Mar Biol (2011) 158:995–1003
DOI 10.1007/s00227-011-1625-7

ORIGINAL PAPER

Effects of salinity on multiplication and transmission of an intertidal trematode parasite

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Received: 9 November 2010 / Accepted: 6 January 2011 / Published online: 20 January 2011

Abstract Salinity levels vary spatially in coastal areas, depending on proximity to freshwater sources, and may also be slowly decreasing as a result of anthropogenic climatic changes. The impact of salinity on host–parasite interactions is potentially a key regulator of transmission processes in intertidal areas, where trematodes are extremely common parasites of invertebrates and vertebrates. We investigated experimentally the effects of long-term exposure to decreased salinity levels on output of infective stages (cercariae) and their transmission success in the trematode *Philophthalmus* sp. This parasite uses the snail *Zeacumantus subcarinatus* as intermediate host, in which it asexually produces cercariae. After leaving the snail, cercariae encyst externally on hard substrates to await accidental ingestion by shorebirds, which serve as definitive hosts. We found that at reduced salinities (25 or 30 psu), the cercarial output of the parasite was lower, the time taken by cercariae to encyst was longer, fewer cercariae successfully encysted and encysted parasites had lower long-term survival than at normal seawater salinity (35 psu). The strong effect of salinity on the replication and transmission of this parasite suggests that there may be sources and sinks of transmission to birds along coastal areas, depending on local salinity conditions. Also, unless it evolves to adapt to changing conditions, the predicted reduction in salinity as a consequence of climate change may have negative impact on the parasite's abundance.

Communicated by S. D. Connell.

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Introduction

Intertidal areas are subject to rapid and sharp environmental changes due to the daily exposure to air and water caused by tides. Salinity levels, though roughly constant in the open ocean, are highly variable in intertidal habitats, in both space and time. They can range from 2 to 3 psu due to freshwater input from precipitation, rivers and surface runoff, up to 60 psu following evaporation from shallow pools at low tide (Adam 1993; Berger and Kharazova 1997). On longer time scales, predicted climate change may lead to altered precipitation regimes and rising sea levels following the thawing of freshwater ice stores at the poles, both of which are likely to affect the salinity levels experienced by intertidal organisms (IPCC 2007). Not surprisingly, salinity is a key abiotic factor influencing small- and large-scale biotic interactions in intertidal ecosystems (Berger and Kharazova 1997; Ingole and Parulekar 1998). It determines the distribution (Crain et al. 2004), physiological performance (Hylleberg 1975; Pequeux 1995; Shock et al. 2009) and reproductive success (Deschaseaux et al. 2010) of a wide range of organisms living on mudflats or rocky shores. Because of its direct physiological impact on intertidal organisms, salinity may also affect interspecific interactions, such as predation or parasitism.

Parasites are increasingly recognized as important players in intertidal communities (Sousa 1991; Mouritsen and Poulin 2002; Kuris et al. 2008). They, too, can affect the physiology, activity, survival and reproduction of their hosts (Mouritsen and Poulin 2002). The wider consequences of infection include the regulation of host populations (Lafferty 1993; Fredensborg et al. 2005) together with altered trophic relationships resulting in changes in community structure and food web properties (Mouritsen

and Poulin 2005; Thompson et al. 2005; Wood et al. 2007). The relationship between abiotic conditions and the proliferation and transmission of parasites in marine systems has been highlighted in the context of climate change and its possible role as a driver of emerging parasitic diseases (Harvell et al. 2002; Poulin and Mouritsen 2006; Lafferty 2009). However, the focus of research to date has been almost exclusively on the likely impact of higher temperature, especially on the transmission of trematode parasites (Mouritsen et al. 2005; Poulin 2006; Studer et al. 2010). Trematodes are flatworms with complex life cycles involving a series of different hosts; they are also the most common metazoan parasites in intertidal habitats and have been shown to be key determinants of invertebrate population dynamics and community structure (Mouritsen and Poulin 2002; Fredensborg et al. 2005; Thompson et al. 2005; Kuris et al. 2008). It is important to understand how the proliferation and transmission success of these ubiquitous parasites are affected by environmental factors like salinity, which vary widely in space and on both short and long time scales.

