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ORIGINAL PAPER

Cercarial survival in an intertidal trematode: a multifactorial experiment with temperature, salinity and ultraviolet radiation

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Abstract Parasite transmission takes place in the context of a multitude of simultaneously fluctuating environmental factors. As a particularly vulnerable step in the transmission, trematode cercariae are directly exposed to ambient conditions during their search for a host. Here, we investigated the survival of cercariae of the intertidal trematode *Maritrema novaezealandensis* in a multifactorial experiment (2×2×2 design) with temperature (20 and 30 °C), salinity (35 and 40 practical salinity units (psu)) and ultraviolet radiation (UVR; exposed and not exposed) as main factors. All factors had significant effects, with cercariae dying faster at the higher temperature, increased salinity and when exposed to UVR. Full activity ceased within ~6 h in all treatments at 30 °C, except at 35 psu under no exposure to UV; in all other treatments full activity was maintained for >6 h. Several factor interactions were identified, of which the interactive negative effect of temperature and UVR was the most important. These results imply that conditions during the main transmission window of *M. novaezealandensis* are highly challenging for cercariae. Our findings highlight the importance of considering multiple environmental factors in the study of parasite transmission to gain a more ecologically relevant understanding of transmission dynamics.

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 particularly pronounced, occurring at 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 and Worm 2002; Przeslawski 2005; Przeslawski et al. 2005; Russell and Phillips 2009a; Russell and 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 or overestimating the ecological impacts of environmental conditions.

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 and Poulin 2002; Mouritsen and Poulin 2010; Sousa 1991). The transmission process

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of trematodes and other endohelminth parasites is dependent on usually more than one free-living larval stage, which are directly exposed to and influenced by ambient conditions (Pietrock and Marcogliese 2003). Cercariae are the infective transmission stage leaving the first intermediate host in order to infect a second intermediate host. These cercariae are short-lived; because they do not feed, conditions encountered will determine the amount of time they stay alive until their energy reserves are used up (e.g. Lawson and Wilson 1980).

For marine trematodes, environmental factors found to affect the survival of cercariae include temperature (e.g. Mouritsen 2002; Studer et al. 2010; Thieltges and Rick 2006), salinity (Lei and Poulin 2011; Studer and Poulin 2012a) and ultraviolet radiation (Studer et al. 2012b). A range of other environmental factors such as pH, oxygen, water depth, light or pollutants have also been shown to affect the survival and/or infectivity of the free-living stages of endohelminth parasites in aquatic ecosystems (see Pietrock and Marcogliese 2003 and references therein). However, parasite transmission has received little attention in the context of more ecologically relevant experimental approaches, including also regarding the importance of biotic factors (Thieltges et al. 2008). Mouritsen (2002), Koprivnikar and Poulin (2009) and Koprivnikar et al. (2010) have attempted to conduct experiments on the output and survival of cercariae in multifactorial designs in order to better account for the complexity of factors occurring in nature. Their research has shown that there are indeed factor interactions which could be of importance under natural settings.

In previous studies investigating the separate effects of temperature, salinity and UVR on the transmission 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 (approximately 170 μm in length including tail, Martorelli et al. 2004) was identified as the only step that was significantly affected by all three factors (Studer et al. 2012b; Studer and Poulin 2012a; Studer et al. 2010). 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 (Fredensborg et al. 2004). 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 potential factor interactions affecting organisms in nature. 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.

Materials and methods

The experiment was a fully factorial 2×2×2 design (temperatures, 20 and 30 °C; salinities, 35 and 40 practical salinity units (psu); and 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 $\mathrm{kJ\,m^{-2}}$ (UVB) and 59.25 $\mathrm{kJ\,m^{-2}}$ (UVA), with a PAR energy flux of 250–350 $\mu\mathrm{mol\,m^{-2}}$. Plexiglas filters were used which were either transparent or non-transparent to UVR (no UV treatment, 81 % PAR, 5.2 % UVA and 0 % UVB; UVR treatment, 84.5 % PAR, 84.6 % UVA and 80.6 % UVB; for filter properties and transmission profiles, see Lister et al. 2010). Additionally, cellulose di-acetate filters were employed which absorb UVC radiation emitted from the 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, Studer and Poulin 2012b). 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).

First intermediate *Zeacumantus subcarinatus* snail hosts 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 the emergence 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 (ten replicate wells per treatment) of 96-well plates (wells 7× 10 mm; total volume 320 µl), which corresponded to an addition of approximately 25 cercariae per well. The volume in each well was standardised to 100 µl of the respective salinity solution. 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). Functional survival of cercariae was checked after 2, 4, 6 and 8 h of continuous exposure. Cercariae were classified visually as fully active, sluggishly motile or immotile/dead. In terms of ecological relevance, full activity is the most important category as it corresponds to infectivity. Hence, 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 and factor interaction.

