amphipods (n = 48 per salinity) were first transferred into plastic containers with water of the respective salinity (approx. 300 ml of the respective solution), and then individually put in wells of two replicate well plates filled with 75 µl of water of the respective salinity. A cercarial mixture was obtained from snails kept at the different salinities which was added to each well (at 25 psu, 50 µl, at 30, 35 and 40 psu, 75 µl were added corresponding to approx. 20 cercariae (± 1.09, 1.02, 0.89, 0.50 (± SE) at 25, 30, 35 and 40 psu); all added volumes then standardised to 75 µl). Amphipods and cercariae were then

incubated for 2 h at 25°C under constant illumination. Subsequently, amphipods were placed in two replicate plastic containers (approx. 300 ml natural aerated seawater) per treatment and left for 2 d at 16°C until dissected. The number of parasites infecting the amphipods was assessed. Effects of salinity, sex and size of amphipods on the proportion of parasites successfully infecting amphipods was analysed with a Generalised Linear Model (GLM) fitted with a quasi-binomial error structure.

Susceptibility of amphipods to infection. Susceptibility of amphipod hosts to infection was investigated by exposing amphipods to different salinities for 24 h before adding untreated cercariae, i.e. cercariae derived from stock snails kept in natural seawater, and then comparing the infection success of these cercariae. After the 24 h exposure of amphipods to different salinities in plastic containers filled with 300 ml, amphipods (n = 37 per treatment in two replicate groups per salinity) were transferred into wells containing 75 µl of water of the respective salinity. From a cercarial mixture (generated from 40 snails kept in stock aquaria), 25 µl was added to each amphipod, which corresponded to an addition of 20 ± 1.19 cercariae per amphipod. Well plates were then incubated under constant illumination for 2 h at 25°C and were subsequently processed and the data analysed as described for the infectivity experiment.

Survival of infected and uninfected amphipods and development of parasites within amphipod hosts. Infected and uninfected amphipods were exposed to different salinities and their survival under these conditions was monitored. For this, uninfected amphipods were placed individually in wells filled with 75 µl natural aerated seawater (n = 90 amphipods in two replicate well plates per salinity). A cercarial mixture was prepared using 40 snails kept in stock aquaria. Of this mixture, 75 µl was added to half of the amphipods (corresponding to an addition of approx. 15 cercariae per amphipod) while the same volume of pure seawater was added to the controls. Well plates were incubated for 4 h at 20°C under constant illumination. Amphipods were then transferred into plastic containers filled with aerated seawater (approx. 300 ml) and stored overnight at 16°C to allow the cercariae to fully penetrate the hosts. The following day, amphipods were distributed to two replicate aquaria per salinity (volume 6.5 l, half filled; aerated; at 19 ± 1°C). Survival of the amphipods was subsequently checked 2 - 3 times a day for 30 d. Dead amphipods were recovered and dissected to assess their infection status, infection intensity (number of metacercariae per amphipod) and the developmental stage of the parasites (early immature metacercariae, late

immature metacercariae, early cyst, mature cyst, according to Keeney *et al.*, 2007b). After 30 d, all remaining amphipods were dissected. The risk of dying during the experiment was analysed with a Cox proportional hazard regression model with salinity, sex, size and infection status of amphipods as predictor variables. A Spearman's Rank correlation was used to assess the effect of infection intensity on the survival time of infected amphipods in the experiment.

4.4 Results

Cercarial output from snails

Salinity had a significant effect on the average number of cercariae that emerged from infected snails (square root transformed; GLM, $F_{3, 38} = 7.99$, $p < 0.001$), with output of cercariae increasing with increasing salinity (Fig. 4.1). The simulated salinity increase that half of the snails were exposed to in the 25, 30 and 35 psu treatments did not have a significant effect on cercarial output (square root transformed; GLM, $F_{1, 30} = 2.36$, $p = 0.135$). The average output per snail and per incubation ranged from 15.9 ± 4.6 at 25 psu to 111.6 ± 24.6 at 40 psu (mean ± standard error, as for all subsequent results). Shell length of snails used was not significantly different between treatments (average shell length 12.8 ± 0.10 mm; ANOVA, $F_{6, 81} = 0.43$, $p = 0.86$).

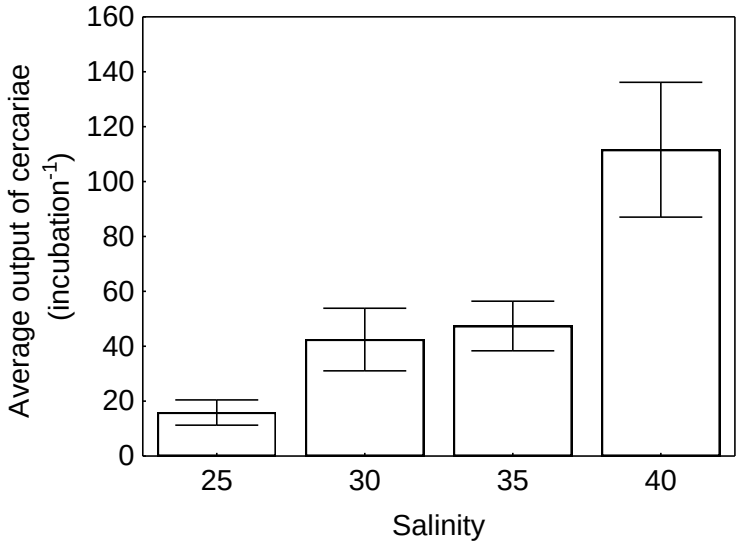


Figure 4.1 Effect of salinity (25, 30, 35 and 40 psu) on the mean (± SE) output of cercarial transmission stages of *Maritrema novaezealandensis* from infected *Zeacumantus subcarinatus* snails per incubation (6 weekly incubations; n = 27, 22, 25 and 14 at 25, 30, 35 and 40 psu).

Cercarial survival

The salinity at which the cercariae were incubated, time and their interaction all had a significant effect on the proportion of fully active cercariae (arcsine-square root transformed, Table 4.1). Full activity of cercariae ceased within about 9 h at all salinities except at 40 psu (incubation temperature: 25°C). Overall, the lower the salinity, the faster full activity decreased (Fig. 4.2).

Table 4.1 Effects of salinity (25, 30, 35 and 40 psu) on the activity of cercariae of *Maritrema novaezealandensis* (proportion of fully active cercariae; arcsine-square root transformed; n = 12 replicates per salinity). Results from a repeated measures ANOVA (with multivariate within-subjects results).

Factor	**df**	**MS**	**F**	**p**
Between subjects				
Salinity	3	0.48	11.74	< 0.001
Error	44	0.04		
Within subjects				
Time	3	17.43	1120.86	< 0.001
Time x salinity	9	0.42	16.66	< 0.001
Error	132	0.01		

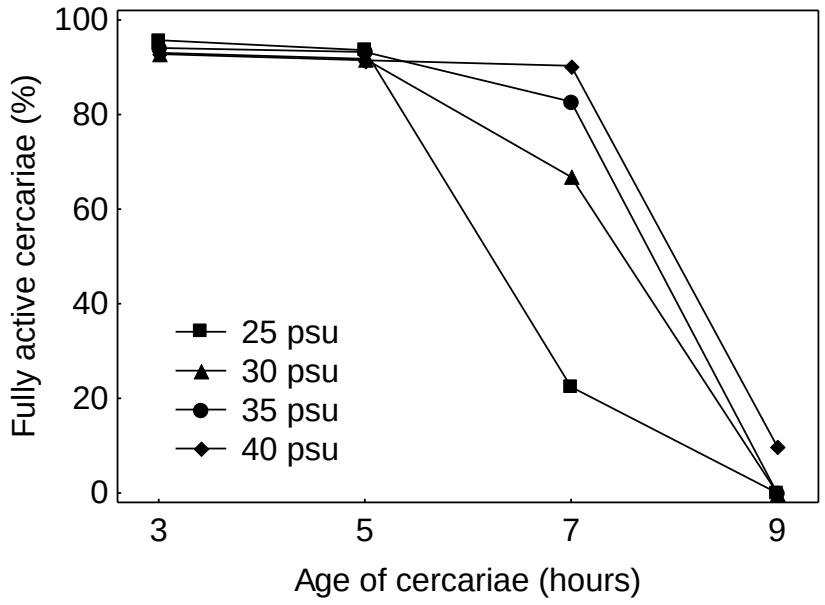


Figure 4.2 Survival of the cercariae of *Maritrema novaezealandensis* at different salinities (25, 30, 35 and 40 psu) shown as the percentage of fully active cercariae at the average age of 3, 5, 7 and 9 h.

Infectivity of cercariae

Salinity had a significant effect on the proportion of parasites successfully infecting amphipod hosts (GLM, quasi-binomial; $F_{3, 184} = 3.47$, $p = 0.017$). The proportion of successful parasites was highest at 25 psu, but was due to the presence of outliers in one replicate (0.34 ± 0.06). The proportion in the other replicate (0.10 ± 0.02) was similar to those in all other treatments (0.14 ± 0.02 at 30 psu, 0.13 ± 0.02 at 35 psu and 0.13 ± 0.02 at 40 psu). If the analysis is repeated after the exclusion of three outliers with unusually high infection levels, there is no significant effect of salinity on the proportion of cercariae successfully infecting amphipods ($F_{3, 181} = 0.93$, $p = 0.426$). Overall, 81% of the amphipods were infected with a mean infection intensity of 2.8 ± 0.2 parasites per amphipod. Sex of amphipods did not significantly affect the proportion of successful parasites ($F_{1, 184} = 0.33$, $p = 0.564$), whereas size of amphipods did with smaller size classes being infected by a larger proportion than larger size classes ($F_{3, 184} = 3.02$, $p = 0.031$) (0.17 ± 0.03 (n = 53), 0.18 ± 0.02 (n = 84), 0.01 ± 0.01 (n = 41) and 0.06 ± 0.01 (n = 14) parasites per amphipod for the 3.0, 3.5, 4.0, 4.5 ± 0.25 mm size classes).

Susceptibility of amphipods

Salinity did not significantly affect the proportion of parasites that successfully infected amphipods that had previously been exposed to different salinities (GLM, quasi-binomial; $F_{3, 139} = 0.69$, $p = 0.562$). Overall, 92% (25 psu), 95% (30 psu), 78% (35 psu) and 87% (40 psu) of the amphipods got infected (mean infection intensity across all amphipods 2.6 ± 0.2; n = 148). Sex of amphipods did not significantly influence the proportion of successful parasites ($F_{1, 139} = 0.39$, $p = 0.534$), whereas size did ($F_{4, 139} = 2.54$, $p = 0.043$). The smallest amphipods (2.5 ± 0.25 mm) had the highest proportion (0.19 ± 0.05; n = 9) and the largest had the lowest (0.05 ± 0.02, n = 5), while intermediate size classes were infected by very similar proportions of successful parasites (0.13 ± 0.01 (n = 44), 0.13 ± 0.01 (n = 65), 0.11 ± 0.02 (n = 25), respectively for the 3.0, 3.5, and 4.0 ± 0.25 mm size classes).

Survival of infected and uninfected amphipods

The risk of amphipods dying was significantly affected by salinity, with a higher risk at 35 and 40 psu (model: $\chi^2 = 59.88$, df = 4, $p < 0.001$; Table 4.2). Survival in the experiment ranged from 10.7 ± 0.8 d at 25 psu (n = 79) and 10.8 ± 0.7 d at 30 psu (n = 77), to 8.5 ± 0.5 d at 35 psu (n = 81) and 9.4 ± 0.5 d at 40 psu (n = 84). While size of amphipods also significantly affected the risk of dying with larger ones having a higher proportional risk than

smaller ones, sex and infection status of amphipods did not (Table 4.2). There was a weak positive correlation between the intensity of infection and survival time of infected amphipods (Spearman's $\rho = 0.15$, $p = 0.01$), indicating that more heavily infected amphipods survived slightly longer than lightly infected ones. In total, 345 amphipods were dissected (180 females, 158 males, 7 unsexed) of which 44% were infected with a mean infection intensity of 5.9 ± 0.4 parasites per infected amphipod.

Table 4.2 Effect of salinity, sex, size and infection status of amphipods on the mortality risk. Results from a Cox proportional hazard model.

Factor	**Cox's parameter**	**SE**	**p**
Salinity	0.02	0.01	0.037
Amphipod sex	-0.13	0.15	0.391
Amphipod size	0.53	0.09	< 0.001
Infection status	-0.01	0.11	0.939

Parasite development

The salinity at which the amphipods were kept affected the amphipod's survival and concomitantly the parasite's development. At 25 and 30 psu, amphipod survival was longer and parasite development progressed more. A substantial number of parasites were recovered as mature cysts at 30 psu (23%, n = 230) compared to 0% at 25 and 40 psu (n = 181 and 214, respectively) and 1% at 35 psu (n = 218). At 25 psu, 22% of the parasites at least reached the early cyst stage, whereas at 35 and 40 psu, over 90% of the metacercariae recovered were still immature. Overall, 863 metacercariae were counted (47% early immature metacercariae, 39% late immature, 8% early cysts and 7% mature cysts).

4.5 Discussion

Salinity has been shown to be an influential environmental factor for parasitism and diseases in brackish and estuarine ecosystems (e.g. Haskin & Ford, 1982; Thieltges *et al.*, 2010; Zander, 1998). Salinity tolerance of parasites and their hosts can vary greatly and can therefore influence the interaction between them. For example, the distribution of marine endoparasites can be limited by the salinity tolerance of hosts as certain marine parasites have been suggested to have a higher tolerance than their hosts (Möller, 1978). However, it has

also been reported that the salinity tolerance of parasite larvae can constrain parasite incidence in hosts that tolerate a greater range of salinities than their parasites (e.g. Haskin & Ford, 1982; Reisser & Forward, 1991). In our study, salinity also differentially affected the parasite *M. novaezealandensis* and its second intermediate amphipod host. While output of cercariae from snail hosts and their survival was highest at normal to increased salinities, survival of amphipod hosts was most prolonged at lower salinities.

Effects on the parasite

Output of cercariae from infected snails was significantly affected by salinity, with increasing numbers of cercariae emerging with increasing salinity. This finding is in accordance with most previous studies and also indicates a roughly two-fold increase in cercarial emergence with an increase of about 10 psu (Mouritsen, 2002; Rees, 1948; Sindermann & Farrin, 1962; Sindermann, 1960). The main transmission window of *M. novaezealandensis* is thought to be during low tides on warm sunny days when the water in tide pools warms up (Fredensborg *et al.*, 2004b; Koprivnikar & Poulin, 2009; Studer *et al.*, 2010), conditions implying normal to elevated salinity levels due to evaporation. Although the effect of the short-term salinity increase in our experiments (incubation salinity equal or greater than acclimatisation salinity) was not significant, the long-term response to the different salinities strongly indicated that cercarial production and emergence are highest at normal to increased salinities. As for temperature, this can be interpreted as an optimal transmission strategy, whereby the release of cercariae is timed to conditions during which the chance of transmission to the next host is maximised (Mouritsen, 2002).

However, our finding differs from the results described by Koprivnikar & Poulin (2009) for the same parasite species (*M. novaezealandensis*) and snail host (*Z. subcarinatus*). One possible explanation for this discrepancy is that short-term responses to salinity are different from long-term responses (snails exposed to different salinity levels for days in Koprivnikar & Poulin (2009), and for weeks in the present study). In a preliminary experiment assessing cercarial emergence of *M. novaezaealandensis* at different salinities without previous acclimatisation, salinity also had a significant effect on cercarial emergence but, in accordance with Koprivnikar & Poulin (2009), the lowest number emerged at the highest salinity (A. Studer, unpubl. data). This clearly emphasizes the need to consider and distinguish between short and long-term experimental responses. Long-term exposure to different salinities may lead to changes in snail physiology in turn affecting the response of

the parasite once the host has acclimatised to new conditions, which may take a few days (Berger & Kharazova, 1997).

Survival of cercariae also increased with increasing salinity, suggesting that conditions considered to be optimal for the transmission of this parasite species are not only promoting larger numbers of cercariae being released into the environment, but also prolonging their survival. This would further increase the chance of successful transmission. Shorter survival at low salinities might be an issue during heavy rainfall at low tide during summer, or for infected snails located close to a freshwater inflow, and may be indicative of low salinity being a barrier for the distribution of this trematode. This finding contrasts with studies which did not report an effect of salinity on the longevity of *Maritrema subdolum* and other marine cercariae (Mouritsen, 2002; Prokofiev, 1999; Rees, 1948; Stunkard & Shaw, 1931) (but see Lei & Poulin, 2011).

Considering the main transmission window for *M. novaezealandensis*, an increase in infectivity with increasing salinity was expected. Excluding the unusual values from one replicate at 25 psu, our results suggested that long-term acclimatisation of infected snails to different salinities did not influence the functionality of the cercariae they release. Infectivity of cercariae has been shown to increase with increasing cercarial density up to a threshold before dropping off (Evans & Gordon, 1983). Based on the results described above for cercarial production and output, this should have translated into increasing infectivity with increasing salinity and it remains unclear why this was not observed.

Effects on the host

Effects of salinity on amphipod hosts differed from the effects described for the parasite. Although susceptibility of *P. novizealandiae* amphipods to infections was not significantly affected, possibly because they had ample time to adjust to the respective osmotic conditions, their survival was. In contrast to patterns observed for the parasite, amphipods survived longer at lower than at normal to increased salinities. This may be consistent to some extent with studies on adult littoral amphipods which have shown that these organisms tolerate a relatively wide range of salinities (e.g. Dorgelo, 1974, 1976; McLusky, 1971), and the fact that crustaceans living in habitats of changing salinities possess tolerance mechanisms to cope with varying osmotic conditions (Pequeux, 1995). This result

may also suggest that these amphipods are also capable of living in more estuarine ecosystems.

However, the longer survival of amphipods at lower salinities also allowed parasite development to progress further. The difference in the percentages of mature cysts at low salinities clearly illustrated the importance of indirect effects of environmental factors. Such an effect was also observed for temperature (Studer *et al.*, 2010). The presence of mature cysts in the ecosystem is crucial for transmission, as only mature cysts can successfully establish in a definitive bird host. In addition, the infectivity and susceptibility experiments also indicated that smaller amphipods were getting more infected than larger ones. Although such an effect of amphipod size has not been found in any previous study (Fredensborg *et al.*, 2004b; Studer *et al.*, 2010), this may indicate that differences in osmotic regulatory mechanisms exist in amphipods of different size classes, which can lower the resistance of smaller individuals to infections.

Net effects

Assuming no other major ecological factor influences the inferences made, the net effects of salinity on the transmission process of *M. novaezealandiae* from first to second intermediate host can be summarised as follows. At low salinities (25 and 30 psu) relatively few cercariae emerge from infected snails and their survival is relatively short. While susceptibility of amphipods to infection is equal across all salinities, the survival of the amphipods is comparatively long at low salinities and the development of the parasite within infected amphipods thus positively influenced. Such conditions would be encountered during periods of rain or in close proximity to freshwater inflows and would benefit the amphipod hosts, but less so the parasite. Incorporating predicted environmental changes due to climate change (IPCC, 2007), altered rainfall patterns (e.g. increased regional rainfall) as well as a long-term, large scale freshening of the oceans due to melting of ice, may negatively influence the transmission of *M. novaezealandensis* and therefore the continuation of the life cycle at least in some areas.

In contrast, at normal to elevated salinities (35 and 40 psu) which would be encountered during periods considered optimal for transmission, more cercariae are being produced and emerge from snail hosts and their survival is better compared to low salinities. Higher transmission to amphipod hosts under these conditions is likely. The survival of amphipods is

also directly negatively affected and the increased transmission of the parasite may further increase their risk of mortality. During more frequent and/or more extreme heat waves or in areas that are predicted to become more arid (IPCC, 2007), evaporation may become more pronounced in tide pools and high salinity levels and high temperatures may occur over longer low tide series. These conditions would be favourable for parasite transmission but not for the amphipods.

However, it needs to be stressed that fluctuations in intertidal habitats are highly complex as multiple environmental factors vary simultaneously. For all results described (from experiments conducted at constant temperatures and salinities) and all inferences made, actual effects under natural conditions may be different. For example, interactive effects of salinity and temperature in particular are highly likely, and temperature may further enhance or reduce any effects of salinity on the parasite and/or the host.

In conclusion, salinity had differential effects on the various steps of the transmission process of *M. novaezealandensis*. Overall, high salinity levels benefited the transmission of the parasite in terms of output and survival of cercariae, whereas low salinities mostly benefited the amphipods but also had an indirect positive effect on parasite development within amphipod hosts. However, during the main transmission window of *M. novaezealandensis*, thermal and osmotic conditions seem optimal for the parasite but may entail direct and indirect negative effects on amphipods. Hence, not only temperature, but also salinity should be considered a potential key regulator of the transmission dynamics of *M. novaezealandensis* in nature.

CHAPTER FIVE

Effects of ultraviolet radiation on the transmission process of an intertidal trematode parasite

5.1 Abstract

The transmission process of parasites takes place under exposure to a range of fluctuating environmental factors. One of these factors is solar radiation including prevailing levels of ultraviolet radiation (UVR) (280 - 400 nm). Here, we investigated the effects of ecologically relevant levels of UVR on the transmission process of the intertidal trematode parasite *Maritrema novaezealandensis* from its first intermediate snail host (*Zeacumantus subcarinatus*) to its second intermediate amphipod host (*Paracalliope novizealandiae*). This included an assessment of the output of parasite transmission stages (cercariae) from infected snail hosts, the survival and infectivity of cercariae, the susceptibility of amphipod hosts to infection (laboratory experiments) and the survival of infected and uninfected amphipod hosts (outdoor experiment) when exposed to photosynthetically active radiation only (PAR, 400 - 700 nm; no UV), PAR+UVA (320 - 700 nm) or PAR+UVA+UVB (280 - 700 nm). Survival of cercariae and the susceptibility of amphipod hosts to infection were the steps of the transmission process that emerged as being significantly affected by UVR. Survival of cercariae decreased strongly in a dose-dependent manner, while susceptibility of amphipods increased after exposure to UVR for a prolonged period. Exposure to solar radiation and in particular UVR is thus negatively affecting both, the parasite as well as its amphipod host, and should therefore be considered an influential component in parasite transmission and host-parasite interactions in intertidal ecosystems.

5.2 Introduction

Solar ultraviolet radiation (UVR) (wavelengths; UVB: 280 - 320, UVA: 320 - 400 nm) has always been a strong selective force in aquatic communities (Hansson & Hylander, 2009; Sommaruga, 2003; Williamson *et al.*, 2001). As an integral part of a complex array of fluctuating environmental factors in these ecosystems, UVR varies considerably in time (e.g. seasonal) and space (e.g. latitude, water depth; Tedetti & Sempere, 2006). Although life on earth has evolved in the presence of UVR, recent stratospheric depletion in ozone has altered the selective pressure that UVR, especially UVB, may exert (Lesser & Barry, 2003 and references therein). Moreover, on-going and predicted climate changes are expected to further exacerbate exposure of organisms to UVB in aquatic ecosystems (Haeder *et al.*, 2007).

UVR, especially UVB, is predominantly known for its potentially deleterious effects, damaging biological macromolecules and cellular structures including enzymes, membranes, DNA and RNA (e.g. Dahms & Lee, 2010; Day & Neale, 2002; Haeder *et al.*, 1998; Vincent & Neale, 2000) (see also Paul & Gwynn-Jones, 2003). Ecologically relevant effects at the whole-organism level include decreases in fecundity, growth, development, mobility and survival rate, all of which can translate into changes in species composition at the community and ecosystem level (e.g. Bancroft *et al.*, 2007; Bothwell *et al.*, 1994; Haeder *et al.*, 1998; Hansson & Hylander, 2009). As a consequence, organisms have evolved a range of behavioural (e.g. migration), physiological (e.g. accumulation of UV absorbing compounds) and molecular mechanisms (e.g. DNA damage repair), to avoid, minimise or repair damage induced by exposure to UVR (Dahms & Lee, 2010 and references therein; Hansson & Hylander, 2009; Roy, 2000; Sinha & Haeder, 2002).

Biological and ecological effects of UVR have been well studied in aquatic systems (e.g. Bancroft *et al.*, 2007; de Mora *et al.*, 2000; Haeder *et al.*, 1998; Hansson & Hylander, 2009; Helbling & Zagarese, 2003). Organisms vary considerably in their susceptibility to UVR (e.g. Bancroft *et al.*, 2007). Hence, it is very likely that at least some of their interactions with other organisms would be influenced by UVR (see examples in Paul & Gwynn-Jones, 2003; Sommaruga, 2003). However, studies about the effects of UVR on aquatic organisms have largely ignored species interactions, particularly those between parasites and their hosts. Despite the fact that parasitism is known to be of high ecological relevance (e.g. Hudson *et al.*, 1998; Lafferty *et al.*, 2006; Mouritsen & Poulin, 2010), knowledge on the effects of UVR

on parasites and diseases in aquatic systems has remained very limited (see Sommaruga, 2003).

The sensitivity of a range of parasites from aquatic environments to UVR, however, has been acknowledged (see Marcogliese, 2001 and references therein). UVR is expected to be particularly problematic in clear, shallow waters and to parasites with small, delicate free-living stages that are extremely susceptible to environmental conditions (MacKenzie *et al.*, 1995). Sub-lethal and lethal effects of UVR on transmission stages of important human parasites (e.g. *Schistosoma* spp., *Cryptosporidium parvum*) have been investigated using artificial lights (Ariyo & Oyerinde, 1990; Connelly *et al.*, 2007; Ghandour & Webbe, 1975; Prah & James, 1977; Ruelas *et al.*, 2006, 2007, 2009; Standen & Fuller, 1959). Many of the older studies fail to specify doses but their irradiation times and the distances from lamps to the bench/unit irradiated indicate that relatively high doses were administered, including exposure to high energy UVC radiation (< 280 nm) which does not reach the surface of the earth and hence does not affect organisms under natural conditions (Madronich *et al.*, 1998). Despite these limitations, results from these studies consistently confirm strong negative effects of UVR on survival as well as infectivity of parasites.

However, most of these studies generally do not consider ecologically relevant interactions between a parasite and its host(s). Using a more inclusive approach, Ruelas *et al.* (2006, 2007, 2009) investigated the effects of UVR on the transmission process of *Schistosoma mansoni* as well as the parasite's aquatic snail host (*Biomphalaria glabrata*). Their results indicated that not only the parasite, but also juvenile and infected snails were vulnerable to UVR exposure, but that both, snail and parasite were also able to repair UV-induced DNA damage. This clearly highlights the importance of direct and indirect effects of UVR on the interaction between parasites and hosts, suggesting that the overall effect of UVR on a particular system may be highly complex and thus difficult to predict. This complexity between environmental conditions, UVR, parasites and hosts is best illustrated by the example of *Saprolegnia*-associated mortality in amphibians (Sommaruga, 2003). Outbreaks of this oomycete have been associated with large scale climatic fluctuations and possibly ozone depletion: amphibian embryos developing in water bodies are exposed to different levels of UVR depending on the extent of precipitation and hence prevailing water levels. This can influence not only their survival, but also their susceptibility to infection (Kiesecker & Blaustein, 1995; Kiesecker *et al.*, 2001).