Here, we investigate the impact of salinity on the asexual multiplication and transmission success of the trematode *Philophthalmus* sp. (Digenea: Philophthalmidae), an eye fluke infecting the nictitating membrane in the eyes of aquatic birds. The parasite uses the intertidal mud snail, *Zeacumantus subcarinatus* (Batillariidae), as its first intermediate host (Martorelli et al. 2008). This small gastropod is commonly found in soft-sediment intertidal zones and estuarine areas around New Zealand and is therefore exposed to varying salinities. *Philophthalmus* sp. occupies the visceral mass of the snail host and causes its complete castration. Within the snail, the parasite undergoes asexual multiplication to generate large numbers of free-swimming infective stages known as cercariae, which leave the snail. Unlike of the cercariae of most other trematodes, which seek and encyst within a second intermediate host, those of *Philophthalmus* sp. encyst on hard substrates, such as the shells of bivalves or on seaweed (Nollen and Kanev 1995). Upon contact with a suitable substrate, cercariae lose their tail and secrete a pear-shaped cyst around their body that is fixed to the substrate; at this stage, the parasites become metacercariae. Transmission to a bird definitive host is achieved when the latter ingests a substrate bearing metacercariae. Excystment is then triggered by thermal stimulation in the throat of the bird and is followed by the migration of the metacercariae towards the bird's eyes, where the parasites become adult worms (Nollen and Kanev 1995). The main avian hosts probably include the red-billed gull (*Larus novaehollandiae scopulinus*) and/or the mallard duck (*Anas platyrhynchos*), both of which are common at our study site. Eggs produced by these worms are released in tears or via direct contact of the eyes with water. Following egg hatching, a small ciliated larva finds and infects a snail, thereby completing the life cycle.

An earlier study suggested that *Philophthalmus* sp. is sensitive to a short-term (<5 days) exposure to reduced salinity, which caused an increase in the emergence of cercariae from snail hosts (Koprivnikar and Poulin 2009). However, this experiment only indicated that sudden salinity stress prompts cercariae to leave their host, as seen in other trematodes (Mouritsen 2002); it did not determine whether long-term exposure to altered salinity affects the rate at which the parasite multiplies asexually within the snail to produce cercariae. Also, it did not address the next two critical steps in the transmission process: encystment success of the cercariae and long-term survival of encysted metacercariae. These are the key determinants of infection risk for shorebirds as a function of spatial differences in the average salinities to which snails are exposed and in the context of decreases in salinity expected from climate change.

In the present study, (1) we quantified the effects of salinity on the number of *Philophthalmus* sp. cercariae produced per unit time by infected *Z. subcarinatus* snails over several months; (2) we determined how exposure to different salinities affects the time taken by cercariae to encyst after they leave their snail host, and their overall encystment success; and (3) we quantified how salinity affects the long-term survival of encysted metacercariae. Data on the influence of salinity on the transitional developmental stages of *Philophthalmus* sp. will provide a baseline for understanding the spatial and temporal epidemiology of this common parasite of shorebirds.

Materials and methods

Snails (*Z. subcarinatus*) were collected by hand on five different occasions at Lower Portobello Bay, Otago Harbour, New Zealand (45°47′S, 170°42′E), in February–March 2010. On this mudflat, the salinity experienced by snails is approximately 34–35 psu at high tide, but it varies sharply in both space and time at low tide. Sharp salinity gradients exist among tide pools on a scale of just a few metres, with salinity approaching 0 psu in areas affected by inflow of freshwater; following heavy rain, the size of these areas increases, such that salinity in one spot varies from one low tide to the next, with low-tide salinities generally lower in the rainier seasons of autumn and winter (A. Studer, University of Otago, personal communication). Salinity can exceed 35 psu in individual tide pools due to evaporation on hot sunny days, but values above 37–38 psu have never been recorded (A. Studer, personal communication). Thus, in this habitat, snails are more likely to experience salinities below normal seawater for prolonged periods than they are of

experiencing high salinity. Snails were collected from the intertidal zone during low tide and transported back to the laboratory in 2-l plastic containers.

In the laboratory, snails were kept in several well-aerated 3-l containers with natural seawater, sea lettuce (*Ulva* sp.) and sediment collected from Lower Portobello. After 2 days of acclimatisation to laboratory conditions, snails were individually placed into 5-ml plastic wells in a 12-well tissue culture plate, with each well containing 3 ml of natural seawater and incubated at 25°C for 18 h under constant illumination. Incubation was carried out to promote cercarial emission to identify snails that were infected with *Philophthalmus* sp. as warmer temperatures trigger the emergence of cercariae (Mouritsen 2002). After the incubation, individual wells were examined under a dissecting microscope for the presence of cercariae. Snails infected by other trematode species were discarded, while snails not shedding any cercariae were placed into a separate container holding seawater together with sediment and sea lettuce, and then incubated again 3 days after the initial shedding procedure to confirm their infection status. Snails infected with *Philophthalmus* sp. were kept in a 2-l plastic container holding 200 ml of sediment, ample sea lettuce and 1 l of artificial seawater (Red Sea Salt®) at 35 psu, i.e. normal salinity. Out of a total of 1,810 snails, 84 (4.6%) were infected by *Philophthalmus* sp.

