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Interactive effects of parasitic infection and ocean acidification on the calcification of a marine gastropod

Colin D. MacLeod*, Robert Poulin

Department of Zoology, University of Otago, PO Box 56, Dunedin 9054, New Zealand

ABSTRACT: The interactive effects of ocean acidification (OA) and parasitic infection have the potential to alter the performance of many marine organisms. Parasitic infection can affect host organisms' response to abiotic stressors, and vice versa, while the response of marine organisms to stressors associated with OA can vary within and between taxonomic groups (host or parasite). Accordingly, it seems likely that the combination of infection stress and the novel stressors associated with OA could alter previously stable host–parasite interactions. This study is a detailed investigation into the changes to shell growth, dissolution, and tensile strength in the New Zealand mud snail *Zeacumantus subcarinatus* caused by trematode infection in combination with exposure to simulated OA conditions. This study also tests the effects of reduced pH on snails infected by 3 different trematode species to investigate potential species-specific effects of infection. After a 90 d exposure to 3 pH treatments (pH 8.1, 7.6, and 7.4), acidified seawater caused significant reductions in shell growth, length, and tensile strength in all snails. Trematode infected snails displayed increased shell growth and dissolution and reduced shell strength relative to uninfected conspecifics. In all measured variables, there were also significant differences between snails maintained at the same pH but infected by different species of parasite. These results indicate that parasitic infection has the potential to alter host organisms' response to OA and that the magnitude of this effect varies among parasite species.

KEY WORDS: Ocean acidification · Parasite · Calcification · Marine gastropod · Intertidal

INTRODUCTION

Over the past 10 yr, ocean acidification (OA) research has developed from short-term, single species experiments that manipulated only seawater pH (e.g. Bibby et al. 2007) to complex, long-term experiments that investigate the effects of combined abiotic stressors and biological interactions in simulated OA conditions (pH and temperature, Melatunan et al. 2011; pH and UV, Melnychuk et al. 2012; pH and salinity, Dickinson et al. 2013; competition, Hofmann et al. 2012; predation, Allan et al. 2013; parasitism, MacLeod & Poulin 2015). In the majority of OA experiments, the effects of acidified seawater on marine organisms are negative (reviewed in Doney et al. 2009, Kroeker et al. 2013), although the degree of sensitivity exhibited by marine species is highly variable even between sympatric or phylogenetically related species (Lardies et al. 2014, Zhang et al. 2014, MacLeod & Poulin 2015). Such differential tolerances could disrupt inter- and intra-specific interactions of ecologically important marine species (De Laender et al. 2014). The interactions between marine parasites and their hosts are particularly vulnerable to stressors associated with OA. The external transmission stages of marine parasites can be negatively affected by changing environmental conditions (Pietrock & Marcogliese 2003), and some species exhibit significantly increased mortality of free-living transmission stages when exposed to acidified seawater (MacLeod

*Corresponding author: colin.macleod@postgrad.otago.ac.nz

& Poulin 2015). Internal parasite life-stages, such as the sporocysts, rediae, and metacercariae of trematodes, rely on host organisms for nutrients and/or physical protection, while metacercariae also function as a transmission vector to the next stage or host in their life cycle (Galaktionov & Dobrovolskij 2003). The greater the number of host species involved in a parasite's life-cycle, the higher the probability that one will prove susceptible to stressors associated with OA. If either the free-living transmission stages of parasites, or any host species involved in the parasite's life-cycle, are negatively affected by OA, the abundance of marine parasites could be reduced and the important regulatory role that they play in many ecosystem processes (Combes 1996, Mouritsen & Poulin 2002) could be altered.

In addition to the direct effects of OA, the combination of parasitic infection and stressors associated with OA could cumulatively alter the performance of host species, further affecting the host-parasite relationship. OA can increase the metabolic demands placed on host species, as the increased concentration of hydrogen ions in seawater causes a corresponding increase in the energy required to maintain internal acid/base homeostasis (Pörtner et al. 2004). Calcifying host organisms also experience higher metabolic demands while forming calcium carbonate ($CaCO_3$) structures in acidified seawater (reviewed in Parker et al. 2013), as the same chemical processes that increase hydrogen ion concentration in seawater also decrease the availability of the carbonate ions that play a central role in the biological synthesis of $CaCO_3$ (see Calcification box). Parasitic infection can also increase the energetic requirements of host organisms by damaging or consuming host tissue (Cheng 1963), increasing energetically costly behaviour (Sousa 1991), or by absorbing nutrients directly from the hosts body (Cheng & Snyder 1963). Just as additional abiotic stressors such as hypoxia have exacerbated the effects of OA on marine organisms (Rosa & Seibel 2008, Gobler et al. 2014), the interaction of parasitic infection and stressors associated with OA may have synergistic negative effects. Indeed, exposure to acidified seawater has already been linked to altered immune response in marine molluscs (Bibby et al. 2008, Dupont & Thorndyke 2012). As parasites are a ubiquitous component of all marine environments, and many of the marine organisms that act as hosts are calcifiers, an understanding of how the effects of acidified seawater and parasitic infection will interact is necessary to fully understand the future ecosystem effects of OA (MacLeod & Poulin 2012, Kroeker et al. 2014).

Trematode parasites are found in most marine environments and use between 2 and 4 host species to complete their life cycle (Galaktionov & Dobrovolskij 2003). A typical trematode life cycle involves: (1) a definitive host, most often a seabird or large fish, where sexual reproduction occurs; (2) a first intermediate host, almost universally a gastropod, where asexual reproduction occurs; and (3) a second intermediate host in which resting cysts (metacercariae) are formed and await ingestion by the definitive host to complete the parasites life cycle. Trematode transmission between first and second intermediate hosts is achieved by free-living transmission stages (cercariae) which emerge from the snail and either actively or passively infect the second intermediate host. In addition to the first intermediate gastropod host, many of the second intermediate hosts of trematode parasites are calcifiers, e.g. bivalves and crustaceans. Consequently, the trematode life cycle may be affected by the 2 main stressors associated with OA, i.e. reduced pH (host and parasite) and reduced carbonate ion concentration (host only).

