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FEATURE ARTICLE

Impacts of ocean acidification on multiplication and caste organisation of parasitic trematodes in their gastropod host

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Abstract Ocean acidification is predicted to impact the structure and function of all marine ecosystems in this century. As focus turns towards possible impacts on interactions among marine organisms, its effects on the biology and transmission potential of marine parasites must be evaluated. In the present study, we investigate two marine trematode species (*Philophthalmus* sp. and *Parorchis* sp., both in the family Philophthalmidae) infecting two marine gastropods. These trematodes are unusual in that their asexually multiplying stages within snails display a division of labour, with two distinct castes, a large-bodied morph producing infective stages and a smaller morph playing a defensive role against other competing parasites. Using a potentiometric ocean acidification simulation system, we test the impacts of acidified seawater (7.8 and 7.6 pH) on the production of free-living infective stages (cercariae), the size and survival of encysted resting stages (metacercariae), and the within-host division of labour measured as the ratio between numbers of the two morphs. In general, low pH conditions caused an increase in cercarial production and a reduction in metacercarial survival. The ratio of the two castes within snail hosts tended to shift towards more of the smaller defensive morphs under low pH. However, the observed effects of reduced pH were species specific and not always unimodal. These results suggest that ocean acidification can affect the biology of marine parasites and may also impact transmission success and parasite abundance of some trematodes, with possible consequences for marine communities and ecosystems.

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Introduction

Since the preindustrial period, emissions of CO_2 in the atmosphere have increased drastically, with atmospheric levels rising each year by 0.5 % (Solomon et al. 2007). As oceans play a major role in the processes driving the natural carbon cycle, the current input of atmospheric CO_2 is buffered by oceanic uptake, amounting to nearly a third of carbon added to the atmosphere from human activity in the past 200 years (Fabry et al. 2008; Doney et al. 2009). Due to the equilibrium between atmospheric CO_2 and dissolved oceanic CO_2 at the water surface, the non-negligible input of dissolved CO_2 is modifying seawater chemistry by changing the balance of different chemical reactions (Dickson et al. 2007). These chemical changes lead to a reduction in carbonate ion concentrations, but an increase in concentrations of bicarbonate ions and hydrogen ions, thus lowering pH (i.e. $pH = -\log [H^+]$), a phenomenon known as ocean acidification (OA). The main consequences for marine ecosystems are a reduction in average seawater pH and a decline in the availability of calcium carbonate ($CaCO_3$) used by many groups of marine organisms, such as molluscs, echinoderms, crustaceans, corals, and many species of plankton that synthesise calcified structures (Orr et al. 2005). Moreover, the increase in hydrogen ions in seawater affects acid–base homoeostasis in several marine organisms (Pörtner et al. 2004), leading to more energy required for maintenance of intra- and extra-cellular balances. Surface-ocean pH has fallen by approximately 0.1 units to its present 8.1 pH value (Doney et al. 2009; Fabry

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et al. 2008; Riebesell et al. 2010) and is predicted to drop to approximately 7.6 by the year 2100 and to 7.4 by 2300 (Intergovernmental Panel on Climate Change (IPCC) 2014).

Research on the effects of OA is now founded on precise understanding of carbonate chemical reactions and the use of realistic OA simulation systems (Riebesell et al. 2010). From an ecological perspective, there is growing interest in the more indirect effects of OA, for instance, effects on interspecific interactions such as predation, competition and parasitism (Kroeker et al. 2013; McCormick et al. 2013; MacLeod and Poulin 2015). Parasites are often neglected in marine ecological research because of their small size. However, parasites are diverse and abundant, and now known to impact host population dynamics, interspecific interactions, community structure, food web complexity and ecosystems energetics (Sousa 1991; Poulin 1999; Mouritsen and Poulin 2002; Thompson et al. 2005; Kuris et al. 2008). There are many reasons to believe OA could impact parasites either directly or indirectly through their hosts, in ways that could change their interactions with their hosts and their influence on ecosystems (MacLeod and Poulin 2012).

In intertidal environments, parasitic trematodes are particularly abundant and known to affect the biology of the two or three host species involved in their complex life cycle. Typically, adult worms live in the gut of a vertebrate definitive host, such as a bird or fish, from where they release eggs in host faeces (Galaktionov and Dobrovolskij 2003). Snails serve as first intermediate host, becoming infected after accidentally ingesting eggs or by free-swimming larvae hatched from eggs. Following asexual (clonal) multiplication within the snail host, free-swimming infective stages, or cercariae, emerge from snails in large numbers and seek a second intermediate host, which they penetrate and in which they encyst as metacercariae, to await ingestion by a suitable definitive host, in which they develop into adult worms. In some trematode species, however, there is no second intermediate host; cercariae encyst on various substrates and become metacercariae, awaiting ingestion by a definitive host (Galaktionov and Dobrovolskij 2003). Multiple studies have documented how sensitive cercariae and metacercariae attached to external substrates are to abiotic conditions, such as temperature (Studer et al. 2010; Morley and Lewis 2013), salinity (Lei and Poulin 2011) and pH (Koprivnikar et al. 2010; MacLeod and Poulin 2015).