Experiment 1: cercarial output

Infected snails were exposed to different salinity levels, and the irreversibly encysted metacercariae were counted on a weekly basis to determine the long-term effects of salinity on *Philophthalmus* sp. cercarial output. Three salinity treatments (25, 30 and 35 psu) were used, with six replicates per group and each replicate containing three snails infected with *Philophthalmus* sp. (total number of snails for each treatment = 18). Each replicate consisted of three snails housed in a 2-l plastic container. We chose 35 psu because it corresponds to normal seawater on intertidal mudflats (Knauss 1978; Cheng et al. 1993), whereas the lower two salinities are associated with areas affected by freshwater inputs.

Each container held 200 ml of sediment collected from Lower Portebello Bay and approximately 2.5 g of sea lettuce. Before use, the sediment was sieved (mesh size = 2.0 mm) to remove unwanted material, rinsed thoroughly with freshwater and then dried. Stock solutions of saltwater for the three treatments (25, 30, 35 psu) were made in three separate well-aerated 20-l containers using artificial sea salt (Red Sea Salt®). A volume of 800 ml of the respective stock solutions was then added to each of the containers; the latter were each equipped with a bubbler and covered to prevent evaporation.

For the experiment, 54 snails were chosen at random out of those infected with *Philophthalmus* sp. These snails were placed individually in 5-ml wells holding 3 ml of saltwater and incubated at 25°C under constant illumination for 24 h to induce shedding of all mature cercariae. This ensured that the snails used were all rid of fully developed cercariae at the onset of the experiment. Snails were measured using vernier calipers (maximum shell length, ±0.02 mm), sorted into size classes and assigned evenly on that basis among replicates and treatments. This ensured that average snail size did not differ among treatments.

In order to measure *Philophthalmus* sp. cercarial output, three standard microscope slides were placed on the sediment in the middle of each container, providing a hard substrate for cercarial encystment. At weekly intervals, slides were removed for metacercarial counts and replaced with new ones. All slides were examined under a dissecting microscope where the permanently encysted metacercariae (Fig. 1) were counted. These counts provided relative, and not absolute, measures of cercarial production, since some metacercariae may have encysted on the sides of the containers and a few (though unlikely) may have accidentally been consumed by grazing snails; they were nevertheless suitable for comparisons among salinity treatments.

The experiment was conducted at room temperature in a laboratory with uncovered windows, thus exposing snails to natural light and moderate temperature fluctuations. Salinity levels were checked with a refractometer every other day for the first 2 weeks; no deviation from the assigned salinity was observed. This experiment lasted 12 weeks with encysted metacercariae being counted weekly. Every second week, 50% of the water was replaced with water from the stock solution together with a fresh piece of sea lettuce.

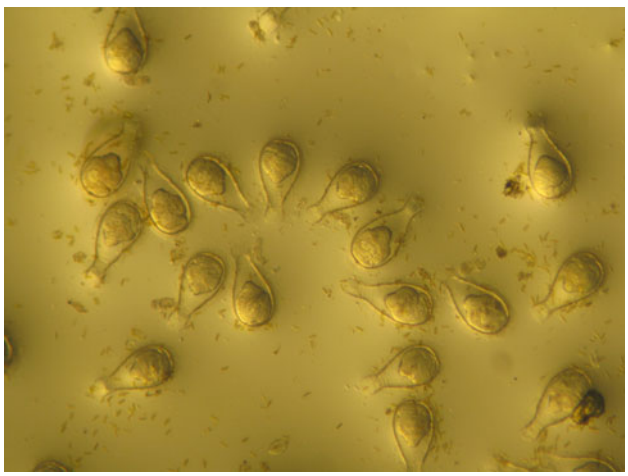

Fig. 1 Several encysted *Philophthalmus* sp. metacercariae attached to a glass microscope slide

Experiment 2: cercarial encystment

To examine the effects of salinity on the encystment success of free-swimming *Philophthalmus* sp. cercariae, 30 infected snails not used in experiment 1 were acclimatised to three salinity levels (25, 30 and 35 psu; 10 snails per treatment) for a total of 9 weeks. For each salinity, the 10 snails were kept together in well-aerated 2-l plastic containers with 200 ml of sediment, approximately 2.5 g of sea lettuce and 800 ml of artificial seawater at the appropriate salinity, prepared as in experiment 1. Sea lettuce was replaced, and 50% of water was changed every 2 weeks. After 9 weeks of salinity exposure, three snails from each group were chosen at random and placed in a 5-ml Petri dish with artificial seawater of the same salinity. The Petri dishes were then incubated at 25°C for 2 h to induce cercarial emergence. The three snails from a given salinity were not separated in order to obtain a genetically diverse mix of cercariae.

