



Differential tolerances to ocean acidification by parasites that share the same host



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ABSTRACT

Ocean acidification is predicted to cause major changes in marine ecosystem structure and function over the next century, as species-specific tolerances to acidified seawater may alter previously stable relationships between coexisting organisms. Such differential tolerances could affect marine host–parasite associations, as either host or parasite may prove more susceptible to the stressors associated with ocean acidification. Despite their important role in many ecological processes, parasites have not been studied in the context of ocean acidification. We tested the effects of low pH seawater on the cercariae and, where possible, the metacercariae of four species of marine trematode parasite. Acidified seawater (pH 7.6 and 7.4, 12.5 °C) caused a 40–60% reduction in cercarial longevity and a 0–78% reduction in metacercarial survival. However, the reduction in longevity and survival varied distinctly between parasite taxa, indicating that the effects of reduced pH may be species-specific. These results suggest that ocean acidification has the potential to reduce the transmission success of many trematode species, decrease parasite abundance and alter the fundamental regulatory role of multi-host parasites in marine ecosystems.

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1. Introduction

During the past 15 years, ocean acidification (OA) has been recognised as a major threat to all marine ecosystems (Raven et al., 2005; Doney et al., 2009). The current average pH of surface ocean waters is 8.1, and is predicted to fall to between 8.06 and 7.77 by the year 2100, and to approximately 7.41 by 2300 (Intergovernmental Panel on Climate Change (IPCC), 2014). This reduction in seawater pH is caused by an increased uptake of carbon dioxide (CO₂) by oceanic surface waters due to the increased concentration of atmospheric CO₂ (Raven et al., 2005). Once dissolved in seawater, CO₂ undergoes a series of chemical reactions that ultimately increases the concentration of hydrogen ions, reducing seawater pH, and decreases the concentration of carbonate ions, reducing the saturation state of seawater with respect to calcium carbonate. This process has the potential to negatively affect many marine species (Kroeker et al., 2013) by increasing the metabolic demands of maintaining inter- and intra-cellular acid/base homeostasis (Pörtner et al., 2004) and the biosynthesis of calcium carbonate structures (Parker et al., 2013). Tolerance to stressors associated with OA varies across and within taxonomic groups (Doney et al., 2009), suggesting that OA may destabilise

interactions between coexisting species, i.e., competitive, predatory or parasitic relationships. Parasitic relationships often involve multiple, phylogenetically diverse, host organisms, which increases the likelihood that we may observe differential tolerances to OA between species involved in the life cycle of a parasite (MacLeod and Poulin, 2012). Such differential tolerances have the potential to alter previously stable host–parasite interactions in the marine environment. Any changes to the calcifying ability of marine organisms caused by OA may further disrupt host–parasite associations, as many parasite hosts are molluscs. The increased metabolic costs of forming calcified structures in an acidified environment could reduce the energy available to parasite species and negatively affect the development of intramolluscan marine parasites.

Parasites play a major role in regulating host populations and, as a result, ecosystem biodiversity, structure and function (Combes, 1996). Consequently, many parasite species have been exposed to simulated anthropogenic stressors to predict the indirect effects of these stressors on host populations: temperature (Jensen and Mouritsen, 1992), salinity (Koprivnikar and Poulin, 2009), ultra-violet radiation (Studer et al., 2012) and trace metals (Morley et al., 2002). Studies have shown that anthropogenic stressors can alter many aspects of host–parasite interactions such as host population density (Mouritsen et al., 2005), parasite reproduction (Poulin, 2006), host immune function (Morley et al., 2006) and parasite transmission success (Morley et al., 2005). While the

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effects of reduced pH on marine parasites have received little attention, the responses of freshwater parasites to reduced pH have been documented in ecosystems affected by acid rain or in sites of naturally low pH (see review in Marcogliese, 2001). In freshwater systems, low pH was correlated with reduced parasite species richness, although it is unknown if the observed effects were due to a pH-mediated reduction in parasite transmission success, increased mortality of first intermediate hosts or a combination of factors. Given the findings of these studies, it seems likely that reduced pH will also affect parasites in marine environments.

In coastal ecosystems, trematodes (phylum Platyhelminthes) are the most dominant parasite group (Mouritsen and Poulin, 2002), with some species infecting up to 80–100% of individuals in host populations (Fredensborg et al., 2005). Trematode parasites commonly use three different host species to complete their life cycle: the first intermediate host, site of asexual reproduction; the second intermediate host, site of external or internal cyst (metacercaria) formation; and the definitive host, site of sexual reproduction. The trematode life cycle requires the consumption of parasite eggs by the first intermediate host or active infection by free-swimming miracidia, active or passive infection of the second intermediate host by free-swimming cercariae, and the consumption of an infected organism (second intermediate host) by the definitive host. Consequently, it should be possible to trace any effects of anthropogenic stressors on parasite fitness through the trematode prevalence and intensity of infection in multiple host species.

Four stages of the trematode life-cycle are directly exposed to environmental conditions: eggs, miracidia, cercariae and externally formed metacercariae. These life stages are lecithotrophic, i.e., they do not feed once they have emerged from the definitive or first intermediate host (Pietrock and Marcogliese, 2003), and any increase in metabolic demands caused by exposure to an acidified environment may reduce the energy available for locating and infecting the next host in their life-cycle. This article focusses on the cercarial and external metacercarial transmission stages of marine trematode parasites.

Any change to seawater pH could increase the amount of energy expended by cercariae to maintain extra- and intra-cellular acid/base homeostasis and cause a corresponding reduction in cercarial longevity. We use the term longevity to define the active period of cercariae, either prior to death or prior to the formation of external metacercarial cysts, depending on the life-history of the trematode species. Energy expended by cercariae during transmission will also have an effect on the energy available to metacercariae, possibly reducing their growth or life span. Any reduction in the longevity of cercariae or the viability of metacercariae will lower the probability of the parasite reaching the next host in its life-cycle, potentially reduce the proportion of parasites that successfully reach the definitive host, and ultimately may cause a decline in the overall abundance of the parasite.