Here, we focus on how OA affects a recently discovered facet of trematode biology. In some species, the asexually multiplying stages within a snail intermediate host, known as rediae, come in two morphotypes representing distinct castes: a larger-bodied reproductive caste and a smaller non-reproductive caste (Hechinger et al. 2011; Leung and Poulin 2011; Miura 2012; Nielsen et al. 2014). Much evidence indicates that this represents a true division of labour, with the small rediae acting as 'soldiers' defending the redial colony against other parasites trying to establish in the same snail host, and the large reproductive rediae produce cercariae that will leave the snail to complete the life cycle (Hechinger et al. 2011; Leung and Poulin 2011; Lloyd and Poulin 2012, 2014; Mouritsen and Halvorsen 2015). As in social insects like ants and termites (Oster and Wilson 1978), the ratio of small-to-large rediae is likely to be optimised to yield maximum fitness, i.e. cercarial output, for the colony. Earlier research has shown that external stressors can indeed affect the caste ratios of social trematode colonies (Lloyd and Poulin 2013).

The present study investigates two trematode species, both of the family Philophthalmidae: *Philophthalmus* sp. (Martorelli et al. 2008) and *Parorchis* sp. (O'Dwyer et al. 2014), which use the New Zealand gastropods *Zeacumantus subcarinatus* and *Austrolittorina cincta*, respectively, as first intermediate host. Both are very common parasites of gastropods in New Zealand intertidal habitats, and their rediae infect the gonad and digestive tissue of the host, leading to its castration (Fredensborg et al. 2005). Both species display a division of labour inside the first intermediate host (Leung and Poulin 2011; Lloyd and Poulin 2012; Kamiya and Poulin 2013; O'Dwyer et al. 2014). Cercariae of *Philophthalmus* sp. and *Parorchis* sp. do not use a second intermediate host but instead leave the snail to encyst on a hard substrate, such as the shell of other gastropods or seaweed (Lei and Poulin 2011; Neal and Poulin 2012; O'Dwyer et al. 2014), in the process losing their tail and becoming metacercariae. The survival of cercariae of both species, and metacercariae at least in the case of *Philophthalmus* sp., is significantly lower under conditions of reduced pH (MacLeod and Poulin 2015). However, the effects of OA on caste ratio and overall trematode colony fitness in these trematodes with division of labour remain unexplored.

The main objective of this study was to experimentally assess the impact of OA on colony organisation and fitness in these two trematodes. Specifically, we test the effects of OA on cercarial output, metacercarial size, metacercarial survival, and within-host colony structure measured as the relative numbers (ratio) of the two castes. Cercarial production and metacercarial survival are key steps in parasite transmission, and thus, our results have bearing not only on the social biology of these trematodes, but also on the infection risk they pose for birds and snails.

Methods

Snail collection and parasite identification

In February and March 2015, *Z. subcarinatus* and *A. cincta* snails were collected by hand at low tide on the intertidal

mudflat of Lower Portobello Bay (LPB), Otago Harbour, South Island, New Zealand (45°80′S, 170°66′E). Prior to the study, all snails were maintained in well-aerated 2 L seawater aquaria (20 °C, >95 % oxygen, 8.05 pH) and fed sea lettuce (*Ulva* spp.) ad libitum. After 2 days, *Z. subcarinatus* and *A. cincta* snails were screened for trematode infection by inducing the release of cercariae. *Z. subcarinatus* snails were placed in individual seawater-filled plastic wells and incubated at 25 °C for 2–3 h under constant illumination (Mouritsen 2002; Fredensborg et al. 2005). *A. cincta* snails were placed in seawater-filled wells and kept in constant motion overnight (Shaker Plate, 80 rpm), followed by a 2-h motionless period (McCarthy et al. 2002; O'Dwyer et al. 2014). All wells were then examined under a dissecting microscope to determine the presence or absence of cercariae, and identify the species of infecting trematode based on cercarial morphology (Martorelli et al. 2008; O'Dwyer et al. 2014). Only *Z. subcarinatus* and *A. cincta* snails infected by philophthalmid parasites (*Philophthalmus* sp. and *Parorchis* sp., respectively) were kept for the final experiments. Infected *Z. subcarinatus* (61) and *A. cincta* (103) snails were then measured (±0.01 mm), individually labelled with numbered tags (Bee Works, Orillia, Canada), and randomly allocated to one of three pH treatments (8.0, 7.8, and 7.6 pH).

Ocean acidification simulation system

Snails were exposed to acidified or unmodified seawater (8.0, 7.8 and 7.6 pH) in a potentiometrically regulated OA simulation system, which used CO_2 gas as an acidifying agent (see description in MacLeod et al. 2015). This system consisted of three identical units, each composed of one open-top 120-L tank [870 mm (L), 600 mm (W), 295 mm (H)], a chiller unit, a pump/filtration unit and a TUNZE pH/CO_2 controller system (glass electrodes, pH meter and solenoid switch unit) connected to a 33 kg cylinder containing 100 % food grade CO_2 gas. The glass electrodes were immersed in each culture tank and connected to the gas cylinder via the pH meter and solenoid switch unit. When seawater pH rose above preset values, the solenoid switch was activated, allowing CO_2 gas to flow into the seawater. All electrodes were calibrated with synthetic saltwater buffers, *2-amino-2-hydroxy-1,3-propanediol* (TRIS) and *2-aminopyridine* (AMP), prepared in accordance with Dickson et al. (2007). To maintain appropriate seawater chemistry, i.e. to offset any effects of evaporation or shell dissolution, 20 L of seawater was replaced in each tank every 2 days.