After cercarial emergence, the snails were immediately removed, leaving behind free-swimming cercariae. For each salinity treatment, 10 cercariae were transferred with a plastic pipette from the Petri dish into a 5-ml well in a 12-well culture plate to monitor their ability to encyst. There were four replicates per treatment, resulting in four 5-ml wells each holding 10 cercariae for each of the three salinity levels. Each well was filled with 3 ml of artificial seawater of the appropriate salinity. Every 20 min following their transfer to a well, cercariae were examined under a dissecting microscope. Numbers of cercariae in each well that were either free-swimming or encysted on the well bottom were recorded. The experiment lasted a total of 340 min, with 17 separate counts at 20-min intervals for each of the three salinity treatments.

Experiment 3: metacercarial survival

To determine how salinity affects the longevity of *Philophthalmus* sp. metacercariae in the external environment, a microscope slide was chosen at random out of the three slides from each of the 18 containers from experiment 1, after the fifth week (total = 18 slides, i.e. six for each salinity level). The six slides from the same treatment were kept in separate 450-ml containers at their respective salinity (25, 30, 35 psu). The containers were covered, with aeration provided by one bubbler per container. They were kept in the same room as those from experiment 1 and exposed to similar light and temperature conditions.

The total number of metacercariae on each slide was recorded at the start of the experiment (note: 100% of metacercariae were alive on all slides at the onset), with the initial number of metacercariae per slide ranging from 31 to 360. In subsequent counts, only live metacercariae were recorded, and these numbers were expressed as proportions of surviving metacercariae out of the initial number. Each slide was examined weekly under a dissecting microscope for 6 weeks following the start of the experiment. A metacercaria was considered to be dead if its pear-shaped cyst was empty and alive if the parasite could be seen clearly within the cyst.

Statistical analysis

All analyses were performed using the statistical packages JMP and SPSS. For experiment 1, cercarial counts, i.e. numbers of cercariae per slide ($n = 648$ slides), were log10 $(x + 1)$ transformed to meet the assumptions of normality and homogeneity of variance. Transformed counts were used as the dependent variable in an ANOVA with two main factors (week and salinity), including a nested design (containers nested within salinity) and an interaction term between salinity and week. In addition, a nested ANOVA was carried out to test for differences in the sizes of snails held in the 18 separate containers, with salinity as the main effect and container identity nested within salinity levels.

A repeated measures ANOVA was used in experiment 2 to assess differences in rates of encystment of cercariae among salinity treatments. The counts were converted into proportions encysted before being arcsine-transformed. The repeated measure was time (i.e. 17 separate 20-min intervals), salinity was the main factor (25, 30 and 35 psu) and the proportion of cercariae encysted was the dependent variable. The proportions of surviving metacercariae recorded from experiment 3 over the 6 weeks were arcsine-transformed and then also used in a repeated measures ANOVA, using weeks (6 levels) as the repeated measures variable, salinity was the main factor and the proportion of live metacercariae was the dependent variable. For both repeated measures ANOVAs, tests for data sphericity or equality of variances between sampling intervals were carried out using Mauchly's test criterion. If sphericity could not be assumed, P values were adjusted with the Greenhouse–Geisser correction. If salinity had a significant effect, we conducted pairwise comparisons with Tukey's HSD post hoc test.

Results

Experiment 1: cercarial output

Overall, most slides recovered during the experiment accumulated fewer than 50 metacercariae in a one-week period, although some amassed several hundred (Fig. 2). The nested ANOVA revealed an overall effect of salinity on the number of cercariae produced ($F_{2,\ 597} = 5.36$,