Although no research has been conducted on the effects of OA on cercarial longevity in accordance with the best practice acidification techniques outlined by Riebesell et al. (2010) (see comment on Koprivnikar et al. (2010) in Section 4), there has been a significant amount of research that investigated the effects of OA on the larvae of many non-parasitic marine organisms. There are many functional and morphological parallels between cercariae and marine larvae: both represent a link between adults of their species and new settlement sites, are often lecithotrophic, and are vulnerable to changing environmental conditions due to their small size and large surface area to volume ratio (Zimmer et al., 2009; Morley, 2011). The predominant results of the exposure of non-parasitic larvae to low pH seawater are reduced survival (Talmage and Gobler, 2011; Gonzalez-Bernat et al., 2013), metabolic depression (Pimentel et al., 2014) and tissue damage

(Frommel et al., 2011). In light of the morphological parallels between non-parasitic larvae and cercariae, cercariae may be similarly affected by low pH seawater.

This study used a custom-designed OA simulation system to investigate the effects of acidified seawater (pH 7.6 and 7.4, 12.5 °C) on the cercarial longevity of four species of trematode parasites: *Maritrema novaezealandensis*, *Philophthalmus* sp., *Parorchis* sp. and *Galactosomum* sp. Two of these species, *Philophthalmus* sp. and *Parorchis* sp., form metacercarial cysts on hard substrates such as shell (Neal and Poulin, 2012), which allowed us to also test the effect of reduced pH on metacercarial survival. *Maritrema novaezealandensis* cercariae infect several crustacean species as second intermediate hosts (Martorelli et al., 2004), while cercariae of the genus *Galactosomum* typically infect teleost fish (Beuret et al., 2000). As the majority of marine non-parasitic species exposed to low pH respond variably, yet negatively (Kroeker et al., 2013), we predicted that cercarial longevity and metacercarial survival would be reduced by exposure to acidified seawater, and that we would also find substantial differences between the responses of each trematode species. The effects of reduced pH on the longevity and survival of all parasites is discussed in the context of OA-mediated changes to parasite abundance in marine ecosystems, while differential tolerances between parasite species are discussed in relation to the physicochemical environment encountered by cercariae and metacercariae during the transmission process.

2. Materials and methods

2.1. Host–parasite system

Three of the four trematode species used in this experiment – *M. novaezealandensis*, *Galactosomum* sp. and *Philophthalmus* sp. (described in Martorelli et al., 2004) – infect the mud snail *Zeacumantus subcarinatus* as first intermediate host, while the fourth species, *Parorchis* sp. (described in O'Dwyer et al., 2014), infects the littorinid snail, *Austrolittorina cincta*. *Zeacumantus subcarinatus* and *A. cincta* are herbivorous grazers and have a wide distribution along the New Zealand coast. Both snail species were collected from Lower Portobello Bay (LPB), New Zealand (45°49'50"S, 170°40'17"E), where they are found in very high densities. Based on a year-long observational study that took weekly pH measurements, the average seawater pH at LPB is 8.083 ± 0.122 , in good agreement with the present-day average for oceanic pH, although pH variability is extreme (pH 7.599–9.186, unpublished data). *Zeacumantus subcarinatus* is found in a wide range of habitats in the Bay – sandy sediment, mud flats and rocky shore – while *A. cincta* predominantly inhabits the upper rocky shore.

2.2. Parasite collection and preparation

Approximately 2000 *Z. subcarinatus* and 100 *A. cincta* snails were collected at LPB in July 2013 and subsequently screened for trematode infection by exposing snails to physical conditions that trigger cercarial emergence. *Zeacumantus subcarinatus* snails were placed in warmed seawater (25 °C) and exposed to constant light, while *A. cincta* snails were placed in unmodified seawater, exposed to constant light, and kept in constant motion (shaker plate, 80 rpm). Trematode species were identified by inspecting cercariae under a dissecting microscope and comparing cercarial morphology with published descriptions of all parasite species (Martorelli et al., 2008; O'Dwyer et al., 2014). Snails that were positively identified as infected with a parasite of interest were maintained at room temperature (approximately 18–20 °C) for 1 week before

being screened a second time, thus reducing the probability of selecting snails that were infected by two parasite species. All snails selected for the experiment were then marked with individual identification labels (Bee Works, Orillia, Canada), maintained at room temperature in aerated seawater (approximately pH 8.1, 20 °C) and fed sea lettuce (*Ulva* sp.) ad libitum.

Prior to the cercarial trials, the snails were placed in 5 mL wells of acidified or unmodified seawater (pH 8.1, 7.6 or 7.4) and cercarial emergence was triggered using the protocol described above. To maintain continuity in the pH conditions cercariae experienced, the pH of seawater in each well matched the pH treatment to which the cercariae would be exposed, e.g. if cercariae were to be exposed to pH 7.4, infected snails were placed in wells filled with pH 7.4 seawater. To standardise the age of cercariae used in the longevity trials, only cercariae that emerged from host snails in the first hour were included in the experiment.

During the collection of *Philophthalmus* sp. and *Parorchis* sp. cercariae, many metacercariae of both species formed cysts on the interior surface of the plastic wells (approximately 30 per well for both species). These metacercariae were used to evaluate metacercarial survival under acidified conditions by placing the plastic wells in the appropriate culture tank (see Section 2.3), i.e., metacercariae that were formed in pH 7.4 seawater were placed in the pH 7.4 treatment culture tank.