The pH treatments used in this study (8.0, 7.8 and 7.6 pH) were chosen based on the annual average seawater pH at LPB (8.03, MacLeod 2015) and the 'Low CO_2 emission' (RCP2.6) and 'Business-as-usual' (RCP8.5) scenarios for the year 2100 outlined in the 2014 Intergovernmental Panel on Climate Change. The chiller units were used to maintain seawater temperature at 12 °C to simulate the annual mean seawater temperature at LPB (MacLeod 2015). To monitor the system's performance, salinity, temperature and pH were measured every 2 days with a YSI multimeter (temperature and salinity) and a Denver pH meter (±0.001 pH). In addition, dissolved inorganic carbon (DIC) and total alkalinity (A_T) were measured every 20 days (Table 1) in 1 L seawater samples taken from each tank. DIC was measured coulometrically, and A_T potentiometrically, using certified reference material (Batch #137) provided by the laboratory of Professor Andrew Dickson, University of California, San Diego. These data also allowed us to validate the performance of the system by independently calculating seawater pH using custom software (Hunter 2007). To avoid a 'tank effect', snails were rotated among tanks every 20 days, with the tanks reset to the appropriate pH at the time of transfer, such that the snails spent an equal amount of time in each of the three tanks while experiencing only their assigned pH value.

Within each tank, infected snails were maintained in cylindrical nylon-mesh chambers [7.6 cm (L), 8.65 cm (D)] that allowed the flow-through of acidified or unmodified seawater. Chambers containing *Z. subcarinatus* were fully submerged in seawater, while chambers containing *A. cincta* were partially submerged; partial or full submersion reflected the habitat of these snail species, i.e. the lower or upper intertidal zone. A small rock taken from LPB was added to all chambers as ballast; partial submersion of *A. cincta* snails was achieved by attaching buoyant material to the outside of the chamber. In both types of chamber, a 10 cm × 10 cm piece of sea lettuce (*Ulva* spp.) was also

Table 1 Mean values (±SD) of all measured and calculated parameters used to characterise the carbonate chemistry of unmodified and acidified seawater

Treatment	pH	Temp. (°C)	Salinity	Oxygen (%)	Alkalinity (μmol kg^{-1})	DIC (μmol kg^{-1})	pH (calc.)	PCO_2 (calc.)	Aragonite (Ω, calc.)
pH 8.0	8.00 ± 0.02	12.2 ± 0.6	34.9 ± 0.3	111 ± 19	2300 ± 30	2171 ± 74	7.91 ± 0.08	578 ± 120	1.68 ± 0.3
pH 7.8	7.84 ± 0.04	12.1 ± 0.8	35.3 ± 0.6	114 ± 9	2297 ± 24	2203 ± 31	7.78 ± 0.09	795 ± 178	1.29 ± 0.3
pH 7.6	7.63 ± 0.06	12.1 ± 0.7	35.1 ± 0.4	112 ± 6	2314 ± 14	2267 ± 44	7.59 ± 0.06	1277 ± 17	0.88 ± 0.1

Temp., temperature; DIC, dissolved inorganic carbon; Ω, saturation state; calc, calculated parameters

included, and replaced each week, as a food source for the snails.

Cercarial production

To measure the production of mature cercariae by snails exposed to acidified or unmodified seawater, we stimulated cercarial shedding at 10-day intervals for 60 days. Prior to the experiment, snails were again placed in seawater-filled plastic wells and incubated at 25 °C for 7 h (*Z. subcarinatus*), or shaken overnight under constant illumination (*A. cincta*), to remove any pre-existing mature cercariae. This process was then repeated at 10-day intervals, albeit for a shorter duration (*Z. subcarinatus*: 2 h; *A. cincta*: 2-h agitation, 2 h motionless). Cercariae produced during these latter incubations were counted under a dissecting microscope and used to investigate the long-term effects of reduced seawater pH on the cercarial production of both philophthalmid species.

Metacercarial survival and size

Philophthalmus sp. and *Parorchis* sp. cercariae released in the cercarial production experiment described above were maintained in acidified or unmodified seawater until they formed metacercarial cysts, which allowed us to quantify the effects of pH on metacercarial survival and cyst size. Forty *Philophthalmus* sp. cercariae per pH treatment were chosen randomly from those generated in the cercarial production trials, distributed equally between 12 Petri dishes [1.2 cm (H), 3.8 cm (D), 4 per pH treatment], allowed to encyst, and returned to their respective pH treatments. As *Parorchis* sp. cercariae rapidly form cysts after emerging from host snails, 50 cysts (per pH treatment) formed in five plastic wells during the cercarial production trials were randomly selected and returned to their respective pH treatments. The metacercarial survival of both species was recorded every 24 h for 30 days. Metacercariae were defined as 'dead' in cysts which were empty or opaque due to degraded tissue, and 'alive' where the metacercariae could be clearly seen within the cyst. Metacercarial survival, recorded as a percentage of metacercariae in each dish or well, was measured in three separate trials for each species, corresponding to the three of the six cercarial production trials described above, i.e. after 20-, 40- and 60-day exposures.