Although many aquatic organisms inhabit high UVR environments, the role of this environmental factor in patterns of diseases and parasitism has only rarely been considered. For example, UVR effects may be particularly relevant in intertidal ecosystems, where conditions for organisms at low tide are almost equivalent to atmospheric conditions (Karentz, 2001; Kramer, 1990), and even more so for those in the Southern hemisphere where incident UVR levels are greatest (McKenzie *et al.*, 1999; Seckmeyer & McKenzie, 1992).

In intertidal ecosystems, trematode parasites are ubiquitous and highly important ecological components (e.g. Lauckner, 1984; Mouritsen & Poulin, 2002b, 2010; Sousa, 1991). In the present study, the transmission process of the intertidal trematode *Maritrema novaezealandensis* was examined. Like most trematodes, *M. novaezealandensis* has a complex life cycle involving several members of an intertidal community (Martorelli *et al.*, 2004). The species in this life cycle are all specific to New Zealand and hence are exposed to the particularly high UVB levels occurring in this region compared to Northern hemisphere regions of the same latitude (McKenzie *et al.*, 1999; Seckmeyer & McKenzie, 1992), and to the transient high levels of UVB during the period of the 'ozone hole' over the Antarctic (McKenzie *et al.*, 2007; McKenzie *et al.*, 2003).

The different stages of the parasite throughout a trematode's life cycle experience vastly different conditions in terms of the solar radiation regimes to which they are exposed. *M. novaezealandensis*, for example, lives as an adult worm in the intestine of mudflat affiliated birds (environment with no direct solar irradiation). Eggs produced pass out with the bird's faeces and persist in the environment until they may get ingested by a first intermediate snail host (*Zeacumantus subcarinatus*) foraging on mudflats. Within a snail's opaque shell, a miracidium hatches from an egg and develops into a sporocyst, probably shielded from solar irradiance. Within the sporocysts, asexual reproduction occurs and large numbers of larval transmission stages (cercariae) are produced, which emerge from an infected snail under optimal conditions in order to infect a second intermediate crustacean host. Cercariae are small (approx. 170 - 200 µm including tail, Koehler & Poulin, 2010; Martorelli *et al.*, 2004), short-lived (< 24 h), non-feeding transmission stages and in the case of *M. novaezealandensis*, they are also translucent. These cercariae are directly exposed to and influenced by environmental conditions (Chapter Three and Four). After infecting a second intermediate crustacean host, such as the amphipod *Paracalliope novizealandiae* used in this study, the parasite experiences different levels of exposure to solar radiation depending on the

opaqueness or transparency of the carapace of its crustacean host. Within a crustacean, the parasite develops into an encysted stage (metacercaria). The life cycle is completed when a crustacean harbouring a mature metacercaria is ingested by a definitive bird host and successfully establishes in its intestine.

In this study, we investigated the effects of ecologically relevant levels of UVR on the transmission process of *M. novaezealandensis* from its first intermediate snail host *(Z. subcarinatus)* to its second intermediate amphipod host *(P. novizealandiae).* In laboratory and outdoor experiments, the effects of UVR on (1) the emergence of cercariae from snail hosts, (2) the survival of cercariae, (3) the infectivity of cercariae, (4) the susceptibility of the amphipod host to infection, and (5) the survival of infected and uninfected amphipods were assessed (for a conceptual figure summarising these steps see Chapter Three). Our aim was to identify steps in this transmission process that are particularly sensitive to UVR, and to evaluate overall net effects of this environmental factor on the transmission of *M. novaezealandensis* from its first to its second intermediate host.

5.3 Materials and Methods

Parasite and host material. First intermediate snail hosts (*Z. subcarinatus*) were collected from a high prevalence site (Lower Portobello Bay, Otago Harbour) and screened for infections with *M. novaezealandensis* as described in Studer *et al.* (2010). For all experimental infections, uninfected second intermediate amphipod hosts (*P. novizealandiae*) were collected from Hooper's Inlet (Otago Peninsula) several days prior to an experiment in order to allow for acclimatisation to laboratory conditions. At Hooper's Inlet, first intermediate snail hosts are absent and therefore, amphipods are not infected naturally (neither by *M. novaezealandensis* nor by any other trematode metacercariae) (Chapter Two; Bryan-Walker *et al.*, 2007; Fredensborg *et al.*, 2004b; Koehler & Poulin, 2010). After their use in experiments (see below), amphipods were stored in small plastic containers containing 300 ml of aerated seawater and a strip of sea lettuce (*Ulva* sp.) until measured (size classes: 2.5, 3.0, 3.5, 4.0, 4.5 ± 0.25 mm), sexed and dissected under a dissecting microscope to assess the number of parasites present (infection status and intensity). All experimental infections as well as the cercarial survival experiment were conducted using 96-well plates (wells 7 x 10

mm; total volume 320 µl). For all experimental infections, a cercarial mixture from 40 infected snails was obtained (see details in Studer *et al.*, 2010).

Experimental design and laboratory set-up. All experiments except the amphipod survival experiment (see details below) were conducted as 3 x 2 designs including three UV treatments (i.e. exposure to photosynthetically active radiation (PAR) only (no UV; 400 - 700 nm), PAR+UVA (UVA; 320 - 700 nm) or PAR+UVA+UVB (UVA+B; 280 - 700 nm)) and two exposure durations (i.e. doses). UV treatments were achieved using Plexiglas filters transmitting PAR (81%), only minimal UVA (5.2%) and no UVB (0.0%) (no UV treatment); PAR (77.9%), UVA (46.5%), and only minimal UVB (0.1%) (UVA treatment); PAR (84.5%), UVA (84.6%) and UVB (80.6%) (UVA+B treatment) (see Lister *et al.*, 2010b or Appendix for transmission profiles of filters).

The laboratory set-up included one UVB (Atlas 07-29006), two UVA (Dr. Kern® excellent RA 80W), and three full spectrum light tubes (Phillips Alto TLD 58W/840), which were suspended 45 cm above the bench top and which were wrapped in cellulose acetate to absorb any UVC radiation being emitted. The spectral output of the lamps was measured with a LiCor Li-1800UW spectroradiometer (Table 5.1, Figure 5.1). Data given in Table 5.1 do not include reductions by filters and only provide lamp output irradiances from 300 - 700 nm. Compared to ambient local levels of solar irradiance during midday peaks in summer, maximum irradiances from our laboratory set-up were relatively low for UVB, UVA and PAR (Table 5.1). All doses administered in each experiment can be calculated based on the dose per hour given in Table 5.1. All low dose exposures (shorter exposures) were administered during the second half of the high dose treatments (longer exposure) and all laboratory experiments were conducted at 20°C. Statistical analyses are described in each of the following subsections. We checked for differences between replicates and transformed data where necessary to ensure the assumptions of the statistical tests were met.

Table 5.1 Irradiances in the laboratory set-up: dose per hour (kJ m^{-2}) and maximum irradiance (W m^{-2} s^{-1}) for UVB, UVA and PAR. Ultraviolet B radiation (UVB), 300 - 320 nm; ultraviolet A radiation (UVA), 320 - 400 nm; ultraviolet radiation (UVR), 300 - 400 nm; photosynthetically active radiation (PAR), 400 - 700 nm; ratio of ultraviolet B radiation to visible light (UVB:PAR). For comparison, ambient maximum irradiances measured locally. *Data from Lamare *et al.* (2007).

	Laboratory set-up		**Ambient***
	Dose per hour	**Max. irradiance**	**Max. irradiance**
	kJ m^{-2}	W m^{-2} s^{-1}	W m^{-2} s^{-1}
UVB (300 - 320 nm)	5.84	1.62	3.02
UVA (320 - 400 nm)	63.27	17.58	49.72
Total UVR (300 - 400 nm)	69.12	19.20	
PAR (400 - 700 nm)	185.09	51.41	892.9
UVB:PAR ratio		0.03	

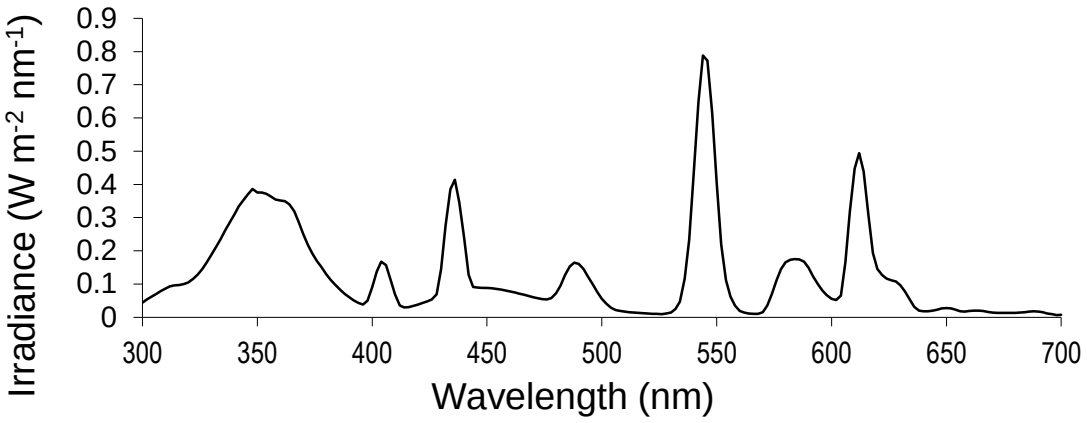


Figure 5.1 Spectral output (300 - 700 nm) of all light tubes combined in the laboratory set-up (W m^{-2} nm^{-1}).

Output of *M. novaezealandensis* cercariae from first intermediate snail hosts.

Output of cercariae of *M. novaezealandensis* from infected *Z. subcarinatus* snails was assessed as a short-term response by counting the number of cercariae shed from snail hosts during exposure to the different UV treatments at 20°C. There were two trials each with 16 replicate snails per treatment. For the exposures, snails were individually placed in 1.5 ml Eppendorf tubes filled with 1 ml of filtered seawater. Tubes were left open, covered with the respective filters and then exposed for 3 or 6 h. After incubation and removal of the snails, tubes were centrifuged (5 min, 20,817 g) and 900 µl of the seawater exchanged with 70%

ethanol to preserve the samples. The supernatant was checked for the presence of cercariae before disposal. Samples were counted under a dissecting microscope. A General Linear Model (GLM) was used to test for the effects of the trial, UV treatment, trial x UV treatment and dose on the number of emerged cercariae (log transformed).

Survival of cercariae. For this experiment, the survival and activity of cercariae was monitored when exposed to the different UV treatments and doses. At the start of the experiment, 40 µl from a cercarial mixture (average age of cercariae 30 min) were added to 24 replicate wells on two 96-well plates per treatment corresponding to an addition of approx. 45 cercariae per well. Cercariae were then exposed for either 2 or 4 h to one of the UV treatments. After exposure, well plates were placed under visible light (Phillips 40 W) for further monitoring. Survival and activity was categorised as fully active, sluggishly motile or immotile/dead. Cercariae were checked at an average age of 3, 5, 7, 9 and 12 h. A repeated measures ANOVA was used to test for the effect of the UV treatment and dose on the proportion of fully active cercariae (arcsine-square root transformed) at an average age of 3, 5, 7 and 9 h. Due to violation of the assumption of sphericity, multivariate results for within subject effects are reported. Additionally, LD_{50} values were calculated to assess the time in each treatment for 50% of cercariae to lose their full functional activity. This was done by fitting logistic functions to the proportion of fully active cercariae data of each treatment and dose and then calculating the inflection point.

Infectivity of cercariae. Infectivity, i.e. the proportion of cercariae that successfully infects second intermediate amphipod hosts (*P. novizealandiae*), was assessed after exposure of cercariae to the UV treatments and doses. For this, 48 uninfected amphipods (≥ 2.5 mm body size) per treatment were put individually in wells on two 96-well plates containing approx. 75 µl of filtered seawater. A cercarial mixture was obtained which was divided into wells of 12-well plates (4 ml per well; two wells per treatment) and then exposed for either 30 min or 1 h to respective UV treatments. Based on aliquot counts prior to exposure, 50 µl of the irradiated cercarial mixtures were added to each amphipod which corresponded to an addition of approx. 25 ± 1.72 (± SE) cercariae per amphipod. Amphipods and irradiated cercariae were then incubated for 2 h at 25°C under constant illumination with visible light (Philips 40 W). After incubation, amphipods were transferred into plastic containers filled with seawater and dissected under a dissecting microscope 7 d after the experiment. A GLM was used to test for the effect of UV treatment, dose, UV x dose, as well as sex and size of

amphipods on the proportion of parasites successfully infecting amphipods (arcsine-square root transformed).

Susceptibility of amphipods to infections. Susceptibility of amphipod hosts was studied by exposing amphipods to the UV treatments before adding non-irradiated cercariae, and then comparing the infection success of the cercariae in these hosts. This experiment consisted of two separate trials. In the first trial, amphipods were exposed for 30 and 60 min. In the second trial, amphipods were exposed for 3 and 6 h. In each trial, 48 amphipods per treatment were transferred individually into wells of two 96-well plates per treatment (in approx. 75 µl natural seawater) prior to exposure. A cercarial mixture was prepared which was then added to individual amphipods at the end of the exposure period (first trial: 60 µl of the cercarial mixture corresponding to approx. 13 ± 0.72 cercariae per amphipod; second trial: 30 µl corresponding to approx. 20 cercariae per amphipod). Well plates containing irradiated amphipods and cercariae were then incubated for 2 h at 25°C under visible light. After incubation, amphipods were transferred into plastic containers and dissected 7 d later. The two trials were analysed separately using GLM's assessing the effect of the UV treatment, dose, UV x dose, as well as size and sex of amphipods on the proportion of cercariae (arcsine-square root transformed) successfully infecting the amphipods. In the second trial, this was followed by a Tukey's post hoc comparison between the UV treatments.

Survival of infected and uninfected amphipods. This experiment was conducted under ambient conditions and assessed the effect of UVR on the survival of uninfected and infected amphipods using the same filters as specified above (treatments: no UV, UVA, UVA+B). The experiment was conducted over a 24 d period at the Portobello Marine Laboratory, Otago Harbour, New Zealand in austral summer. UVA and UVB levels during the experiment were recorded at the laboratory's weather station (UVA SKU 420 and UVB SKU 430 sensors, Skye Instruments Ltd). Mean total daily doses during this period were 1938.67 ± 131.63 kJ m^{-2} of UVA and 113.98 ± 8.12 kJ m^{-2} of UVB, with maximum irradiances of 44.1 (UVA) and 2.83 W m^{-2} s^{-1} (UVB). For the experimental infections, 48 uninfected amphipods per replicate container (three containers per treatment) were individually put into wells of 96-well plates. A cercarial mixture was obtained, of which 50 µl were added to half of the amphipods (corresponding to an addition of approx. 28 cercariae per amphipod), whereas the same volume of filtered seawater was added to controls. Amphipods were then incubated for 2 h at 25°C under constant illumination with visible light. Infected

and uninfected amphipods were left in separate plastic containers (1 l; aerated seawater) overnight to allow the parasites to complete infection. Infected and uninfected amphipods (24 each per container, sexes matched) were then distributed into white opaque rectangular plastic containers (1 l) and covered with the respective filter. Containers were placed outdoors and received filtered flow through seawater. Survival of amphipods was subsequently monitored and container positions rotated twice a day. Dead amphipods recovered were measured, sexed and dissected under a dissecting microscope to assess the number of parasites present in each amphipod. After 24 d, all remaining amphipods were dissected. The risk of dying during the experimental period was analysed with a Cox proportional hazard regression model with UV treatment, sex, size and infection status of amphipods as predictor variables.

5.4 Results

Cercarial output from snails

There was substantial variability of cercarial output among individual snails (range 0 - 2738 cercariae per snail per incubation), with a mean output (± standard error as in all following results) of 152 ± 31 cercariae per snail (at 20°C). Thus, no clear pattern was found for the effect of the UV treatment or dose on the number of cercariae emerging from infected snails (log number of cercariae; GLM, UV treatment: $F_{2, 185} = 1.44$, $p = 0.239$; dose: $F_{1, 185} = 1.14$, $p = 0.287$). While there was also no significant difference between trials ($F_{1, 185} = 3.29$, $p = 0.072$), there was a significant interaction between trial and UV treatment ($F_{2, 185} = 3.45$, $p = 0.034$), indicating an inconsistency of the UVR effects between trials. Sizes of snails used did not differ between the trials or UV treatments (ANOVA, trials: $F_{1, 191} = 1.16$, $p = 0.284$; UV treatment: $F_{2, 191} = 1.18$, $p = 0.309$).

Cercarial survival

UVR negatively influenced the survival of cercariae in a dose-dependent manner. Mortality was highest for cercariae exposed to a high dose of both, UVA+B (all dead at an average age of 7 h), and lowest for the cercariae exposed to a low dose of no UV (23% still fully active at an average age of 9 h). Survival after administration of high doses was reduced in all cases when compared to the low dose (Fig. 5.2), even for exposure to visible light only. This effect was exacerbated when cercariae were exposed to UVR with survival under UVA+B < UVA. The repeated measures ANOVA carried out on the proportions of fully

active cercariae (arcsine-square root transformed) indicated a significant effect of all predictor variables and their interactions (between subjects; UV treatment: $F_{2, 138} = 301.76$; dose: $F_{1, 138} = 774.62$; UV x dose: $F_{2, 138} = 28.82$; within subjects; time: $F_{3, 414} = 3566.28$; time x UV: $F_{6, 414} = 93.68$; time x dose: $F_{3, 414} = 270.32$; time x UV x dose: $F_{6, 414} = 92.77$; for all $p < 0.001$). LD_{50} values calculated from these data revealed the following times at which 50% of cercariae lost their full functional activity: 8.1 h (low no UV dose), 6.3 h (high no UV dose), 7.6 h (low UVA), 5.7 h (high UVA), 6.5 h (low UVA+B) and 3.9 h (high UVA+B).

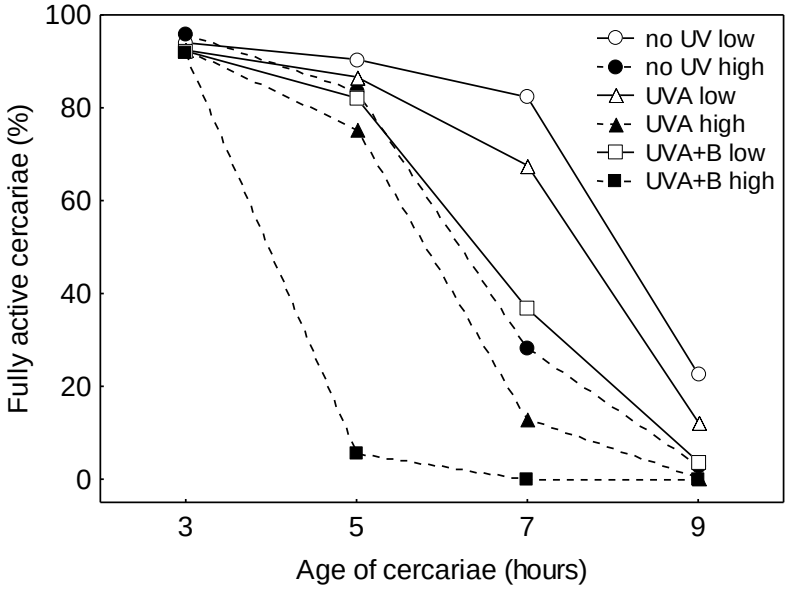


Figure 5.2 Survival (i.e. full activity as a percentage) of *Maritrema novaezealandensis* cercariae at an average age of 3, 5, 7 and 9 h post-emergence from infected snails, when exposed to either a low or a high dose of no UV, UVA or UVA+B radiation.

Infectivity

While UV treatment and dose did not have a significant effect on the proportion of parasites successfully infecting the amphipods (arcsine-square root transformed proportion of successful parasites, GLM; UV treatment: $F_{2, 253} = 2.65$, $p = 0.073$; dose: $F_{1, 253} = 0.13$, $p = 0.725$), their interaction did ($F_{2, 253} = 8.39$, $p < 0.001$). Results indicated that for cercariae exposed to no UV or only UVA, proportions of successful parasites were higher after a high dose than a low dose (no UV: 0.11 ± 0.02 (low) to 0.13 ± 0.02 (high); UVA: 0.08 ± 0.02 (low) to 0.13 ± 0.02 (high), whereas the proportions for cercariae exposed to UVA+B decreased (from 0.11 ± 0.01 (low) to 0.06 ± 0.01 (high)). While size of the amphipods did not have a significant effect, sex did, with males being more infected than females ($F_{1, 253} = 4.11$,

p = 0.044). A total of 264 amphipods were dissected of which 74% were infected. The mean number of parasites per infected amphipod was 1.6 ± 0.2, indicating only low levels of successful transmission overall.

Susceptibility of amphipods to infections

UVR influenced the susceptibility of amphipods in terms of the proportion of cercariae successfully infecting the amphipods, but only after several hours of exposure to UVR (Fig. 5.3). The pattern observed suggests that, after reaching a certain threshold of exposure to UVR, susceptibility of amphipods increases. After only relatively short exposures (30 and 60 min, trial one), UV treatment or dose did not have a significant effect on the susceptibility of amphipods (Fig. 5.3). In this trial, 94% of a total of 155 amphipods dissected were infected (average number of parasites per amphipod: 4.0 ± 0.2). After 3 and 6 h (trial two), there was a significant effect of the UV treatment, but not of any other factor tested (arcsine-square root transformed proportion of successful parasites, GLM; UV treatment: $F_{2, 165} = 6.84$, p = 0.001, dose: $F_{1, 165} = 1.01$, p = 0.316; UV x dose: $F_{2, 165} = 0.20$, p = 0.822; sex: $F_{1, 165} = 0.17$, p = 0.683, size: $F_{4, 165} = 0.42$, p = 0.794). The Tukey's post hoc test revealed a significant difference between the no UV treatment and the UVA and UVA+B treatments ($p < 0.05$), but not between the UVA and UVA+B treatments (Fig. 5.3). In total, 176 amphipods were dissected in this second trial of which 86.5% (no UV, n = 52), 96.3% (UVA, n = 54), 91.4% (UVA+B, n = 70) were infected. The average number of parasites per amphipods was 3.0 ± 0.4 (no UV), 5.5 ± 0.7 (UVA) and 4.9 ± 0.4 (UVA+B).

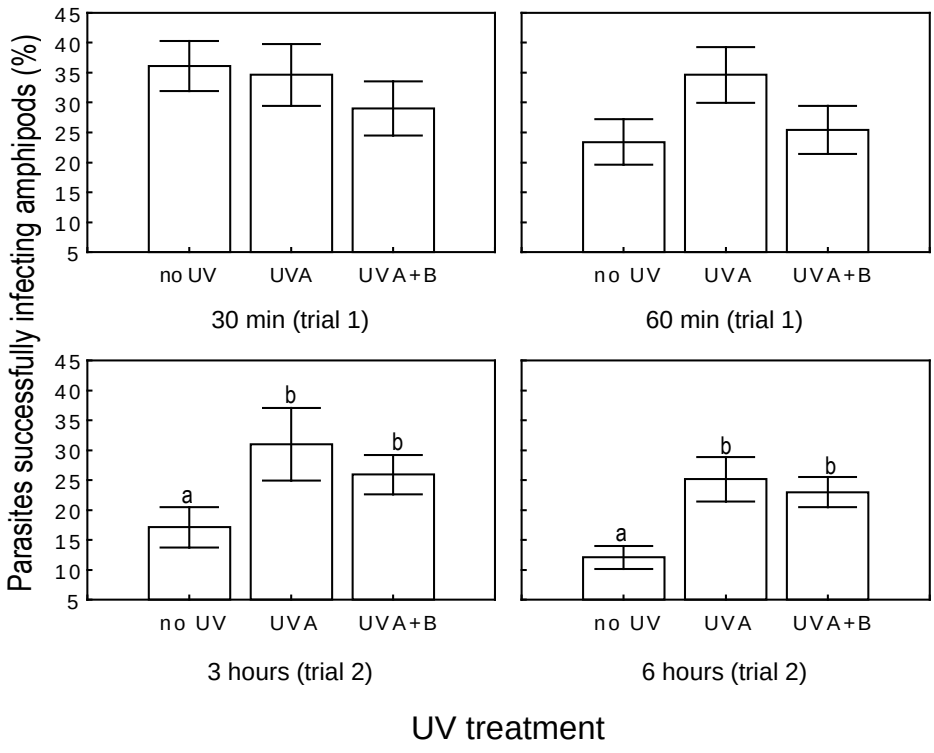


Figure 5.3 Susceptibility of *Paracalliope novizealandiae* amphipods to infection with *Maritrema novaezealandensis*, measured as the proportion of parasites successfully infecting the amphipods after a low or a high dose exposure of amphipods to no UV, UVA or UVA+B radiation (mean ± SE). Exposure times in trial one were 30 and 60 min. In trial two, a separate experiment, amphipods were exposed for 3 and 6 h. Trials are not directly comparable. The letters denote significant differences between treatments according to Tukey's post hoc test (significance level $p < 0.05$).

Amphipod survival

There was no significant difference in the risk of dying for amphipods exposed to different UV treatments during the 24 d outdoor experiment (Table 5.2; model $\chi^2 = 38.58$, df = 4, $p < 0.001$). In contrast, sex, size and infection status had significant effects with infected amphipods, male and larger amphipods having a higher risk of dying than uninfected ones, females or smaller size classes. There was a weak negative correlation between the number of days alive and the number of parasites present in infected amphipods (Spearman's $\rho = -0.21$, $p = 0.063$), suggesting that more heavily infected amphipods died slightly earlier than those with light infections. A total of 293 amphipods were dissected (139 females, 150 males, 4 unknown) of which 43% were infected (no UV: 38% (n = 116), UVA: 48% (n = 83), UVA+B: 44% (n = 88) with a mean infection intensity of 5.1 ± 0.4 parasites per infected amphipod.

Table 5.2 Results of the Cox proportional hazard model assessing the effects of the UV treatment (no UV, UVA, UVA+B), sex, size and infection status of *Paracalliope novizealandiae* amphipods on the risk of dying during the outdoor experiment.