2.3. Experimental apparatus

In order to expose cercariae and metacercariae to acidified seawater, a modular ocean acidification simulation system was designed (MacLeod et al., 2015). Three seawater aquaria were constructed, each consisting of a 120 L culture tank (870 mm (L) × 600 mm (W) × 295 mm (H)), a pump and filtration unit, a refrigeration unit and a pH regulation unit (see Supplementary Fig. S1). pH, measured on the total hydrogen ion scale, was adjusted with 100% CO₂ gas and monitored potentiometrically with glass electrodes calibrated with saltwater buffers (2-amino-2-hydroxy-1,3-propanediol (TRIS) and 2-aminopyridine (AMP)). Temperature was actively controlled using the flow-through chiller unit, while total alkalinity (A_T) and salinity were passively controlled by the regular addition of unmodified seawater (20 L/48 h); light levels were also standardised across all culture tanks. Seawater in the three culture tanks was maintained at 12.5 °C, 32 (Practical Salinity Scale) and at one of the three pH treatment levels: pH 7.4, 7.6 and 8.1. The pH 7.6 and 7.4 treatments were selected based on predictions in the IPCC report (2014); temperature and salinity values were selected based on average conditions in the habitat of *Z. subcarinatus* and *A. cincta* (unpublished data). We also validated the potentiometric regulation of pH by measuring A_T and dissolved inorganic carbon (DIC) in seawater samples taken from each culture tank, and used that data to calculate pH with the software package SWCO2 (http://neon.otago.ac.nz/research/mfc/people/keith_hunter/software/swco2/) (Table 1).

Two novel pieces of equipment were constructed to expose the cercariae to acidified seawater. The larger cercariae (*Galactosomum* sp., length: 0.76–2.11 mm (Martorelli et al., 2008), *Philophthalmus* sp., length: 0.8–1.0 mm (Martorelli et al., 2008), and *Parorchis* sp., length: 0.66–1.077 mm (O'Dwyer et al., 2014)) were placed in

cylindrical chambers fixed in floating platforms (20 cm × 10 cm) in each culture tank. Each chamber consisted of a length of plastic tubing (20 mm (L) × 10 mm (D)) covered at one end by 25 μm gauge nylon mesh. Sixteen chambers were inserted vertically into each floating platform with the mesh-covered end facing downward. When the platforms were floated in the culture tanks, the lower half of the chambers was submerged, immersing the cercariae in seawater. To record the proportion of living and/or unencysted cercariae, each platform was periodically lifted out of the tank within a larger container of seawater taken from the same culture tank; both containers were placed under a dissecting microscope and the number of living and/or unencysted cercariae recorded. As cercariae of all trematode species included in this study appeared to be phototactic (our personal observations), the light emitted by the dissecting scope was used as a stimulus to assess the status of the cercariae. *Maritrema novaezealandensis* and *Galactosomum* sp. cercariae that responded to the light with movement were classified as alive, while those that did not move were classified as functionally dead. *Philophthalmus* sp. and *Parorchis* sp. cercariae were never categorised as dead, as they either responded to the light or had formed a cyst.

For the smaller cercariae of *M. novaezealandensis* (length: 0.145–0.190 mm (Martorelli et al., 2004)), plastic culture plates containing 12 wells were modified so that they could be partially submerged in the culture tanks. After cercariae were loaded into the wells with acidified or unmodified seawater, the rim of each well was coated in a layer of petroleum jelly and a glass plate pressed down simultaneously onto the top of all 12 wells. The seal provided by the petroleum jelly prevented the acidified seawater from being affected by ambient pCO₂ and the glass plate allowed for a visual count of cercariae to be made at inspection times; partial submersion of the plate allowed the temperature to be kept constant. When recording the survival of *M. novaezealandensis* cercariae, the wells were lifted out of the tanks within containers of seawater taken from the same tank, placed under a dissecting scope and the number of surviving cercariae recorded. At the end of each 12 h experiment, the pH in each well was measured to check for deviation from the initial pH. In all cases, the pH of seawater in the wells remained within 0.01 units of target values.

2.4. Cercarial longevity

Between 1 August and 31 October 2013, three cercarial longevity trials were conducted for each parasite species. Each trial consisted of a 12 h exposure of all parasites species to all pH treatments, and was carried out over 6 days (approximately 2 days per week over a 30 day period). The duration of the trials (12 h) was based on preliminary data that showed significant reductions in cercarial longevity of all trematode species within that time frame, a literature review of cercarial longevity under control conditions (e.g., Studer and Poulin, 2013), and on the assumption that cercarial transmission in some species would occur while cercariae were isolated in seawater pools at low tide (6–8 h; our personal observations). Observations of the active period of cercariae prior to death (*M. novaezealandensis* and *Galactosomum* sp.) and the active period of cercariae prior to encystment (*Parorchis* sp. and *Philophthalmus* sp.) were combined under the term longevity, as

Table 1

Mean values (±S.D.) of all measured and calculated parameters used to characterise the carbonate chemistry of unmodified and acidified seawater.

Treatment	pH (measured)	Temp. (°C)	Salinity	Alkalinity (μmol kg ⁻¹)	DIC (μmol kg ⁻¹)	pH (calculated)	pCO ₂ (Calculated)
pH 8.1	8.09 ± 0.03	12.5 ± 0.3	31.7 ± 0.6	2361 ± 10	2138 ± 11	8.12 ± 0.03	365 ± 30
pH 7.6	7.60 ± 0.03	12.6 ± 0.6	31.9 ± 0.6	2389 ± 7	2351 ± 16	7.64 ± 0.04	1304 ± 115
pH 7.4	7.40 ± 0.03	12.6 ± 0.5	31.3 ± 0.6	2375 ± 12	2397 ± 13	7.45 ± 0.04	1980 ± 110

Temp., temperature; DIC, dissolved inorganic carbon.