To identify potential trade-offs between quantity and quality in cercarial production and metacercarial cyst formation in an acidified environment, the surface area of metacercarial cysts was calculated after each of the three cercarial production trials also used to assess metacercarial survival. Digital photographs of 15 cysts per parasite species per pH treatment were taken during each trial, using a digital camera (Olympus, DP25) connected to a dissecting microscope (×10). The length and width of each cyst were measured with ImageJ software and used to calculate total surface area.

Caste ratio

To determine whether exposure to acidified seawater altered the caste ratio of *Philophthalmus* sp. or *Parorchis* sp. rediae, i.e. the number of small, defensive morphotypes divided by the number of large, reproductive morphotypes (Lloyd and Poulin 2013), all snails were dissected after the 60-day exposure and the number of small and large rediae recorded. During dissection, the visceral mass of each snail was separated from the shell and carefully teased apart to release all rediae. The rediae were then gently pressed between two glass slides after being stained with neutral red stain, which increased the visibility of individual rediae. Small and large morphotypes, identified according to Leung and Poulin (2011), were counted in each snail, and these data were used to calculate the caste ratio.

Statistical analyses

We used a generalised linear mixed effect model with Poisson error structure to analyse the effects of exposure to acidified seawater on the cercarial production of both snail species. Fixed effects were pH, Time and Snail Length, with 'Snail ID' and 'Tank ID' as random effects. Random effects were used to account for tank switching (Tank ID) and repeated measurements of the same snail (Snail ID). A second generalised linear mixed effect model, this time with a binomial error structure, was used to analyse the effects of pH on metacercarial survival. As *Parorchis* sp. metacercariae exhibited no mortality over the 30-day observation period, analysis was limited to *Philophthalmus* sp. In the second model, fixed effects were pH and Time, with 'Trial ID' and 'Box ID' as random effects to account for the inclusion of data from three separate trials (Trial ID) and repeated measurements of the same containers of metacercariae (Box ID). For both generalised linear mixed effect models, the significance of fixed effect variables was set at $P < 0.05$.

ANOVA and post hoc Tukey HSD tests were used to compare the distribution of metacercarial sizes among pH treatments in each of the three trials, and Pearson correlations were used to detect any variation through time, i.e. between trials. As the caste ratio data did not meet the criteria for parametric analysis, comparisons were made using a Kruskal–Wallis test and its associated post hoc Nemenyi tests in the R package *PMCMR* (v1.1; Pohlert 2015). The average number of soldier and reproductive

rediae in individual snails was also compared between pH treatments using the same nonparametric tests. Spearman's correlations were used to test for a relationship between the caste ratio and the shell length of host snails. Only the initial shell length was used in our analyses, as we observed no significant differences between the initial and final shell length (Wilcoxon test: *Philophthalmus*: $P = 0.9616$/*Parorchis*: $P = 0.4495$). All analyses were completed using the programming language R (version 3.0.1) and the packages *PMCMR* (Pohlert 2015) and *lme4* (Bates et al. 2014).

Results

Cercarial production

The production of *Philophthalmus* sp. cercariae by infected *Z. subcarinatus* snails was significantly affected by Time and the interaction of pH and Time, but not by pH or Shell Length (Fig. 1a; Table 2). In the most extreme conditions (7.6 pH), cercarial production increased significantly during the 60-day trial ($r = 0.12$, $P = 0.02159$), while in the control treatment (8.0 pH) cercarial production exhibited a significant decrease ($r = -0.21$; $P = 0.01397$). Changes in cercarial production by snails maintained in 7.8 pH seawater were highly variable and nonsignificant. *Parorchis* sp. cercariae production by infected *A. cincta* snails was significantly affected by Time, Shell Length, and the interaction between pH and Time (Fig. 1b; Table 2). The change in cercarial production over time in all pH treatments was significant and positive: 8.0 pH, $r = 0.54$, $P < 0.01$; 7.8 pH, $r = 0.33$, $P < 0.01$; 7.6 pH, $r = 0.59$, $P < 0.01$. Due to snail mortality, there were small but significant differences in the average shell length of *A. cincta* snails in the 8.0 pH treatment (17.28 mm) relative to those maintained in the 7.8 pH (16.87 mm) and 7.6 pH (16.78 mm) treatments (Kruskal–Wallis: $P = 0.0011$).

Metacercarial survival and size

The survival of *Philophthalmus* sp. metacercariae was significantly affected by pH, Time, and the interaction of pH and Time (Table 3). Metacercariae exposed to 7.6 pH seawater exhibited significantly lower survival relative to those in control conditions ($P = 0.008$), while no significant differences were found between 7.8 and 7.6 pH or 8.0 and 7.8 pH treatments. The differential survival exhibited by metacercariae maintained in acidified or unmodified seawater was more pronounced at 15 days (8.0 pH—58 %, 7.7 pH—45 %, 7.6 pH—28 %) than at 30 days (8.0 pH—23 %,