Trematode infection of the gastropod host typically causes complete sterilisation, either by destroying reproductive tissue or by manipulating host physiology (Lafferty & Kuris 2009). Trematode infection can alter the tolerance of host snails to abiotic stressors (e.g. Cruz-Mendoza et al. 2006), change feeding behaviours (Wood et al. 2007), and affect habitat choice (Bates et al. 2011). It is assumed that these modifications are adaptive for the parasite, i.e. they increase its probability of reaching its next host (Curtis 1987). In some cases, infection has also resulted in changes to shell growth rate and shell morphology in host snails (Mouritsen & Jensen 1994, Probst & Kube 1999, Hay et al. 2005). Three hypotheses have been proposed to explain increased shell growth, or gigantism, resulting from trematode infection: (1) an adaptive strategy of the host to deprive the parasite of energy that would usually be used for reproduction; (2) a non-adaptive consequence of the excess energy available to the snail after sterilisation, i.e. the parasite does not utilise all the energy that would otherwise have been used for reproduction (reviewed in Minchella 1985); and (3) an adaptive strategy of the parasite to increase the space available for asexual reproduction (McCarthy et al. 2004). In all hypotheses, infection plays a role in altering the rate of calcification of infected host snails and may interact with the changes to the calcification process caused by OA. OA has caused increased shell dissolution (Nienhuis et al. 2010), reduced shell repair rates (Coleman et al. 2014), and elevated metabolic rates (Lardies et al. 2014) in marine gastropods. Ac-

cordingly, the trematode–snail relationship is an ideal model system to investigate the combined effects of OA and infection stress.

This study exposed groups of mud snails *Zeacumantus subcarinatus* infected with 1 of 3 species of trematode parasite *Maritrema novaezealandensis, Philophthalmus* sp., or *Acanthoparyphium* sp., and an uninfected group to acidified seawater. The aim of this study was to investigate changes to shell growth, dissolution, and tensile strength in infected and uninfected snails exposed to acidified seawater, and to identify intra-specific differences in measured parameters based on the species of infecting parasite. We predicted that acidified seawater will negatively affect shell growth and strength and increase shell dissolution of uninfected snails. However, as the effects of parasitic infection may interact antagonistically with the effects of OA, i.e. by increasing growth, it is unclear how the combined effects of infection and OA will alter the production of $CaCO_3$ structures by infected individuals.

Calcification box

Many marine organisms form calcium carbonate ($CaCO_3$) in extra- or intra-cellular sites to construct calcified structures such as shells or plates (Weiner & Dove 2003). The biological formation of this mineral is described by the equation:

$$Ca^{2+} + 2HCO_3^- \rightleftharpoons CaCO_3 + CO_2 + H_2O$$

where Ca^{2+} are calcium ions and HCO_3^- are bicarbonate ions. Bicarbonate ions are supplied by the breakdown of metabolically generated CO_2 or taken from surrounding seawater (Roleda et al. 2012). The calcifying organism must also maintain saturated concentrations of carbonate ions (CO_3^{2-}) at the site of $CaCO_3$ formation so that the newly formed structures do not dissolve (Raven et al. 2005). The dissolution of $CaCO_3$ is described by the equation:

$$CaCO_3 \rightleftharpoons Ca^{2+} + CO_3^{2-}$$

and quantified by the expression:

$$\Omega = [Ca^{2+}][CO_3^{2-}] / k$$

where omega (Ω) represents the saturation state of $CaCO_3$ and k is a dissolution constant specific to the polymorph of $CaCO_3$ formed by the calcifying organism. Saturation states less than 1 ($\Omega < 1$) indicate that the dissolution of $CaCO_3$ is thermodynamically favoured (Gattuso & Buddemeier 2000). Aragonite and calcite are the most common polymorphs of $CaCO_3$ used by marine organisms, and aragonite is more soluble in acidified seawater than calcite (Doney et al. 2009).

As the increased dissolution of atmospheric CO_2 into seawater increases the concentration of bicarbonate ions and decreases the concentration of carbonate ions, the calcification process is compromised though dissolution rather than impaired synthesis.

MATERIALS AND METHODS

Trematode–snail system

The mud snail *Zeacumantus subcarinatus* is found in extremely high densities in many intertidal habitats along the coast of New Zealand (Fredensborg et al. 2005) and experiences a wide range of pH conditions on a diel scale (C. D. MacLeod unpubl. data). In Lower Portobello Bay (LPB) (45° 49′ 50″ S, 170° 40′ 17″ E), the collection site of snails used in this study, 8 species of trematode parasite are known to use Z. *subcarinatus* snails as a first intermediate host (Leung et al. 2009). In the following experiments, *Z. subcarinatus* individuals were divided into 4 infection categories: uninfected snails and those infected with *Maritrema novaezealandensis*, *Philophthalmus* sp., or *Acanthoparyphium* sp.

Snail collection and parasite identification

Approximately 2000 *Z. subcarinatus* snails were collected at LPB in July 2013 and subsequently screened for trematode infection by exposing snails to warmed seawater (25°C) and constant light, the physical conditions that trigger cercarial emergence. Trematode species were identified by inspecting cercariae under a dissecting microscope and comparing cercarial morphology with published descriptions of parasite species (Martorelli et al. 2008). Snails that were positively identified as infected with a parasite of interest were maintained at room temperature (~18–20°C) for 1 wk before being screened a second time, thus reducing the probability of selecting snails that were infected by 2 parasite species. All snails selected for the experiment were then marked with individual identification labels (Bee Works), maintained at room temperature (20°C) in aerated seawater (~pH 8.1) and fed sea lettuce *Ulva* spp. ad libitum.