Factor	**Cox's parameter**	**SE**	**p**
UV treatment	-0.13	0.10	0.177
Amphipod sex	-1.42	0.26	< 0.001
Amphipod size	-0.55	0.12	< 0.001
Infection status	0.44	0.16	0.006

5.5 Discussion

The transmission of parasites *in situ* is taking place under exposure to prevailing environmental conditions, including incident solar irradiance. These conditions can influence the parasite, but also the host, and may thus affect their interaction. In the present study, two steps of the transmission process investigated were affected by UVR: the survival of the cercarial transmission stages of *M. novaezealandensis* and the susceptibility of amphipod hosts to infection. This indicates that UVR should be considered an important ecological modulator of parasite transmission in natural systems.

Effects on the parasite

There was a strong negative dose-dependent effect of exposure to UVR on the survival of cercariae. This result is supported by an additional experiment run under ambient conditions on a warm sunny day which also showed a marked decline in survival and activity when comparing cercariae exposed to no UV, only UVA or UVA+B: after 2 h of exposure in 96-well plates, 97% (no UV), 84% (only UVA) and 14% (UVA+B) of cercariae were fully active, whereas after 3 h, only 61% (no UV), 4% (only UVA) and 0% (UVA+B) were still fully active (A. Studer, unpubl. data). In the laboratory experiment, even exposure to a high dose of no UVR (i.e. PAR only) reduced the survival of cercariae when compared to a low dose (Fig. 5.2). This suggests that once cercariae emerge into the environment, a phenomenon which does not itself seem to be conclusively influenced by UVR (see Results), one of the factors determining the life span of these cercariae will be the amount of solar radiation and particularly of UVR that prevails at that time. The high vulnerability and the reduced survival of *M. novaezealandensis* cercariae are consistent with other studies reporting an increased mortality of parasitic transmission stages after exposure to UVR (e.g. Ariyo & Oyerinde,

1990; Prah & James, 1977), and with the general notion of large, negative effects of UVR on the survival of a range of organisms (e.g. Bancroft *et al.*, 2007).

In contrast to the clear effect on cercarial survival, results regarding the output and infectivity of cercariae were less straightforward. The effect of UVR on cercarial output from snails may only become apparent after longer exposures, in the order of weeks or months; thus we can only conclude here that short-term exposure of the snail host had no clear immediate effect. Regarding infectivity, we expected to find a reduced capacity of cercariae to complete penetration of amphipod hosts after exposure to UVR, similar to what has been described for miracidia (parasite transmission stage hatching from eggs) of *Schistosoma* sp. These were shown to have an impaired host penetration capability due to the loss of activity after exposure to UVR, whereas the developmental potential of those that successfully infected a host was normal (Prah & James, 1977). Our findings indicated that exposure to a high dose of UVA+B result in lower proportions of cercariae capable of infecting amphipods when compared to cercariae exposed to high doses of no UV or UVA only. However, we have been unable to confirm the response observed in the present experiment in any subsequent trial conducted (A. Studer, unpubl. data) and thus no consistent effect of UVR has been found on the subtle functional aspect of infectivity of *M. novaezealandensis*. It is possible that UVR may not harm cercariae in their functionality until a certain threshold is reached, after which not just infectivity, but survival as such is impaired. However, different approaches may be needed to reach more conclusive results on these aspects of the transmission process.

Effects on the host

Susceptibility of *P. novizealandiae* amphipods to infection was increased after a prolonged exposure to UVR (Fig. 5.3) compared to prolonged exposure to no UV. A range of mechanisms could account for this observation. For example, exposure to UVR may exert immediate behavioural or physiological stress reactions that allow for easier location of the host and subsequent penetration by the cercariae. Alternatively, UVB has been shown to cause epidermal tissue damage in fish (e.g. Ewing *et al.*, 1999; McArdle & Bullock, 1987; McFadzen *et al.*, 2000; Sommaruga, 2003 and references therein). This may increase a host's susceptibility to bacterial or parasitic infections which may cause further tissue damage, increasingly weakening the functionality of the epidermal tissue as a physical barrier (Kramer, 1990 and references therein). Similar effects may facilitate penetration of cercariae in

organisms such as crustaceans. Alternatively, UVR is known to be immunosuppressive in some organisms (e.g. Patz *et al.*, 1996; Salo *et al.*, 1998); thus stress reactions in crustacean hosts due to the exposure to UVR may suppress immune responses and thus limit the ability of a host to deal with parasites after their successful penetration.

Various species of zooplankton and benthic invertebrates are known to be sensitive to UVR (e.g. Bothwell *et al.*, 1994; Leech & Williamson, 2000). UVR has also been shown to affect the survival, fecundity and sex ratio in some intertidal copepods, but these effects were highly species-specific (Chalker-Scott, 1995). Amphipods are translucent to UVR and therefore intertidal species are thought to be particularly prone to stress due to the fluctuating exposure levels (Obermueller *et al.*, 2005). Nonetheless, no effect of UVR was found on the survival of *P. novizealandiae* amphipods in our study. Infected amphipods had, however, an increased risk of dying during this experiment, especially those with high infection intensities, which is in contrast to what was previously observed for the effects of temperature (Studer *et al.*, 2010). Organisms have evolved strategies to cope with UVR and the occurrence of these mechanisms often depends on the radiation levels to which organisms are normally exposed (e.g. Gleason & Wellington, 1995; Helbling *et al.*, 2002b; Siebeck *et al.*, 1994). *Paracalliope novizealandiae* lives in high UVR environments, i.e. shallow intertidal soft-sediment habitats and tidal pools during low tide, and thus should be adapted to high levels of UVR. Several protective mechanisms may contribute to this. Vegetation present in this habitat allows for behavioural adaptations (i.e. seek protective shading). Also, these amphipods contain photo-protective compounds such as mycosporine-like amino acids (A. Studer and V. Cubillos, unpubl. data) and it is likely that other protective compounds (e.g. carotenoids) or repair mechanisms are present, similar to what has been described for other marine, herbivore amphipod species (e.g. Helbling *et al.*, 2002a; Obermueller *et al.*, 2005).

Net effects

The net effect of UVR on the transmission process of a parasite from one host to the next depends on the differential response and sensitivity of each species and step involved. Based on our results, we suggest that the negative effect of UVR on the survival of *M. novaezealandensis* cercariae is the most pronounced response, but that this negative effect on cercarial survival might be compensated, at least to some degree, by the increased susceptibility of amphipod hosts to infection. Thus, overall, no clear impact of UVR on the transmission process would be expected. In order to validate this, an additional outdoor

experiment was conducted assessing the success of cercariae at infecting amphipods under natural light conditions (i.e. cercariae and amphipods exposed together in wells of 96-well plates to no UV, only UVA or UVA+B for 2 h on a clear summer day using the same filters as described above). Neither UV treatment, nor sex or size of amphipods in this un-replicated side-experiment were significantly affecting the proportion of successful parasites or the number of parasites infecting the amphipods (A. Studer, unpubl. data), hence supporting our conclusion of an overall undetectable effect of UVR.

UVR does not act in isolation under natural conditions (see e.g. Przeslawski *et al.*, 2005). Our study is therefore limited by the fact that interactive effects with other environmental factors, especially temperature, were not incorporated and that many experiments were only conducted under laboratory conditions. For example, during optimal conditions for the transmission of *M. novaezealandensis* in the field, i.e. during low tide on warm sunny days when water in the shallow tide pools warms up, temperature and UVR may synergistically affect the organisms and their interaction differently than what has been described here. In particular, cercarial survival is likely to be strongly negatively affected by synergistic effects of UVR and temperature (see Chapter Seven); however, increased infectivity at an optimum temperature level (Studer *et al.*, 2010) may on the other hand counterbalance this reduced survival of cercariae. Such interactions as well as many other aspects of the ecological role of UVR for parasitism and disease transmission in marine ecosystems (as well as other ecosystems) remain to be elucidated. Appropriate experiments should be conducted in laboratory settings closely matching relevant ambient field conditions, and preferably, whenever feasible, should be repeated under natural conditions.

In summary, we found that UVR negatively influenced both, the parasite and its amphipod host. The survival of cercariae was reduced and the susceptibility of amphipod hosts to infection increased. UVR should therefore be considered an important ecological component in the transmission process of intertidal and possibly other parasites. Although it remains unclear how these effects may manifest in nature, the overall net effect of UVR on the host-parasite system studied here may be considered neutral, with the negative effect on cercarial survival being compensated by the increased susceptibility of amphipods.

CHAPTER SIX

Effects of ultraviolet radiation on an intertidal trematode parasite: an assessment of damage and protection

6.1 Abstract

Trematode parasites are integral components of high ultraviolet radiation (UVR) environments such as intertidal ecosystems. Although these parasites mostly live within hosts, their life cycle involves free-living larval transmission stages such as cercariae which are directly exposed to ambient conditions. UVR has previously been shown to considerably reduce the survival of cercariae. Here, we investigated potential mechanisms of protection and damage related to UVR in the intertidal trematode *Maritrema novaezealandensis*. Firstly, the presence of sunscreen compounds (i.e. mycosporine-like amino acids (MAA)) was quantified in the parasite tissue producing cercariae within a snail host, as well as in the free-swimming cercariae themselves. Secondly, levels of oxidative stress in cercariae after exposure to UVR were investigated (i.e. protein carbonyls, catalase and superoxide dismutase). Thirdly, the DNA damage (i.e. cyclobutane-pyrimidine dimers) was compared between cercariae exposed and not exposed to UVR. And lastly, the functional recovery capacity (survival, infectivity) was assessed for cercariae kept in light conditions versus dark after exposure to UVR. We confirmed the presence of MAA's in cercariae-producing tissue from within snail hosts, but were unable to do so in cercariae directly. Results further suggested that exposure to UVR induced high levels of oxidative stress in cercariae which was accompanied by a reduction in the levels of protective antioxidant enzymes present. We also identified higher levels of DNA damage in cercariae exposed to, compared to those not exposed to UVR. Moreover, no clear indication of a light-dependent capacity was found to restore the functionality of cercariae after exposure to UVR. We conclude that cercariae are highly susceptible to UVR damage and that they have very little scope for protection against or repair of UV-induced damage

6.2 Introduction

Ultraviolet radiation (UVR) is an important environmental factor fluctuating at various spatial and temporal scales (e.g. Hansson & Hylander, 2009). In biological terms, UVR (UVB: 280 - 320, UVA: 320 - 400 nm) represents the most reactive part of the incident solar irradiance and is known to have a broad spectrum of deleterious genetic and cytological effects in aquatic organisms (e.g. Dahms & Lee, 2010; Haeder *et al.*, 1998, 2007; Sinha & Haeder, 2002; Vincent & Neale, 2000). Of all aquatic environments, the intertidal zone is probably among the most physiologically stressful, with organisms living exposed to multiple fluctuating stressors including high levels of UVR (e.g. Karentz, 2001; Kramer, 1990; Przeslawski *et al.*, 2005). In intertidal ecosystems, there is little or no attenuation of UVR through the water column and consequently, conditions at low tide are similar to atmospheric conditions. Trematode parasites are an integral component in these ecosystems, being the most common parasite group (Lauckner, 1984; Mouritsen & Poulin, 2002b, 2010). Trematodes have complex life cycles involving several members of an intertidal community as hosts. Their transmission from one host to the next relies on free-living larval stages directly exposed to and influenced by prevailing environmental conditions (Pietrock & Marcogliese, 2003). Despite the biological and ecological importance of both parasites and UVR, little is known about the damaging effects of UVR on parasites, or the latter's capacity to protect against, or cope with, UV-induced damage or stress.

The damaging effects of UVR occur on the molecular, cellular and physiological level. Direct absorption of UVR can degrade or transform molecules such as proteins, lipids or nucleic acids, which may result in the impairment or complete loss of their biological function (Vincent & Neale, 2000). For example, direct absorption of UVB by DNA molecules results predominantly in the formation of cyclobutane-pyrimidine dimers (CPD's) and to a lesser extent in 6-4 photoproducts (e.g. Lamare *et al.*, 2006; Lesser *et al.*, 2003; MacFadyen *et al.*, 2004; Malloy *et al.*, 1997). The formation of CPD's is dose-dependent and varies between taxa (Lamare *et al.*, 2007). CPD levels resulting from sub-lethal doses of UVB may inhibit normal development of embryos and larvae and thus affect their survival and fitness (Dahms & Lee, 2010). Indirectly, UVR can be absorbed by molecules leading to an accelerated production of reactive oxygen species (ROS) (Lesser, 2006). ROS are powerful oxidants, some of which can diffuse through cellular membranes and react with other components leading to cytological damage. If the production of ROS exceeds a certain threshold,

oxidation of DNA, proteins and membrane fatty acids occurs (Lesser, 2006). This damage is also known as oxidative stress and is considered to be a highly sensitive biomarker for a range of environmental stressors including UVR (Burritt, 2008; Burritt & MacKenzie, 2003; Lesser, 2006; Lister *et al.*, 2010a). Oxidative stress can further translate into ecologically relevant effects, including impaired development and reduced survival of damaged organisms (Lesser, 2006).

However, most organisms have evolved a range of mechanisms for protection against UVR, such that the net effect of UVR on an organism is a combination of damage, repair and the energetic costs associated with the protective strategies (Vincent & Neale, 2000). The range of strategies to cope with UVR and its potentially deleterious effects include the intracellular accumulation of UV screening compounds such as mycosporine-like amino acids (MAA's) or other UV absorbing compounds (e.g. carotenoids) (Dahms & Lee, 2010; Rastogi *et al.*, 2010; Roy, 2000; Shick & Dunlap, 2002). MAA's play a dual role, protecting not only against UVR, but also against oxidative damage (Shick & Dunlap, 2002). These compounds are synthesised by bacteria, fungi and algae and are transferred trophically to other organisms (Rastogi *et al.*, 2010 and references therein; Shick & Dunlap, 2002). MAA transfer is also known to take place via symbiotic associations or through maternal provision to eggs (e.g. Adams & Shick, 2001; Carroll & Shick, 1996; Karentz, 2001; Rastogi *et al.*, 2010; Shick & Dunlap, 2002). Other preventive mechanisms include antioxidants (e.g. the enzymes catalase and superoxide dismutase), which scavenge ROS and help reduce oxidative stress, and the capacity to repair UV-induced DNA damage (e.g. Dahms & Lee, 2010; Kim & Sancar, 1993; Lesser, 2006; Malloy *et al.*, 1997; Sinha & Haeder, 2002). The two primary repair mechanisms for UV-induced DNA damage are photo-reactivation and the light-independent nucleotide excision repair which can also take place in the dark (e.g. Dahms & Lee, 2010; Sancar, 1994; Sinha & Haeder, 2002). Photo-reactivation is likely the oldest and simplest form of DNA repair and is widespread in aquatic organisms (Haeder & Sinha, 2005; Weber, 2005). Photo-reactivation repairs primarily CPD's via the enzyme photolyase, which depends on light as its source of energy.

Most studies combining the effects of UVR and parasites or pathogens are studies of induced immune reactions, vaccine development or sterilisation of drinking water or food (e.g. Allam & Hadid, 2009; Bintsis *et al.*, 2000; Hijnen *et al.*, 2006; Lagapa *et al.*, 2001; Rochelle *et al.*, 2004). The role of UVR as an ecological factor has only rarely been

investigated in host-parasite interactions. As an exception (also see previous chapter, Perrot-Minnot *et al.*, 2010), studies by Ruelas *et al.* (2006, 2007, 2009) on effects of UVB irradiation on an important human parasite, the trematode *Schistosoma mansoni* and its snail host (*Biomphalaria glabrata)*, have shown negative effects for both the parasite and the snail (see also previous chapter). This included reduced survival of snails as well as abnormal and reduced development of parasites after penetration of the snail host following irradiation. Moreover, these authors provided evidence that not only the snail was able to repair DNA damage by photo-reactivation (Ruelas *et al.*, 2006), but also the parasite (Ruelas *et al.*, 2007). Their results indicated that photo-reactivation, rather than nucleotide excision repair, was the primary mechanism of UVB-induced DNA damage.

Here, we used the intertidal microphallid trematode parasite *Maritrema novaezealandensis* as a model system. The complex life cycle of this parasite includes first intermediate snail hosts (*Zeacumantus subcarinatus*), second intermediate crustacean hosts such as the amphipod *Paracalliope novizealandiae* and definitive bird hosts (Martorelli *et al.*, 2004). The transmission process from the first to the second intermediate host is via translucent, free-living, non-feeding, short-lived (< 24 h) larval transmission stages called cercariae (approx. 100 µm body length, 100 µm tail length, 20 µm body depth and 10 µm tail depth, Koehler *et al.*, 2011). These cercariae are produced asexually in the parasite's sporocysts within infected snails and emerge into the environment to infect second intermediate hosts. It is thought that the optimal conditions for transmission of this parasite occur on warm days during low tides when shallow soft-sediment tide pools warm up (Chapter Two and Three, Bates *et al.*, 2010; Fredensborg *et al.*, 2004b); conditions typically coinciding with maximal levels of UVR (i.e. late spring to late summer). A previous study investigating *M. novaezealandensi*s showed that the survival of cercariae was strongly reduced by UVR in a dose-dependent manner (Chapter Five).

Because the survival of cercariae is a key step in the life cycle of this parasite, the aims of this study were to investigate potential protective mechanisms available to cercariae during the free-living phase when they are exposed to UVR, as well as routes of damage. Firstly, we searched for protective UV absorbing compounds (especially MAA's) in sporocyst tissue within snail hosts as well as in cercariae directly. Secondly, we investigated oxidative damage to proteins in cercariae after exposure to UVR as a biomarker of UV-induced oxidative stress, as well as activity of protective antioxidant enzymes. Thirdly, we assessed differences in the

relative concentrations of CPD's in cercariae exposed or not exposed to UVR. And fourthly, we evaluated the light-dependent capacity of cercariae to recover functionality (i.e. survival and infectivity) after exposure to UVR. The data provided here add important new knowledge on the direct effects of UVR on parasites, but also on the biotic interaction between parasites and their hosts.

6.3 Materials and Methods

UV absorbing compounds. In a preliminary HPLC (high pressure liquid chromatography) - mass spectrophotometry analysis, the presence of UV absorbing compounds and MAA's in particular was detected in a small number of samples of cercariae-producing tissue from within snail hosts (sporocysts). Therefore, an outdoor experiment was conducted to assess temporal changes in the concentrations of UV absorbing compounds in sporocysts of infected snails over a six month period. For this, white rectangular 1 l plastic containers were placed outdoor at the end of austral winter (July 2009). Containers were filled with a thin layer of sediment and 30 snails infected with *M. novaezealandensis* were added to each container. Snails had a constant supply of sea lettuce *Ulva* sp., a species of algae not containing MAA's (Carefoot *et al.*, 2000; Lamare *et al.*, 2004). Containers were covered with Plexiglas filters, either blocking UVR (i.e. photosynthetically active radiation (PAR; 400 - 700nm) only, no UV treatment), being transparent to PAR and some UVA (UVA treatment), or being transparent to PAR, UVA and UVB (UVA+B treatment) (three replicate containers per treatment). The same filters were used as in the previous chapter (i.e. no UV treatment: 81% PAR, 5.2% UVA, 0.0% UVB; UVA treatment: 77.9% PAR, 46.5% UVA, 0.1% UVB; UVA+B treatment: 84.5% PAR, 84.6% UVA, 80.6% UVB) (see Lister *et al.*, 2010b or Appendix for the transmission profiles of the filters). Containers were randomly distributed (and occasionally rotated) on an outdoor shelf receiving flow through filtered seawater. After three (October 2009) and six months (January 2010), snails were randomly sub-sampled, dissected and the parasite tissue from within the snails frozen at -80°C. Additionally and because experimental snails did not shed enough cercariae, samples of cercariae were obtained by incubating snails collected from the field in January 2010 (i.e. austral summer) at 25°C under constant illumination. Seawater containing emerged cercariae was transferred into Eppendorf tubes and centrifuged in order to remove excess seawater, then also frozen at -80°C until processing.

For the extraction and analysis of UV absorbing compounds, samples (five replicate tissue samples per container and five samples containing cercariae) were dried using a SpeedVac (Savant) (45°C for 24 h), then ground and weighed. Samples were frozen at -80°C until extraction of MAA was carried out by adding 1 ml of 100% HPLC grade methanol (MeOH) per 10 mg of sample. Due to varying amounts of dry material per sample, the volume of MeOH added was adjusted according to each sample weight. Samples were then sonicated in an ultrasonic bath for 8 min and stored at 4°C wrapped in aluminium foil for approx. 24 h. This was followed by filtering of samples using nylon syringe filters (0.22 µm). An aliquot of 20 µl of the methanolic extraction from each sample was injected into an HPLC (Dionex/Ultimate 3000) using a C8 Phenosphere analytical column (Phenomnenx; 250 x 4.6 mm; pore size 5 µm) and eluted in a mobile phase composed of 89.9% MilliQ water + 10% MeOH + 0.1 formic acid at a flow rate of 1 ml min^{-1}. All samples injected into the HPLC were analysed at 310 and 334 nm using a UV/VIS detector. Peaks identified in the chromatograms were corroborated by mass spectrometry (Bruker TOF).

HPLC revealed a mixture of two to three compounds making up the main peak at 310 nm (Fig. 6.2). One compound was identified as mycosporine-glycine (λ_{max} = 310; molecular weight = 245 Da, ε = 28100), whereas another main compound had a molecular weight of 261 Da (hereafter referred to as unknown UV-absorbing compound UC-261). To estimate the concentration of these compounds, the entire peak area was used thus combining all compounds making up the main peak. At 334 nm, the main compound identified was the MAA porphyra-334 (P-334; λ_{max} = 334; molecular weight 346 DA; ε = 42300). Concentrations were estimated using calibration curves which were generated from isolated standards obtained through semi-preparative HPLC (C18 column, Phenomenex - Luna 250 x 10 mm; pore size 5 µm). For this, UC-261 was extracted from the parasite tissue of infected *Z. subcarinatus* snails and P-334 was isolated from *Nyctiphanes australis* (krill). The concentration of each isolated standard was then estimated using the respective molar extinction coefficient (ε) and the corresponding absorbance at their maximum wavelength.

For the statistical analysis, the concentrations of the compounds in sporocyst tissue obtained from infected snails were square root transformed in order to meet the assumption of normality and homogeneity of variances. A repeated measures ANOVA was then used to assess the effect of the UV treatment on the concentration of the compounds absorbing at 310 nm and at 334 nm after three and six months, followed by Tukey's post hoc tests. Container

was initially included as a factor but had no significant effect and thus was omitted from the analysis and the results presented.

Analysis of oxidative stress and antioxidant enzymes. The extent of oxidative stress in cercariae experiencing different regimes of UVR was assessed in an experiment conducted under ambient conditions. To achieve the UV treatments, the three types of Plexiglas filters were used as described earlier. To obtain the free-living cercarial transmission stages, 40 snails infected with *M. novaezealandensis* were put into 50 ml UVR opaque plastic jars filled with seawater (four replicate samples per treatment). Snails were then incubated for 1 h at 25°C under constant illumination (using cold light). After incubation, the seawater containing the emerged cercariae was transferred into new 50 ml plastic jars. Four replicate baseline samples were processed immediately (see below). The remaining samples were exposed outdoors on a cloudless summer day under one of the three types of filters (no UV, UVA, or UVA+B) for 2 h (UVB dose: 16.1 kJ m^{-2}; not considering the reductions by the filters).

Samples were processed by centrifuging the seawater containing cercariae in 50 ml tubes (Nalgene Centrifuge Ware) for 30 min at 18,000 rpm (38,724 g; 4°C). Seawater was removed and cercariae re-suspended in 5 ml of 50 mM potassium phosphate buffer (pH 7.4). Samples were subsequently analysed for total protein content, protein oxidation (i.e. protein carbonyls) and the levels of activity of antioxidant enzymes (i.e. catalase, CAT, and superoxide dismutase, SOD). For this, cercariae were centrifuged as above and re-suspended in 0.5 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 1.5% NaCl and 1 mM phenylmethylsulphonyl, transferred into 1.5 ml Eppendorf tubes and then lysed by sonication at 4°C. The protein extracts were again centrifuged for 30 min at 14,000 rpm (21,000 g) in a microfuge and the supernatants were frozen at -80°C prior to analysis. The protein contents of the extracts were determined using a Lowry protein assay (Fryer *et al.*, 1986). Protein carbonyl levels in the protein extracts were analysed via reaction with 2.4-dinitrophenylhydrazine (Reznick & Packer, 1994) with minor adaptations (Burritt, 2008). Catalase (EC 1.11.1.6) was assayed using the chemiluminescent method of Maral *et al.* (1977), as adapted by Janssens *et al.* (2000). Superoxide dismutase (EC 1.15.1.1) was analysed using the microplate assay described by Banowetz (2004) with minor modifications. All assays were carried out with a PerkinElmer (Wallac) 1420 multilabel counter, controlled by a PC and fitted with a temperature control cell (set to 25°C) and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer).

Differences in concentrations of protein carbonyls, CAT and SOD between the different UV treatments were analysed using ANOVAs followed by Tukey post hoc tests. Data were transformed where necessary (log transformation of protein carbonyl concentrations) to meet the assumptions of the parametric test.

DNA damage in cercariae. In this outdoor experiment, the amount of DNA damage in terms of the relative concentration of cyclobutane-pyrimidine dimers (CPD) per mega base of DNA was measured after exposing cercariae to ambient conditions. The concentration of CPD's formed was compared in cercariae exposed or not exposed to UVR. In order to obtain the cercariae, 35 infected snails from stock aquaria were placed per 50 ml UV opaque plastic jars filled with seawater and then incubated at 25°C under constant illumination (cold light) for 1 h. After incubation, the seawater containing the cercariae was transferred to new 50 ml plastic jars. Two samples were used as a baseline and processed immediately (see below). The remaining samples were either exposed to ambient conditions under a UV-blocking filter (no UV), or UV transparent filters (UVA+B; four samples per treatment; same filters used as described previously). After 1 h of exposure (corresponding to a UVB dose of 12.0 kJ m^{-2}), samples were processed by centrifuging the seawater containing cercariae twice in sequence; first in 50 ml Nalgene tubes for 25 min at 18,000 rpm (38,724 g; 4°C) and then for 10 min in 1.5 ml Eppendorf tubes at 14,000 rpm (20,817 g; 4°C). After removal of seawater and addition of 1 ml of salt-saturated DMSO buffer to each tube, samples were stored at -80°C until further analysis.