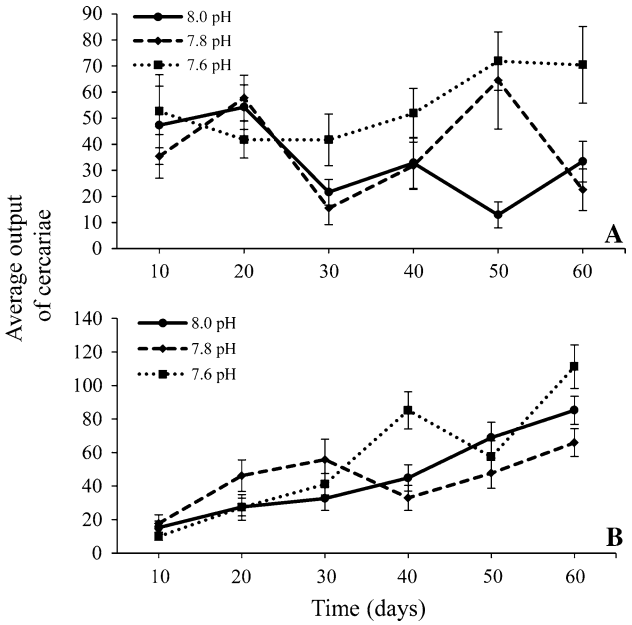

Fig. 1 Mean cercarial production (±SE) of *Philophthalmus* sp. (**a**) and *Parorchis* sp. (**b**) from infected snails over a 60-day exposure to acidified or unmodified seawater (8.0, 7.8 and 7.6). Sample sizes were: *Philophthalmus* sp., 23 (8.0 pH), 15 (7.8 pH), 23 (7.6 pH); *Parorchis* sp., 33 (8.0 pH), 34 (7.8 pH), 34 (7.6 pH)

Table 2 Generalised linear mixed effect model output for the analysis of cercarial production by two trematodes in snails exposed to acidified or unmodified seawater (8.0, 7.8, and 7.6 pH) for 60 days

Species	Fixed effect	*df*	*F* value	*P* value	Random factors	% variance
Philophthalmus sp.	pH	2	2.844	0.059	Tank ID	<0.01
	Time	5	107.472	**<0.01**	Snail ID	19.67
	pH × time	10	125.626	**<0.01**		
	Length	1	0.042	0.837		
Parorchis sp.	pH	2	2.699	0.068	Tank ID	<0.01
	Time	5	1028.910	**<0.01**	Snail ID	17.86
	pH × time	10	163.257	**<0.01**		
	Length	1	16.225	**<0.01**		

Bold indicates significant main effects ($P < 0.05$). The proportion of the remaining variance accounted for by the random factors is also shown

Table 3 Generalised linear mixed effect model output for the analysis of survival of metacercariae exposed to acidified or unmodified seawater (8.0, 7.8 and 7.6 pH) for 30 days

Species	Fixed effect	*df*	*F* value	*P* value	Random factors	% variance
Philophthalmus sp.	pH	2	84.746	**<0.01**	Trial ID	7.68
	Time	1	13.344	**<0.01**	Box ID	11.82
	pH × time	2	3.239	**0.040**		

Bold indicates significant main effects ($P < 0.05$). The proportion of the remaining variance accounted for by the random factors is also shown

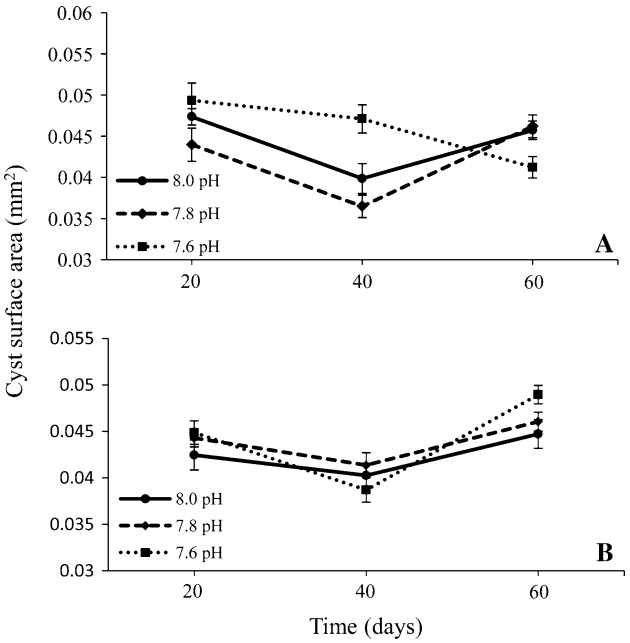

Fig. 2 Surface area (±SE) of *Philophthalmus* sp. (**a**) and *Parorchis* sp. (**b**) metacercarial cysts produced during a 60-day exposure to acidified or unmodified seawater (8.0, 7.8 and 7.6 pH). $N = 15$ for each snail species/pH combination

Table 4 ANOVA output for the analysis of variation in metacercarial size after exposure of infected snails to acidified or unmodified seawater (8.0, 7.8 and 7.6 pH) for 60 days

Species	Fixed effect	*df*	*F* value	*P* value
Philophthalmus sp.	pH	2	4.093	**0.019**
	Time	2	9.98	**<0.01**
	pH × time	4	6.892	**<0.01**
Parorchis sp.	pH	2	1.526	0.221
	Time	2	19.428	**<0.01**
	pH × time	4	1.707	0.153

Bold indicates significance ($P < 0.05$)

7.8 pH—12 %, 7.6 pH—11 %). In contrast, survival of *Parorchis* sp. metacercariae was 100 % for the duration of the experiment, in all pH treatments.