OA simulation system

In order to expose snails to acidified seawater, a modular OA simulation system was designed (MacLeod et al. 2015). Three seawater aquaria were constructed, each consisting of a 120 l culture tank (870 × 600 × 295 mm [length × width × height]), a pump and filtration unit, a refrigeration unit and a pH regulation unit (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m537p137_supp.pdf). pH, measured on the total hydrogen ion scale,

was adjusted with 100% CO_2 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 every 48 h); light levels were also standardised across all culture tanks. Seawater in the 3 culture tanks was maintained at 12.5°C, 32 (practical salinity scale) and at one of the 3 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 Intergovernmental Panel on Climate Change (IPCC) report (2014); temperature and salinity values were selected based on average conditions in the habitat of *Z. subcarinatus* (C. D. MacLeod unpubl. 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 (Hunter 2007) (Table 1).

Experimental design

The combined effects of parasitic infection and exposure to acidified seawater on *Z. subcarinatus* individuals were investigated by exposing snails from each infection category to all pH treatments. In each pH treatment, ~30 snails from each infection category were distributed evenly between 5 cylindrical nylon mesh chambers (height = 8 cm, diameter = 8.5 cm), and the chambers submerged in unmodified or acidified seawater for a period of 90 d. Throughout the 90 d period, snails were provided with a constant supply of sea lettuce *Ulva* spp. To account for any unrecorded and unwanted variation in the performance of a particular culture tank and associated apparatus, i.e. tank effect, the pH assigned to each culture tank was changed at 30 and 60 d and snails transferred between tanks. Consequently, all snails experienced constant pH conditions and spent equal amounts of time in each culture tank. In addition to transferring snails between culture tanks, the position of each chamber was changed within each tank every 4 d, so that all chambers spent an equal period of time at each of the 20 positions available in the culture tanks. The periodic movement of snail chambers was carried out so that no snails spent an unequal amount of time in close proximity to the CO_2 inflow point, although frequent testing of seawater pH did not identify any pH gradient in the culture tanks.

Shell growth and dissolution

To document shell growth and dissolution, all snails were photographed (Olympus camera, DP25) in a standardised orientation and at a fixed magnification (6.4×) before and after exposure to acidified or unmodified seawater. Total shell length was measured in both sets of images using ImageJ software and changes to total shell length calculated. Prior to the experiment, all snails were also soaked for 24 h in a saltwater solution of Calcein™ (120 mg l^{-1}) to provide a measure of shell growth independent of dissolution. Calcein™ is a soluble fluorochrome which is incorporated into growing calcified structures and produces a fluorescent band which can be treated as a baseline for subsequent shell growth (Riascos et al. 2007). After the 90 d exposure to acidified or unmodified seawater, all snails were imaged under UV light (Leica camera, DFC350), and ImageJ software was used to measure the average distance in micrometres between the baseline fluorescent band and the new growing edge of the shell (Fig. 1).

Tensile strength

The tensile strength of each snail shell was tested using a texture analyser TA.HD*plus* (Stable Micro

Table 1. Mean values (±SD) of all measured and calculated parameters used to characterise the carbonate chemistry of unmodified and acidified seawater. Temp: temperature; DIC: dissolved inorganic carbon; pCO_3: partial pressure of CO_3; Ω_a: saturation state of aragonite

Treatment	pH (measured)	Temp. (°C)	Salinity	Alkalinity (µmol kg^{-1})	DIC (µmol kg^{-1})	pH (calculated)	pCO_2 (calculated)	Ω_a
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	2.52 ± 0.2
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	0.94 ± 0.1
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	0.62 ± 0.1

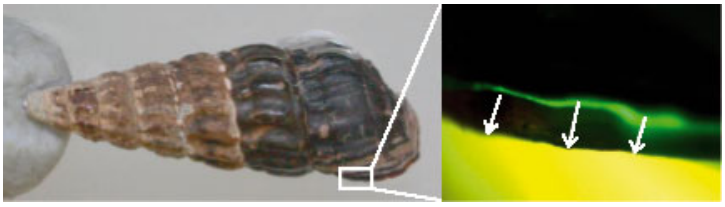

Fig. 1. Example of the images used to measure new shell growth. Insert shows the fluorescent band produced by Calcein™ stain that was used to mark the start of shell growth at the beginning of the 90 d period

Systems) that measured the maximum force in grams required to break a shell sample (Fig. S2 in the Supplement). Two sub-sets of data were gathered using this technique: the tensile strength of shell formed during the 90 d exposure to unmodified or acidified seawater and shell that had been formed prior to exposure. To ensure standardisation of measurements across snails, a P/2N needle probe (Stable Micro Systems) was attached to the texture analyser and used in conjunction with a small custom-made clamp to hold snails in a standardised orientation, ensuring that the same point on all snail shells was tested in each trial.

Identification of $CaCO_3$ polymorph

The polymorph of $CaCO_3$ used by *Z. subcarinatus* was identified using X-ray diffraction (XRD). Shell samples were placed in an agate mortar and pestle with a small amount of 95% ethanol and ground to a fine slurry. The ethanol and shell mixture was then spread onto a 2 × 2 cm glass slide and allowed to dry completely. Slides were placed in a PANalytical X'Pert Pro MPD X-ray diffractometer (PW3040/60 XRD, °2Θ, 3-80° scan, copper [Cu] anode) and the polymorphs of $CaCO_3$ identified by comparing X-ray diffraction patterns to a reference library of known minerals. Two shell samples from each infection category and pH combination (n = 24) were processed using this technique. For each sample the X-ray diffractometer produced a graph which indicated the presence of known minerals (see Fig. S3 in the Supplement). Aragonite was the only polymorph of $CaCO_3$ found in all *Z. subcarinatus* shells.