Results

Temperature was identified as the most important factor of all factors studied (see results and effect sizes in Table 1). In all cases, cercariae died faster at the higher temperature—at both salinities and when exposed or not exposed to UVR (Fig. 1). However, each of the factors individually had a significant effect on the survival of cercariae. Cercariae lost their functionality faster at the higher salinity level and under exposure to UVR. This was also reflected in the overall mean percentage of survival of cercariae (Fig. 2). Mean survival was consistently lower at 30 °C compared to 20 °C. At 20 °C, exposure to UVR decreased mean survival whereas no such effect was evident at 30 °C. Moreover, mean survival was reduced at 40 psu compared to 35 psu.

Several factor interactions could be identified. 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 interactive 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. 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. 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 × salinity (Table 1).

Table 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* value	*p* value	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 × UV	1	0.34	15.01	<0.001	0.172
Temperature × salinity	1	0.06	2.51	0.118	0.034
UV × salinity	1	0.02	0.72	0.397	0.01
Temperature × UV × 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 × temperature	3	3.00	204.97	<0.001	0.751
Time × UV	3	0.21	9.15	<0.001	0.177
Time × salinity	3	0.01	0.91	0.441	0.006
Time × temperature × UV	3	0.17	11.84	<0.001	0.146
Time × temperature × salinity	3	0.11	6.19	0.001	0.1
Time × UV × salinity	3	0.03	2.41	0.074	0.031
Time × temperature × UV × salinity	3	0.06	2.90	0.041	0.056
Error (time)	216	0.01			