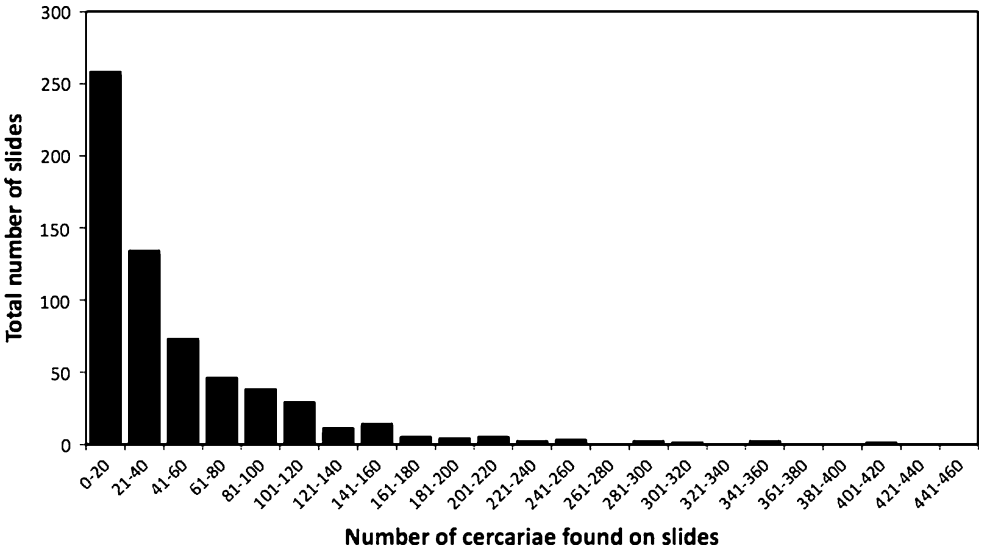

Fig. 2 Frequency distribution of the number of *Philophthalmus* sp. metacercariae encysted per slide per week, among all 648 slides used in the course of the whole experiment

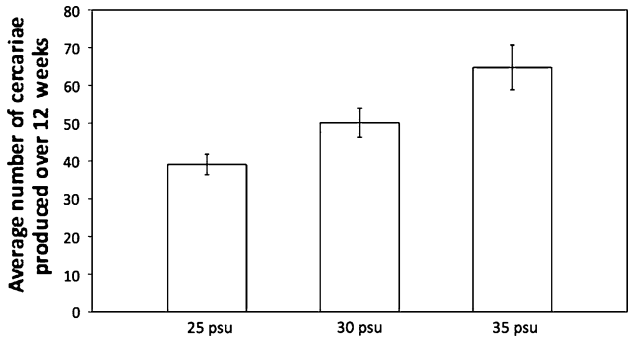

Fig. 3 Mean number (±standard error among replicates) of *Philophthalmus* sp. metacercariae attached per slide for the three salinity levels (25, 30 and 35 psu) across the whole exposure period

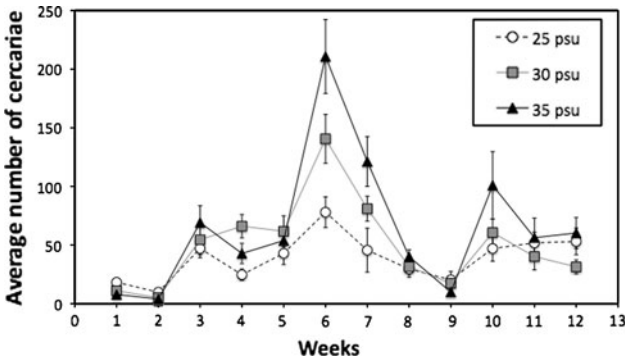

Fig. 4 Average number (±standard error among replicates) of *Philophthalmus* sp. metacercariae attached per slide each week for the three salinity levels (25, 30 and 35 psu) over the 12 weeks of exposure

$P = 0.0049$). Thus, salinity significantly affected cercarial output; on average, cercarial production was highest in the snails exposed to normal salinity levels of 35 psu and decreased with decreasing salinity (Fig. 3). Based on Tukey's tests, mean cercarial production did not differ between the 25 and the 30 psu treatments, but cercarial production at 35 psu was significantly higher than at 25 or 30 psu. Overall, 40% fewer cercariae were produced at 25 psu than at 35 psu (Fig. 3).

This analysis also revealed an effect of time ($F_{11,\ 597} = 48.71$, $P < 0.0001$), with the number of cercariae produced differing from week to week for all three salinity treatments (Fig. 4). An interaction effect ($F_{22,\ 597} = 3.06$, $P < 0.0001$) between salinity and week also emerged, implying that over the 12 weeks of treatment, cercarial production showed greater sensitivity to salinity on some occasions than others (Fig. 4). Lastly, there was also a container effect ($F_{15,\ 597} = 5.30$, $P < 0.0001$), which is accounted for by the nested design.