Statistical analysis

All response variables: shell growth, shell length, tensile strength (new growth) and tensile strength (old growth), were analysed using 3 different linear mixed effect models. In the first model, each response variable was analysed separately using pH, infection category, and initial shell length as fixed effects, and 'Chamber ID' as a random effect. This model provided an overview of the effect of pH and infection category on each response variable. Initial shell length was included as a proxy for snail age, as we assumed that age may alter calcification ability. 'Chamber ID' was included as a random effect to compensate for any bias introduced by maintaining multiple snails in the nylon mesh chambers. The output of this model type is summarised in Table 2. The first model was also used to calculate intra-class correlation (ICC) of 'Chamber ID' to quantify the repeatability of data recorded between groups of snails maintained in different nylon chambers:

$$\mathrm{ICC} = \left[\frac{(\text{between} - \text{individual variance})}{(\text{between} - \text{individual variance}) + (\text{within} - \text{individual variance})}\right] \times 100 \quad (1)$$

An ICC score of 0% indicates no repeatability of measurements between groups, 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 multiple chambers in the same pH treatment.

The second model was used to analyse changes in each response variable within each infection category. This model used the data from a single infection category as the response variable and included pH and initial shell length as fixed effects and 'Chamber ID' as a random effect. The output of this model type is summarised in Tables S1–S4 in the Supplement. These models also tested for the significant differences between pH treatments within infection categories. The third model used the data from each pH treatment as the response variable, with infection category and initial shell length as fixed effects and 'Chamber ID' as a random effect. The output of this model type provided a test for the significant differences between infection categories within pH treatments.

All analyses were conducted using R version 3.1.0 (R Development Core Team 2014) and the function *lmer* in the package lme4 v. 1.1-7 (Bates et al. 2014). The *powerTransform* function in the package *car* v. 2.0-21 (Fox et al. 2014) was used if any response variable required transformation to meet the assumptions of normality. Data generated by the function *lmer* are presented in the standard output format of analyses of variance (ANOVA). However, the func-

tion *lmer* does not provide a value for the degrees of freedom required to calculate p-values using a *t*- or *F*-statistic, so degrees of freedom were estimated from a linear model of the same data, without the random effect 'Chamber ID', and used to calculate p-values using *t*- and *F*-statistics from the original *lmer* model. Fixed effects were considered significant if p-values were less than or equal to 0.05.

RESULTS

Shell growth

The shell growth of *Zeacumantus subcarinatus* was significantly affected by pH, infection category, and the interaction of these factors (Table 2). Within infection categories, pH significantly affected the shell growth of all snails, while initial shell length was a significant factor in the shell growth of uninfected snails only (Table S1). In most infection categories, shell growth was significantly lower in the pH 7.4 treatment relative to the pH 7.6 and 8.1 treatments (Fig. 2A). The only exceptions to this trend were snails infected with *Philophthalmus* sp., which exhibited significantly reduced shell growth in both acidified treatments (Fig. 2A). There were significant differences in shell growth between infection categories in pH 8.1 and 7.6 treatments (Fig. 2B). In the pH 8.1 treatment, uninfected snails exhibited significantly lower shell growth than *Maritrema novaezealandensis*-infected snails, whilst snails infected with *Acanthoparyphium* sp. and *Philophthalmus* sp. showed intermediate shell growth compared to the former treatment groups. In the pH 7.6 treatment, snails infected by *M. novaezealandensis* and *Acanthoparyphium* sp. exhibited significantly higher shell growth than *Philophthalmus* sp.-infected snails, while uninfected snails grew significantly less than those infected by *M. novaezealandensis*. At pH 7.4, there were no significant differences in shell growth between any infection categories. Intra-class correlation scores generated by 'Chamber ID' showed that the repeatability of average snail shell growth between chambers was 10%, indicating that repeatability was low and pseudoreplication not a confounding factor in these data.

Table 2. Outputs for the linear mixed effect analysis of shell growth, length, and breaking force (old and new shell growth). Sample size for each analysis is given in parentheses. IC: infection category; length: length of snail shells at the beginning of the 90 d trial. Significant p-values are shown in **bold**

		df	MS	*F*	p
Shell growth (313)	pH	2	161.1	59.1	**<0.001**
	IC	3	13.6	5.0	**0.002**
	Length	1	2.7	1.0	0.313
	pH × IC	6	7.3	2.7	**0.012**
	pH × length	2	0.7	0.2	0.770
	IC × length	3	3.2	1.2	0.313
Shell length (313)	pH	2	16.2	56.6	**<0.001**
	IC	3	103.6	34.5	**<0.001**
	Length	1	13.2	13.2	**<0.001**
	pH × IC	6	0.2	0.6	0.684
	pH × length	2	0.4	1.4	0.234
	IC × length	3	2.3	8.1	**<0.001**
Breaking force (263) (new growth)	pH	2	3.3901	37.8777	**<0.001**
	IC	3	0.5603	6.2598	**<0.001**
	Length	1	0.1320	1.4745	0.229
	pH × IC	6	0.0776	0.8665	0.520
	pH × length	2	0.0153	0.1711	0.843
	IC × length	3	0.0410	0.4580	0.712
Breaking force (263) (old growth)	pH	2	29.2344	37.4814	**<0.001**
	IC	3	4.7599	6.1027	**<0.001**
	Length	1	1.0996	1.4098	0.236
	pH × IC	6	0.7216	0.9252	0.477
	pH × length	2	0.1492	0.1913	0.826
	IC × length	3	0.3285	0.4211	0.738

Changes in shell length

Changes in the shell length of *Z. subcarinatus* were significantly affected by pH, infection category, initial shell length, and the interaction of initial shell length and infection category (Table 2). Within infection categories, pH significantly affected all groups, while initial shell length was a significant factor for *Acanthoparyphium* sp.-infected snails only (Table S2). In all infection categories except *M. novaezealandensis*, there were significant differences in the change in shell length between all pH treatments; only the pH 8.1 and 7.4 treatments differed significantly in the *M. novaezealandensis* group (Fig. 3A). In all pH treatments, uninfected snails, and those infected by *Philophthalmus* sp., exhibited significantly smaller changes in shell length than *M. novaezealandensis* and *Acanthoparyphium* sp.-infected snails, while there was also a significant difference between the latter 2 groups; however, in the pH 8.1 treatment, changes in shell length for uninfected snails were positive (Fig. 3B). Intra-class correlation scores generated by 'Chamber ID' showed that the repeatability of average changes in