DNA damage in terms of CPD's was quantified in an enzyme-linked immunoabsorbent assay (ELISA). For this, DNA was isolated from the cercariae using a commercially available extraction kit and protocol (Bioline). The ELISA was carried out following Mori (1991), as modified by Schmitz-Hoerner (2003). Briefly, 10 ng of DNA from each sample was used per well in protamine sulphate (0.003%)-coated 96-well polyvinyl chloride microtiter plates, with three replicate wells assayed per sample. In each well, DNA was added to PBS (phosphate-buffered saline) denatured by boiling at 100°C for 10 min followed by 10 min on ice and incubation overnight. Plates were washed five times before 150 µl of 2% fetal bovine serum in PBS-T was added followed by 1.5 h of incubation and rinsing. To each well, 100 µl of 0.001% TDM-2 primary monoclonal antibody was added, and incubated for 30 min, after which wells were rinsed. 100 µl of rabbit anti-mouse IgG secondary antibody was added to each well and incubated for 30 min followed by rinsing. Then, 100 µl of 0.0001%

streptavidin horseradish peroxidase conjugate was added to each well and incubated for 30 min followed by rinsing. All rinsing steps were done five times using 0.05% Tween-20 in PBS and all incubations were at 38°C. Colour development was achieved by addition of Sigma Fast reagents (Sigma) and colour was read (at 405 nm) after 5 min using a plate reader. Absorption readings were adjusted for background absorption levels. The concentration of CPD's was assessed by calculating values based on the absorbance of standards included on the plate with known UVB exposure doses of 0, 2.5, 5.0 and 7.5 J m^{-2}.

Light-dependent recovery capacity of cercariae. While free-living cercariae are exposed to environmental conditions, light-dependent repair mechanisms were hypothesised to potentially ameliorate the damage caused by UVR. Two separate laboratory experiments were thus conducted to assess the capacity of the cercariae of *M. novaezealandensis* to recover their functionality after exposure to UVR. After UVR treatment of cercariae (dose administered during a 1 h exposure, not considering reductions by filters: UVB: 5.84, UVA: 63.27, PAR: 185.09 kJ m^{-2}), cercariae were either incubated under white fluorescent light (Phillips 40W) (using a transparent plastic bag) or kept in the dark (using a black, non-transparent plastic bag), and differences in survival and infectivity were assessed. The same laboratory set-up as described in the previous chapter and the same filters (UVA+B treatment) were used as described above.

Survival experiment. 60 µl of a cercarial mixture (prepared using 40 snails; for details on preparation see Studer et al. 2010) were added to 12 replicate wells on two replicate 96-well plates per treatment (corresponding to an addition of approx. 35 cercariae per well). Cercariae were then exposed for 1 h under the UVA+B transparent filters and subsequently placed in the either transparent (light treatment) or black plastic bag (dark treatment) under white fluorescent light. Survival and activity of the cercariae was monitored at an average cercarial age of 2, 4, 6, 10 and 12 h. As in previous chapters, survival and activity categories distinguished were fully active, sluggishly motile and immotile/dead. The effect of the light/dark treatment on the proportion of fully active cercariae (arcsine-square root transformed) at an average age of 2, 4, 6 and 10 h was statistically analysed with a repeated measures ANOVA.

Infectivity experiment. Uninfected *P. novizealandiae* amphipods were placed individually into wells of 96-well plates (48 amphipods on two replicate well plates per

treatment). A cercarial mixture prepared from 40 snails was distributed into four small Petri dishes (7 ml per dish) and exposed under the UVA+B transparent filters for 1 h. After exposure, the mixture was combined again and 50 µl was added to each amphipod (corresponding to an addition of approx. 20 cercariae per amphipod). Amphipods were then incubated for 3 h in a transparent or black plastic bag (temperature in both plastic bags approx. 21 - 22°C) illuminated by white fluorescent light. After incubation, amphipods were transferred into plastic containers (300 ml) filled with seawater (aerated) and provided with sea lettuce *Ulva* sp. After 8 d, surviving amphipods were measured, sexed and dissected under a dissecting microscope. The number of parasites per amphipod was counted. The effect of the light/dark treatment, as well as sex and size of amphipods, on the proportion of cercariae successfully infecting amphipods (arcsine-square root transformed) was statistically analysed with a General Linear Model (GLM).

6.4 Results

UV absorbing compounds

In the vast majority of samples, four peaks were identified by HPLC (see Fig. 6.1 for retention times). Two of them were MAA's, namely mycosporine-glycine and porphyra-334 (P-334). The two other peaks were not identified (including UC-261). For the main compounds with a maximum absorbance at 310 nm, no differences in the concentrations were found after three months of exposure between treatments (Fig. 6.2). After six months, concentrations were significantly lower in the no UV and UVA treatments, but not in parasite tissue from snails exposed to UVA+B, in which concentrations remained highest and were not significantly different from the concentration after three months (Tukey's post hoc, $p = 0.932$) (see also Table 6.1). Compared to the mean concentrations of these compounds in parasite tissue of UV unexposed snails after three months, concentrations after six months had decreased to 44.7% (no UV), 49.7% (UVA) and 82.0% (UVA+B).

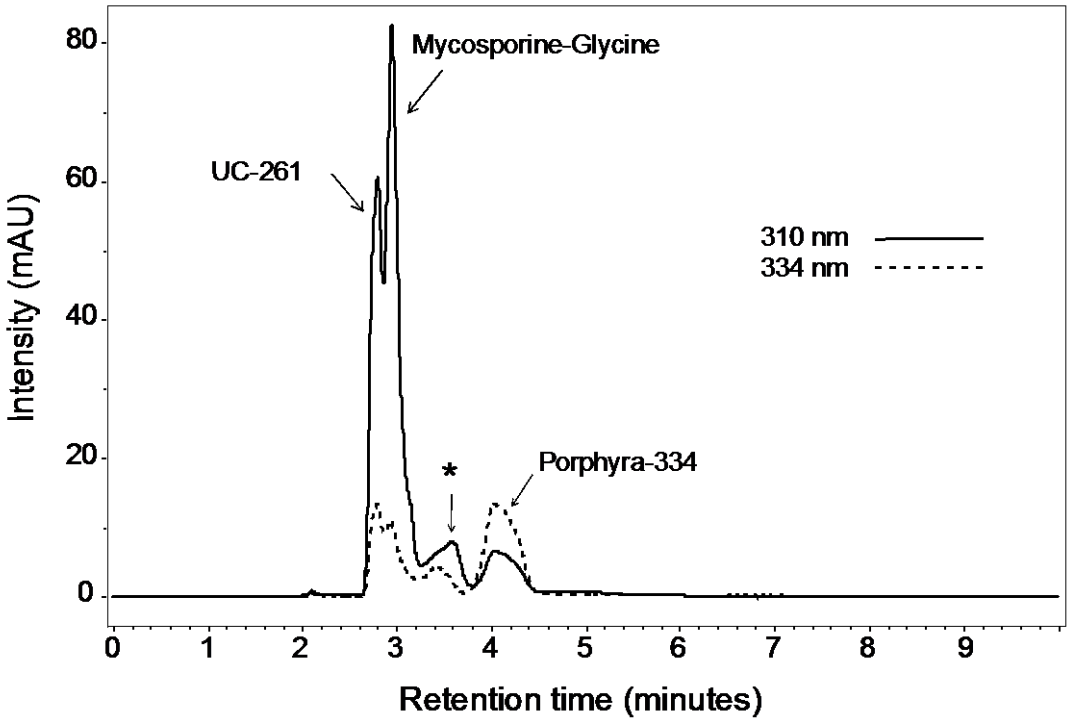


Figure 6.1 HPLC chromatograph of a MeOH extract of *Maritema novaezealandensis* tissue (sporocysts) from within an infected *Zeacumantus subcarinatus* snail host. Four peaks are present: at 310 nm (solid line), the main peak was mainly composed of an unidentified UV absorbing compound (UC-261) and mycosporine-glycine, whereas at 334 nm, the main peak was porphyra-334. Another small peak (*) was apparent with a higher absorbance at 310 nm.

Table 6.1 Results of the repeated measures ANOVA testing the effect of the UV treatment (no UV, UVA or UVA+B) on the concentration of compounds absorbing at 310 nm and 334 nm, respectively, in parasite tissue of infected snail hosts after three and six months (n = 15 per treatment).

	Factor	**df**	**MS**	**F**	**p**
310 nm	**Between subjects**				
	UV	2	27.58	3.24	0.050
	Error	40	8.51		
	Within subjects				
	Time	1	329.05	25.31	< 0.001
	Time x UV	2	42.76	3.29	0.048
	Error	40	13.00		

	Factor	df	MS	F	p
334 nm	**Between subjects**				
	UV	2	1.17	3.89	0.029
	Error	40	0.30		
	Within subjects				
	Time	1	3.09	10.40	< 0.001
	Time x UV	2	0.38	1.28	0.288
	Error	40	0.30		

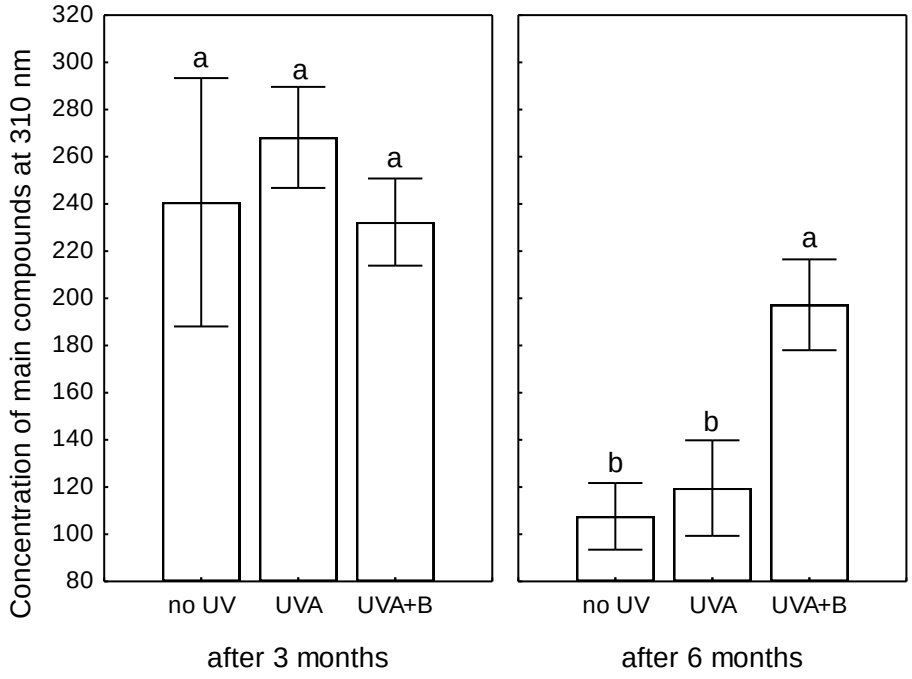


Figure 6.2 Concentrations (mean ± SE) of the compounds with λ_{max} = 310 nm (mycosporine-glycine, UC-261 and in few samples another unidentified compound; nmol mg^{-1} dry weight) after three and six months exposure to ambient conditions in different UV treatments (no UV, UVA, UVA+B; n = 15 per treatment). The data were analysed with a repeated measures ANOVA followed by a Tukey's post hoc test: the letters denote significant differences between treatments if not equal (significance level $p < 0.05$).

For P-334, UV treatment and time both had a significant effect on concentrations (Table 6.1). However, the only significant difference was between the concentrations in parasite tissue from snails not exposed to UV after three months and those exposed to UVA+B after six months (Tukey's post hoc, $p = 0.001$). Concentrations of P-334 compared to the concentration in parasite tissue from snails not exposed to UV after three months increased to 126.6% (no UV), 142.5% (UVA) and 178.1% (UVA+B) (Fig. 6.3). Concentrations of P-334 were, however, much lower than concentrations of compounds absorbing at 310 nm.

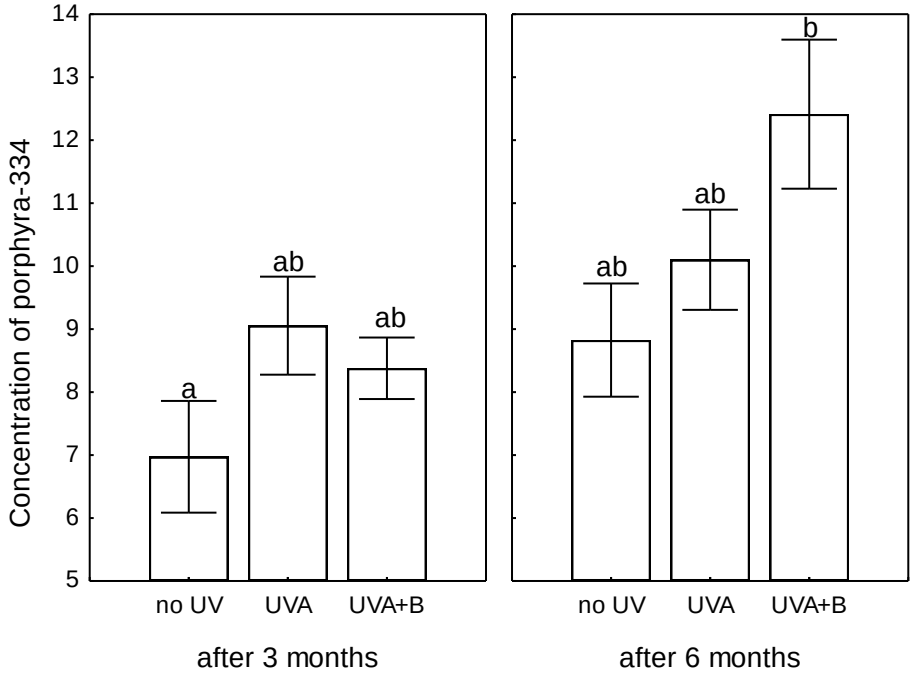


Figure 6.3 Concentrations (mean ± SE) of porphyra-334 (nmol mg^{-1} dry weight) after three and six months exposure to ambient conditions in different UV treatments (no UV, UVA, UVA+B; n = 15 per treatment). The data were analysed with a repeated measures ANOVA followed by a Tukey's post hoc test: the letters denote significant differences between treatments (significance level $p < 0.05$).

In contrast to the presence of MAA's in sporocysts, these compounds could not be detected in cercariae directly. While HPLC revealed the presence of unidentified UV absorbing compounds in three out of five samples, no MAA's were identified by mass spectrometry.

Oxidative stress

Exposure of cercariae to the UV treatments resulted in significantly different concentrations of protein carbonyls, as well as activity levels of catalase and superoxide dismutase enzymes (ANOVA; log transformed, protein carbonyls: $F_{3, 12} = 100.42$; CAT: $F_{3, 12} = 14.01$; SOD: $F_{3, 12} = 63.65$; all $p < 0.001$). Levels of protein carbonyls increased significantly by more than five-fold in cercariae exposed to UVR compared to baseline levels and both, UVA and UVB radiation induced protein oxidation in these organisms (Fig. 6.4). However, already in the no UV (i.e. PAR only) treatment there was a considerable increase in protein carbonyl levels compared to baseline levels. A similar pattern emerged for the activity levels of antioxidant enzymes (i.e. CAT and SOD). When comparing the UVA+B treatment

to the baseline, enzyme activity was reduced by almost four-fold (CAT) and by more than six-fold (SOD). Again, both UVA and UVB negatively affect these enzymes, but the no UV (PAR only) treatment already caused a substantial decrease in activity levels of these enzymes (Fig. 6.4).

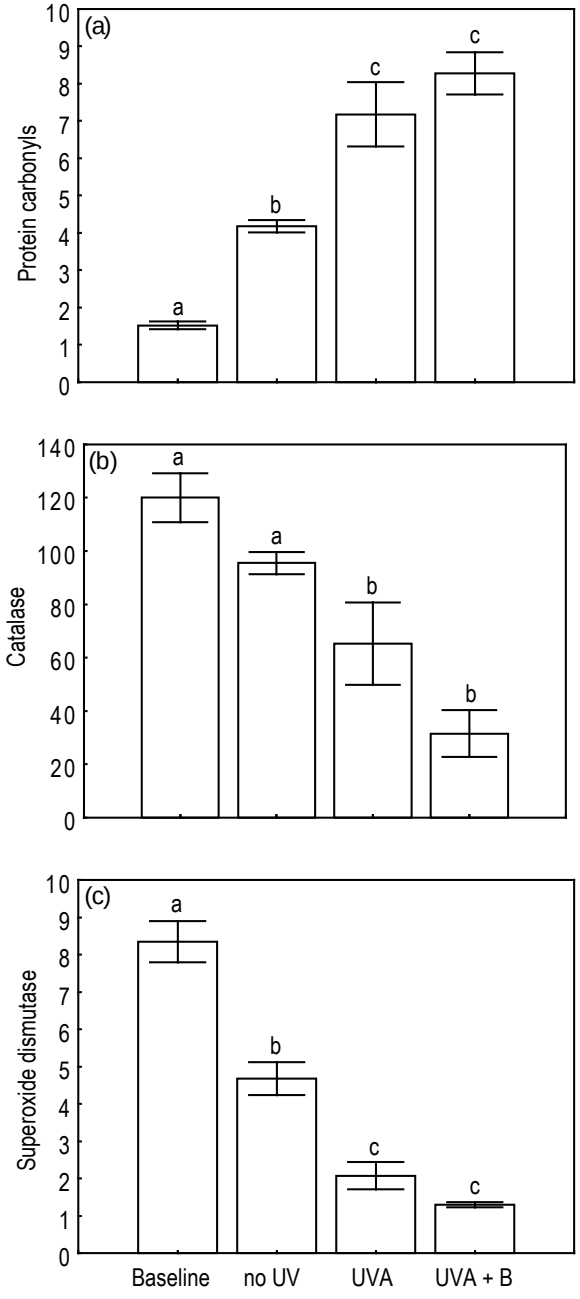


Figure 6.4 Concentrations (mean ± SE) of (a) protein carbonyls (nmol mg^{-1} of protein), (b) catalase (µmol H_2O_2 mg^{-1} protein min^{-1}) and (c) superoxide dismutase (u mg^{-1} protein min^{-1}), in baseline samples and after exposure to no UV, UVA or UVA+B radiation (n = 4 per treatment). The letters denote significant differences between treatments if not equal, according to Tukey's post hoc tests (significance level $p < 0.05$).

DNA damage in cercariae

There was a slight increase in the concentration of CPD's per megabase of DNA for cercariae exposed to UVA+B (0.30 ± 0.005) compared to cercariae not exposed to UV (0.21 ± 0.002), with the average concentration of the two baseline samples (0.19) being similar to the concentration in cercariae not exposed to UV.

Functional recovery capacity of cercariae

There was no significant difference in the survival of cercariae kept in light versus dark after exposure to UVR (repeated measures ANOVA; arcsine-square root transformed proportion of fully active cercariae; between subjects, effect of light/dark: $F_{1, 46} = 0.85$, $p = 0.362$; within subjects, time: $F_{3, 138} = 2471.33$, $p < 0.001$, time x light/dark: $F_{3, 138} = 0.64$, $p = 0.591$). Full activity of cercariae ceased within 10 h regardless of treatment. There was a slight trend in terms of infectivity, i.e. a higher proportion of cercariae successfully infecting amphipods, for infections taking place under light (21.9 % successful in the light treatment versus 17.5 % in the dark), which was however also not significant (GLM, arcsine-square root transformed proportion of successful parasites; $F_{1, 44} = 1.55$, $p = 0.220$). Amphipod sex significantly influenced the proportion of successful cercariae, with males being more infected than females ($F_{1, 44} = 4.62$, $p = 0.037$), while size of amphipods did not ($F_{3, 44} = 0.31$, $p = 0.815$).

6.5 Discussion

Larval stages and planktonic eggs of aquatic organisms are ecologically very important but are also particularly at risk of UV-induced damage (Adams & Shick, 1996, 2001; Lister *et al.*, 2010b). However, organisms have a range of mechanisms and strategies to counteract the potentially detrimental effects of UVR (e.g. Dahms & Lee, 2010; Roy, 2000; Shick & Dunlap, 2002). In this study, the larval cercarial transmission stage of the intertidal trematode parasite *M. novaezealandensis* was investigated in terms of UVR related mechanisms for protection, damage and repair. In contrast to larval stages of marine organisms, cercariae were shown to have only limited capacity to prevent UV-induced damage through the provision of MAA's and to cope with oxidative stress. In addition, DNA damage was increased in cercariae exposed to UVR and no difference was found in the functional recovery of cercariae, if, after exposure to UVR, they were kept in light or dark conditions.

UV absorbing compounds

Regarding the concentrations of UV absorbing compounds in parasite tissue (sporocysts) within snail hosts, significant differences between treatments emerged only after six months of exposure to ambient conditions. After this period, concentrations of UV absorbing compounds remained high only in snails exposed to UVA+B for the compounds with a maximum absorbance at 310 nm. On the other hand, concentrations of P-334 were slightly increased, especially in snails exposed to UVA+B. Mycosporine-glycine is one of the most abundant MAA's found in marine organisms (Bandaranayake, 1998), and alone or in combination with the other unidentified compounds with a maximum absorbance at 310 nm, would be expected to provide effective protection across a range of UVB wavelengths. In contrast, despite the fact the concentrations were also significantly affected by the UV treatment, concentrations of P-334 in sporocysts were consistently much lower than concentrations of compounds absorbing at 310 nm. Increases in MAA content in response to ambient UVR has been shown in a number of organisms (see review by Shick & Dunlap, 2002), but it has also been shown that this may not be a universal pattern in marine organisms (e.g. Adams & Shick, 1996; Hoyer *et al.*, 2001; Shick & Dunlap, 2002; Shick *et al.*, 2002).

During the experiment, snails had to acquire MAA's through their diet, i.e. diatoms or algae growing in the containers, and parasites through feeding on the snail hosts. It is unlikely that absorbance by the parasite was selective. In an infected snail host, the sporocysts replace the gonads and may divert any resources which the snail would allocate to this organ. Both loss and accumulation of MAA's in the parasite tissue is thus likely to reflect the respective concentrations in the snail itself and hence the concentrations in phytoplankton growing in the containers under the different light conditions over the entire period of the experiment (see Whitehead *et al.*, 2001). Alternatively, due to changing conditions (in particular temperature) over the six month period, it is also possible that snails were selectively metabolising certain compounds therefore accounting for some of the observed patterns.

In addition to the results described here, the preliminary analysis of a small number of parasite tissues samples from infected snail hosts collected from the field also identified the presence of the MAA shinorine in sporocysts in two out of three samples (A. Studer and V. Cubillos, unpubl. data), thereby extending the range of known MAA's extracted from *M. novaezealanbdensis* tissue from within *Z. subcarinatus* snail hosts.

Despite the presence of these MAA's in sporocysts, we could not find any evidence that these may be transferred to cercariae for protection against UVR during their free-living transmission phase. The dual protection these compounds provide (absorption of UVR as well as antioxidant activity) (Dunlap & Yamamoto, 1995; Shick & Dunlap, 2002), hence does not seem to be available to cercariae, at least not in concentrations high enough to be detectable by the methods used. However, the small size of the *M. novaezealandensis* cercariae and consequently the small amount of tissue we were working with may be responsible for our limited ability to detect the low concentrations in which these compounds may be present.

Nonetheless, the small size of cercariae as well as their transparency may be directly responsible for the absence of MAA's. Small size appears to limit the beneficial use of sunscreen compounds (Garcia-Pichel, 1994). Garcia-Pichel (1994) developed a model describing the degree to which organisms can self-shade based on their size and the concentration of sunscreen compounds. According to this model, we estimated (assuming a body radius of 10 µm, a tail radius of 4.7 µm and a water content of cercariae of 70%), that even if cercariae contained the same amount of MAA's as present in the sporocyst tissue (mycosporine-glycine (245 Da) and porphyra-334 (346 Da)), this would only protect them against a maximum of 6% of the incoming radiation. Consequently, unrealistic amounts of UV absorbing compounds would be required to achieve effective protection for an organism the size and nature of these cercariae.

Oxidative stress and DNA damage

As indicated by the increasing levels of protein oxidation, exposure to UVR induced oxidative stress in cercariae. Compared to the baseline samples, even exposure to no UV (i.e. only PAR) increased levels of protein carbonyls in cercariae, and this was further exacerbated under exposure to UVA and UVA+B. This increase in oxidative damage of proteins was, however, not accompanied by an increase in the activity of antioxidant enzymes. While CAT and SOD activity levels in baseline samples were still mostly comparable to those found in other marine organisms (e.g. Buchner *et al.*, 1996; Korkina *et al.*, 2000; Lister *et al.*, 2010b), no increase could be detected in the cercariae of *M. novaezealandensis*, despite the fact that activity levels of antioxidant enzymes are usually increased in response to oxidative stress (Dykens & Shick, 1982; Shick *et al.*, 1995). Rather than an increase in activity, activities of both CAT and SOD declined in response to UVR, indicating that enzymes lost their functionality during exposure. These results from an outdoor experiment were replicated in a

laboratory experiment, confirming the observed patterns (A. Studer and D.J. Burritt, unpubl. data). Therefore, UV-induced damages to cercariae after reaching a certain threshold are bound to be substantial and are highly likely to contribute to the high mortality of cercariae after exposure to UVR (previous chapter).

It has been observed in a behavioural study, that cercariae of *M. novaezealandensis* during their first hour after emergence from snail hosts prefer "light" microhabitats, whereas afterwards they prefer "dark" ones (A.V. Koehler, pers. communication). Thus, it is possible that oxidative stress accumulating during this first hour of exposure to ambient condition is perceived by the parasite and serves as a signal to induce avoidance of light, i.e. to move into the dark. Ultimately, this is what the parasite needs to do in order to locate crustacean hosts such as crabs, inside which it would thereafter live in a more or less opaque internal environment. If this transition does not take place, then the negative effects of UV-induced damages may exceed a threshold with direct consequences for the survival of cercariae. In addition to the oxidative stress parameters assessed in this study, deleterious effects may also include damage to DNA, as observed in the increased concentrations of CPD's in cercariae exposed compared to those not exposed to UVR.

Functional recovery capacity of cercariae

Survival and infectivity of cercariae in our experiments did not differ significantly between cercariae kept in light and those kept in dark conditions after exposure to UVR. This indicated that no evidence for an effective or efficient photo-dependent mechanism could be detected in cercariae to restore functionality after exposure to UVR, which may prolong the survival and hence their chance of successful transmission. Based on the observations described above, this is not unexpected. Nonetheless, experiments were conducted using light from a 40 W light bulb and the quality of this light may not have been optimal; exposing cercariae to natural light might be a better approach. However, it is also possible that light-independent repair mechanisms were responsible for the non-significant differences observed. In particular the dark excision of DNA damage clearly warrants further research as it should be the preferred mechanism for a parasitic organism living in an opaque environment after successful penetration of a host. As described earlier, it seems likely though that UVR effects on cercariae of *M. novaezealandensis* are relatively decisive rather than subtle, and that slight differences in functionality may be difficult to detect.