The other nested ANOVA, on snail shell lengths, revealed no significant differences in snail sizes among the three salinity treatments ($F_{2,\ 53} = 0.19$, $P = 0.981$) or among the containers within treatments ($F_{15,\ 53} = 0.27$, $P = 0.996$).

Experiment 2: cercarial encystment

A significant overall effect of salinity on cercarial encystment rates was revealed by the repeated measure analysis (tests for between-subjects effects: $F_{2,\ 9} = 29.60$, $P < 0.0001$), indicating that encystment rates depended on the salinity to which cercariae were exposed (Fig. 5). All cercariae exposed to 35 psu had encysted by 180 min, well before the end of the experiment. In contrast, only 63% and 25% of cercariae were encysted at the end of the experiment when exposed to 30 and 25 psu, respectively (Fig. 5). Tukey's tests indicated that differences in encystment rates between 35 psu and the other two treatments were highly

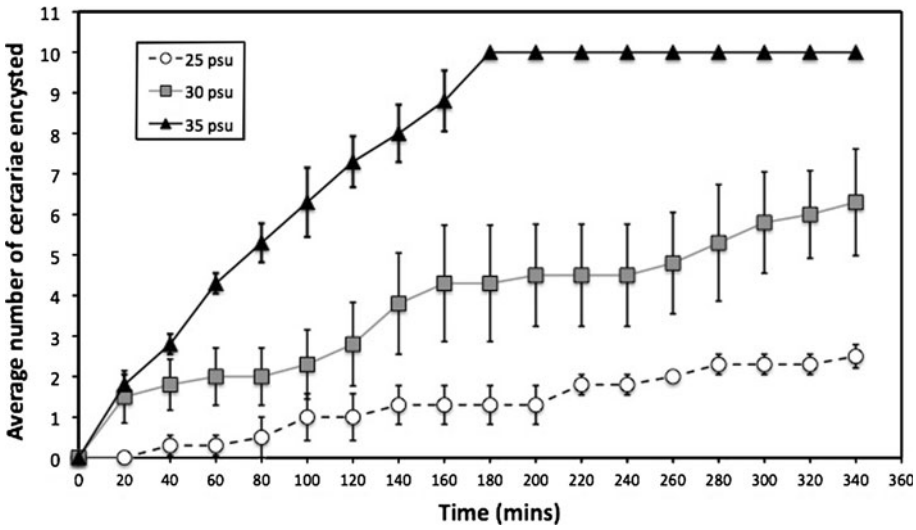

Fig. 5 Cumulative average number (±standard error among replicates) of *Philophthalmus* sp. cercariae that encysted as a function of time when exposed to the different salinity levels (25, 30 and 35 psu)

significant. However, there was no difference between the 25 and the 30 psu treatments ($P = 0.052$).

Mauchly's test indicated that data sphericity had been violated. Therefore, degrees of freedom for within-subjects effects were corrected using the Greenhouse–Geisser method. The results indicated a significant effect of time of sampling on how many cercariae had encysted (Greenhouse–Geisser: $F_{3.76,\ 33.83} = 75.58$, $P < 0.0001$) (Fig. 5). The within-subjects test also revealed an interaction between time of sampling and salinity (Greenhouse–Geisser: $F_{7.52,\ 33.83} = 9.01$, $P < 0.0001$), indicating that encystment rates changed differently among the different salinities over the 17 sampling occasions.

Experiment 3: metacercarial survival

The repeated measures analysis of metacercarial survival rates revealed that overall, there was a significant difference in survival among the salinity treatments (tests of between-subject effects: $F_{2,\ 15} = 9.02$, $P = 0.003$) (Fig. 6). Survival rates were similarly low at the lower salinity levels of 25 and 30 psu, and significantly lower than at 35 psu (based on Tukey's tests). Again, Mauchly's test indicated that the assumption of data sphericity had been violated, and degrees of freedom were therefore corrected using the Greenhouse–Geisser method. Results show that the survival rates in all treatments decreased over time (Greenhouse–Geisser: $F_{3.14,\ 47.08} = 419.56$, $P < 0.0001$) and that there was a significant interaction between salinity and time (Greenhouse–Geisser: $F_{6.28,\ 47.08} = 4.28$, $P = 0.001$). Thus, the survival of encysted metacercariae dropped at different rates for different salinities over the six-week experiment (Fig. 6).