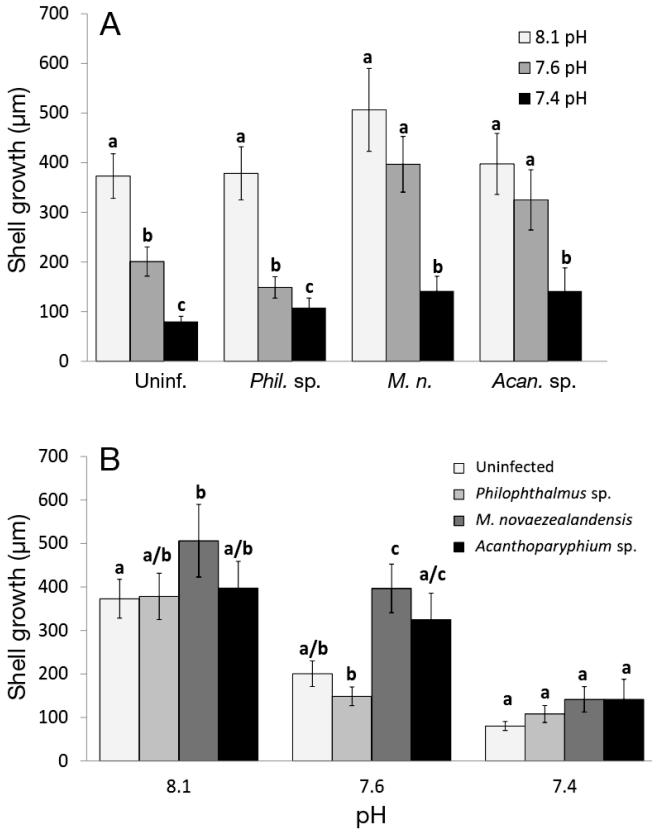

Fig. 2. Mean shell growth (±SE) of snails exposed to acidified and unmodified seawater for 90 d. Data grouped by (A) pH treatment and (B) infection category. Lowercase letters indicate significant differences between treatments. Sample sizes for uninfected snails = 31 (pH 8.1), 23 (pH 7.6), 19 (pH 7.4), and snails infected with *Philophthalmus* sp. = 27 (pH 8.1), 25 (pH 7.6), 17 (pH 7.4), with *Maritrema novaezealandensis* = 27 (pH 8.1), 27 (pH 7.6), 17 (pH 7.4), and with *Acanthoparyphium* sp. = 27 (pH 8.1), 22 (pH 7.6), 23 (pH 7.4)

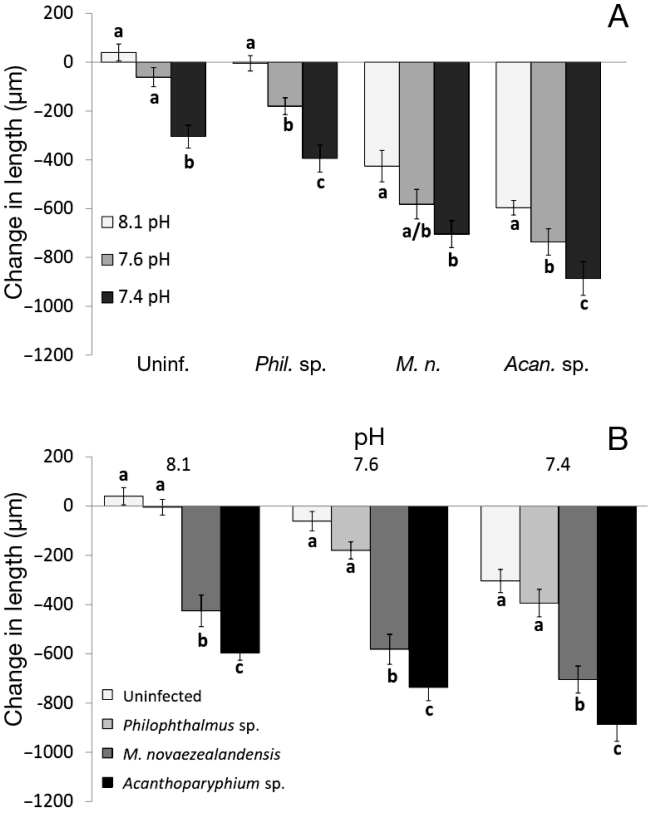

Fig. 3. Mean change in shell length (±SE) of snails exposed to acidified and unmodified seawater for 90 d. Data grouped by (A) pH treatment and (B) infection category. Lowercase letters indicate significant differences between treatments. Sample sizes for uninfected snails = 31 (pH 8.1), 23 (pH 7.6), 25 (pH 7.4), and snails infected with *Philophthalmus* sp. = 27 (pH 8.1), 27 (pH 7.6), 24 (pH 7.4), with *Maritrema novaezealandensis* = 23 (pH 8.1), 27 (pH 7.6), 24 (pH 7.4), and with *Acanthoparyphium* sp. = 28 (pH 8.1), 29 (pH 7.6), 26 (pH 7.4)

snail shell length between chambers was 7.8%, indicating that repeatability was low and pseudoreplication not a confounding factor in these data.