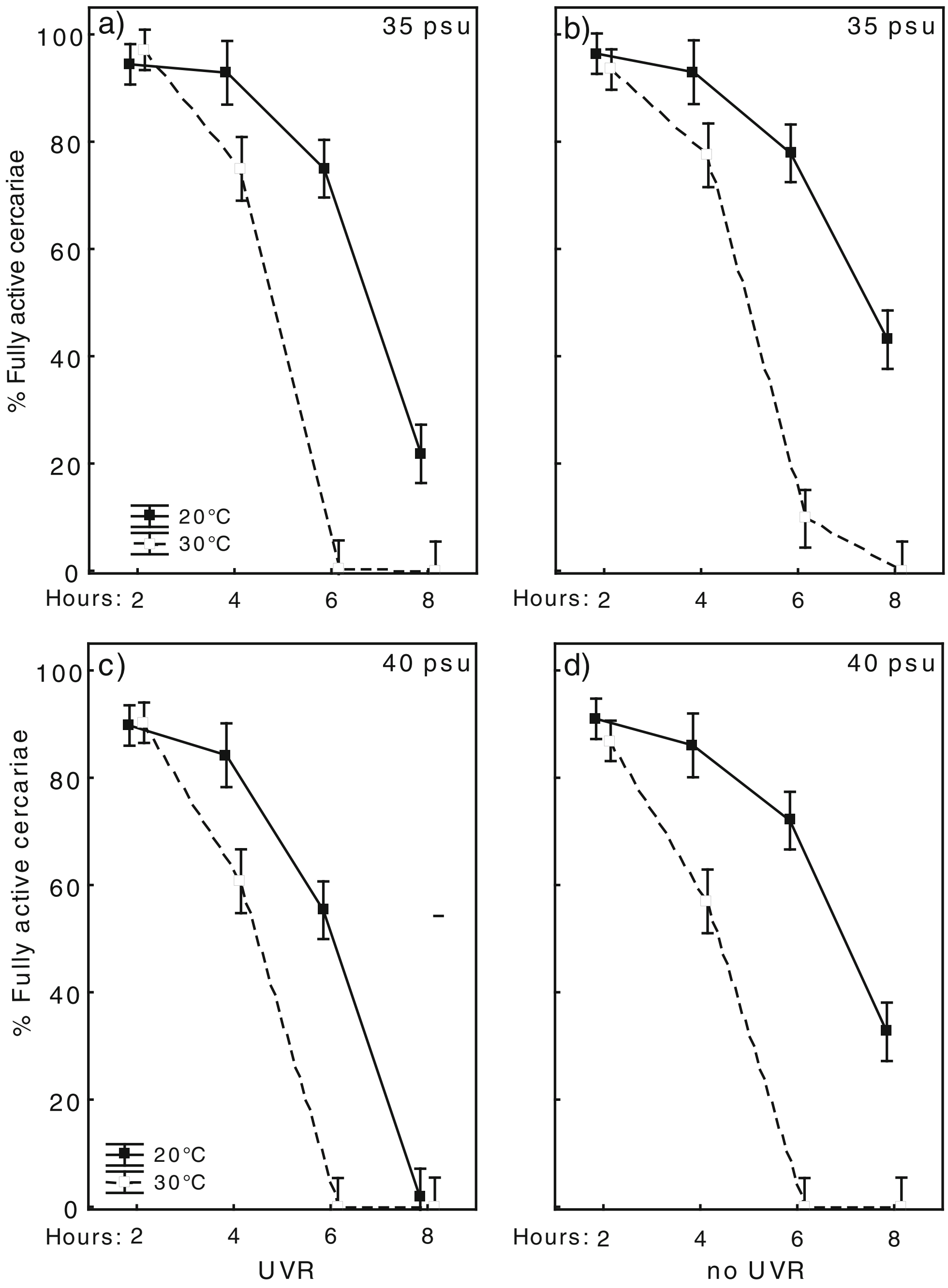

Fig. 1 Survival, i.e., mean percentage of functionally active individuals, of the cercariae of *M. novaezealandensis* at 2, 4, 6 and 8 h post emergence from infected snail hosts. Results from a multifactorial experiment (2×2×2 design) with temperature, salinity and UVR as factors (i.e., 20 and 30 °C, 35 and 40 psu and exposed or not exposed to ultraviolet radiation (*UVR*; *n*=10 per treatment; showing 95 % confidence intervals)

Discussion

Temperature, salinity and UVR are environmental factors which have been shown to interactively affect the biota of intertidal ecosystems (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 several significant interactions among the factors investigated, including interactions with time.

Results from single-factor experiments (Studer et al. 2012b; Studer and Poulin 2012a; Studer et al. 2010) 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

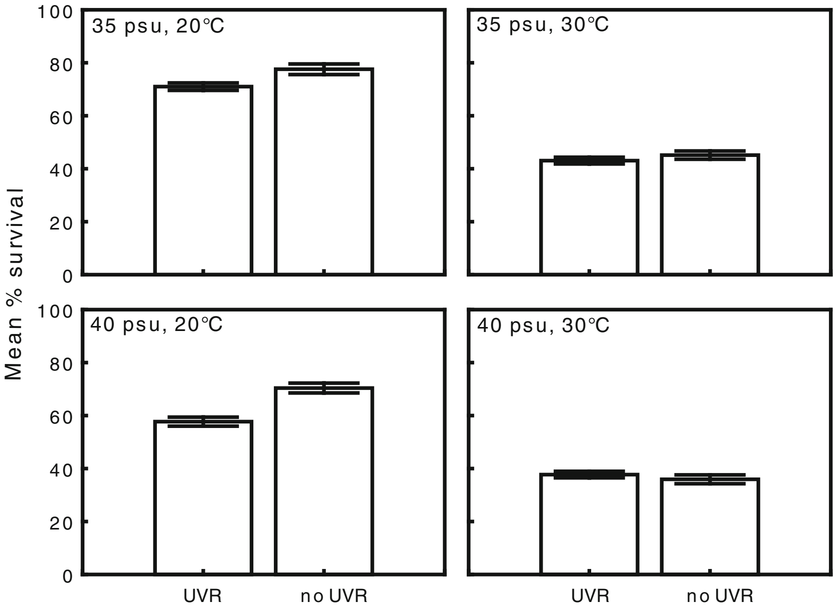

Fig. 2 Overall mean (±SE) percentage of survival of the cercariae of *M. novaezealandensis* across 8 h of experimental exposure to 20 or 30 °C, 35 or 40 psu and exposed or not exposed to ultraviolet radiation (*UVR*, *no UVR*)

exposure to UVR, but also indicated a higher mortality in the high salinity treatment with an even higher effect size for salinity than for UVR (Table 1). The observed effect of temperature is consistent across a range of previous studies from different systems (Lowenberger and Rau 1994; McCarthy 1999; Mouritsen 2002; Pechenik and Fried 1995; Thieltges and 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 and Fried 1995). The increased mortality under UVR exposure is also consistent with previous findings and may be mainly due to UV-induced oxidative damage and the lack of protective mechanisms in cercariae (Studer et al. 2012a). 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 was 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 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. In comparison, Mouritsen (2002) found a significant interaction between temperature and salinity for the emergence of cercariae of *Maritrema subdolum*, but not for their longevity, whereas Koprivnikar et al. (2010) found interactions between temperature, salinity and time, as well as between pH, salinity and time, but only for one of two species investigated.

Based on our 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. 2004; 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 reduced survival at this temperature being counterbalanced by 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 physiological challenge to these larval stages. Considering the effects of all factors, exposure 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 cercariae.

However, further multifactorial experiments are 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 dynamics would be affected in the actual environment. 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.

The consideration of more ecologically relevant environmental complexity in parasite and pathogen transmission studies is needed for species from all ecosystems, as has been highlighted from a range of examples including parasitism and diseases of amphibians, oysters and fish (e.g. Kiesecker et al. 2001; Lenihan et al. 1999; Valtonen et al. 1997). However, our understanding of multiple-factor interactions and their consequences for transmission dynamics remains limited. Addressing this gap is crucial for a better understanding of transmission dynamics and host–parasite interactions in general, especially in the context of global climate change and other human-induced alterations of ecosystems (see also Lafferty 2009).

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 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 *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.

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