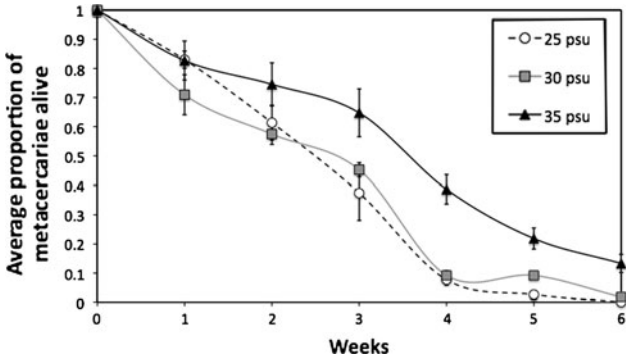

Fig. 6 Average proportion (±standard error among replicates) of surviving *Philophthalmus* sp. metacercariae over the 6 weeks of treatment for each of three salinity levels (25, 30 and 35 psu)

Discussion

Using *Philophthalmus* sp. in its snail host as model system, this study is one of the first to look at the effects of long-term exposure (weeks as opposed to hours or days) to different salinities on parasite replication and transmission success. Philophthalmid trematodes are common parasites of snails and shorebirds around the world (Kanev et al. 2005), and therefore our results are likely to apply to other ecosystems. In addition, although trematodes of other families generally encyst within a second intermediate host and not on exposed substrate, the observed impact of salinity on cercarial output within the snail host might apply to any trematode. Here, we found that salinity influenced the number of cercariae produced, the time they needed to encyst, how many succeeded at encysting and how long encysted metacercariae survived. Overall, it is very clear that all these key facets of parasite transmission

peaked in the treatment corresponding to the normal salinity of seawater, i.e. 35 psu, with lower salinities causing substantial decreases in parasite replication and its success outside the snail host.

Firstly, long-term exposure to reduced salinity leads to a decrease in the output of cercariae from infected snails. The replication capacity of trematodes within snails is usually positively correlated with host size (Kalantan et al. 1997); however, snail size did not differ among treatments in our experiment, reinforcing the conclusion that salinity caused the observed patterns. These results are not easy to reconcile with recent investigations into the effects of salinity on cercarial emergence, since previous studies used very short (hours to days) exposure periods. For instance, Mouritsen (2002) used a 12-h exposure to various salinities and temperatures and found that cercarial output by the trematode *Maritrema subdolum* in the marine snail *Hydrobia ulvae* was only influenced by salinity at certain temperatures. Koprivnikar and Poulin (2009) also used short-term (24 h) exposure to different temperature and salinity combinations to show that cercarial emergence of *Philophthalmus* sp., the same species as that in the present study, was highest when snails were exposed to the lower salinity of 30 psu. Because of their short-term nature, it is possible that cercarial emergence in those studies was merely the result of osmotic stress experienced by the host, as opposed to alterations in rates of within-snail parasite replication of the kind only detectable in a long-term study like the present one. Our cercarial output experiment also revealed significant week-to-week variation in addition to salinity effects. This may have been due to intrinsic cycles in cercarial production or to small fluctuations in room temperatures over the duration of the experiment, since trematode cercarial production and emergence are known to be extremely sensitive to temperature (Poulin 2006). Nevertheless, all replicates of all three treatments were exposed to the same room conditions, and the salinity effect thus remains.

Mouritsen (2002) suggested that for the trematode *M. subdolum*, a change in salinity might be used by the parasite as an emergence cue that would coincide with an optimal transmission window to its next host. However, given the transmission mode of *Philophthalmus* sp., other explanations must be sought for the relationship between salinity and cercarial output. One possible mechanistic explanation could be linked to the capacity of gastropods to tolerate lower salinity levels. Activity rates of marine gastropods are affected by various abiotic factors including salinity. Although marine gastropods occur across a wide range of salinities, their activity can be dramatically reduced and they may retreat into their shells at salinities lower than normal seawater (i.e. <35 psu) (Shumway 1979; Cheung and Lam 1995). Decrease in activity, measured by oxygen consumption, is an energy conservation strategy adopted by snails in suboptimal conditions to protect their tissues from osmotic stress (Cheung and Lam 1995). This can impact their trematode parasites and translate into lower cercarial output (Anderson et al. 1976). Hence, lower salinity could have lead to reduced activity in *Z. subcarinatus* resulting in fewer cercariae emerging in our experiment. An alternative explanation is that the capacity of the parasite itself to tolerate lower salinity outside the snails may affect how many cysts were recovered per slide. The lower cercarial encystment rates seen in this study at lower salinities can partially explain the cercarial output results, since these are based on cyst counts.