Shell strength

The tensile strength of shell formed by *Z. subcarinatus* prior to and during the 90 d exposure to unmodified or acidified seawater was significantly affected by pH and infection category (Table 2). For all infection categories, the tensile strength of newly formed shell structures was significantly affected by pH (Table S3), while the strength of shell formed prior to the experimental period was significantly affected by pH in infected snails only and by initial shell length in snails infected with *M. novaezealandensis* (Table S4). The tensile strength of newly formed shell was significantly lower in the pH 7.4 treatment relative to the pH 8.1 treatment in all infection categories, although the effect of pH 7.6 was different for each group. In uninfected snails, the pH 7.6 treatment was significantly different from both pH 8.1 and 7.4 treatments. In snails infected with *Philophthalmus* sp., the pH 7.6 treatment was significantly lower than that of the controls, whilst in snails infected with *M. novaezealandensis*, there was significantly greater tensile strength in the pH 7.6 than the pH 7.4 treatment, and in *Acanthoparyphium* sp.-infected snails, there were no significant differences in the tensile strength of snails in the pH 7.6 and those of either pH 7.4 or control treatments (Fig. 4A). Within pH treatments, there were significant differences between *M. novaezealandensis*-infected snails and uninfected snails at pH 8.1 and between *M. novaezealandensis*-infected snails and all other

infection categories at pH 7.4 (Fig. 4B). The tensile strength of shell formed prior to the experimental period was significantly greater in control (pH 8.1) conditions relative to the pH 7.6 and 7.4 treatments in all infected categories, with the exception of *M. novaezealandensis*, for which the pH 7.4 treatment was statistically similar to both pH 7.6 and 8.1. There were no significant differences recorded in the tensile strength of pre-existing shell of uninfected snails at any pH treatment (Fig. 5A). Within pH treatments, there were significant differences at pH 8.1 between uninfected snails and those infected with *Philophthalmus* sp. and *Acanthoparyphium* sp. (Fig. 5B). Intra-class correlation scores generated by 'Chamber ID' showed that the repeatability of average changes in shell strength between chambers was 0.8% for new shell growth and 0.8% for old shell growth, indicating that repeatability was very low and pseudoreplication not a confounding factor in these data.

DISCUSSION

The goals of this study were to investigate the combined effects of acidified seawater and parasitic infection on the calcifying ability of the mud snail *Zeacumantus subcarinatus* and to compare the responses of snails infected with different species of trematode parasite. In all measured parameters (shell growth, dissolution, and strength), there were significant negative effects of reduced pH, i.e. reduced shell growth and strength, and increased shell dissolution. Significant differences in all parameters were also recorded between infected and uninfected individuals, and between snails infected with different species of parasite. The combined effects of acidified seawater and parasitic infection were most notable in the moderate pH treatment (pH 7.6), as the relative responses of snails from all infection categories recorded at 8.1 pH were significantly altered when

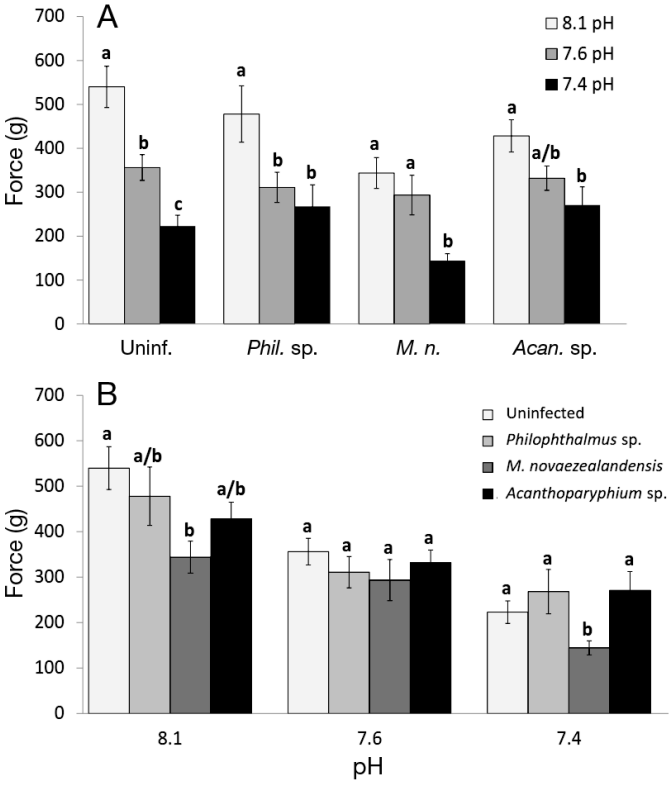

Fig. 4. Mean force (±SE) required to break shell formed during the 90 d exposure to acidified and unmodified seawater. Data grouped by (A) pH treatment and (B) infection category. Lowercase letters indicate significant differences between treatments. Sample sizes for uninfected snails = 25 (pH 8.1), 23 (pH 7.6), 22 (pH 7.4), and snails infected with *Philophthalmus* sp. = 22 (pH 8.1), 22 (pH 7.6), 21 (pH 7.4), with *Maritrema novaezealandensis* = 22 (pH 8.1), 22 (pH 7.6), 19 (pH 7.4), and with *Acanthoparyphium* sp. = 23 (pH 8.1), 23 (pH 7.6), 19 (pH 7.4)