Table 2
The order in which cercariae of each individual parasite were exposed to the three pH treatments used in activity trials. Numbers in parentheses indicate which sub-group of 16 host snails were used to provide cercariae on a given day.

	pH 8.1	pH 7.6	pH 7.4
Day 1	<i>Galactosomum</i> sp.(1–8)	<i>Maritrema novaezealandensis</i> (1–8) <i>Parorchis</i> sp. (1–8)	<i>Philophthalmus</i> sp. (1–8)
Day 2	<i>Galactosomum</i> sp. (9–16)	<i>M. novaezealandensis</i> (9–16) <i>Parorchis</i> sp. (9–16)	<i>Philophthalmus</i> sp. (9–16)
Day 12	<i>Philophthalmus</i> sp. (1–8)	<i>Galactosomum</i> sp. (1–8)	<i>M. novaezealandensis</i> (1–8) <i>Parorchis</i> sp. (1–8)
Day 13	<i>Philophthalmus</i> sp. (9–16)	<i>Galactosomum</i> sp. (9–16)	<i>M. novaezealandensis</i> (9–16) <i>Parorchis</i> sp. (9–16)
Day 21	<i>M. novaezealandensis</i> (1–8) <i>Parorchis</i> sp. (1–8)	<i>Philophthalmus</i> sp. (1–8)	<i>Galactosomum</i> sp. (1–8)
Day 22	<i>M. novaezealandensis</i> (9–16) <i>Parorchis</i> sp. (9–16)	<i>Philophthalmus</i> sp. (9–16)	<i>Galactosomum</i> sp. (9–16)

either reduced survival or an increased rate of encystment will reduce the functional life-span of the cercariae.

To avoid any unwanted variation in longevity and survival data caused by a particular seawater aquarium, i.e., a tank effect, each module was assigned a different pH treatment in each of the three trials. In all trials, cercariae were collected from 16 host snails per parasite species; we assumed that only one parasite individual, i.e., one genotype derived from a single miracidium, was present in each host snail. Host snails were also divided into two groups of eight and used to supply cercariae on different days during the longevity trials (see Table 2). This approach was intended to avoid the effect of any unknown factor, present on a particular day, affecting the longevity data generated for a given species of parasite. During each complete trial, cercariae were collected from each snail and used to stock two chambers per pH treatment, i.e., 32 chambers per parasite species per pH treatment ($n = 384$). The average number of cercariae per chamber varied between parasite species: *Galactosomum* sp., 11; *Philophthalmus* sp., 10; *Parorchis* sp., 13; and *M. novaezealandensis*, 34. The number of living and/or unencysted cercariae in each chamber was recorded at the beginning of the 12 h period (see Section 2.3) and then recorded every 2 h ($t = 0, 2, 4, 6, 8, 10$ and 12 h). The number of living and/or unencysted cercariae at each time point was used to calculate the change in proportion of living and/or unencysted cercariae in each chamber over the 12 h period.

2.5. Metacercarial survival

Because the metacercariae of *Philophthalmus* sp. and *Parorchis* sp. were produced as a by-product of cercarial collection and were monitored over a longer period than the cercariae (12 days), only two metacercarial survival trials were completed between 1 August and 31 October 2013. Each trial consisted of 32 wells (approximately 30 metacercariae per well, two wells per individual parasite i.e., per infected snail) immersed in each pH treatment for 12 days ($n = 192$). After recording the initial number of metacercariae in each well, the number of living cysts was recorded every 2 days for 12 days ($t = 0, 2, 4, 6, 8, 10$ and 12 days) and used to calculate the change in the proportion of surviving metacercariae in each well during the 12 day period. Cysts were categorised as “living” when defined metacercarial features could be clearly seen with a dissecting microscope and “dead” when the cysts appeared opaque (due to degraded tissue) or empty.

2.6. Statistical analysis

Generalised linear mixed effect models with binomial error structures were used to analyse the effects of reduced pH on

cercarial longevity and metacercarial survival, within and between parasite species; this type of model structure allowed us to use untransformed data and random effect variables. All models used “Parasite ID” and “Chamber ID” or “Well ID” as random effects, thus compensating for repeated measures of parasites taken from the same snail (Parasite ID), and repeated measures of the cercariae in each chamber (Chamber ID) or the metacercariae in each well (Well ID). An overall analysis, used to detect differences between parasite species, included pH, time, parasite species and median size of cercariae as fixed effects, and the proportion of living and/or unencysted cercariae in each chamber (*M. novaezealandensis*, *Philophthalmus* sp., *Galactosomum* sp. and *Parorchis* sp.), or the proportion of living metacercariae in each well (*Philophthalmus* sp. and *Parorchis* sp.), as the response variable. In models used to detect the effects of pH on individual parasite species, the fixed effects were pH and time, and the response variable was the proportion of living and unencysted cercariae in each chamber (*M. novaezealandensis*, *Philophthalmus* sp., *Galactosomum* sp. and *Parorchis* sp.) or the proportion of living metacercariae in each well (*Philophthalmus* sp. and *Parorchis* sp.). Significant differences in cercarial longevity and metacercarial survival were detected between pH treatments for all parasite species using general linear mixed effect models, and are reported at 6 and 12 h (cercariae) and days 6 and 12 (metacercariae) in Table 3 and Supplementary Table S1. Fixed effects were considered significant if P values were less than or equal to 0.05. Analysis was completed using R version 3.1.0 (R Development Team, <http://www.r-project.org/>; 2014-11-11) and the function *glmer* in the package *lme4* v. 1.1-7 (<http://cran.r-project.org/web/packages/lme4/index.html>).

The intra-class correlation (ICC) of “Parasite ID”, “Chamber ID” and “Well ID” was calculated to quantify the repeatability of longevity and survival data recorded between parasite individuals, cercarial chambers and metacercarial wells:

Table 3
The mean percentage of active/unencysted cercariae in each pH treatment after 6 and 12 h.

	6 h			12 h		
	8.1	7.6	7.4	8.1	7.6	7.4
<i>Galactosomum</i> sp.	92%	90%	80% ^{a,b}	81%	73% ^a	52% ^{a,b}
<i>Maritrema novaezealandensis</i>	83%	78%	57% ^{a,b}	54%	41% ^a	21% ^{a,b}
<i>Parorchis</i> sp.	61%	54% ^a	30% ^{a,b}	43%	38%	22% ^{a,b}
<i>Philophthalmus</i> sp.	60%	56%	42% ^{a,b}	54%	51%	35% ^{a,b}

^a Significantly different from the pH 8.1 treatment.