Secondly, as just mentioned, cercariae took longer to encyst, and fewer of them successfully encysted at salinities below that of normal seawater. Although the effects of salinity on the encystment rates of cercariae that encyst freely have not been previously investigated, the variation in cercarial encystment rates observed here is not surprising given the sensitivity of parasite free-living infective stages to external conditions (Pietrock and Marcogliese 2003). For instance, Koprivnikar et al. (2010) found species-specific cercarial survival rates as a function of salinity in two marine trematodes, *Euhaplorchis californiensis* and *Acanthoparyphium spinulosum*, both using the same snail intermediate host. In our study, the mechanism that caused cercariae to delay their encystment at low salinity is unclear. However, the low number of encysted metacercariae seen at both 30 and 25 psu by the end of the experiment was not the result of mortality, since unencysted cercariae were still exhibiting jerking motions, which is the normal swimming behaviour of philophthalmid cercariae. Lower salinity might then interfere with the onset of cyst formation, possibly by imposing osmotic stress through water intake and a diffusion of salts (Stunkard and Shaw 1931). Whatever the mechanism, after emerging from the gastropod host, cercariae are exposed to a very different environment where conditions are highly variable. Once at the mercy of abiotic factors and biotic factors such as predation, the ability of *Philophthalmus* sp. to encyst on a suitable substrate as quickly as possible is paramount to their survival and transmission. Slower encystment can also have further consequences for survival later in the life cycle, as metacercarial viability may be significantly reduced the longer it takes the cercariae to encyst (Graczyk and Shiff 1994).

Finally, metacercarial survival also decreased at lower salinities. Unlike most other trematodes, the life cycle of philophthalmids is unusual as it does not involve a second intermediate host. Instead, *Philophthalmus* sp. cercariae encyst on hard substrates where they transform into metacercariae. The only protection they obtain from the external environment comes in the form of a cyst in which

metacercariae await ingestion by a definitive host. Although the proportion of surviving metacercariae decreased over the six-week duration of the experiment for all salinity levels, those at 25 and 30 psu incurred higher mortality compared with those exposed to normal seawater salinity. Clearly, the cyst does not protect enclosed metacercariae from long-term osmotic stress at lower salinities. Perhaps, the cyst wall itself is not formed properly at low salinities, leading to its subsequently reduced protective ability. Alternatively, the cyst structure may be inadequate to seal the parasite from the outside water regardless of salinity. In some trematodes, like *Notocotylus attenuatus*, which also encysts on outside surfaces, the thick cyst is effective at protecting metacercariae from toxic metal ions (Evans 1982). However, in *N. attenuatus*, the cyst opening has a plug present (Southgate 1971; Dixon 1975), whereas in philophthalmid trematodes, the posterior aperture in the cyst is never plugged (Thakur and Cheng 1968; Nollen and Kanev 1995). The absence of a plug in *Philophthalmus* sp. means that metacercariae are not entirely sealed off from the external environment, a possible explanation for their decreased survival at low salinities.

In conclusion, lower salinity has negative impacts on all aspects of the biology of the parasite *Philophthalmus* sp. that we investigated in this study. Its replication rate in snail intermediate hosts, and its subsequent performance in the outside environment are all reduced at salinities below that of normal seawater, suggesting that low salinity would reduce transmission success to shore birds. On the basis of these findings, we may predict that among the habitats where the snail *Zeacumantus subcarinatus* occurs and where birds come to feed, estuaries exposed to lower average salinities may act as sinks and mudflats outside estuaries may act as sources (or foci) in the transmission dynamics of the parasite. On smaller spatial scales, such as seen on many mudflats where freshwater run-off causes localised areas of reduced salinity and strong gradients in salinity across just a few metres, the conditions might select for parasite genotypes capable of altering the salinity preferences of their snail hosts. Although this is something well within the range of host behavioural modifications reported for trematode parasites (Moore 2002; Poulin 2010), this possibility remains to be tested experimentally. Finally, in the context of the lower salinity levels predicted for certain parts of the world by some climate-change scenarios, we might expect strong selection acting on *Philophthalmus* sp. to adapt to the new conditions and become tolerant of reduced salinities. Parasites are important components of natural ecosystems, in particular trematodes in intertidal communities (Mouritsen and Poulin 2002). We certainly require a deeper understanding of how abiotic factors impact their epidemiology, and our study is one of the first long-term exposure studies on trematodes to tackle this issue.

Acknowledgments We thank Anja Studer, Tommy Leung and Christoph Matthaei for assistance with logistical and statistical aspects of this study and Melanie Lloyd, Devon Latoa and Nathan Brown-Haysom for assistance during snail collection.

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