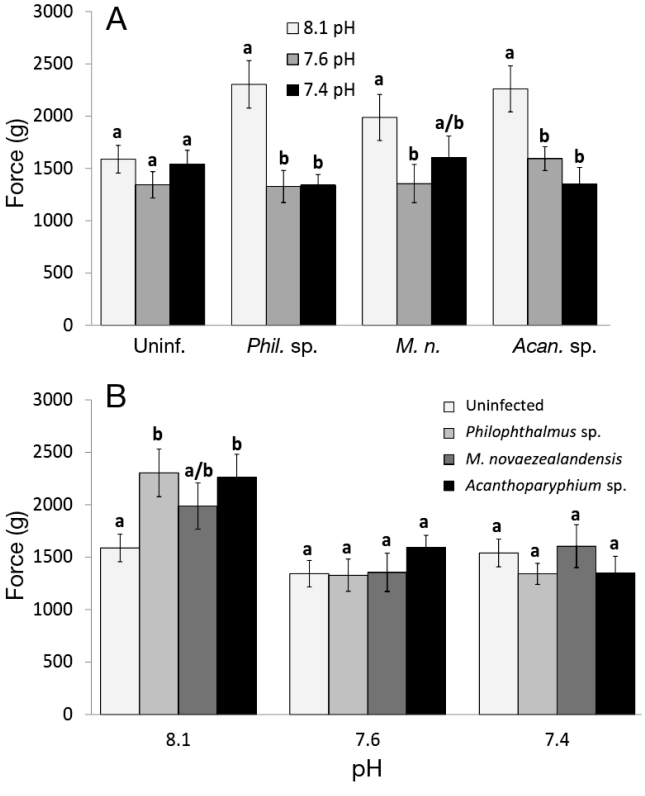

Fig. 5. Mean force (±SE) required to break shell formed prior to the 90 d exposure to acidified and unmodified seawater. Data grouped by (A) pH treatment and (B) infection category. Lowercase letters indicate significant differences between treatments. Sample sizes for uninfected snails = 25 (pH 8.1), 23 (pH 7.6), 22 (pH 7.4), and snails infected with *Philophthalmus* sp. = 22 (pH 8.1), 22 (pH 7.6), 20 (pH 7.4), with *Maritrema novaezealandensis* = 22 (pH 8.1), 22 (pH 7.6), 19 (pH 7.4), and with *Acanthoparyphium* sp. = 23 (pH 8.1), 23 (pH 7.6), 19 (pH 7.4)

seawater pH was reduced to 7.6. These data indicate that the interaction of stressors associated with OA and trematode infection have the potential to change the calcifying ability of *Z. subcarinatus*. Conversely, differences between infection categories were not observed when snails were exposed to pH 7.4 seawater, suggesting there is a tipping point beyond which the effects of acidified seawater mask any effects of trematode infection on the calcifying ability of *Z. subcarinatus*. These results indicate that trematode infection currently alters the ability of host organisms to form calcified structures and that these effects will be modified by the continued acidification of oceanic waters.

Snails in all infection categories exhibited positive shell growth in all pH treatments, although growth was significantly reduced in pH 7.6 and 7.4 seawater (Fig. 2A). These results show that *Z. subcarinatus* has the ability to form $CaCO_3$ in seawater undersaturated with respect to aragonite ($\Omega_a < 1$), the only polymorph of $CaCO_3$ present in the shell of this species of snail (see 'Identification of $CaCO_3$ polymorph'). Shell growth in seawater undersaturated with respect to aragonite indicates that *Z. subcarinatus* snails possess cellular processes capable of creating a saturated solution of carbonate ions in extracellular spaces, despite exposure to undersaturated ambient conditions (Weiner & Dove 2003). *Z. subcarinatus* snails may possess this ability because they are periodically exposed to similar conditions in their natural habitat, e.g. Ω_a = 0.293 and pH = 7.599 (C. D. MacLeod unpubl. data). The observed reductions in shell growth across acidified treatments may be caused by the increased energetic cost of establishing the extra-cellular zones of carbonate ion saturation, or by the dissolution of newly formed calcified structures as they become exposed to undersaturated ambient conditions.

While the observed reductions in shell growth may have a relatively simple underlying mechanism, i.e. changes in the availability of carbonate ions in seawater, the cause of differences in shell growth observed between infection categories of snails exposed to the same pH may be more complex. Increased shell growth of snails infected with trematode parasites, also referred to as gigantism, has been discussed at length in the parasitology literature (Minchella 1985, Mouritsen & Jensen 1994, Gorbushin 1997, Probst & Kube 1999). Gigantism has been attributed to differential energy costs of infection versus reproduction (Gorbushin 1997) or to differences in the reproductive life histories of trematode parasites (Minchella 1985). Gastropods infected by trematode parasites are typically sterilised as the parasite grows within the infected snails. The energy consumed by parasitic infection may be less than the energy required for reproduction by uninfected individuals, allowing infected snails to invest surplus energy in shell growth. This could explain why *Maritrema novaezealandensis*-infected snails exhibited greater shell growth than uninfected individuals at pH 8.1 and 7.6, although it fails to explain why there was no difference in growth rates between uninfected snails and those infected with *Acanthoparyphium* sp. and *Philophthalmus* sp. The varied life histories of the 3 trematode parasites may further explain the observed differences, or lack thereof, between infection categories. Once reaching the gastropod host, trematode parasites form rediae or sporocysts, depending on the trematode species, both capable of asexual reproduction. Although both reproductive morphs produce clonal cercariae, rediae actively consume host tissue while sporocysts absorb nutrients through their tegument (Cheng 1963). Consequently, the feeding strategy of rediae may require the host to invest additional energy into tissue repair relative to hosts infected with sporocyst-producing parasites. In the group of trematode species used in this study, *M. novaezealandensis* cercariae are produced by sporocysts while *Acanthoparyphium* sp. and *Philophthalmus* sp. cercariae are produced by rediae. If the active consumption of tissue and the subsequent cost of repair increase the energetic cost of infection by a rediae-producing parasite, this may explain why snails infected with *Acanthoparyphium* sp. and *Philophthalmus* sp. exhibited lower growth compared to snails infected with *M. novaezealandensis*. If this explanation is correct, the energetic cost of repair may be comparable to the cost of reproduction, potentially explaining the similarity in growth rates of uninfected snails and those infected with rediae-producing trematode species. If gigantism and/or parasite reproductive life history do explain the differences observed between infection categories in control conditions, these factors must be altered by acidified seawater, as the relationship between the shell growth of snails in different infection categories changes between pH 8.1 and 7.6 treatments (Fig. 2B). At pH 7.6, *M. novaezealandensis*-infected snails nonetheless exhibit the greatest shell growth, but the relative differences between uninfected snails and those infected with *Philophthalmus* sp. and *Acanthoparyphium* sp. are altered. As the type of reproductive morph present in the snail host is obviously unchanged by seawater pH, it is likely that the change in the relative growth

rates of snails in different infection categories was caused by altered energy availability. If snail hosts must invest more energy in maintaining acid/base homeostasis, there would be less energy available for shell growth. Similarly, exposure to acidified seawater can reduce extracellular pH (Reipschläger & Pörtner 1996), exposing parasites to pH stress and causing an increase in the energy required by the parasite to maintain acid/base homeostasis. Consequently, the altered responses of snails from the same infection category exposed to different seawater pH levels may also indicate differential tolerances of parasite species to acidified conditions.