The size of *Philophthalmus* sp. metacercarial cysts was significantly affected by pH, Time, and the interaction of pH and Time (Fig. 2a; Table 4), and post hoc analysis showed a significant difference between cysts formed at 7.8 and 7.6 pH ($P = 0.014$). At 8.0 and 7.8 pH, cyst size decreased between day 20 and 40 and increased between day 40 and 60 (Fig. 2a); in both treatments, these changes were statistically significant ($P < 0.05$ in all pairwise comparisons). At 7.6 pH, cyst size significantly decreased between day 20 and 40 and between day 40 and 60 (day 20–40, $P < 0.01$; day 40–60: $P = 0.035$). The size of metacercarial cysts formed by *Parorchis* sp. was significantly affected by Time (Table 4), but there was no clear relationship between cyst size and pH treatment (Fig. 2b).

Caste ratio

The caste ratio of small-to-large rediae of *Philophthalmus* sp. was significantly higher in the 7.8 pH treatment relative to the 7.6 and 8.0 pH treatments (Table 5; Fig. 3a). This result was caused by a significantly higher number of small rediae, and a lower number of large rediae, at 7.8 pH compared to the 8.0 and 7.6 pH treatments (Fig. 4a). In addition, caste ratio was significantly and negatively correlated with snail length for *Philophthalmus* sp. (Spearman correlation: $r = -0.27$, $P < 0.01$), indicating that larger snails contained relatively more reproductive rediae than soldier rediae. This correlation was particularly evident for snails in the 8.0 and 7.6 pH treatments (Spearman correlation: 8.0 pH, $r = -0.19$, $P = 0.02$; 7.6 pH, $r = -0.57$, $P < 0.01$), with a steeper negative slope for the more acidified treatment. The caste ratio of *Parorchis* sp. rediae decreased as pH was reduced and was significantly lower in the 7.6 pH treatment relative to control conditions (Table 5; Fig. 3b). These results correspond to a variable decrease in the number of small and large rediae in hosts exposed to acidified seawater relative to hosts maintained in the control treatment (Fig. 4b). Overall, there was a higher caste ratio, i.e. a greater number of small versus large rediae in *Z. subcarinatus* (mean ± SD: 1.23 ± 0.62) compared to *A. cincta* (mean ± SD: 0.33 ± 0.19).

Discussion

Since the chemical processes underpinning ocean acidification (OA) have been elucidated and its inexorable

Table 5 Kruskal–Wallis tests and associated post hoc Nemenyi comparisons for the analysis of variation in caste ratio after exposure of infected snails to acidified or unmodified seawater (8.0, 7.8 and 7.6 pH) for 60 days

Species	Chi-squared	*df*	*P* value
Philophthalmus sp.	33.188	2	**<0.01**
Paired comparisons	8.1 pH–7.8 pH		**<0.01**
	7.8 pH–7.6 pH		**<0.01**
	8.1 pH–7.6 pH		0.25
Parorchis sp.	7.499	2	**0.024**
Paired comparisons	8.1 pH–7.8 pH		0.260
	7.8 pH–7.6 pH		0.471
	8.1 pH–7.6 pH		**0.017**

Bold indicates significance ($P < 0.05$)

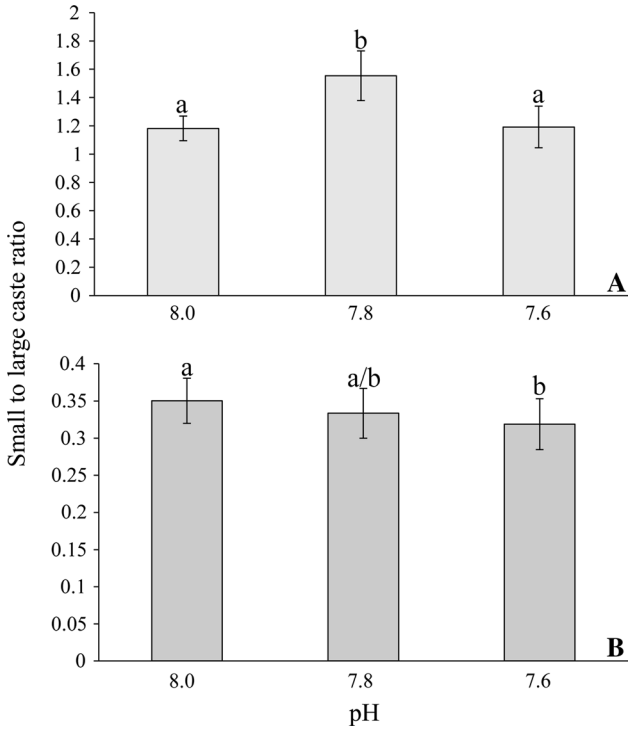

Fig. 3 Average caste ratio (±SE) of *Philophthalmus* sp. (**a**) and *Parorchis* sp. (**b**) parasites after exposure of host snails to acidified or unmodified seawater (8.0, 7.8 and 7.6 pH) for 60 days. *Lowercase letters* indicate significant differences between treatments. Sample sizes were: *Philophthalmus* sp., 23 (8.0 pH), 15 (7.8 pH), 23 (7.6 pH); *Parorchis* sp., 33 (8.0 pH), 34 (7.8 pH), 34 (7.6 pH)