^b Significantly different from the pH 7.6 treatment.

$$ICC = \left(\frac{\text{(between – individual variance)}}{\text{(between – individual variance) + (within – individual variance)}} \right) * 100$$

An ICC score of 0% indicates no repeatability of measurements between individuals and a score of 100% indicates identical measurements, i.e., pseudoreplication. Calculating ICC scores allowed us to assess the independence, or lack thereof, of data points taken from the same parasite, i.e., two chambers containing cercariae or metacercariae from the same parasite individual (i.e., same infected snail), and between cercariae and metacercariae taken from different parasite individuals but maintained in the same pH treatment (Supplementary Table S2).

3. Results

The cercariae of all parasite species exhibited reduced longevity over the 12 h monitoring period in all treatments (Figs. 1–4). Cercarial longevity was significantly affected by pH and time in all parasite species, and by the interaction of pH and time in *Galactosomum* sp., *Philophthalmus* sp. and *Parorchis* sp. (Table 4). In the 7.6 pH treatment, significant reductions in cercarial longevity were recorded in *Parorchis* sp. after 6 h, and in *Galactosomum* sp. and *M. novaezealandensis* after 12 h. In the 7.4 pH treatment, all parasite species showed significantly reduced cercarial longevity at both time points (Table 4). The inclusion of the fixed terms “Parasite species” and “median size of cercariae” showed that the species of parasite had a significant effect on cercarial longevity ($F_{3,8025} = 182.4$, $P < 0.001$), while the size of cercariae did not ($F_{1,8025} = 0.093$, $P = 0.76$) (note: the denominator of the F -statistic indicates the combined number of observations at seven time points over 12 h for four parasite species in three replicate trials). Post hoc analysis of longevity data showed significant differences between all parasite species ($P < 0.001$ in all paired comparisons).

The metacercarial survival of *Philophthalmus* sp. also decreased over time in all treatments and was significantly affected by pH, time and the interaction of these factors (Table 3). Exposure to pH 7.6 and 7.4 seawater caused significantly reduced metacercarial survival at 6 and 12 days (Fig. 5, Supplementary Table S1). The metacercariae of *Parorchis* sp. exhibited no mortality during the 12 day observational period and were excluded from analysis.

ICC scores generated by “Parasite ID”, “Chamber ID” and “Well ID” showed that the average repeatability of longevity or survival data was: 19% between samples of the same individual parasite; 54% between chambers containing groups of cercariae from different individual parasites; and 27% between chambers containing

groups of metacercariae from different individual parasites (Supplementary Table S2). These results indicate that repeatability is generally moderate; therefore dependence of data points (pseudoreplication) is not a major confounding issue in this study.

4. Discussion

To date, Koprivnikar et al. (2010) is the only known study that exposed cercariae to acidified seawater, although the authors

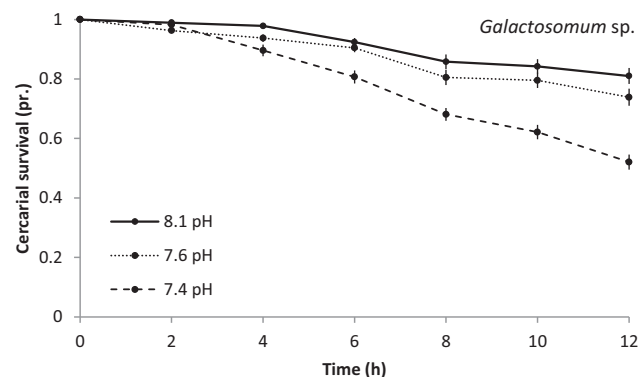


Fig. 2. Survival of *Galactosomum* sp. cercariae exposed to pH 8.1, 7.6 and 7.4 seawater. Data points represent mean proportions (pr.) of surviving cercariae \pm S.E. ($n = 96$).

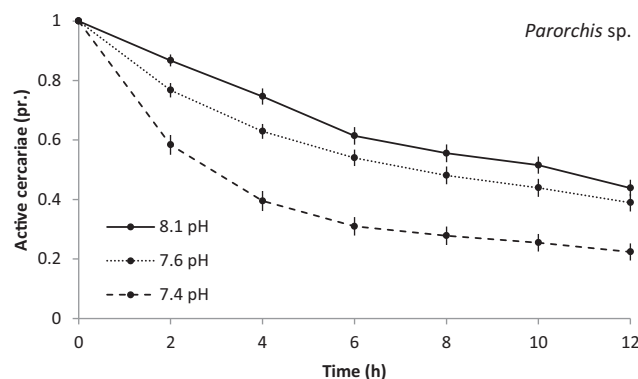


Fig. 3. Activity of *Parorchis* sp. cercariae exposed to pH 8.1, 7.6 and 7.4 seawater. Data points represent mean proportions (pr.) of active and unencysted cercariae \pm S.E. ($n = 96$).

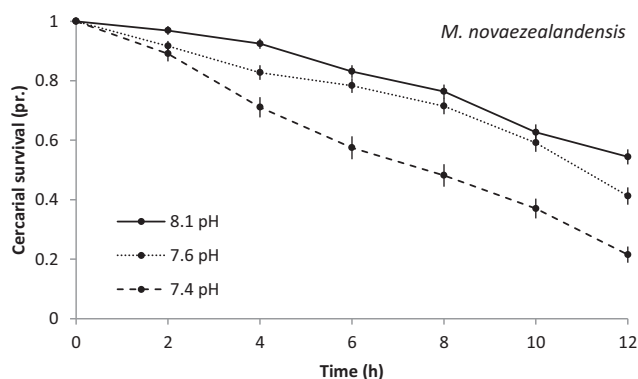


Fig. 1. Survival of *Maritrema novaezealandensis* cercariae exposed to pH 8.1, 7.6 and 7.4 seawater. Data points represent mean proportions (pr.) of surviving cercariae \pm S.E. ($n = 96$).