The results presented here indicate that negative effects of UVR on these free-living parasitic larvae may be even more pronounced than effects on non-parasitic marine larvae or eggs. Cercariae are small, equipped with only limited energy reserves (e.g. Johnson *et al.*, 2010) and their survival is usually less than 24 h (depending on environmental conditions such as temperature and UVR, Chapter Three and Five). Due to these limited resources and the high energetic costs of some of the protective and repair mechanisms, no costly investments may be made in this relatively short-lived cercarial transmission stage. Our observations may thus be more consistent with what has been described for sperm of marine free spawners, which is more susceptible to damage caused by UVR than eggs, embryos or larvae (Dahms & Lee, 2010; Lu & Wu, 2005). Sperm lack UV-absorbing compounds such as MAA's, possess limited antioxidant potential and have a comparatively low DNA repair capacity (e.g. Adams *et al.*, 2001; Aitken *et al.*, 1998), and a decline in motility after exposure to UVR can contribute to reduced fertility (Dahms & Lee, 2010). In the case of cercariae, their reduced survival and activity when exposed to UVR may limit their chance of successful transmission and therefore the continuation of their life cycle.

The high vulnerability of the small and translucent *M. novaezealandensis* cercariae may be offset by the parasite producing them in large numbers. This strategy is common in nature, especially for parasites. Moreover, for the transmission process taking place in nature, certain environmental characteristics may further increase the number of successful transmission events. For example, vegetation in tide pools where snail and crustacean hosts are aggregated provides some shelter from direct insolation. On the other hand, temperature in particular is bound to play an important role, affecting the transmission process on many levels (Studer *et al.*, 2010), and conditions of increased temperature are highly likely to further increase the negative effect of UVR on cercariae (Chapter Seven).

Interestingly, other intertidal trematode cercariae are not produced in similarly large numbers as *M. novaezealandensis* and cercariae of some species are coloured, i.e. pigmented (e.g. observed in other trematode species infecting *Z. subcarinatus*). Moreover, another trematode species infecting *Z. subcarinatus* snails (i.e. *Philophthalmus* sp.) does not seek a second intermediate host but encysts on hard substrates (Lei & Poulin, 2010), therefore remaining exposed to ambient conditions once emerged from a snail host awaiting ingestion by the next host. Comparison of the results presented here with other parasite species of the

same snail host or other species inhabiting intertidal ecosystems across UVR gradients would be highly informative and may have the potential to reveal many novel aspects of parasitism.

To summarise, the free-living transmission stage of the intertidal trematode parasite *M. novaezealandensis* is clearly at the mercy of environmental conditions (Pietrock & Marcogliese, 2003), especially with regards to UVR: neither do these cercariae contain detectable levels of UV absorbing compounds for protection against UVR, nor do they appear capable of dealing with increasing oxidative stress during exposure to UVR. Moreover, exposure to UVR also induced DNA damage and we did not find any evidence for a light-dependent recovery capacity which may possibly prolong their functionality in the environment under exposure to UVR. Results presented here thus consistently confirmed that UVR has the potential to impair cercariae and cause disruption of cellular processes, and that cercariae of *M. novaezealandensis* possess only little capacity, if any, to prevent and cope with such effects.

CHAPTER SEVEN

Survival of an intertidal trematode cercaria: a multifactorial experiment with temperature, salinity and ultraviolet radiation

7.1 Abstract

Parasite transmission takes place in the context of a multitude of simultaneously fluctuating environmental factors. As a particularly vulnerable step in the transmission process, trematode cercariae and other free-living endohelminth stages are directly exposed to ambient conditions during their search for a host. Here, we investigated the survival of the cercariae of the intertidal trematode *Maritrema novaezealandensis* in a multifactorial experiment (2 x 2 x 2 design) with three main factors, i.e. temperature, salinity and ultraviolet radiation (UVR), which fluctuate substantially over space and time in intertidal habitats. All three factors had significant effects on the survival of the cercariae, with cercariae dying faster at the higher temperature, increased salinity and when exposed to UVR. Several factor interactions were identified, of which the interactive negative effect of temperature and UVR, based on effect sizes, was the most important. Salinity was the only factor without consistent significant interactive effects. These results imply that conditions during the main transmission window of *M. novaezealandensis* are physiologically highly challenging for cercariae. Our results highlight the importance of considering multiple environmental factors in the study of parasite transmission in order to gain a more ecologically relevant understanding of disease dynamics in natural systems.

7.2 Introduction

Natural variations and fluctuations have been shaping ecosystems over evolutionary time scales. However, changes in abiotic and biotic factors are occurring in this period of global change at unprecedented rates, scales and combinations (Vitousek *et al.*, 1997). These changes include a whole range of variables, well beyond climatic aspects only, which affect individual species as well as species interactions. The consideration of multiple environmental factors is of great importance in order to more accurately address ecologically relevant consequences of these factors in general and of global change in particular.

In intertidal ecosystems, natural fluctuations of environmental factors are pronounced, occurring on different spatial scales as well as various time scales. Compared to high tide, conditions at low tide may include a substantial warming of shallow water bodies, a concomitant increase in salinity due to evaporation and a direct exposure to ambient solar irradiation. Organisms in these ecosystems therefore experience a variety of challenging conditions including thermal, osmotic and ultraviolet radiation (UVR; 280 – 400 nm) stress (e.g. Przeslawski *et al.*, 2005). A range of studies have investigated the effects of multiple environmental factors on intertidal or other marine organisms (e.g. Fredersdorf *et al.*, 2009; Lenihan *et al.*, 1999; Lotze & Worm, 2002; Przeslawski, 2005; Przeslawski *et al.*, 2005; Russell & Phillips, 2009a; Russell & Phillips, 2009b), describing complex interactions not predictable from single factor experiments. Conditions in intertidal habitats may become more extreme or stochastic due to on-going global changes, including global warming and stratospheric ozone depletion. Therefore, a better understanding of the effects of multiple environmental factors is an important step towards accounting for natural complexity, thus limiting the risk of underestimating the ecological impacts of environmental conditions.

This is of particular relevance for the transmission of parasites. In intertidal ecosystems, trematodes are an important and ubiquitous component linking several members of an intertidal community through their complex life cycles (e.g. Lauckner, 1984; Mouritsen & Poulin, 2002b, 2010; Sousa, 1991). The transmission process of trematodes is dependent on usually more than one free-living larval parasitic stage directly exposed to ambient conditions (Pietrock & Marcogliese, 2003). In previous studies investigating the effects of temperature, salinity and UVR on the transmission process of the intertidal trematode *Maritrema novaezealandensis* from its first intermediate snail host (*Zeacumantus subcarinatus*) to one of

its second intermediate crustacean hosts (*Paracalliope novizealandiae*), the survival of cercariae (short-lived, non-feeding, translucent larval transmission stage, approx. 170 µm in length including tail, Martorelli *et al.*, 2004) was identified as the only step that was significantly affected by all three factors (Chapter Three, Four and Five). Results from these single factor experiments indicated that cercariae died faster when exposed to either high temperatures or high UVR, and that survival of cercariae was not compromised at normal to increased salinities (but was reduced at lower salinities).

The aim of the present study was to investigate the survival of the cercariae of *M. novaezealandensis* with a multifactorial experiment in order to identify factor interactions between temperature, salinity and UVR. Cercarial survival is a crucial step in the overall transmission success of a trematode. The number of cercariae successfully transmitting has important ramifications for infection levels (prevalence and infection intensities) in second intermediate crustacean hosts and will influence survival of these hosts through intensity-dependent mortality (e.g. Fredensborg *et al.*, 2004b). To date, most studies on the survival of cercarial transmission stages have focussed on single environmental factors which allow investigating a broader range of factor levels, but which do not account for the complexity of conditions encountered by these organisms in nature. Parasite transmission especially in marine environments has received little attention in the context of more ecologically relevant experimental approaches. The experiment described here is one of few attempting to include such environmental complexity, as well as a factor rarely considered as an environmental component in this context, namely UVR.

7.3 Materials and Methods

The experiment was a fully factorial 2 x 2 x 2 design (temperatures: 20 and 30°C; salinities: 35 and 40 practical salinity units (psu); exposed or not exposed to UVR). The experiment was conducted in two temperature controlled cabinets fitted with fluorescent tubes (Phillips TL20 W for UVB; Phillips TL40 W for UVA; Phillips, Aquarella for photosynthetically active radiation (PAR; 400 - 700 nm)), such that the doses administered per hour were 8.9 kJ m^{-2} (UVB) and 59.25 kJ m^{-2} (UVA), with a PAR energy flux of 250 - 350 µmol m^{-2}. Plexiglas filters were used which were either transparent or non-transparent to UVR (no UV treatment: 81% PAR, 5.2% UVA, 0% UVB; UVA+B treatment: 84.5% PAR,

84.6% UVA, 80.6% UVB) (for filter properties and transmission profiles see Lister *et al.*, 2010b or Appendix). Additionally, cellulose di-acetate filters were employed which absorb UVC radiation emitted from lamps. Temperatures were chosen to simulate an average summer tide pool condition at low tide (20°C) and an exceptionally warm, but realistic condition (30°C; Chapter Two). The two salinity solutions were prepared using artificial sea salt (Red Sea salt®). The salinity levels were chosen to mirror roughly the normal salinity level (35 psu), as well as an increase in salinity at low tide on hot sunny days due to evaporation (40 psu). The level of exposure to UVR was chosen to simulate summer conditions experienced by these organisms in the field in accordance with Lamare *et al.* (2007).

Zeacumantus subcarinatus snails were collected from Lower Portobello Bay, Otago Harbour, South Island (New Zealand) and screened for infection. Those found shedding *M. novaezealandensis* cercariae were maintained in the laboratory until the onset of the experiment. To obtain cercariae, 40 infected snails for each salinity were distributed into eight replicate Petri dishes containing 7 ml of the respective salinity solution and incubated for 1 h at 25°C under constant illumination (cold light source), which induced shedding of fully developed cercariae from infected snails. The cercariae were then combined (one mixture for each salinity) in order to generate a genetically mixed array of cercariae to be used in the experiment. Sixty microliters of the 35 psu cercarial mixture and 75 µl of the 40 psu mixture were then added into individual wells (10 replicate wells per treatment) of 96-well plates (wells 7 x 10 mm; total volume 320 µl), which corresponded to an addition of approx. 25 cercariae per well. The volume in each well was standardised to 100 µl of the respective salinity. Well plates were then covered with the filters for the appropriate irradiation regime and placed within the temperature cabinets (at 20 or 30°C, respectively). Survival and activity of cercariae were checked after 2, 4, 6 and 8 h of continuous exposure. Cercariae were classified as fully active, sluggishly motile or immotile/dead.

The proportions of fully active cercariae (arcsine-square root transformed to meet the assumptions of the statistical test used) after 2, 4, 6 and 8 h were analysed with a repeated measures ANOVA. For within-subject effects, multivariate results are reported since Mauchley's test of sphericity was significant, and this assumption therefore violated. Effect sizes (partial η^2) are presented in order to provide an estimation of the strength of each factor or factor interaction.

7.4 Results

Temperature was identified as the most important factor of all factors studied (see results and effect sizes in Table 7.1). In all cases, cercariae died faster at the higher temperature - at both salinities and when exposed or not exposed to UVR (Fig. 7.1). However, each of the factors individually had a significant effect on the survival and activity of cercariae. Cercariae also lost their functionality faster at the higher salinity and under exposure to UVR.

Table 7.1 Results from the repeated measures ANOVA on the effects of temperature, salinity and UVR on the survival and functional activity of *Maritrema novaezealandensis* cercariae (proportion of fully active cercariae, arcsine-square root transformed; n = 10 per treatment; with multivariate within-subject results).

Factor	**df**	**MS**	**F**	**p**	**Effect size**
Between subjects					
Temperature	1	14.10	617.90	< 0.001	0.896
UV radiation	1	0.41	17.87	< 0.001	0.199
Salinity	1	1.61	70.66	< 0.001	0.495
Temperature x UV	1	0.34	15.01	< 0.001	0.172
Temperature x salinity	1	0.06	2.51	0.118	0.034
UV x salinity	1	0.02	0.72	0.397	0.01
Temperature x UV x salinity	1	0.13	5.66	0.020	0.073
Error	72	0.02			
Within subjects					
Time	3	20.90	883.21	< 0.001	0.955
Time x temperature	3	3.00	204.97	< 0.001	0.751
Time x UV	3	0.21	9.15	< 0.001	0.177
Time x salinity	3	0.01	0.91	0.441	0.006
Time x temperature x UV	3	0.17	11.84	< 0.001	0.146
Time x temperature x salinity	3	0.11	6.19	0.001	0.1
Time x UV x salinity	3	0.03	2.41	0.074	0.031
Time x temperature x UV x salinity	3	0.06	2.90	0.041	0.056
Error (Time)	216	0.01			

Furthermore, there was a significant interaction between temperature and UVR, as well as a significant three-way interaction including all factors investigated. Salinity was the only factor which did not consistently have significant effects. Nevertheless, at 40 psu the proportion of fully active cercariae dropped to zero when exposed to UVR, at both temperatures; this was the only case where full activity ceased for cercariae kept at 20°C during the 8 h experiment (Fig. 7.1c). At 30°C, cercariae lost their full activity within 6 h, except at 35 psu and when cercariae were not exposed to UVR (Fig. 7.1b). The within-subjects results of the repeated measures ANOVA also indicate a significant time effect, with the proportion of fully active cercariae decreasing rapidly with time. The time effect was significantly interacting with most main factors and factor interactions (except with salinity and UV x salinity) (Table 7.1).

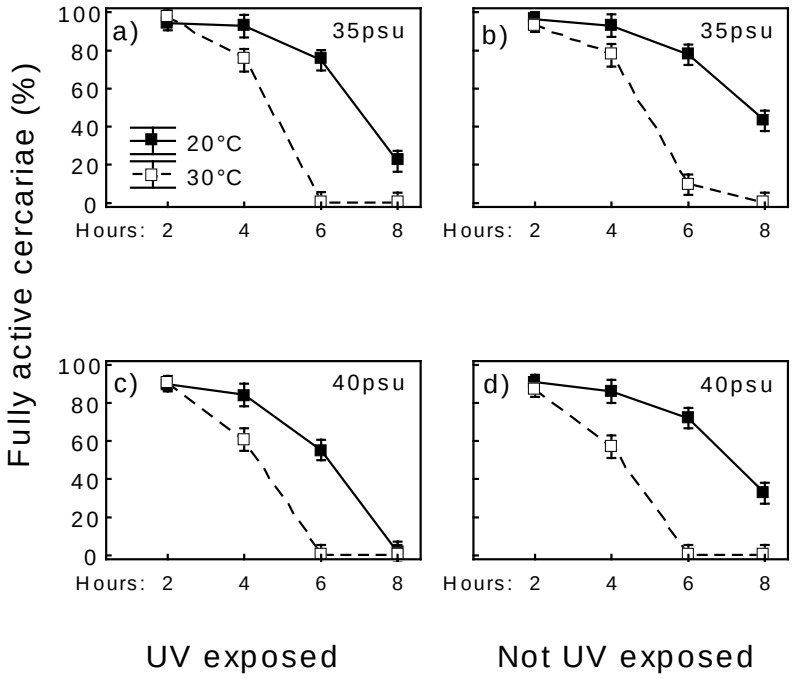


Figure 7.1 Survival, i.e. mean (± SE) proportion of functionally active individuals, of the cercariae of *Maritrema novaezealandensis* at 2, 4, 6 and 8 h post emergence from infected snail hosts. Results from a multifactorial experiment (2 x 2 x 2 design) with temperature, salinity and UVR as factors (i.e. 20 and 30°C, 35 and 40 psu, and exposed or not exposed to UVR; n = 10 per treatment).

7.5 Discussion

In intertidal ecosystems, temperature, salinity and UVR are known environmental stressors which have been shown to interactively affect the biota of these habitats (e.g. Przeslawski *et al.*, 2005). The transmission of intertidal parasites such as trematodes takes place within this environmental complexity, with the survival of cercariae being a key step in the life cycle of these parasites. The results presented here confirmed strong effects of especially temperature, but also salinity and UVR on the survival of the cercarial transmission stage of the intertidal trematode *M. novaezealandensis*. More importantly, the multifactorial design revealed significant interactions among the three factors investigated (Table 7.1, Fig. 7.1).

Results from single factor experiments (Chapter Three, Four and Five) indicated that cercariae died faster the higher the temperature and the more exposed to UVR they were, and that survival was not compromised at normal to increased salinities. The results from the multifactorial experiment presented here confirmed an increased mortality with increasing temperature and exposure to UVR, but also indicated a higher mortality in the high salinity treatment with an even higher effect size for salinity than that of UVR (Table 7.1). The observed effect of temperature is consistent across a range of previous studies (e.g. McCarthy, 1999; Mouritsen, 2002; Thieltges & Rick, 2006) and is based on increased metabolic rates and hence the faster depletion of the limited energy reserves available to cercariae under increased temperatures (Pechenik & Fried, 1995). The increased mortality under UVR exposure is also consistent with previous findings (Chapter Five) and may be mainly due to UV induced oxidative damage (Chapter Six). In contrast, the reason for the discrepancy between this study and our previous salinity study remains unclear, but supports the notion that predictions based on single factor experiments may not reflect outcomes of multifactorial experiments. Despite this, salinity was the only factor that did not consistently have a significant effect (e.g. no interaction between UVR and salinity).

Out of all factor combinations investigated, the two-way interaction between temperature and UVR seemed to be the most important, i.e. had the largest effect size. Interactive effects of temperature and UVR have been described in a range of other studies (e.g. Hoffman *et al.*, 2003; Przeslawski *et al.*, 2005). Including the effect of time, there was, however, also a significant interaction between temperature and salinity. Moreover, there was

a significant interaction between all three factors (also across time), highlighting the importance of considering multiple environmental factors in the study of parasite transmission.

Based on these findings, the conditions encountered by *M. novaezealandensis* cercariae during the main transmission window need to be revisited. Conditions during this window should be optimal for the transmission process to be successful (Combes *et al.*, 2002). The main transmission window of *M. novaezealandensis* is thought to occur during low tide in warmer months when water in tide pools warms up (Bates *et al.*, 2010; Fredensborg *et al.*, 2004b; Studer *et al.*, 2010). This triggers the emergence of the cercarial transmission stages from infected first intermediate snail hosts. Temperatures around 25°C are considered optimal for the overall transmission success (Studer *et al.*, 2010). Under these conditions, cercariae emerge from snail hosts in large numbers, with their slightly reduced survival at this temperature being counterbalanced by slightly higher infectivity (Poulin, 2006). However, conditions of optimal (as well as beyond optimal) temperatures often coincide with exposure to high levels of ambient solar irradiance during spring and summer, and may also be accompanied by slightly increased salinities, therefore posing an additional osmotic challenge. Considering the effects of all factors, exposure of cercariae to these conditions may lead to a considerably lower transmission success of *M. novaezealandensis* cercariae when compared to temperature effects only, due to the interactive negative effects of increased temperature and exposure to UVR and/or increased salinity on the survival of the cercariae.

However, further multifactorial experiments would be needed to investigate how other aspects of the transmission process would be affected, such as cercarial output from first intermediate snail hosts, infectivity of cercariae or susceptibility of the second intermediate hosts. Other factors adding to the complexity of natural systems may also be influential, thus making it difficult to predict how the overall transmission process would be affected in the actual habitat. For example, ambient vegetation in tide pools may provide shading for *M. novaezealandensis* cercariae from ambient UVR, therefore reducing the negative effect of this factor. Moreover, abiotic factors which have not been studied to date in *M. novaezalandensis* (e.g. dissolved oxygen or pH) may further modify the effects and interactions described here.

In conclusion, all three environmental factors investigated in this multifactorial experiment strongly affected the survival of the *M. novaezealandensis* cercariae. Of all factors

studied, temperature had the most pronounced effect while the interaction between temperature and UVR emerged as the factor interaction with the highest effect size. Other factor interactions were identified, including the complex three-way interaction between all factors investigated. Conditions during the main transmission window of this parasite are thus considered physiologically highly challenging for the survival of *M. novaezealandensis* cercariae. The results described highlight the importance of considering interactions between multiple environmental factors in order to account for the complexity of natural systems in which parasites and their hosts are integrated. Due to the complex interactions among environmental factors, predictions made based on single factor experiments need to be interpreted with great care, which is of particular relevance in all climate change research.

CHAPTER EIGHT

Biotic interference in parasite transmission: Can the feeding of anemones compensate an increased risk of parasitism in amphipods at high temperatures?

8.1 Abstract

The transmission of parasites is embedded in the complexity of natural systems and is influenced not only by prevailing abiotic conditions but also the presence of community members. In particular, temperature affects the number of transmission stages released into the environment as well as their survival and infectivity. Temperature, however, also influences the metabolic rate and feeding activity of non-host organisms. We tested the hypothesis that at higher temperatures the increased feeding activity of the anemone *Anthopleura aureoradiata* interferes with the transmission of the intertidal trematode parasite *Maritrema novaezelandensis* by reducing the number of cercarial transmission stages successfully infecting *Paracalliope novizealandiae* amphipod hosts. In a microcosm experiment with two temperatures (15 and 22°C) and three densities of anemones (0, 5 and 10 per microcosm), only a significant effect of temperature was found, with more parasites infecting surviving amphipods at the higher temperature. The effect of anemone density was not significant, although there was a trend towards fewer parasites infecting amphipods when anemones were present at 22°C. Our results show that although there may be some interference by a non-host species, this effect might not be strong enough to mitigate increased transmission success at higher temperatures. However, mortality of amphipods at 22°C with low and medium anemone density may reflect an increased transmission pressure and thus parasite-induced mortality, therefore masking the actual outcome of this experiment based on surviving amphipods. All findings, however, point towards a higher risk of parasite-induced mortality of small crustaceans with global warming.

8.2 Introduction

The transmission of free-living stages of parasites such as larval endohelminths takes place within the continuously changing complexity of the environment. During transmission, these larval parasite stages are directly affected by ambient biotic and abiotic conditions. For example, members of the community of organisms in which the transmission processes are embedded, can alter the transmission success of (or the disease risk from) a parasite through consumption of infective stages (Johnson *et al.*, 2010; Johnson & Thieltges, 2010; Thieltges *et al.*, 2008b). Simultaneously, prevailing abiotic conditions provide a framework in which biotic processes such as parasite transmission take place. These conditions not only directly affect a parasite, but all the hosts and non-hosts of a community and therefore also the interactions between them.

In marine ecosystems, non-host predators and filter-feeders such as anemones and molluscs have been found to prey on the transmission stages of trematode parasites (Kaplan *et al.*, 2009; Mouritsen & Poulin, 2003; Prinz *et al.*, 2009; Thieltges *et al.*, 2008a; Thieltges *et al.*, 2009), which are the dominant parasite group in coastal ecosystems (Mouritsen & Poulin, 2002b). For instance, the anemone *Anthopleura aureoradiata* living on the shell of cockles (*Austrovenus stutchburyi*) has been shown to decrease parasite transmission to these cockles by preying on trematode cercariae which use cockles as intermediate hosts (Mouritsen & Poulin, 2002b). The same anemone has also been shown to ingest cercariae of the trematode *Maritrema novaezealandensis* (the parasite species used in the present study) and thus reduce the transmission success of this parasite from its first intermediate snail host to second intermediate crab hosts (Hopper *et al.*, 2008).

Abiotic factors, in particular temperature, are well known to influence survival and infectivity of trematode transmission stages (e.g. Pietrock & Marcogliese, 2003). Furthermore, the output of trematode cercariae from first intermediate mollusc hosts is also strongly influenced by temperature, with generally more cercariae emerging with increasing temperature (Poulin, 2006), at least up to an optimum temperature level (Studer *et al.*, 2010). Poulin (2006) therefore suggested that trematodes may find favourable conditions for transmission more often in a warming world. However, in a warmer world, the feeding demands of predators or filter-feeders will also increase due to the temperature dependence of metabolic rates (e.g. Schmidt-Nielson, 1997; Stone & Johnston, 2000; see also Sanford, 2002

and references therein for a range of intertidal consumers). Hence, an increase in transmission success might actually be compensated by an increased consumption of infective stages under warmer conditions (Thieltges *et al.*, 2008b).

The combined effect of high temperature and increased parasite transmission is known to have caused a massive parasite-induced die-off of amphipod hosts in an intertidal soft-sediment ecosystem with drastic ecosystem-wide consequences (Jensen & Mouritsen, 1992; Mouritsen *et al.*, 1998). Considering that the mortality of crustacean hosts, in particular *Paracalliope novizealandiae* amphipods, induced by *M. novaezealandensis* is also known to be intensity-dependent (Bates *et al.*, 2010; Fredensborg *et al.*, 2004b), the transmission success of this parasite will also determine the impact of the parasite on affected amphipod populations and possibly the entire crustacean host community. In light of on-going and predicted climate changes, especially global warming, a better understanding of the complex interactions of abiotic and biotic factors on parasite transmission is therefore of great importance.

The objective of this study was to investigate the combined effect of temperature and the presence of anemones at different densities, on the transmission of the trematode parasite *M. novaezealandensis* to an amphipod host (*P. novizealandiae*). We determined whether the presence a known predator of these cercariae has the potential to protect amphipods from increasing parasite loads in a warming climate. If cercariae are consumed by anemones, then it was hypothesised that the consequences of increasing temperatures may not actually lead to an increase in parasite transmission stages successfully infecting hosts, as increased anemone feeding rates may reduce the number of transmission stages remaining in the system.

8.3 Materials and Methods

A microcosm experiment was conducted as a 2 x 3 design with two temperatures (15 and 22°C) and three densities of *A. aureoradiata* anemones (0, 5 or 10 anemones per microcosm). Anemones were collected from Hooper's Inlet (Otago Peninsula, New Zealand) a week prior to the experiment in order to allow for adequate acclimatisation to laboratory conditions and in order to minimise their feeding. On local mudflats, these anemones are very patchily distributed and occur in dense aggregations in some areas whereas they are absent in

others. Thus, our experimental densities reflected a range of natural densities in the field. Infected first intermediate snail hosts (*Zeacumantus subcarinatus*) were used from stock aquaria (snails collected from Lower Portobello Bay, Otago Harbour, New Zealand) in order to obtain the cercarial transmission stages (see below). Uninfected *P. novizealandiae* amphipods were also collected from Hooper's Inlet a few days prior to the experiment. In this locality, *Z. subcarinatus* snails are absent and amphipods have never been found infected by any trematode species (Bryan-Walker *et al.*, 2007; Fredensborg *et al.*, 2004b; Studer *et al.*, 2010). The temperature levels used in this experiment were chosen to mirror conditions where transmission and activity of organisms are low (15°C) and conditions where transmission should be optimal and feeding activity of organisms elevated (22°C) (Studer *et al.*, 2010).