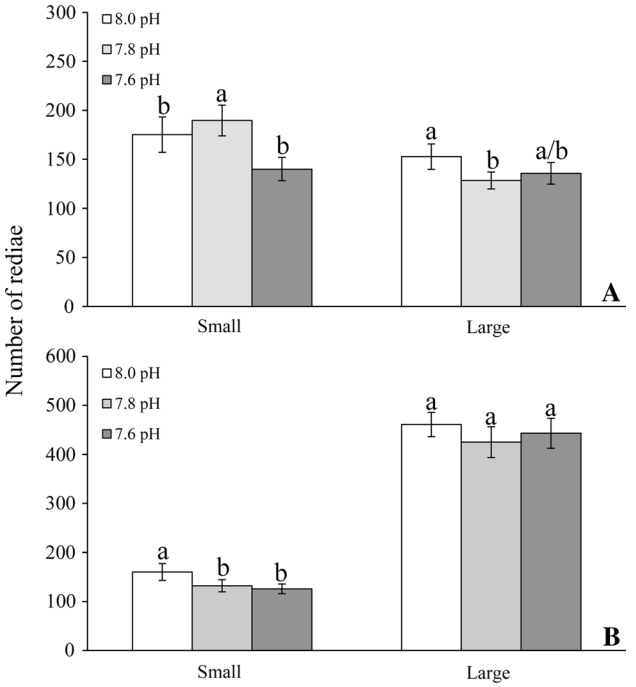

Fig. 4 Average number (±SE) of small and large rediae of *Philophthalmus* sp. (**a**) and *Parorchis* sp. (**b**) present in host after exposure of infected snails to acidified or unmodified seawater (8.0, 7.8 and 7.6 pH) for 60 days. *Lowercase letters* indicate significant differences between treatments. Sample sizes were: *Philophthalmus* sp., 23 (8.0 pH), 15 (7.8 pH), 23 (7.6 pH); *Parorchis* sp., 33 (8.0 pH), 34 (7.8 pH), 34 (7.6 pH)

progress exposed (Caldeira and Wickett 2003), research on the ecological impacts of OA has flourished (Riebesell and Gattuso 2015). However, the interactive effects of parasitism and OA on marine organisms have generally been ignored (MacLeod and Poulin 2012). The very first study on parasites using a realistic OA simulation system showed a significant decrease in cercarial longevity for different trematode species (including *Philophthalmus* sp. and *Parorchis* sp.) under low pH conditions, accompanied by a reduction in metacercarial survival for *Philophthalmus* sp. (MacLeod and Poulin 2015). A further study uncovered OA impacts on transmission success in a different trematode species (Harland et al. 2015). Using the same OA simulation system as those previous studies, we explored the impact of long-term exposure to low pH on caste ratio of redial stages within intermediate hosts for two philophthalmid species known to have a social structure with division of labour (Hechinger et al. 2011; Leung and Poulin 2011; Miura 2012; Nielsen et al. 2014). Our findings reveal further impacts of OA on parasite biology, not always unimodal and often species specific.

The presence of two distinct castes has been well described in the within-snail redial colonies of both *Philophthalmus* sp. and *Parorchis* sp. This division of labour among redial stages within intermediate snail hosts can be influenced by environmental changes, especially by biotic factors such as inter-specific competition (Lloyd and Poulin 2012, 2013, 2014). However, influences of abiotic factors on caste ratio in trematode colonies remain poorly understood. In the case of *Parorchis* sp. living in

A. cincta snails, reduced pH affected the colony composition by causing a reduction in the ratio of small-to-large rediae, with fewer small non-reproductive rediae at 7.6 pH. Assuming that small rediae of *Parorchis* sp. have the same defensive function as small rediae of *Philophthalmus* sp. and are able to feed on sporocysts or/and rediae of other parasite species trying to co-infect the same host (Lloyd and Poulin 2012, 2014), a decrease in the number of small rediae might represent a response to environmental stress threatening colony survival. Thus, stress from low pH conditions may shift investments towards reproduction, such that over 60 days, as small and large rediae died naturally, only large rediae were formed to replace them. The effect of low pH conditions may also be indirect, as the host may also be severely affected by low pH, resulting in less energy for the parasite colony. Either way, caste ratio should respond to changing environmental conditions, because social trematode colonies act as 'superorganisms', and plasticity in colony traits, such as caste ratio or colony size, is shaped by natural selection to respond optimally to environmental stressors (Hasegawa 1997). In the case of *Philophthalmus* sp. in the snail *Z. subcarinatus*, caste ratios were higher at 7.8 pH than at higher or lower pH. This suggests a shift towards investment in colony defence (more small rediae) under mild external stress, but towards reproduction, as in *Parorchis* sp., under more extreme stress. Our results also show a decrease in caste ratio in larger snails, confirming a pattern identified earlier (Leung and Poulin 2011). Therefore, the effects of OA on caste ratio in these trematodes differ slightly between species.