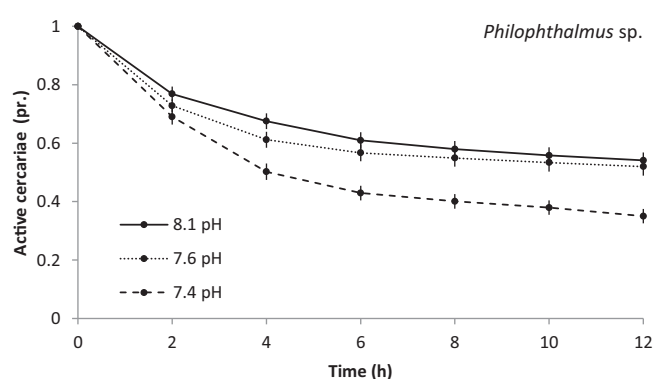


Fig. 4. Activity of *Philophthalmus* sp. cercariae exposed to pH 8.1, 7.6 and 7.4 seawater. Data points represent mean proportions (pr.) of active and unencysted cercariae \pm S.E. ($n = 96$).

Table 4
ANOVA outputs from the generalised linear mixed effects models used to analyse the activity of cercariae and survival of metacercariae exposed to three pH treatments (8.1, 7.6 and 7.4) for 12 h. $n = 32$ per trial for each parasite species in each pH treatment.

Species	Factor	df	Trial 1		Trial 2		Trial 3	
			F	P	F	P	F	P
<i>Galactosomum</i> sp. (cercariae)	pH	2	18.2	<0.001	19.7	<0.001	12.5	<0.001
	Time	1	281.3	<0.001	226.9	<0.001	178.7	<0.001
	pH*Time	2	6.6	0.001	2.6	0.075	6.6	0.001
<i>Maritrema novaezealandensis</i> (cercariae)	pH	2	22.1	<0.001	19.4	<0.001	25.5	<0.001
	Time	1	211.5	<0.001	66.8	<0.001	247.9	<0.001
	pH*Time	2	31.0	<0.001	2.4	0.091	2.2	0.109
<i>Philophthalmus</i> sp. (cercariae)	pH	2	8.4	<0.001	9.34	<0.001	6.1	0.002
	Time	1	211.9	<0.001	167.1	<0.001	94.3	<0.001
	pH*Time	2	4.4	0.013	3.7	0.025	5.1	0.006
<i>Parorchis</i> sp. (cercariae)	pH	2	17.7	<0.001	12.1	<0.001	8.6	<0.001
	Time	1	197.0	<0.001	167.2	<0.001	144	<0.001
	pH*Time	2	10.5	<0.001	16.9	<0.001	11.3	<0.001
<i>Philophthalmus</i> sp. (metacercariae)	pH	2	542.9	<0.001	428.5	<0.001	–	–
	Time	1	291.4	<0.001	235.8	<0.001	–	–
	pH*Time	2	76.8	<0.001	65.4	<0.001	–	–

Bold indicates significance ($P < 0.05$).

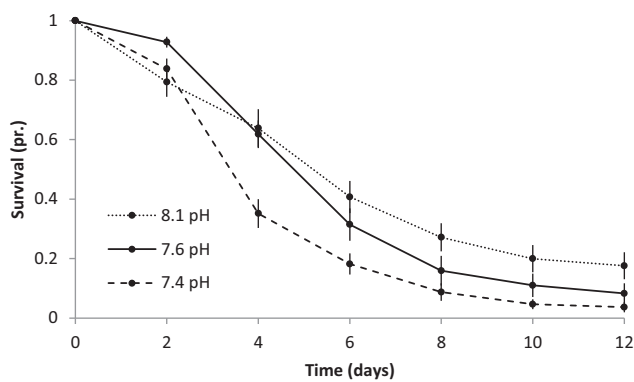


Fig. 5. Survival of *Philophthalmus* sp. metacercariae exposed to pH 8.1, 7.6 and 7.4 seawater. Data points represent mean proportions (pr.) of surviving metacercariae \pm S.E. ($n = 64$).

found no direct effect of pH on cercarial survival. However, that study included only one pH treatment (pH 7.8) and the acidification techniques used were not in accordance with the “Guide to best practices for ocean acidification research and data” reporting (Riebesell et al., 2010). Accordingly, the present article describes, to our knowledge, the first rigorous test of cercarial longevity and metacercarial survival in acidified seawater. We chose pH treatments based on current surface ocean conditions (pH 8.1), the range of predicted conditions for the year 2100 (pH 8.07–7.77), and the estimated oceanic pH for the year 2300 (pH \sim 7.4) (IPCC, 2014). In accordance with current recommendations for OA simulation systems, we reduced pH by bubbling CO₂ gas directly into temperature-controlled seawater, measured pH on the total hydrogen ion scale, and recorded A_T and DIC throughout the study to validate pH control and fully characterise the carbonate chemistry of modified seawater (Dickson et al., 2007; Riebesell et al., 2010; MacLeod et al., 2015).

The cercarial longevity of all parasite species was significantly reduced at pH 7.4, while the cercariae of *Parorchis* sp., *Galactosomum* sp. and *M. novaezealandensis* also exhibited significantly reduced longevity at pH 7.6. Species-specific responses to reduced pH can also be seen in Figs. 1–4, and statistical analysis confirmed that parasite species identity significantly influenced the longevity of cercariae exposed to acidified seawater. *Philophthalmus* sp. metacercariae exhibited significantly reduced

survival at pH 7.6 and 7.4 (Fig. 5), while the metacercariae of *Parorchis* sp. showed no mortality in any treatment over 12 days. These results clearly support our hypothesis that cercariae and metacercariae of all parasite species would be negatively affected by reduced pH and that the severity of these effects would be species-specific.

The majority of OA research to date has focussed on establishing such species-specific responses to acidified seawater, as a tolerance of pH stress may indicate phenotypic plasticity that will enable certain species to survive changing oceanic conditions. The adaptive abilities of marine organisms are influenced by the environmental conditions they experience throughout their lifespan, i.e., an organism will retain the ability to adapt to a stressor if it regularly experiences that stressor (DeWitt and Scheiner, 2004).