Either 0, 5 or 10 anemones were placed into round plastic containers (300 ml total volume; 8.5 cm high; 7 cm diameter at the bottom; i.e. density of anemones per 38.5 cm^2) which were filled with 100 ml of seawater (five replicate containers per treatment). Containers were then incubated at either 15 or 22°C for 6 h according to the duration of a low tide period. Towards the end of this period, amphipods were added to the containers (n = 10 per container). Also, infected snails (n = 40; five snails in eight replicate Petri dishes containing 7 ml of seawater) were incubated for 1 h at 25°C under constant illumination, triggering the release of cercarial transmission stages. The seawater containing emerged cercariae was then combined in order to use a genetic mixture of parasites in the experiment. Approx. 400 cercariae (average age of about 1 h; max. life span < 24 h) from this cercarial mixture were then added to each container. Under constant illumination, containers with anemones, amphipods and cercariae were then incubated for 24 h at the respective temperature (i.e. 15 or 22°C). Subsequently, amphipods were transferred into new plastic containers filled with approx. 300 ml of aerated seawater, provided with a strip of sea lettuce (*Ulva* sp.) for food and shelter and stored at 15°C for 2 - 3 d. Surviving amphipods were then sexed, measured (size classes: 2.5, 3.0, 3.5, 4.0 and 4.5 ± 0.25 and > 4.75 mm) and dissected under a dissecting microscope in order to determine the number of parasites infecting each amphipod.

Due to mortality of amphipods, we tested for the effect of temperature and anemone density on the number of surviving amphipods per replicate using a Generalised Linear Model fitted with a Poisson error structure. Only amphipods that survived until dissection were included in the subsequent analysis, for which amphipods from the different replicates had to

be pooled. A General Linear Model (Gaussian error structure) was used to test for the effects of temperature, anemone density, their interaction, sex and size class of amphipods on the number of parasites infecting the surviving amphipods (square root transformed).

8.4 Results

The number of surviving amphipods was significantly affected by temperature, but not anemone density, with a mean number of 7.27 ± 0.42 and 4.40 ± 0.55 (± standard error as for all following results) amphipods surviving at 15 and 22°C, respectively (Generalised Linear Model, Poisson; temperature: $\chi^2 = 11.57$, df = 1, p = 0.001; anemone density: $\chi^2 = 2.16$, df = 2, p = 0.339). The number of surviving amphipods was lowest in the low and medium anemone density treatments at 22°C (Fig. 8.1). In total, 175 amphipods were recovered (74 females, 99 males, 2 unknown; at 15°C: n = 31 (0 anemone/container), n = 43 (5 anemones/container), n = 35 (10 anemones/container); at 22°C: n = 22 (0 anemone/container), n = 14 (5 anemones/container) and n = 30 (10 anemones/container)), of which 96.6% were infected.

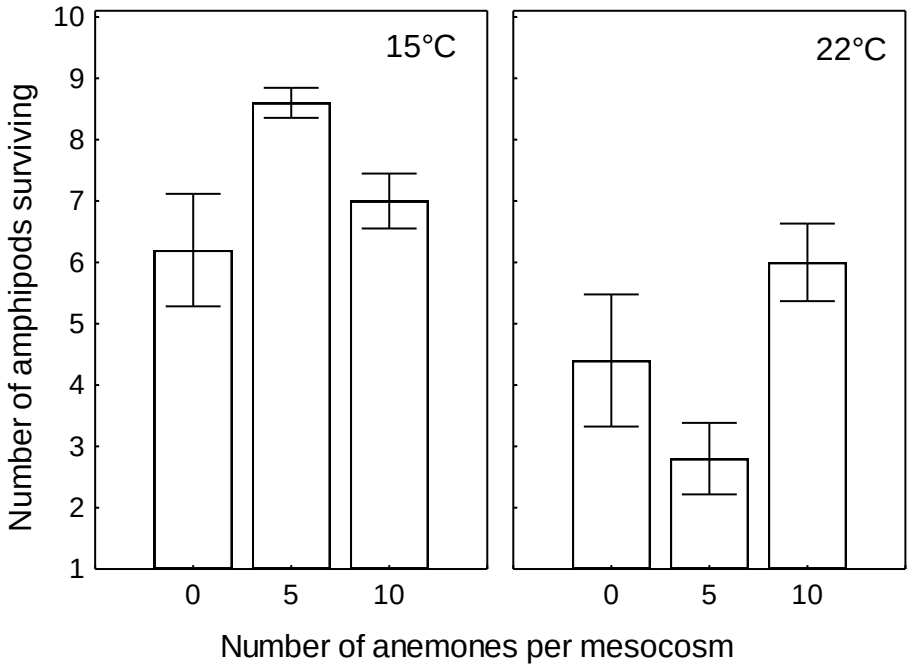


Figure 8.1 Mean number (± standard error) of amphipods surviving until dissection at two temperatures (15 and 22°C) and three densities of *Anthopleura aureoradiata* anemones (0, 5 or 10 anemones per microcosm). There were five replicate containers in each treatment to which 10 amphipods were originally added.

Temperature, but not anemone density (or their interaction), had a significant effect on the number of parasites infecting surviving amphipods (Table 8.1). At 22°C, amphipods harboured on average 9.5 ± 0.7 parasites, whereas those at 15°C had 6.3 ± 0.4 (Fi . 8.2). At 15°C, the presence of anemones had no influence on the transmission, whereas some effect, although not significant, was apparent at 22°C with slightly reduced numbers of parasites in treatments where anemones were present in medium and high densities (Fig. 8.2). Sex and size of amphipods did not significantly influence the number of parasites infecting the amphipods (Table 8.1).

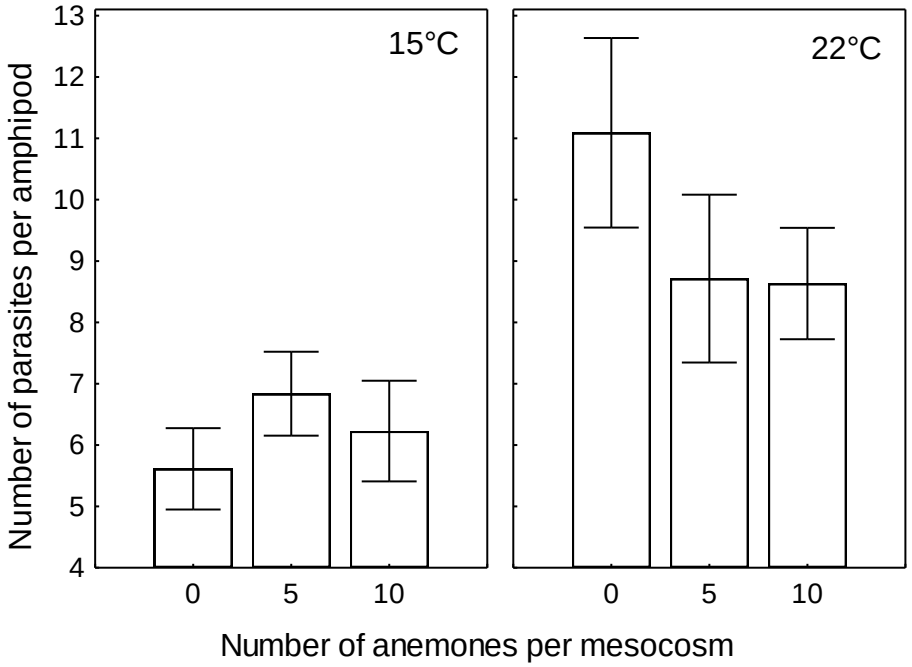


Figure 8.2 Mean number (± standard error) of *Maritrema novaezealandensis* parasites infecting amphipod hosts (*Paracalliope novizealandiae*) at two temperatures (15 and 22°C) and three densities of *Anthopleura aureoradiata* anemones (0, 5, 10 anemones per microcosm).

Table 8.1 Results from a General Linear Model assessing the effects of temperature and density of anemones (including their interaction) as well as amphipod sex and size class, on the number of *Maritrema novaezealandensis* parasites infecting amphipods (square root transformed).

Factors	**df**	**MS**	**F**	**p**
Temperature	1	13.96	16.23	< 0.001
Anemone density	2	0.62	0.72	0.490
Temperature x density	2	1.26	1.47	0.234
Amphipod sex	1	0.05	0.06	0.815
Amphipod size	4	0.48	0.56	0.690
Error	162	0.86		

8.5 Discussion

We hypothesised that a higher feeding rate of *A. aureoradiata* anemones might compensate or reduce the increased transmission success of *M. novaezealandensis* cercariae under increased temperatures. However, our study did not completely support this: the presence of anemones was not strong enough to interfere with the greater transmission success of the parasite to surviving amphipods at higher temperatures. Amphipods at 22°C compared to 15°C were more infected and the presence of anemones did not significantly reduce the number of parasites successfully infecting surviving amphipod hosts.

Given that *A. aureoradiata* anemones were shown to cause a more than four-fold reduction in the number of *M. novaezealandensis* acquired by crab hosts and that they were by far the most effective community member investigated in a previous study (Hopper *et al.*, 2008), it is surprising that we could not conclusively confirm this role for the transmission process to amphipod hosts. A direct assessment of the feeding of anemones at the experimental temperatures was not conducted and thus it remains unclear to what extent temperature indeed influenced the feeding rate of the anemones. However, the number of parasites infecting amphipods in our experiment was highest at 22°C and when no anemones were present (Fig. 8.2), suggesting that the presence of anemones had at least some influence on the transmission process (this was not the case at 15°C). Irrespectively, the result suggests that anemones might not be capable of substantially reducing the number of cercariae present in a system at higher temperatures due to an increased feeding activity.

Nonetheless, it is likely that amphipods that died during the experiment, and were not included in the dissection and analysis due to fast decomposition, may have been the ones that were most infected. The highest number of amphipods died at 22°C when zero or five anemones were present per container (see results, Fig. 8.1). These should be the treatments with the highest expected infection levels, and mortality of amphipods may therefore directly reflect the high infection pressure under these conditions (coupled with an exposure to 22°C for 24 h). This may provide some indirect evidence for a buffering role of anemones if they are present in high densities. Parasite-induced amphipod mortality may thus be responsible for the non-significant outcome of the statistical analysis which only included infection levels of surviving amphipods; actual infection levels of all amphipods right after the experimental

infection may be considerably underestimated. Therefore, the results available from the surviving amphipods may not adequately match the actual outcome of this experiment.

Other mechanisms may however also be responsible for the observed lack of an effect of anemone density. It is possible that the feeding activity or the densities of the anemones was not high enough to interfere with the number of cercariae added to the microcosms. However, densities adequately reflected the natural range and anemones have been shown to feed on *M. novaezealandensis* cercariae in similar experiments conducted in our lab (Hopper *et al.*, 2008). Alternatively, the number of cercariae added may have been too high for the anemones to clearly reduce, therefore masking any effect the anemones may have. However, infected *Z. subcarinatus* snails are known to release much higher numbers of cercariae (Studer *et al.*, 2010) and the number of cercariae added in this experiment is probably rather conservative for natural systems. During the main transmission window of *M. novaezealandensis*, i.e. on warm sunny days when the water in tide pools warms up, several snails can simultaneously release readily developed cercarial transmission stages within a relatively small area, leading to a "burst" of cercariae emerging (Fredensborg *et al.*, 2004b; Keeney *et al.*, 2007b). Under such conditions, amphipods, and any other crustacean, may be exposed to hundreds if not thousands of parasites at a time. In our experiment, the same number of cercariae was added to each micocosm regardless of treatment. In reality, the number of cercariae released into the environment by first intermediate snail hosts would be substantially higher at 22 than at 15°C (Fredensborg *et al.*, 2005; Studer *et al.*, 2010) and therefore also the density of cercariae to which crustaceans would be exposed to. This may further overcome any losses to predation by anemones and thus increase the risk of parasite-induced amphipod mortality under these conditions.

It is concluded that despite the fact that community members have the potential to strongly interfere with and regulate the transmission of trematodes and hence influence infection levels in host organisms, this effect might not be strong enough to counteract increased transmission success of trematode cercariae at higher temperatures. This supports that host species such as amphipods are at an increased risk of intensity-dependent, parasite-induced mortality under increased temperatures, and that this may be particularly the case in an ecosystem with low species diversity and densities. The role of co-inhabiting organisms with ongoing and predicted global changes clearly needs to be further investigated. The combined study of abiotic and biotic factors in disease ecology is relatively novel despite the

fact that climate change, the unprecedented loss of biodiversity, species invasions and increasing diseases and parasitism have all been recognised as major challenges of global concern (e.g. Sala *et al.*, 2000). The acknowledgement that host-parasite interactions are integrated within complex and dynamic ecological communities exposed to a network of fluctuating abiotic conditions must now be followed by targeted research to provide a more realistic understanding of how these components are functionally linked.

CHAPTER NINE

Local effects of a global problem: modelling the risk of parasite-induced mortality in an intertidal trematode-amphipod system

9.1 Abstract

The interactive effects of climate change and parasitism have been of particular concern in terrestrial as well as marine ecosystems, because of potentially important consequences for host populations, communities and entire ecosystems. In marine environments, the absence of historic baseline data on parasitism and disease-related aspects limits our ability to make realistic predictions about the consequences of climate change. Here, we adapted a simulation model developed for a Northern hemisphere intertidal host-parasite system to a comparable system in the Southern hemisphere. We modelled the entire life cycle of the intertidal trematode parasite *Maritrema novaezealandensis* in order to investigate the interactive effect of parasitic infections and increasing temperatures on the population dynamics of *Paracalliope novizealandiae* amphipod hosts, based on climate forecasts for the forthcoming decades. We focussed on a mudflat where prevalence in hosts is known to be high, as these hosts may be particularly vulnerable to further increases in temperature and the consequences of infection. The model was parameterised with data from the field, experiments and the literature. Despite considerable uncertainties associated with the model and its parameterisation, most temperature increases that were predicted to cause the collapse of the modelled amphipod population in the long term lay within the range of predicted warming for the study area. The high vulnerability of the amphipods in the modelled system illustrates a potentially important ecological mechanism, by which consequences of a global problem might manifest on the local level.

9.2 Introduction

Ecological processes are highly influenced by climatic conditions (e.g. Mysterud *et al.*, 2001; Ottersen *et al.*, 2001; Stenseth *et al.*, 2002). As a consequence, anthropogenically induced global changes have already been linked to significant alterations in these processes (Parmesan, 2006; Parmesan & Yohe, 2003; Root *et al.*, 2003; Walther, 2010; Walther *et al.*, 2002). Besides direct effects of environmental conditions on individual organisms or species, it is becoming increasingly apparent that these effects also extend to biotic interactions due to differential impacts on, or responsiveness by, individual ecological components (e.g. Gilman *et al.*, 2010; Walther, 2010). This can lead to altered interaction strengths and may have cascading effects through ecological networks such as food webs (e.g. Ottersen *et al.*, 2001; Stenseth *et al.*, 2002; Walther, 2010). Not only trophic relationships or competitive interactions are affected, but also interactions between parasites and their hosts (Mouritsen & Poulin, 2002a).

The importance of parasites and pathogens in relation to climate change, especially global warming, has been of major concern in many systems (e.g. Dobson *et al.*, 2003; Harvell *et al.*, 2002; Kutz *et al.*, 2005; Poulin & Mouritsen, 2006; Rohr *et al.*, 2011). In marine environments, there has also been considerable concern about increasing diseases and parasitism coinciding with on-going climate change (Harvell *et al.*, 2004; Ward & Lafferty, 2004). However, in the absence of historic baseline data and due to the lack of long-term monitoring programs that include parasites, our ability to assess the influence of climate change on parasitism and diseases is limited. As a consequence, determining whether diseases have been increasing in the ocean can only be attempted indirectly in hindsight (see Ward & Lafferty, 2004). On the other hand, simulation models have been used to forecast how predicted climate change, especially global warming, and parasitism may impact on marine host populations (see Mouritsen *et al.*, 2005).

Parasites are integral components of marine ecosystems (e.g. Mouritsen & Poulin, 2002b; Sousa, 1991). Moreover, parasites play crucial ecological roles, for example by regulating host population dynamics or influencing community structure and food webs (e.g. Hudson *et al.*, 1998; Mouritsen & Poulin, 2002b, 2005, 2010). By definition, parasites depend on one or more hosts to complete their life cycle; each component of a life cycle is likely to be differentially affected by environmental parameters, and thus the effects of climate change on

these systems are likely to be complex as well as species- and context-dependent (Marcogliese, 2001, 2008). Climatic or environmental conditions modulate the extent and intensity of parasitism in hosts (Cattadori *et al.*, 2005; Hudson *et al.*, 2006a; Kutz *et al.*, 2005; Mouritsen & Poulin, 2002a; Poulin & Mouritsen, 2006) through effects on the parasite itself, on its hosts (e.g. host condition and hence susceptibility), and/or on the interaction between hosts and parasites (Harvell *et al.*, 2009; Lafferty, 2009; Rohr *et al.*, 2011). As a consequence, changes in climate or in the local environment are bound to affect levels of parasitism with potentially important repercussions not only for host individuals, populations, communities and ecosystems - but ultimately also for the parasite's ability to complete its life cycle (Dobson *et al.*, 2008; Rohr *et al.*, 2011).

In particular, parasites with complex life cycles and/or those with ectothermic hosts should be disproportionally affected by climate changes such as global warming (Harvell *et al.*, 2002; Marcogliese, 2008). Trematode parasites have complex life cycles and usually depend on three hosts to complete their life cycle. Within the first intermediate mollusc host, trematodes asexually produce large numbers of their free-living transmission stage (cercariae). The fact that the production of these cercariae is highly temperature-dependent has led to the prediction that trematodes may find suitable conditions for transmission more often in a warming world (Poulin, 2006). However, the larger numbers of trematode transmission stages potentially present in a system also raise the risk of parasite-induced mortality of second intermediate hosts (Poulin & Mouritsen, 2006). The impact of a parasite on second intermediate hosts will not only depend on temperature, but also on pre-existing local conditions. For instance, there can be considerable differences between the proportions of infected first intermediate hosts even between populations in close proximity (e.g. Fredensborg & Poulin, 2006). Hence, we would only expect second intermediate hosts to be at risk of parasite-induced mortality with increasing temperatures in areas where prevalence in first intermediate snail hosts is high. However, increasing temperatures may also positively influence the reproduction of hosts, which may counterbalance, to some extent, an increased pressure of parasitism (Neuparth *et al.*, 2002).

In 1990, intertidal *Corophium volutator* amphipods as well as the snail hosts of two microphallid trematode parasites experienced a massive die-off event in the Danish Wadden Sea, which was inferred to have been caused by a combination of unusually high temperatures during a heat wave and the concomitant massive release of parasitic transmission stages of the

trematodes (Jensen & Mouritsen, 1992; Mouritsen *et al.*, 1998). Because of the crucial ecological role of *C. volutator* in stabilising the sediment, the die-off event had substantial ecosystem-wide consequences. This event has lent great support to the importance of the interactive effects of unusual environmental conditions and parasitism on marine host populations. It also stimulated a considerable amount of research on this system (e.g. Mouritsen, 2002; Mouritsen & Jensen, 1997), which led to the construction of a model to assess the system dynamics in the face of predicted climate warming, in particular the effects on the highly vulnerable amphipod hosts (Mouritsen *et al.*, 2005).

In the present study, we applied the same modelling approach to a host-parasite system from the Southern hemisphere (i.e. South Island, New Zealand), which is comparable to the above mentioned European Wadden Sea trematode-amphipod system. Here, the focus was on the intertidal microphallid trematode *Maritrema novaezealandensis* and the hosts associated with its complex life cycle (Martorelli *et al.*, 2004). This parasite, similar to the microphallids in the *C. volutator* system, uses birds attracted to mudflats, such as red-billed gulls (*Chroicocephalus scopulinus*), as definitive hosts in which adult worms live in the intestine and reproduce sexually. *M. novaezealandensis* eggs pass out with the bird's faeces and may get ingested by first intermediate hosts, mudsnails (*Zeacumantus subcarinatus*), foraging on soft-sediment intertidal mudflats. Within a snail host, the parasite replaces the snail's gonads thus castrating it, and begins the asexual production of large numbers of its transmission stages (cercariae). Cercariae emerge from infected snails into the environment in order to infect second intermediate crustacean hosts. A range of crustaceans are suitable second intermediate hosts for *M. novaezealandensis* (Koehler & Poulin, 2010). Here, we focussed on the amphipod *Paracalliope novizealandiae*, which is a highly abundant species on local mudflats and is believed to be an important component of intertidal food webs (Fredensborg *et al.*, 2004b; Thompson *et al.*, 2005). Within a crustacean host, the parasite matures into a cyst stage (metacercaria) and awaits trophic transmission to a final bird host. *Paracalliope novizealandiae* has not only been shown to be highly infected during warm summer months in the field (Chapter Two), but also to be affected by parasite-induced mortality (Bates *et al.*, 2010; Fredensborg *et al.*, 2004b) and to be highly susceptible to high temperatures (Studer *et al.*, 2010). Importantly, these amphipods have never been found infected with any metazoan parasite other than *M. novaezealandensis* within the study area.

The spatial focus of this study was a mudflat where the proportion of first intermediate snail hosts that are infected with *M. novaezealandensis* is particularly high (i.e. Lower Portobello Bay, Otago Harbour; Fredensborg & Poulin, 2006). It is expected that global warming may cause *P. novizealandiae* amphipod populations from high prevalence areas to be not only at an increased risk of exposure to higher mean temperatures and more frequent and extreme heat waves, but also to repeated bursts of parasitic transmission stages, due to the sensitivity of the transmission process of *M. novaezealandensis* to temperature (Chapters Two and Three). This may increase the risk of temperature as well as parasite-induced mortality of amphipods and thus lead to the collapse of local amphipod populations. The parasite, as well as the temperature-mediated mortality, may only lead to a short-term loss of amphipods from a limited area; however, if occurring repeatedly, such events may prevent population recovery and cause local extinctions. Moreover, the loss of a host from a specific locality may ultimately affect the parasite's ability to complete its life cycle (Dobson *et al.*, 2008). Although the model is parameterised for Lower Portobello Bay conditions, its general structure is applicable to any other set of local parameters.

The aim of the present study was thus to model the dynamics of the entire life cycle of *M. novaezealandensis* and in particular the amphipod host population, on a mudflat where prevalence in first intermediate snail hosts is high. How global warming may influence this system was then explored. The questions addressed were: 1) At what temperature increase is the modelled amphipod population of Lower Portobello Bay no longer sustainable? 2) How do changes in amphipod reproduction affect this critical temperature threshold? And 3) what short-term extreme event (heat wave) would be necessary to cause an immediate (i.e. within a year) population collapse?

9.3 Materials and Methods

Model formulation. The study and the model of Mouritsen *et al.* (2005) provided the basis for the current assessment of *M. novaezealandensis* and *P. novizealandiae* amphipods, allowing direct comparison between the two systems. The model links equations simulating the flow of the parasite through its complex life cycle: the dynamics of metacercariae (M) within adult amphipods (A), the population of adult worms (H) in the definitive bird hosts,

and the number of infected snails (I) present in a system. The model was adapted accordingly to the system used in the present study (see Table 9.1 for explanation of symbols):

$$\frac{dM}{dt} = I * \lambda_I * \beta_{IM} - \alpha_A * M - \alpha_{AT} * M - \mu * M/A \quad \text{(Metacercariae)}$$

$$\frac{dA}{dt} = \lambda_{JA} * J - \alpha_A * A - \alpha_{AT} * A - \mu \quad \text{(Adult amphipods)}$$

$$\frac{dH}{dt} = e^{-a*H} * \mu * M/A - \alpha_H * H \quad \text{(Adult worms)}$$

$$\frac{dI}{dt} = \beta_{HI} * \gamma_I * H * \lambda_H - \alpha_I * I \quad \text{(Infected snails)}$$

Table 9.1 Symbol specifications for each parameter used in the model equations.

Model parameters	
λ_I	Number of cercariae produced per infected snail per day
β_{IM}	Proportion of cercariae shed by infected snails that successfully transmit to amphipods and thus remain in the system
γ_{adjust}	Adjustment term for laboratory based estimates of cercarial production and transmission
λ_{JA}	Maturation rate of juvenile amphipods into adults
α_A	Daily mortality rate of amphipods based on a maximum life span
α_{AT}	Daily temperature-dependent mortality rate of amphipods
μ	Number of amphipods ingested per day by definitive hosts
δ	Amphipod breeding activity
λ_A	Rate of amphipod fecundity per individual per day
σ	Density-dependent reduction in amphipod recruitment with increasing population size
α_H	Daily mortality rate of adult worms in the definitive host
a	Density dependent reduction in the proportion of ingested parasites which successfully establish in the definitive host with increasing worm population size
β_{HI}	Proportion of parasite eggs excreted by birds that are ingested by the snail population at 15°C
γ_I	Temperature-dependent modifier of the rate at which snails encounter parasite eggs (calibrated to 1 at 15°C)
λ_H	Fecundity of adult worms per parasite per day
α_I	Daily mortality of infected snails

As in Mouritsen *et al.* (2005), the amphipod population was the only host component dynamically modelled. In the present study, we distinguished between juvenile (J) and adult (A) amphipods (see below), but only considered consequences for the total amphipod population. The juvenile amphipods were included in the model as follows:

$$\frac{dJ}{dt} = A * \delta * \lambda_A * e^{-\sigma A} - \alpha_{AT} * J - \lambda_{JA} * J \qquad \text{(Juvenile amphipods)}$$

Model parameterisation. Data from the field (Chapter Two), experiments (especially Chapter Three, Fredensborg *et al.*, 2004b) and from the literature (especially Mouritsen *et al.*, 2005 and references therein) were used to parameterise the model. Parameter estimates were scaled to 1 m^2 of the sediment surface and expressed as per day. The number of cercariae produced per infected snail per day (λ_I) and the proportion of those cercariae that successfully transmit to amphipods (β_{IM}) are temperature (T)-dependent (Studer *et al.*, 2010). Although cercarial survival is also temperature-dependent (Studer *et al.*, 2010), the decrease in survival with increasing temperature is generally compensated by higher infectivity up to an optimum temperature (approx. 25°C), such that transmission efficiency is relatively constant up to that temperature level (McCarthy, 1999; Poulin, 2006), after which there is a combined negative effect on cercariae (Studer *et al.*, 2010). Hence, cercarial survival was not explicitly included in the model, but is incorporated as a component of the overall transmission efficiency (β_{IM}) (see also Mouritsen *et al.*, 2005). Both the output rate and transmission efficiency of *M. novaezealandensis* cercariae increase up to 25°C, after which they drop off (Studer *et al.*, 2010). These patterns were each described by fitting linear regression lines to the means from 16 to 25°C and from 25 to 30°C, respectively ($r^2 \geq 0.99$). Because these parameters are known to be influenced by other environmental factors (e.g. ultraviolet radiation, Chapters Four and Five) and in order to allow for a correction of the laboratory data to more field relevant rates, an adjustment term (γ_{adjust}) was incorporated in these equations. Cercarial output (λ_I) per infected snail per day was described as:

$\lambda_I = 0$ T < 16°C
$\lambda_I = (13.68T - 215.89) * \gamma_{adjust}$ 16°C < T < 25°C
$\lambda_I = (-17.67T + 571.55) * \gamma_{adjust}$ T > 25°C

And the transmission success rate of cercariae was described as:

$\beta_{IM} = 0$ T < 16°C
$\beta_{IM} = (0.062T - 0.24) * \gamma_{adjust}$ 16°C < T < 25°C
$\beta_{IM} = (-0.039T + 1.39) * \gamma_{adjust}$ T > 25°C

Adult amphipod breeding activity was based on data from the seasonal sampling on Lower Portobello Bay (Chapter Two). Females with eggs can be found all year round but at different proportions (24%, 43%, 31% and 9% in spring, summer, fall and winter, respectively; overall 53% females of which 66% were gravid). The daily rate at which new amphipods are produced (λ_A) was based on a maximum number of four broods per female

amphipod lifetime (estimate consistent with Mouritsen *et al.*, 2005) and an average brood size of 7.7 eggs (Chapter Two). For an assumed nine month amphipod life span (i.e. 270 d, based on Chapter Two and Fredensborg *et al.*, 2004b), the daily rate of offspring production per gravid female thus equalled 0.1141. Juvenile amphipods produced at this rate were assumed to mature into adults (i.e. size ≥ 2.5 mm) within three months, so their instantaneous rate of maturation was set to 0.01. Juveniles are rarely found infected even during summer and if so only with very few parasites (A. Studer, personal observation). Hence, they were included in the model separately, only being affected by natural mortality. The daily mortality rate of adult amphipods (α_A) was a combination of natural mortality (assumed maximum life span of nine months) and parasite-induced mortality dependent on intensity of infection (estimated from Fredensborg *et al.*, 2004b), such that

$\alpha_A = 0.0037 + 0.005(M/A)$

($r^2 > 0.95$)

Additionally, both juveniles and adult amphipods in the model were subjected to temperature-induced mortality (α_{AT}). Mortality of amphipods in the laboratory has been shown to be 100% within 2 h at 34°C, and 100% within 2 d at 30°C (Studer *et al.*, 2010). At temperatures between 20 and 30°C, 50% mortality occurred within about 10 - 12 d. Below 20°C, there was almost no mortality during the experiment, so the default mortality rate is used based on a nine months life span. All survival related parameters and equations were corrected to field-relevant rates based on an eight-fold difference between rates from the laboratory and the estimated nine month survival of amphipods in the field.