Interestingly, *Philophthalmus* sp. and *Parorchis* sp. colonies had very different average ratios of small-to-large rediae. The average of *Philophthalmus* sp. caste ratio was greater than one, indicating more small rediae than large ones per colony, in accordance with previous studies showing that colonies from Lower Portobello Bay achieve higher fitness with a relatively greater proportion of non-reproductive individuals per colony (Lloyd and Poulin 2012). Lower Portobello Bay has the highest prevalence of parasites in *Z. subcarinatus* snails in the Otago region (Martorelli et al. 2008), and interspecific competition for resources and space among trematodes using this snail species is rather strong (Lloyd and Poulin 2012). *Philophthalmus* sp. colonies have been observed several times in competition with *Maritrema novaezealandensis* within co-infected snail hosts, and increase their number of non-reproductive 'soldier' rediae during co-infection (Lloyd and Poulin 2012). The intense competition for snail hosts occurring in Lower Portobello Bay may explain why, regardless pH conditions, a higher number of small rediae of *Philophthalmus* sp. is maintained. In contrast, the average of *Parorchis* sp. caste ratio was much lower than one (around 0.3), translating into a higher number of large reproductive rediae compared to small ones in all three pH conditions. O'Dwyer et al. (2014) described several trematode parasites infecting *A. cincta*, with *Parorchis* sp. being by far the most common one and the others being very rare. This suggests that *Parorchis* sp. faces very little competition for host resources on the rocky shore of Lower Portobello Bay and may not need to maintain a large number of small defensive rediae to protect its colony.

Cercarial production was significantly affected by an interaction between pH and time in both *Philophthalmus* sp. and *Parorchis* sp. Long-term exposure to reduced pH generally leads to an increase in cercarial output from infected snail hosts, a trend particularly pronounced under lower pH conditions. The pattern was not so clear for *Parorchis* sp., possibly due to differences in snail lengths among pH treatments, as snail size influenced cercarial output in this trematode. Nevertheless, colonies of both trematode species increased their cercarial production over time, and particularly under low pH conditions, a finding consistent with the observed gradual shift in colony composition towards relatively more reproductive rediae as a response to external stress. Alternatively, the reduced pH may somehow negatively affect the snail hosts, allowing the parasite to withdraw more energy and produce more cercariae.

However, higher production in *Philophthalmus* sp. is accompanied by a reduction in parasite size. Metacercarial size decreased significantly after 60-days exposure to the lowest pH environment (7.6 pH) indicating the presence of a trade-off between the quantity and the quality of produced cercariae. We suggest that low pH seawater may cause the host to channel more energy to maintain physiological processes, leaving less energy available for the parasite colony and forcing a reduction in parasite size when cercarial output is boosted. No such reduction in metacercarial size was observed in *Parorchis* sp., again indicating that responses to OA are species specific.

Furthermore, reduced pH may influence the survival of encysted metacercariae. As reported earlier (MacLeod and Poulin 2015), metacercarial survival of *Philophthalmus* sp. was negatively affected, as shown by a significant difference between the three different pH conditions over 30 experimental days, with higher mortality at 7.8 and 7.6 pH. Thus, even at the early stage of ocean acidification simulated by 7.8 pH conditions, parasite transmission success can be affected. It is unclear whether greater cercarial output can offset higher metacercarial mortality, and therefore, the consequences of OA for the parasite population, and for infections of birds and snails, remain to be determined. In contrast, *Parorchis* sp. metacercariae showed no differences in survival among pH treatments, maintaining 100 % survival over 30 days in all conditions. Here again, OA impacts on transmission success appear to be species specific. Interspecific differences in metacercarial survival

may arise from differences in cyst membrane and shape between the two species and the habitat of their respective hosts. In the family Philophthalmidae, protective cysts have a thicker outer membrane than those of other trematode families (Dixon 1975). Because of the habitat of their *A. cincta* snail hosts, *Parorchis* sp. metacercariae are more exposed to desiccation at low tide, and therefore, they have a cyst structure more tolerant to abiotic factors, with no opening and a thicker membrane compared to *Philophthalmus* sp. metacercariae (O'Dwyer et al. 2014). In contrast, the *Philophthalmus* sp. cyst has an opening at the neck of the cyst permitting faster infection upon reaching a bird (Nollen and Kanev 1995). The opening allows contact with seawater and provides less protection against environmental variations. These differences suggest that transmission success of *Philophthalmus* sp. can be more affected by acidified seawater than that of *Parorchis* sp.

In parallel with metacercarial survival, faster encystment for cercariae from both philophthalmid species exposed to 7.6 pH was observed compared to that in the control 8.0 pH (Guilloteau, personal observation). This result was consistent with the study of MacLeod and Poulin (2015) showing a significant decrease in cercarial longevity for *Philophthalmus* sp. and *Parorchis* sp. under low pH conditions. Cercariae at low pH have less time to find a suitable substrate to encyst, possibly decreasing their odds of reaching the definitive host.

Our study revealed some impacts of OA on the structure and fitness of social trematode colonies, impacts that are both complex and species specific. It is more difficult to extrapolate these results to predict changes in overall parasite abundance and effects on the hosts. Ocean acidification may alter host susceptibility to infection and several other aspects of the host–parasite interaction not covered here (MacLeod and Poulin 2012). Recent studies showed that low pH conditions also lead to increased energetic costs of host physiological processes, such as acid–base regulation (Kroeker et al. 2014), and are likely to negatively impact the abundance of calcifying organisms like gastropods, due to a decrease in carbonate ions in seawater necessary for their growth (Orr et al. 2005). These effects could change snail host density and modify host population structure, possibly also changing how they interact with predators and parasites (Kroeker et al. 2014). At the same time, OA is just one part of global climate change, and other climate-related factors can affect not only host species, but also parasite biology (see Marcogliese 2001; Poulin 2006). The next research steps should involve the simultaneous assessment of OA and multiple other stressors, and especially the interactions among stressors, on host–parasite associations.

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