The habitat-specific range of seawater pH experienced by marine organisms has been used to predict tolerance of pH stress more reliably than phylogenetic relationships, as organisms taken from habitats that experience a high degree of variability in ambient pH typically exhibit a greater tolerance to reduced pH conditions than conspecifics from more stable habitats (e.g., Lardies et al., 2014; Zhang et al., 2014). Trematode parasites are exposed to the highly variable environment of the intertidal zone during free-living transmission between hosts and experience the internal micro-environment of host bodies post-transmission, e.g., when burrowing through crustacean somatic tissue (Fredensborg et al., 2004) or penetrating fish organs (Beuret et al., 2000). Consequently, it is vital to view the response of cercariae and metacercariae to acidified seawater in the context of the range of pH values they would experience throughout their entire life cycle, not only during direct exposure to seawater.

Due to the preferred habitats of *Z. subcarinatus* and *A. cincta* (low and high intertidal, respectively), and the conditions that trigger cercarial emergence from each species (*Z. subcarinatus* – warm temperature and constant light; *A. cincta* – constant light and motion), we inferred that parasites infecting *Z. subcarinatus* would release cercariae into tidal pools, while parasites infecting *A. cincta* would release cercariae during tidal inundation.

At the collection site of both species of snail, tidal pool seawater has a greater variability in pH than incoming tidal seawater (tidal pool seawater, pH 7.60–9.19; incoming tidal seawater, pH 7.82–8.35, unpublished data). Accordingly, cercariae emerging from *Z. subcarinatus* are exposed to a greater range in seawater pH than cercariae emerging from *A. cincta*. The difference in pH variability

between tidal pool seawater and incoming tidal seawater is due to biotic and abiotic factors that have a greater impact on small volumes of water: photosynthesis, respiration and freshwater influx (Duarte et al., 2013). This differential variability suggests that *Parorchis* sp. cercariae may be less tolerant of low pH conditions than all trematode species infecting *Z. subcarinatus*. This prediction is supported by cercarial longevity data which show that *Parorchis* sp. exhibited the lowest proportion of living and/or unencysted cercariae in both acidified treatments after 6 h, and in the pH 7.6 treatment after 12 h.

After the initial infection of their second intermediate hosts, *Galactosomum* sp. cercariae are exposed to acidic environments within the body of the host which may affect their tolerance to acidified seawater. Cercariae of this genus typically have a dark pigmentation and exhibit a very distinctive undulating swimming action which is believed to mimic the prey of planktivorous fish that act as second intermediate hosts (Pearson, 1973; Rekharani and Madhavi, 1985; Beuret et al., 2000). Once consumed by the fish host, the cercariae pass through the digestive system, where they penetrate the gut wall before tunnelling through somatic tissue to reach the brain. In the brain, cercariae encyst as metacercariae and await a second predation event, i.e., a shore bird consuming the fish, which completes the life cycle of the parasite (Prudhoe, 1949; Culurgioni et al., 2007). As *Galactosomum* sp. cercariae can survive the extreme pH environment of the fish stomach (pH 3.5–6.0, Taylor and Grosell, 2006), they may possess a pre-adaptation for low seawater pH. The presence of this pre-adaptation is supported by the cercarial longevity data, as *Galactosomum* sp. cercariae exhibited the lowest overall reduction in longevity, and the highest proportion of living and/or unencysted cercariae after 12 h, of all parasite species exposed to acidified seawater (Table 3). These results suggest that *Galactosomum* sp. cercariae may be more tolerant of the reduced pH associated with OA than other parasites that infect *Z. subcarinatus*.

Maritrema novaezealandensis uses many species of crustacean as a second intermediate host (Koehler and Poulin, 2010). Cercariae penetrate the outer cuticle and somatic tissue of the crustaceans before encysting in their body cavity (Martorelli et al., 2004). The transmission to their definitive host is achieved when the crustacean is consumed by a shorebird. Extra- and intra-cellular pH in crustacean somatic tissue is maintained at approximately pH 7.2–7.8 (Wheatly and Henry, 1992). Accordingly, we expected *M. novaezealandensis* cercariae to be more tolerant of low pH conditions than *Philophthalmus* sp. or *Parorchis* sp. cercariae, which form metacercariae in the least acidic environment, but less tolerant than *Galactosomum* sp. cercariae. Contrary to our predictions, *M. novaezealandensis* was the least tolerant of low pH conditions (see Fig. 1 and Table 3). This may be due to the relatively small size of the cercariae of *M. novaezealandensis*, approximately an order of magnitude smaller than cercariae of the other three parasite species. *Maritrema novaezealandensis* cercariae may store less energy than other parasite species due to their smaller body size, while a higher surface area to volume ratio may increase their vulnerability to changing ionic conditions, as cercariae attempt to maintain internal ionic equilibrium. Despite the low tolerance of *M. novaezealandensis* cercariae to acidified seawater, the overall prevalence of this parasite may not be significantly altered by OA. Studies that exposed *M. novaezealandensis* to other abiotic stressors (e.g., ultraviolet radiation, Studer et al., 2012) have found that transmission rates to second intermediate hosts remained unaffected, despite high cercarial mortality rates. This incongruity may be explained by the relatively high mean number of cercariae produced by snails infected with *M. novaezealandensis* (2000/week) compared with snails infected with other species of trematode, e.g., *Galactosomum* sp. – 50/week, *Parorchis* sp. – 100/week, and *Philophthalmus* sp. – 60/week (unpublished data). Consequently,

predictions of the effects of acidified seawater on *M. novaezealandensis* may be inaccurate without additional information on the transmission success of this species under simulated OA conditions.