The number of adult amphipods ingested by definitive hosts per day (μ) was set to 6.42 during the main bird season from October to January and 1.28 for the rest of the time. This estimation was based on seasonal differences in bird abundance on the high prevalence mudflat of Lower Portobello Bay (abundance October to January: 156.53 (mostly gulls), rest of the year: 31.28 on an approx. 3.6 ha area; Chapter Two) and an estimated feeding rate of individual birds visiting mudflats adapted from the literature, since no data are available for the study system used here. The estimate (ingestion of 4.1 amphipods per minute per bird during one 6 h low tide per day, i.e. half of the value provided in Mouritsen *et al.*, 2005) was based on the assumption of a relatively low predation pressure by birds on *P. novaezealandensis*, as studies have shown that amphipods in general may only constitute a

relatively small part of the gut content of birds, at least in the case of gulls (McClatchie *et al.*, 1989; Wootton, 1997).

The daily mortality of adult worms in the definitive bird host (α_H) was set at 0.1, based on a 10 d life span (Fredensborg & Poulin, 2005; Ginetsinskaya, 1988; Mouritsen *et al.*, 2005). The rate of adult worm fecundity per parasite per day (λ_H) was set at 55 eggs, based on an in vitro study by Koehler *et al.* (submitted). These parasite eggs are excreted onto the mudflat by the definitive bird hosts. Due to temperature effects on the feeding activity of snails (Hylleberg, 1975), the rate at which uninfected snails encounter parasite eggs (β_{HI}) is also temperature dependent. β_{HI} was set to 0.299 based on the value estimated in Mouritsen *et al.* (2005). Also, the same equations were included in the present model as specified in Mouritsen *et al.* (2005), due to the lack of data for our particular study system. The equations encompassed a modifier term (γ_I) calibrated to equal 1 at 15°C ($r^2 > 0.99$), such that:

$\gamma_I = 0$	$T < 10°C$
$\gamma_I = 0.19T - 1.85$	$10°C < T < 20.3°C$
$\gamma_I = 0.237T - 2.785$	$T > 20.3°C$

The daily mortality of infected snails ($\alpha_I = 0.00106$) was based on a mean life span of uninfected snails of five years and an estimated mortality rate of infected snails of 1.93 times that of uninfected snails (Fredensborg *et al.*, 2005).

Model simulation. Numerical simulations were conducted to explore the model properties. Because no data were available to estimate density-dependence in the amphipods (σ) and the adult worm population (a), as well as the appropriate value for the adjustment term (γ_{adjust}), the model was analysed to determine which parameter combination yielded the model output that best fitted the available field data (Chapter Two). The parameters were simultaneously estimated so that the total amphipod population peaked at 1,500 amphipods m^{-2}, the maximum number of infected snails was about 210 m^{-2} and the maximum mean infection intensity in amphipods was 12 metacercariae per amphipod.

The temperature data used in the present model were collected during a one-year temperature recording period with loggers deployed in tide pools on the Lower Portobello Bay mudflat (Chapter Two). Daily mean temperatures were used here. The effect of an overall increase in temperature was then assessed by increasing the daily mean temperatures year round in intervals of 0.1°C, to determine the new equilibrium dynamics or to identify an

amphipod population crash. The climate change predictions for the study area (i.e. Otago, New Zealand) are, for the full range of scenarios, an increase of 0.6 - 1.3°C until 2040 and 1.3 - 2.8°C until 2090, respectively (http://www.niwa.co.nz/our-science/climate/information-and-resources/clivar/scenarios) (0.7 - 1.1°C by 2040 and 0.9 - 2.6 by 2090 based on Reisinger *et al.*, 2010). The model was run for 80 years in each instance, except for the heat wave assessment. For the heat wave scenario, the model was explored to assess the temperature increase necessary to drive the amphipod population to an immediate collapse (model starting on January 1st, i.e. austral summer; temperature increase on-going).

Because there are a considerable number of sources of variability and possible errors in the model, sensitivity analyses were performed. For this, all model parameters were varied individually ± 25% and ± 50% around their estimated value and the respective critical temperature increase at which the amphipod population is predicted to crash was determined.

The model was elaborated and simulations were run using ModelMaker version 4.0 (Cherwell Scientific Ltd).

9.4 Results

Parameter estimation

The optimised parameter values were 0.0017 for the density-dependent reduction in amphipod recruitment with increasing population size (σ), 337.52 for the density-dependent reduction in adult worms (a), and 0.2009 for the adjustment term correcting laboratory based into field relevant relationships (γ_{adjust}). The adjustment term indicated that the laboratory derived relationships for the production of cercariae and their transmission efficiency were reduced by a factor of about five in the model. Using these values, the total amphipod populations at equilibrium dynamics fluctuated yearly between 1,052 and 1,495 amphipods m^{-2} (Fig 9.1). The number of infected snails fluctuated between 176 and 210 m^{-2}, and the mean infection intensity of amphipods between less than one and twelve metacercariae per amphipod (Fig. 9.2).

Effects of global warming

An increase of the mean daily temperature of 0.7°C was sufficient to drive the modelled amphipod population of Lower Portobello Bay to extinction in the long-term (Fig. 9.1). At an increase of 0.6°C, the amphipod population still maintained itself, fluctuating between 888 and 1,173 amphipods m^{-2} (Fig. 9.1), while the number of infected snails peaked at 257 m^{-2} and the mean infection intensity of amphipods increased to 29 metacercariae per amphipod (Fig. 9.3). With an increase of 0.7°C, a threshold was exceeded which caused a sharp increase in the parasite-related parameters leading to a spike in the number of metacercariae and thus causing the amphipod population to collapse. This is likely due to non-linearities in the model structure (Mouritsen *et al.*, 2005).

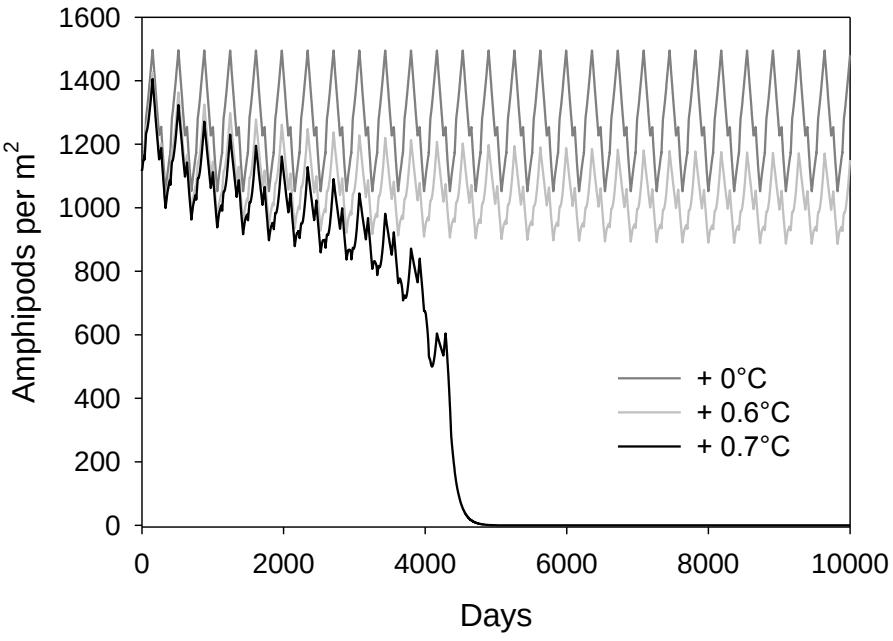


Figure 9.1 Yearly population fluctuations of amphipods per m^2 under different climate warming scenarios. Default model (dark grey line) with population trajectory under current conditions, predicted population trajectory with an increase of the mean daily temperature of 0.6°C (light grey line), and predicted population decline with an increase of 0.7°C (black line). Day 0 denotes January first (austral summer). The model was run over 80 years; only trajectories over 10,000 days (i.e. approx. 27 years) are shown in this graph.

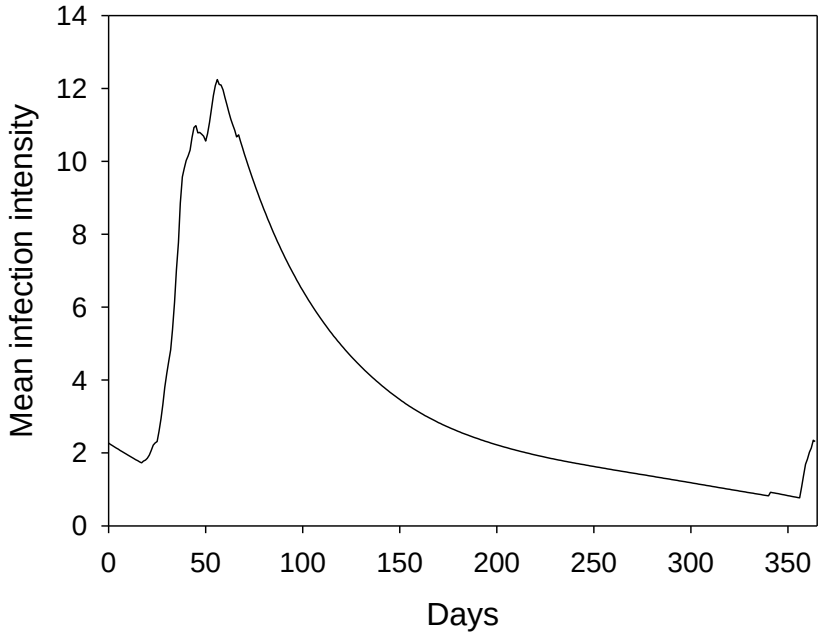


Figure 9.2 Mean number of parasites per infected amphipod (mean infection intensity) over the course of a year at equilibrium population dynamics under current climatic conditions. Day 0 denotes January first (austral summer).

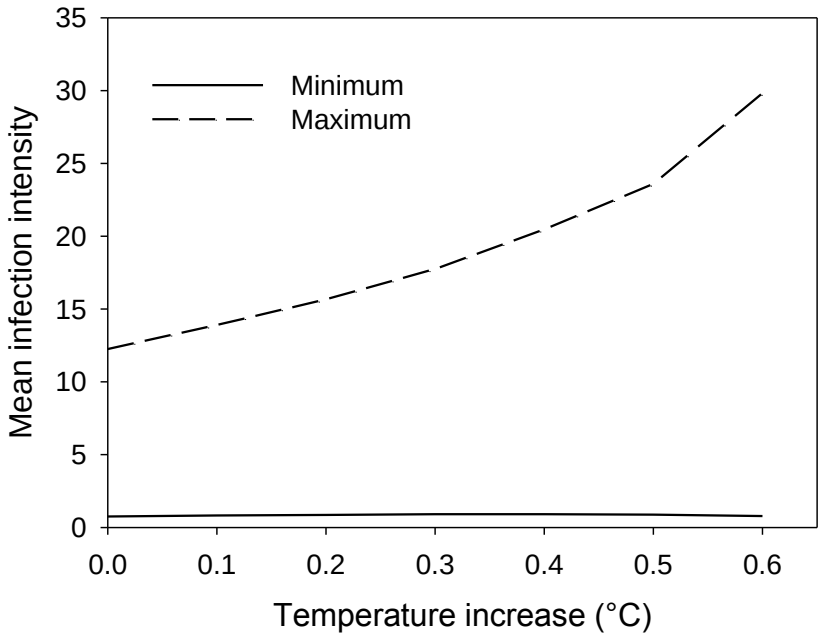


Figure 9.3 Minimum (solid line) and maximum (dashed line) mean infection intensities (number of metacercariae per infected amphipod) with increasing mean daily temperature at equilibrium population dynamics.

The sensitivity analyses showed that when varying the estimated parameter values, the temperature increase at which the amphipod population of Lower Portobello Bay is predicted to collapse in the long-term lies between 0°C and + 3.2°C (Table 9.2). The most influential parameters were all related to the amphipods: the proportion of amphipods breeding, the fecundity of amphipods, the density-dependence of the amphipod population, and the predation of amphipods by birds (Table 9.2).

Table 9.2 Results from the sensitivity analyses on all model parameters, indicating the temperature increase (in °C) at which the amphipod population collapse in the long-term is predicted. All model parameters were varied individually ± 25% and ± 50% around their estimated value.

	Parameter	**-50%**	**-25%**	**±0%**	**+25%**	**+50%**
λ_I	Production of cercariae	1.4	0.9	0.7	0.4	0.2
β_{IM}	Transmission of cercariae	1.4	0.9	0.7	0.4	0.2
γ_{adjust}	Adjustment term	2.2	1.3	0.7	0.2	0
α_A	Amphipod mortality natural	1.5	1.1	0.7	0.1	0
α_A	Amphipod mortality parasite induced	1.3	0.9	0.7	0.4	0.3
α_{AT}	Amphipod mortality temperature induced	0.7	0.7	0.7	0.6	0.6
μ	Amphipod predation by birds	2.4	1.7	0.7	0	0
λ_A	Amphipod fecundity	0	0	0.7	2.3	3.2
δ	Proportion of amphipods breeding	0	0	0.7	2.3	3.2
λ_{JA}	Maturation rate of juvenile amphipods	0.4	0.7	0.7	0.6	0.6
σ	Density-dependence of amphipods	3.0	2.0	0.7	0	0
a	Density-dependence of adult worms	0	0.4	0.7	0.8	1.0
λ_H	Adult worm fecundity	1.4	0.9	0.7	0.4	0.2
α_H	Adult worm mortality	0.6	0.6	0.7	0.7	0.7
β_{HI}	Parasite egg ingestion by snails	1.4	0.9	0.7	0.4	0.2
α_I	Mortality of infected snails	0	0.4	0.7	0.8	1.1

Considering the temperature sensitivity of amphipod reproduction and the importance of these parameters on the predicted increase necessary to drive the amphipod population to extinction, the model was also used to assess at what temperature increase the amphipod population would collapse, 1) when the proportion of amphipods breeding during winter was set equal to the proportion breeding in spring, and 2) when the proportion of breeding amphipods in all seasons, the fecundity of amphipods and the maturation rate of juvenile amphipods were simultaneously increased by 50% each. Increasing winter temperatures have been identified as a main consequence of global warming (IPCC, 2007). Assuming that, as a

consequence, amphipod breeding would be similar in winter and spring, the temperature increase necessary to drive the amphipod population to extinction under such conditions was predicted by the model to be 1.9°C. When simultaneously raising the amphipod reproduction parameters, the amphipod population remained viable up to an increase of 4.9°C, after which it crashed.

Another prediction regarding global warming is the increase in the frequency and severity of extreme events such as heat waves (IPCC, 2007). Hence, the model was also explored to assess the temperature increase necessary to drive the amphipod population to an immediate collapse. Model outputs indicated that the increase would have to be in the order of about 14°C in order to bring the modelled population to an immediate crash (Fig. 9.4), but the population was already pushed to very low levels at substantially lower temperature increases; levels at which extinction due to stochastic effects may readily occur.

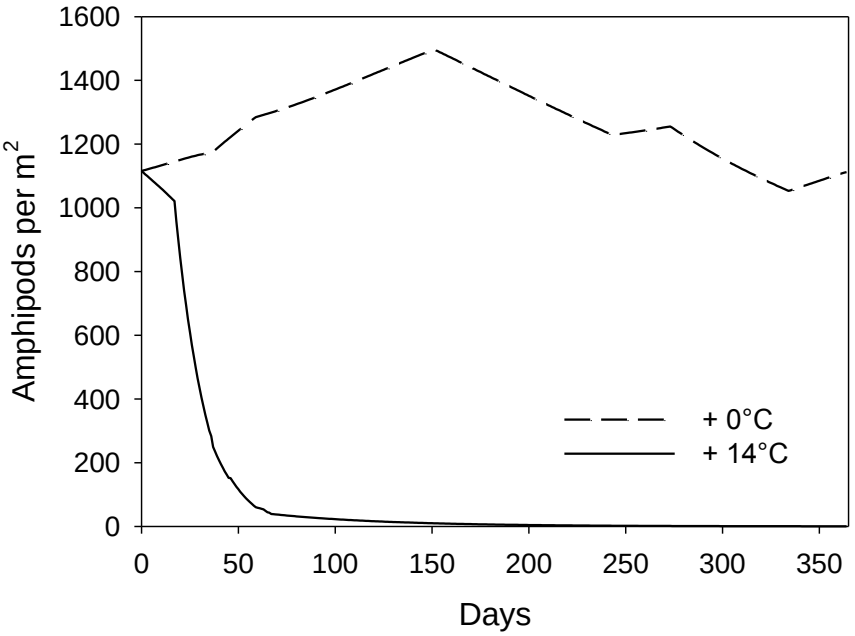


Figure 9.4 Number of amphipods per m^2 over a yearly cycle at default equilibrium dynamics (dashed line) and predicted trajectory of the amphipod population for an increase of 14°C under a heat wave scenario (solid line).

9.5 Discussion

The most striking finding of this study is the sensitivity of the modelled *Paracalliope novizealandiae* amphipod population to the trematode parasite *Maritrema novaezealandensis*. Even increases which are at the lower end of the expected range of temperature increase for the study area, were predicted to affect the amphipod population of the Lower Portobello Bay mudflat, despite the fact that conservative parameter estimates were used throughout the model. Moreover, even when varying the parameters to account for possible variation and errors, most predicted temperature increases at which the amphipod population may collapse, were still within the predicted range of temperatures likely to prevail in the next 80 years. The predictions regarding the collapse of this amphipod population are therefore relatively robust, even if some of the temperatures are beyond those predicted to occur in terms of mean temperature increases over the coming decades.

The model developed for the European *Corophium volutator* amphipod population served as a basis for the present model. In addition to the model developed by Mouritsen *et al.* (2005), the current model was extended to differentiate between juvenile and adult amphipods, and to include temperature-induced amphipod mortality. Also, an adjustment term was included which not only served to correct laboratory derived data for the production and transmission of *M. novaezealandensis* to more field-relevant rates, but also incorporated the effect of other environmental factors (besides temperature) known to influence aspects of the transmission process of this parasite (e.g. Chapter Four and Five).

There were a number of similarities as well as striking differences between the model system studied by Mouritsen *et al.* (2005) and the present system. Both of the systems are known to be highly dependent on environmental factors (Chapters Two, Three and Four, Meissner & Bick, 1999a; Mouritsen, 2002; Studer *et al.*, 2010), hence their sensitivity to climatic fluctuations and changes. In both cases, increasing temperatures are expected to increase parasite transmission as well as parasite-induced mortality (Bates *et al.*, 2010; Fredensborg *et al.*, 2004b; Jensen & Mouritsen, 1992; Mouritsen & Jensen, 1997; Studer *et al.*, 2010). However, *C. volutator* amphipods in the Danish Wadden Sea can reach densities up to 100,000 m^{-2}, while *P. novizealandiae* in the study area occur at much lower densities. The higher density of *C. volutator* is likely to buffer the amphipod population up to higher temperatures, despite the fact that not only the density of infected snails is higher than in the

P. novizealandiae system, but also predation by birds during migration periods in the Wadden Sea. The high density of *C. volutator* is probably responsible for the 'decline' in the amphipod population with increasing temperatures, rather than a long-term collapse as observed in the present study. However, even the *C. volutator* system was predicted to collapse when amphipod reproduction parameters were altered (Mouritsen *et al.*, 2005). Furthermore, the temperature increases necessary to cause the collapse or decline in the amphipod populations were consistent in both models; mostly being within the ranges predicted to occur in each part of the world over the coming decades.

The model presented here was the most realistic we were able to construct based on current knowledge. The model is, however, limited by several issues (see also Mouritsen *et al.*, 2005). For example, the model is limited by the fact that it cannot take into account any other ecological changes that may occur with on-going and predicted climate change, which may directly or indirectly affect the hosts, the parasite and/or the interaction between them. For instance, sea level rise is expected to cause the loss of intertidal habitats, especially in areas where inland movement is restricted. This may fundamentally alter any of the ecological processes occurring in intertidal systems, including those associated with the life cycle of parasites.

In addition, the model does not incorporate the adaptive or evolutionary potential of any of the components involved. Both of these issues are likely to alter the predictions made here, but we are confident that they are unlikely to change them completely. Furthermore, the amphipod population in the default model was handled such that the maximum density observed in the field sampling would be reached (i.e. 1,500 m^{-2}; Chapter Two), though the modelled population did not fluctuate to the extent that would be expected (approx. 500 - 1,500 m^{-2}). The model simulation outcomes may have been different had the fluctuations more closely matched observations from the field. However, it seems reasonable to assume that the system may have been even more sensitive, as zero amphipod density would have been reached with even smaller temperature increases.

There are also considerable uncertainties associated with some of the model parameters due to the lack of appropriate data for some aspects of the study system (see Methods). For example, there is substantial uncertainty associated with the definitive bird hosts, as they are one of the least investigated components of the life cycle of *M. novaezealandensis*. Moreover,

multiple effects of global changes on the occurrence and behaviour of birds are possible, and thus their effects on the study system span a range of possibilities. On one hand, it is possible that the number of birds and the time they spend in the area may increase due to an increased abundance of food (Mouritsen *et al.*, 2005 and references therein). This may prolong the period in which the entire life cycle of *M. novaezealandensis* may take place (see also Chapter Two) and thus may increase the impact of the parasite on the studied amphipod population. On the other hand, the opposite effect is also possible, with fewer birds visiting the areas due to feeding grounds or migration routes shifting elsewhere or due to the loss of suitable feeding grounds following sea level rise (e.g. Galbraith *et al.*, 2002), thus lowering the chance of the completion of the parasite's life cycle. Furthermore, at this stage, only one definitive host species has been identified for *M. novaezealandensis* (Fredensborg *et al.*, 2004a). However, trematodes often show little host specificity at the level of the definitive host and thus other birds attracted to local mudflats may also be involved in the life cycle. Any aspects related to other possible definitive hosts, however, remain unresolved and thus could not be appropriately incorporated into the model. Nevertheless we are still confident that, with the sensitivity analyses, we were capable of capturing a realistic range of possibilities for the variation of the critical temperature increase capable of driving the local amphipod population to collapse.

The consequences of global warming for the reproduction of amphipods are likely to be a key factor for this and comparable systems in nature. Based on the sensitivity analyses and other model simulations (see Results), increasing amphipod reproduction parameters substantially prolonged the viability of the amphipod population, hence increasing the critical temperature for a parasite-induced collapse. Increasing winter temperatures are particularly likely to benefit the amphipods' reproductive output. However, amphipods may only be positively influenced by increasing temperatures (see Neuparth *et al.*, 2002) up to an optimum and many intertidal organisms are thought to already live close to their thermal tolerance levels (e.g. Hofmann & Todgham, 2010; Stillman & Somero, 2000). With increasing temperatures especially during warmer months, periods when temperatures may exceed the optimal temperature range of the amphipods are thus likely to become more frequent, with negative rather than positive consequences for the amphipod's reproductive output (Mouritsen *et al.*, 2005; Wiklund & Sundelin, 2001). Moreover, observations from the field also indicated that large and hence reproductively active amphipods were absent from summer samples and were also considered most at risk for parasite-induced mortality (Chapter Two). However,

little information is available on the thermal biology of *P. novizealandiae* amphipods and thus it remains unclear at this stage at what critical temperature threshold a positive effect may become negative. Based on Studer *et al.* (2010) and Chapter Two, stressful temperature levels for amphipods are likely to be reached even under current conditions during warm periods in summer. Such periods may not only negatively affect the reproduction of amphipods, but also affect their immune response (Le Moullac & Haffner, 2000; Roth *et al.*, 2010) and thus enhance the impact of parasitism. Hence, during an extreme event, the combined effect of the parasite and the high temperatures would be expected to have an immediate, strong negative effect, which may override any positive effect on amphipod reproduction that an increase in mean temperatures over longer time scales may have.