Parorchis sp. and *Philophthalmus* sp. both belong to the family Philophthalmidae and form metacercariae externally on hard substrates (Leung et al., 2009; O'Dwyer et al., 2014). Neal and Poulin (2012) found that *Philophthalmus* sp. cercariae showed a preference for forming metacercariae on the shells of snails that may serve as second intermediate hosts, and completed their life cycle when the shell was consumed by a shorebird. No information is available on the substrate used by *Parorchis* sp. as surrogate for a second intermediate host. Whatever the transmission strategy behind the formation of external metacercariae on hard substrates, cercariae of these species remain exposed to ambient seawater conditions from the time they emerge from their first intermediate host until they reach their definitive host. Consequently, cercariae of *Philophthalmus* sp. are exposed to the range of pH values associated with tidal pools (pH 7.60–9.19), while *Parorchis* sp. cercariae are exposed to the more moderate range of pH associated with tidal seawater (pH 7.82–8.35). Given our hypothesis that environmental variability affects the tolerance of an organism to abiotic conditions such as pH, we predicted that *Philophthalmus* sp. cercariae would be more tolerant of low pH conditions than *Parorchis* sp. cercariae. Indeed, the cercariae of *Philophthalmus* sp. exhibited a longer active period than *Parorchis* sp. cercariae prior to forming metacercarial cysts (Table 3). In the absence of an appropriate substrate, we assumed that cercariae of both trematode species would postpone the formation of metacercariae for as long as their energy stores would allow, and that a shorter active period would imply a greater susceptibility to pH stress.

The protective cysts formed by *Philophthalmus* sp. and *Parorchis* sp. parasites may provide some measure of protection for metacercariae against changing environmental conditions, as protective cysts of this family have a thicker outer membrane than other trematode families (Dixon, 1975). If we view the environmental conditions experienced by these two species as a predictor of tolerance to abiotic stressors, we see that cysts formed by *Philophthalmus* sp. are less exposed to desiccation stress than those of *Parorchis* sp. due to differences in habitat, i.e., low versus high intertidal zones. It would seem reasonable to assume that cysts exposed to desiccation stress may have a thicker membrane than those that are typically submerged for most of their active period, and that a thicker membrane would provide protection from other abiotic factors such as pH. There are also morphological differences between *Parorchis* sp. and *Philophthalmus* sp. cysts which suggest that the latter species may be more vulnerable to changing abiotic conditions. *Philophthalmus* sp. cysts are flask-shaped, with an opening at the neck of the cyst (Howell, 1983). This opening facilitates a rapid excystment in the throat of the avian definitive host so that metacercariae are not destroyed by the host's digestive system (Nollen and Kanev, 1995). Cysts formed by *Parorchis* sp. have no opening and the metacercariae are completely isolated from environmental conditions (O'Dwyer et al., 2014). Consequently, the cysts of *Philophthalmus* sp. may provide less protection against changing abiotic conditions compared with the cysts formed by *Parorchis* sp.

These hypotheses are supported by the metacercarial survival data, as *Philophthalmus* sp. metacercariae exhibited significantly lower survival rates in both acidified treatments, while the metacercariae of *Parorchis* sp. showed no mortality at all over the 12 day period. In fact, *Parorchis* sp. metacercariae were left for up to 30 days in acidified seawater and showed no mortality. In spite of the greater tolerance of *Philophthalmus* sp. cercariae for acidified conditions, the 100% survival observed in *Parorchis* sp. metacercariae indicates that the overall transmission success of *Parorchis* sp. will be less affected by acidified seawater. The differential response

of these confamilial species to reduced pH aptly illustrates the importance of the physicochemical characteristics of an organism's habitat in establishing tolerance to abiotic stressors.

All parasites included in this study exhibited significant reductions in cercarial longevity under acidified conditions. This suggests that the transmission window for all species may be shortened under OA conditions, i.e., the amount of time cercariae have to find and infect a suitable second intermediate host may be reduced. Any reduction in the transmission window could decrease the probability of successfully locating the next host, potentially reducing the abundance of these parasites in all stages/hosts of their life-cycle. A reduction in overall parasite abundance could alter marine ecosystems in significant ways: populations of first intermediate host snail species, typically sterilised by trematode infection (Lafferty and Kuris, 2009), would have a greater reproductive potential; parasite-mediated changes to second intermediate host behaviour, some of which have important ecological ramifications (Thomas et al., 1998), would be minimised; and any host species that experience increased mortality as a consequence of parasitic infection may become more abundant (Jensen and Mouritsen, 1992). However, in attempting to predict the effects of OA on marine ecosystems vis à vis the impact of OA on parasites, it becomes obvious that a holistic view must be taken of host–parasite interactions. As parasites depend on host organisms to survive and complete their life cycles, the responses of both host and parasitic species to the stressors associated with OA must be understood before we can predict the full impact of OA on marine ecosystems.

The reduced longevity of cercariae and survival of metacercariae described in this study offer us a glimpse of the potential effects of OA on host–parasite relationships. The next stage in investigating the effects of OA on parasites should be a thorough assessment of the transmission process, i.e., cercarial transmission success, and the development and survival of metacercariae formed within second intermediate hosts, under low pH conditions. Of course, implicit in this process is an investigation of the response of infected and uninfected second intermediate host species to low pH conditions. Similarly, snails acting as first intermediate hosts may prove equally or more vulnerable to stressors associated with OA, as calcifying organisms such as gastropods are also exposed to an environment that is not conducive to the biosynthesis of calcified structures (Orr et al., 2005).

In the broader context of OA research, it is now also acknowledged that multi-stressor experiments must be conducted to more accurately record the responses of marine organisms to the stressors associated with OA, e.g., manipulating temperature and pH or ultraviolet radiation and pH (Boyd, 2011). The inclusion of additional abiotic variables in experimental design would improve our understanding of how OA will affect marine parasites throughout their life-cycle.

Data presented in this study indicate that cercarial longevity and metacercarial survival are negatively affected by exposure to low pH seawater. This could have dramatic cumulative effects on a wide range of marine organisms due to the multi-host life cycles of trematodes. As parasites have the ability to influence host population behaviour, reproduction and survival, it is vital that parasitology is fully incorporated into the study of the ecosystem effects of OA. Predictions of future ecosystem structure and function under all CO₂ emission scenarios will be incomplete without a greater understanding of the effects of OA on host–parasite interactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpara.2015.02.007>.

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