These considerations also raise another limitation of the model. We used mean daily temperatures across an entire year, ignoring daily temperature fluctuations in the microhabitat where parasite transmission takes place. However, the use of maximum daily temperatures would have exacerbated the high vulnerability of the amphipod population to temperature increases, and thus was not additionally explored. The highest mean daily temperature measured by the temperature loggers was 20.3°C, whereas the maximum value was 26.5°C. The maximum temperature measured directly in the field in tide pools was just over 30°C (Chapter Two). These temperature peaks are, however, only brief and using maximum temperatures may have considerably overestimated the effect of a temperature increase on the modelled amphipod population. Instead, to explore the effect of a large, short-term temperature increase, i.e. simulating a heat wave event, we estimated the temperature increase at which the modelled amphipod population is predicted to collapse within a few months (see Results). An increase of about 14°C was found to be necessary to cause such a collapse of the modelled population, despite the fact that the population was no longer sustainable in the long-term at far lower increases. Considering the maximum temperatures measured in the field, this values lies about 4°C above current maximum temperatures. Due to the high temperature fluctuations in intertidal systems, such extreme values may eventually be reached even if the mean temperature increase is only moderate. However, it remains unclear if the temperature increase would be sustained for long enough to have such drastic consequences.

There are considerable spatial differences in infection levels of first intermediate snail hosts across mudflats, and thus the effect of the parasite on second intermediate host populations is expected to be highly localised. For example, removing the parasite from the

modelled system would push the critical temperature for a collapse in the long-term up to 5.1°C. The difference between the predicted thresholds of +0.7°C in a high prevalence area and +5.1°C in a no parasite area, for the amphipod host population to no longer be sustainable, clearly highlights the importance of the impact of the parasite on local host populations with global warming. Similarly, at the level of an individual mudflat, the organisms involved in the life cycle of *M. novaezealandensis* also tend to be patchily distributed, despite the fact that areas with many snails also tend to have many amphipods (Chapter Two). The model developed here does not take into account such spatial variation. Spatial variation is, however, likely to influence the recovery potential of an affected population. For example, Bates *et al.* (2010) found that low shore amphipods were less infected than their upper shore counterparts. Hence, if upper shore amphipods were to experience a die-off event, low shore amphipods may act as a source population allowing a rapid re-colonisation of an affected upper shore area. Such factors influencing the resilience of a vulnerable amphipod population associated with a mudflat where infection levels are high, including the connectivity of populations across mudflats and thus the potential for dispersal and re-colonisation across larger spatial scales, remain a challenge for future investigations.

Snail first intermediate hosts are also a highly crucial component in the life cycle of *M. novaezealandensis* that require further consideration. When compared to the amphipod hosts, the snail population is expected to be relatively stable. However, the consequences of climate warming on the snails are also uncertain. A moderate increase in temperature may increase the reproduction and development of these snails, hence increasing the number of susceptible snail hosts present in the system. Snail activity, parasite egg encounter and thus parasite recruitment into a snail population may also be increased. Moreover, parasite development within snail hosts would also be accelerated, therefore enhancing the impact of the parasite on second intermediate crustacean hosts. On the other hand, infected snails have a higher mortality than uninfected snails under prolonged exposure to high temperatures (Fredensborg *et al.*, 2005). Under relatively high temperatures and especially during heat wave events, snail mortality may occur, affecting particularly infected individuals. This may lower the density of infected snails in a high prevalence area and thus reduce the impact of the parasite on second intermediate host populations.

Under certain circumstances, the loss of a given species from a system may thus also imply the loss of associated and dependent species, such as parasites (e.g. Dobson *et al.*, 2008). For *M. novaezealandensis*, the loss of a second intermediate host such as the amphipods studied here may not be vital for its overall fitness, due to the redundancy of second intermediate hosts. Different second intermediate crustacean host species are expected to be differentially affected by temperature and the combined effect of temperature and parasites, but only *P. novizealandiae* has been investigated thus far. However, such differential responses would likely affect the host community structure of a high prevalence mudflat with increasing temperatures. For parasites lacking such host redundancy, the loss of a given host from a system may be more substantial. Indeed, even for *M. novaezealandesis,* this may be an issue as this parasite is highly host specific to its first intermediate snail host: a die-off of these snails may thus have serious negative consequences for the sustained presence of the parasite in the system.

In conclusion, the modelled amphipod population was found to be highly vulnerable to temperature increases, with most increases predicted to cause the collapse of the Lower Portobello Bay population lying within the range of temperatures expected for the study area over the coming decades. However, the model is associated with a range of limitations, uncertainties and possible errors, which may be responsible for differences seen between the modelled system and actual occurrences in nature. Nonetheless, although the exact temperature at which the amphipod population would collapse may differ from what is predicted by the current model, it seems likely that the collapse of this amphipod population is, consistent with Mouritsen *et al.* (2005), not a question of if, but rather when. Simulation models provide a powerful tool to explore the possible impacts of climate change on ecological systems and to formulate predictions. However, long-term monitoring efforts are necessary to verify and detect the actual consequences for ecosystems in nature, and urgent actions are imperative to potentially prevent such changes from occurring.

CHAPTER TEN

General discussion

The central question of this thesis was how global changes influence parasitism in marine ecosystems. The intertidal microphallid trematode *Maritrema novaezealandensis* and hosts associated with its complex life cycle were investigated as a model system. Firstly, knowledge of the current seasonal dynamics of the parasite and its hosts in nature provided a baseline for a better understanding of the life cycle dynamics of this parasite (Chapter Two). Secondly, studies of potential environmental modulators (temperature, salinity and ultraviolet radiation (UVR); Chapter Three, Four and Five) of the parasite's transmission between first and second intermediate host provided an estimation of their importance as factors and identified vulnerable steps in the transmission process of the parasite. For UVR, assessments of damage and protection provided novel insights into the mechanisms by which UVR affects the transmission stage of this parasite (Chapter Six). Thirdly, the consideration of multiple abiotic factors (Chapter Seven) and the combined consideration of an abiotic and a biotic factor (Chapter Eight) enabled the identification of interactive effects and the inclusion of more realistic complexity into the study of parasite transmission. Finally, the enhanced knowledge and understanding of the study system enabled the use of a modelling approach in order to investigate how global warming may affect the life cycle dynamics of this parasite and specifically, what the consequences may be for an amphipod host population on a mudflat where prevalence of infection in first intermediate snail hosts is high (Chapter Nine).

The field study (Chapter Two) confirmed that infection levels in first intermediate *Zeacumantus subcarinatus* snail hosts were high in the study locality (Lower Portobello Bay, Otago Harbour, New Zealand). This translated into a strong seasonal pattern in prevalence and infection intensities in second intermediate *Paracalliope novizealandiae* amphipod hosts. The observations supported that temperature is likely to be a main driver in the transmission of this parasite. The study also provided data on the dynamics and availability of all hosts associated with the life cycle. In summer, high infection levels in amphipod hosts coincided with a peak in the abundance of known and potential definitive bird hosts, as well as the highest density of snail hosts, in particular small and still uninfected snails. This indicated that all transmission steps in the life cycle of *M. novaezealandensis* are mainly occurring during summer months and that they are probably greatly accelerated compared to colder months. There was, however, no indication that, as expected for normal circumstances and consistent with Fredensborg *et al.* (2004b), the parasite would exert a strong negative effect on affected amphipod host populations, despite the fact that larger amphipods were not present in our summer samples. However, the high infection levels in amphipod hosts are strong evidence

that infections during summer can occur in bursts (see also Keeney *et al.*, 2007b) and that they have great potential to cause parasite-induced mortality in *P. novizealandiae* amphipods (Bates *et al.*, 2010; Fredensborg *et al.*, 2004b). These observations suggest that raising winter temperatures may increase successful transmission of the parasite during colder months, especially due to the accelerated development of the parasite in snail and crustacean hosts. This also suggests that especially under unusual circumstances such as heat waves, there is a high risk of parasite-induced mortality in second intermediate amphipod hosts especially due to the large numbers of transmission stages being released into the environment (see also Chapter Three).

Climate change is expected to particularly affect ectothermic invertebrates (e.g. Hofmann & Todgham, 2010). Both intermediate hosts of *M. novaezealandensis* are ectotherms. In addition, because parasitic transmission stages are also known to be highly sensitive to environmental conditions (Pietrock & Marcogliese, 2003), the laboratory studies investigating the effects of selected naturally fluctuating environmental factors were focussed on the transmission from first intermediate snail host to second intermediate amphipod host, which takes place via free-living larval cercarial transmission stages. This allowed not only the analysis of individual transmission steps, but also to infer overall net effects of each environmental factor. Due to their substantial variability in intertidal ecosystems, especially at low tide when transmission processes in tide pools would occur, the environmental factors investigated were temperature (Chapter Three), salinity (Chapter Four) and UVR (Chapter Five). All of them emerged as potentially strong modulators of the parasite's transmission process.

Based on the individual responses of each transmission step (Fig. 10.1.), results indicated that overall, granted that temperatures do not exceed a certain optimum, moderately high temperatures are expected to benefit the transmission of *M. novaezealandensis*, including the parasite development within amphipod hosts. This may, however, increase the risk of parasite-induced mortality of amphipod hosts. Moreover, if temperatures exceed an optimum level, direct negative effects on the parasite as well as the amphipods may be pronounced.

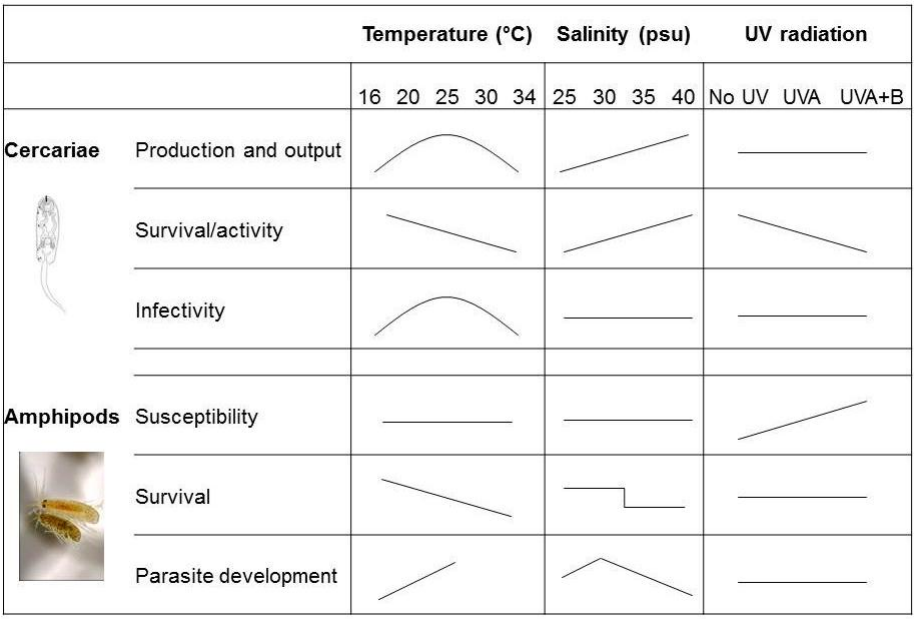


Figure 10.1 Summary of the responses of each step of the transmission process to the range of experimental factors (temperature, salinity and ultraviolet (UV) radiation) and their levels.

Salinity effects on the transmission steps were less pronounced than temperature effects, but indicated that the parasite and its amphipod host are differentially affected (Chapter Four; Fig. 10.1.). Most importantly, it was shown that during conditions which are considered to be optimal for transmission, osmotic conditions would be mainly beneficial for the parasite's longevity in the environment and thus increase the chance of successful transmission to a second intermediate host. This also indicated that low salinities may present a distributional boundary for the parasite.

During the optimum window for transmission (low tide, warm), conditions in tide pools can thus be considered beneficial in terms of both, temperature and salinity (but see Chapter Seven). However, with regards to levels of solar irradiation and in particular UVR, conditions seem less optimal. UVR has rarely been incorporated into ecological parasitology research and thus provided a great opportunity to extend the study of UVR effects into the realm of intertidal parasites. Despite some inconclusive results regarding some steps of the transmission process, UVR particularly emerged as a strong modulator of the survival of cercarial transmission stages, decreasing their survival in a dose-dependent manner (Chapter Five). UVR also increased the susceptibility of amphipod hosts to infection. Hence, UVR has a negative effect on the parasite as well as the amphipod host (Fig. 10.1.), but these effects may be compensatory.

In Chapter Six, some of the potential mechanisms behind the reduction in survival of cercariae after exposure to UVR were analysed. Their high vulnerability to UVR was concluded to stem from the fact that they are very small, translucent and designed for only a short free-living phase. The study revealed that only limited, if any, investments are made into protective mechanisms in these transmission stages. Despite the presence of UV absorbing compounds (especially mycosporine-like amino acids) in the tissue where the cercariae are being produced, no evidence for their presence in cercariae directly could be found. Moreover, cercariae exposed to UVR showed high levels of oxidative stress, as well as an increase in DNA damage (i.e. a higher concentration of cyclobutane-pyrimidine dimers). Hence, UVR effects on the parasite seem relatively decisive, rather than subtle, with pronounced deleterious effects after reaching a certain threshold.

The only step of the transmission process that emerged as being clearly affected by all three environmental factors was the survival of cercariae; hence this step was further investigated with a multifactorial experiment (Chapter Seven). This multifactorial experiment was conducted in order to assess the relative importance of each factor and to identify important factor interactions. Overall, it was found that temperature was the most influential factor studied. Moreover, significant interactions were found, especially the interaction between temperature and UVR, but also the three-way interaction including all environmental factors considered. The salinity effect was not consistent with what was found in Chapter Four, highlighting the need to better incorporate environmental complexity into the study of parasite transmission. Moreover, considering a scenario of increasing temperatures and on-going high levels of UVR due to ozone depletion, it might be expected that the combined negative effect of temperature and UVR on the survival of cercariae may contribute to control infection levels in second intermediate hosts. Considering a scenario of increasing temperatures and lowered UVR due to the recovery of the ozone layer, it might be expected that the strong selective pressure on cercariae may be reduced, thus further benefiting the parasite.

Another step attempting to better account for the complexity in natural systems in which parasite transmission takes place, was the concomitant consideration of a biotic (i.e. the presence of a community member known to be capable of interfering with the transmission process, Hopper *et al.*, 2008) and an abiotic factor (i.e. temperature) (Chapter Eight). The aim of this microcosm experiment was to investigate if a known predator of cercarial transmission

stages (i.e. the anemone *Anthopleura aureoradiata*) was capable of reducing the transmission success of the parasite due to the simultaneously increased feeding activity at elevated temperatures, and therefore plays a buffering role under conditions of increased temperatures. It was not possible to directly confirm this buffering role of anemones and results indicated that amphipods are, regardless of the density of anemones in their immediate environment, at an increased risk of parasite-induced mortality under conditions considered optimal for transmission. Consequently, despite the differential effects of temperature, salinity and UVR, overall, *M. novaezealandensis* will probably find optimal conditions for transmission more often in a warming world - at least up to an optimum temperature level and especially in areas where vegetation provides shading from incident solar irradiation.

Spatially, pronounced patchiness with regards to the parasite's impact on hosts can be expected, particularly due to differences in infection levels in first intermediate snail hosts (Fredensborg *et al.*, 2005; Fredensborg & Poulin, 2006). Effects on second intermediate host populations are expected to be strongest in areas where prevalence in snail hosts is high. Hence, the focus of the field study (Chapter Two) and the modelling (Chapter Nine) was on Lower Portobello Bay. A model was developed to investigate the life cycle dynamics of *M. novaezealandensis* under different mean daily temperature increases and to assess the potential consequences for the amphipod population in this locality. The model simulations confirmed the high vulnerability of the modelled amphipod population with increasing temperatures. Despite the uncertainties and limitations associated with the modelling approach, most temperature increases capable of driving the modelled amphipod population to collapse were within the temperature increases predicted to occur over the coming decades. Impacts on other amphipod populations are highly likely to be site-specific and context-dependent and thus generalisations from one study location can only be made cautiously. For example, low prevalence areas close to Lower Portobello Bay are not expected to be similarly vulnerable (unless the density of snails is very high), wheras other high prevalence areas may be even more strongly affected due to differences in latitude and thus thermal conditions.

Die-off events related to high temperatures or other environmental anomalies have been documented in a range of marine species, including sea grasses, oysters, corals, starfish, sea urchins or abalone (Harvell *et al.*, 1999), as well as amphipods (Jensen & Mouritsen, 1992). Such events can be only brief, repeated, or long-lasting, potentially causing local extinction or at least long-term local modifications (Marcogliese, 2008). The disappearance of a species

may entail consequences for associated species (e.g. Dann *et al.*, 2000) or for an entire ecosystem (e.g. Mouritsen *et al.*, 1998). Due to the dependency of symbionts on the presence of other organisms, the disappearance of a given organism or species thus may also imply the disappearance of its associated symbionts. This is likewise the case for parasites (Dobson *et al.*, 2008; Dunn *et al.*, 2009). Therefore, parasites should not only be considered a source of threat for wildlife populations, but also an ecosystem component considerably at risk of secondary extinction (de Castro & Bolker, 2005; Lafferty & Kuris, 2009a; McCallum & Dobson, 1995). This is of particular relevance for parasites with high host specificity at least in one part of their life cycle and for those parasites with complex life cycles, thus depending on several species to complete their life cycle (Rohr *et al.*, 2011).

Intertidal organisms have been suggested to serve as early warning systems for climate change effects (Helmuth *et al.*, 2006b). Likewise, the sensitivity of trematodes to temperature has also been suggested to provide a tool to monitor the ecological impact of climate change (Marcogliese, 2001; Poulin & Mouritsen, 2006), similar to what has been described for the indicator role of parasites for other environmental changes (Lafferty, 1997). Results from this thesis support these notions, and highlight the need to consider not only the interactions between hosts and parasites, but also their interactions within complex ecological settings.

10.1 Suggestions and directions for future research

1. *M. novaezealandensis* was the model system used in this thesis. However, one species or one host-parasite system does not allow any form of generalisation about the effects of climate changes on parasitism in intertidal ecosystems. Therefore, in depth studies on other systems are required to not only allow a better understanding of the ecological dynamics of a bigger range of intertidal host-parasite systems, but also to eventually allow the identification of general patterns of the consequences of global changes on host-parasite interactions in marine ecosystems.

2. The present thesis focussed on one species of second intermediate host. Despite the fact that amphipods seem particularly vulnerable, another suggestion for future research would be to investigate other crustacean hosts of *M. novaezealandensis* (see Koehler & Poulin, 2010), especially with regards to their responses to temperature and their vulnerability

to parasite-induced mortality, which under natural conditions, has already been inferred to occur for some species (Koehler & Poulin, 2010). How these crustaceans are differentially affected will determine how the combined effect of the parasite and environmental changes will affect the community structure and possibly the entire ecosystem, at least in areas where prevalence in first intermediate snail hosts is high. Future research should thus incorporate the host community, rather than only focussing on individual host-parasite relationships. This may also allow the inclusion of the community level into modelling approaches. Ultimately, a key question that also needs to be addressed concerns the stability and persistence of a particular system as a whole, which requires a better understanding of the resilience of food webs to species loss, including the loss of parasites, the extinction risks of generalists, and the extent to which interactions within a community may influence resilience to climate change (Bascompte & Stouffer, 2009).

3. In this thesis, the focus was on a few environmental factors naturally fluctuating in intertidal ecosystems. However, a range of other factors also fluctuate in intertidal systems (e.g. dissolved oxygen) and in addition to that, marine ecosystems are also subject to a range of other global changes not considered here (e.g. Halpern *et al.*, 2008). For example, ocean acidification has been identified as a major threat to marine systems and organisms and can also disrupt species interactions (Bibby *et al.*, 2007; Dixson *et al.*, 2010; Munday *et al.*, 2009). Clearly, the consequences of altered pH should be investigated also in marine host-parasite systems, especially in systems involving calcifying host organisms. Such assessments should incorporate interactive effects with temperature.

4. An important next step in the study of effects of abiotic environmental factors is the simulation of realistic conditions (see e.g. Roth *et al.*, 2010; Sorte *et al.*, 2010). This includes the consideration of variability, which has been shown to be of substantial importance in the case of parasite transmission (Paaijmans *et al.*, 2010). Despite the fact that mean global temperatures are increasing, short-term temperature variability such as extreme heat waves are potentially very powerful and more important than long-term trends in mean temperatures (Easterling *et al.*, 2000; Jentsch *et al.*, 2007; Parmesan *et al.*, 2000). Moreover, the occurrence of extreme events and non-linear thresholds in disease dynamics and climatic processes can also confound predictability (Harvell *et al.*, 2002). Hence, realistic simulations of extreme events and how they influence ecological processes including transmission dynamics should follow.

5. Future studies may also include the consideration of the interactive effects of environmental factors and infections on, for example, the immune status or immune responses of hosts. Immune systems are known to be affected by environmental conditions (see e.g. Le Moullac & Haffner, 2000; Roth *et al.*, 2010) and would thus be another next step to incorporate into the study of the model system chosen here.

6. The study of ecologically relevant UVR effects on parasites is still at a very early stage and there is scope for many research avenues. For example, patterns described in this thesis should be compared to other parasite species or other host-parasite systems. Due to the large differences in size, colour and transmission strategies of cercariae, effects of UVR may differ considerably between species. Also, many more biomarkers could be assessed to explore the mechanisms by which UVR influences host-parasite interactions. For instance, oxidation of lipids and DNA or other measures of DNA damage could be assessed. Moreover, the capacity to repair DNA damage has been suggested to occur in parasites (Ruelas *et al.*, 2007) and although in the system studied here, there is little evidence for the presence of repair mechanisms, other approaches may be needed for their identification. In particular, the dark repair mechanism of DNA damage may be an important mechanism occurring in metacercariae within crustacean hosts and thus future research could focus on levels of DNA damage in crustacean hosts, which may also differ between crustacean species depending on the transparency of their cuticle. Similarly, it could also be assessed if UV-absorbing compounds may be present in metacercariae. Moreover, parasite species from different latitudes could be compared with regard to their responses to UVR and their capacities to deal with UVR in order to describe more general patterns of UV effects on parasites. Additionally, future studies should consider all UVR effects on host-parasite interactions in the context of the combined effects between UVR and temperature.

7. Climate change is also highly likely to be a strong selection pressure in natural systems (Gienapp *et al.*, 2008), and this is true for hosts as well as their parasites. Single *M. novaezealandensis* clone infections are common in first intermediate *Z. subcarinatus* snail hosts (Keeney *et al.*, 2007a). This may provide an excellent opportunity to investigate how different clones may respond to e.g. temperature and hence may indicate the scope of evolutionary potential of a parasite like *M. novaezealandensis*. Along similar lines, host-parasite systems from different locations along latitudinal gradients may indicate compatibility between allopatric and sympatric host-parasite combinations. Coupled with e.g.

temperature effects, such studies may indicate the potential for hosts or parasites to adapt to changing conditions including exposure to altered host community structures.

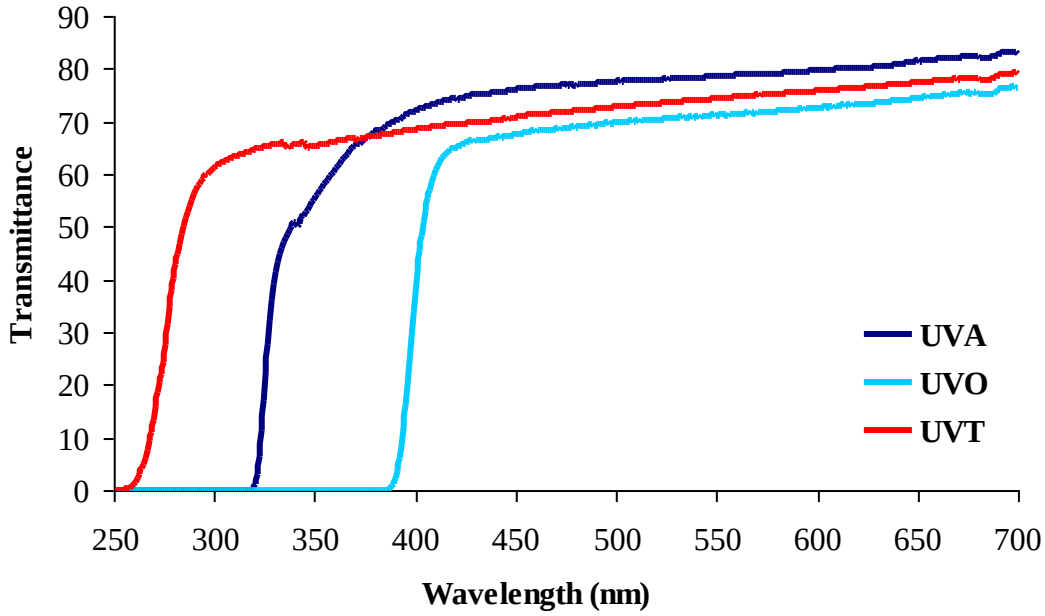
8. One major problem in the study of the ecological consequences of climate change and particularly in the case of parasitism in marine ecosystems is the lack of historical baseline data and the lack of commitment to long-term monitoring programs that include parasites. The absence of such highly valuable long-term data on disease dynamics in marine ecosystems not only limits our general understanding of parasitism and disease in these ecosystems, but also hinders the detection of long-term temporal and spatial trends. Moreover, it also deprives scientists of a tool to communicate with other stakeholders. Long-term monitoring projects should be improved to take a broader ecosystem approach and include measures of parasitism. It would be of great importance to conduct spatial studies in order to identify areas at risk of parasite-induced mortality in certain target host populations or communities (e.g. those with limited recruitment and dispersal abilities), on which monitoring programs may focus. Such efforts are essentially the only chance to gain knowledge on long-term changes in the environment, including the detection of disease-mediated extinction in local populations or changes in community structures due to range expansions or contractions. For example, Lower Portobello Bay may be a candidate for repeated sampling and thus the generation of multi-year datasets on a particular locality which may also allow disentangling short term variation and long-term trends. This would also allow the verification of the predictions made in Chapter Nine. The concomitant identification and assessment of appropriate replicate populations or communities with varying levels of prevalence in first intermediate snail hosts across larger spatial scales may provide additional insight into the effects of the parasite on host populations or communities.

Overall, there is still a great lack of many aspects of disease and parasitism in marine ecosystems (e.g. life cycles) and thus great scope for basic parasitological and epidemiological research. A better understanding of "what is" will ultimately enable to better anticipate what "may be". The complexity of the consequences of climate change on natural systems cannot be addressed with a highly reductionist approach and requires more integrative approaches and interdisciplinary and multidisciplinary collaborations. However, regardless of any such efforts, nothing exempts us from our obligation for immediate actions to prevent further consequences of climate change to manifest, which are threatening the very basis on which we, and other organisms, depend.

Appendix

11.1 Transmission profiles of filters used in the UVR experiments (Chapters Five, Six and Seven)

Spectrophotometer (Jasco V-550) transmission profiles of the experimental Plexiglas filters used to obtain no UV (UVO), UVA, and UVA+B (UVT) treatments. Figure provided by K. N. Lister (see also Lister *et al.*, 2010).



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