The lack of significant differences in shell growth between snails from all infection categories exposed to pH 7.4 seawater may indicate that, in these conditions, the effects of reduced pH mask parasite-induced changes to shell growth. The lack of differences could be caused by: (1) a dramatic reduction of metabolic energy available for calcification, as snails prioritise maintaining acid/base balance; (2) an increase in the metabolic demands of the parasites as they are exposed to pH stress; (3) the dissolution of newly formed calcified shell; or (4) a combination of these.

Despite the positive shell growth observed in all infection categories in all pH treatments, the majority of snails exhibited negative net changes in total shell length over the 90 d exposure to acidified seawater, indicating that shell dissolution was greater than shell growth. Positive changes in shell length were observed only in uninfected snails maintained at pH 8.1, and there was a clear trend of increased dissolution in all infection categories as pH was reduced (Fig. 3B). Shell dissolution in acidified seawater can be explained by the same mechanism underlying changes to shell growth, i.e. reduced carbonate ion concentration. As seawater becomes undersaturated with respect to carbonate ions, the dissolution of $CaCO_3$ structures becomes thermodynamically favoured, and shells not protected by biologically generated zones of carbonate ion saturation begin to dissolve (see Calcification box). However, differences between infection categories cannot be explained completely by the dissolution kinetics of $CaCO_3$. Although all infection categories exhibited shell dissolution in acidified seawater, dissolution was recorded for snails infected with *M. novaezealandensis* and *Acanthoparyphium* sp. in the seawater control treatment, while there were also significant differences between the dissolution responses of snails in different infection categories in all pH treatments (Fig. 3B).

The observed shell dissolution or low shell growth in pH 8.1 seawater may reflect differences in the dissolution kinetics of biogenically synthesised and pure forms of $CaCO_3$, as the saturation states of aragonite refer only to the pure form of the mineral. Alternatively, the observed response to control conditions may reflect the difference between the average pH of bulk water at LPB (~8.1), and the average pH value recorded in the micro-habitat of *Z. subcarinatus* (pH 8.53) (C. D. MacLeod unpubl. data). On the basis that the biogenesis of $CaCO_3$ structures by *Z. subcarinatus* evolved in a more alkaline environment, potentially due to the buffering ability of photosynthetic organisms (Hurd et al. 2009), pH 8.1 may prove corrosive despite its correspondence with an $\Omega_a > 1$. Despite the uncertainty surrounding the dissolution kinetics of biogenically synthesised $CaCO_3$, the reasons for differences in dissolution between infection categories is more likely to be biological, rather than mineralogical, as we detected no difference in the mineral content of shells from different infection categories. Two biological mechanisms could account for the observed differences between infection categories: (1) infection compromises the synthesis of the biological component of calcified shell, and/or (2) changes to the behaviour of infected individuals increases the mechanical damage incurred by the surface of shells, making them more prone to dissolution.

The organic component of biogenically synthesised $CaCO_3$ in calcifying marine organisms is not fully understood (Weiner & Dove 2003), although it may play an important role in dictating the dissolution kinetics of biomineralised $CaCO_3$ by providing a protective coating (Hall-Spencer et al. 2008, Ries 2011). As parasitic infection can affect shell morphology and growth rates in calcifying organisms (Mouritsen & Jensen 1994, Probst & Kube 1999), infection may also compromise a snails ability to synthesise the organic component of its shell and may explain the difference in observed dissolution rates between infection categories. Snail shells could also become more vulnerable to dissolution if the protective layer becomes worn or damaged. When infected, some species of snail have shown a preference for certain types of habitat that maximise the probability of successful transmission for the parasite (Curtis 1987). If infected *Z. subcarinatus* snails exhibit a preference for habitat zones that increase the danger of mechanical damage, they may become more vulnerable to dissolution. As the experimental conditions used in this study did not replicate factors that could cause such mechanical damage, e.g. wave action or rocky substrates, the differential dissolution recorded here

may represent damage that occurred in the field prior to collection.

The differential dissolution rates observed between infection categories suggest that biogenically formed $CaCO_3$ structures are affected by more complex factors than the dissolution kinetics of pure $CaCO_3$ in acidified seawater. The differences found between infection categories also suggest that while snails infected by certain species of parasite may grow faster, some quality of the shell is changed so that it becomes more vulnerable to dissolution in acidified seawater. These parasite-induced changes to the calcification process may make infected individuals more susceptible to the changes to seawater chemistry caused by OA.

The tensile strength of 2 discrete areas of snail shell was tested: shell formed prior to exposure to acidified seawater and shell formed during exposure to acidified seawater. The tensile strength of newly formed shell was interpreted as a measure of the calcifying ability of infected and uninfected snails in control and acidified conditions. Overall, the strength of newly formed shell decreased in acidified seawater (Fig. 4B), which can again be broadly explained with reference to the saturation state of carbonate ions in seawater. After new shell is formed, it becomes exposed to ambient seawater conditions and may exhibit dissolution, causing a reduction in tensile strength. Within the control (pH 8.1) treatment, uninfected snails formed the strongest shell, although this was only significant in the case of one species of infecting parasite (*M. novaezealandensis*). These results further support the hypothesis that infection alters the calcifying ability of *Z. subcarinatus*, such that the shell grows at a greater rate but is in some way of lesser quality. In this case, *M. novaezealandensis*-infected snails, which had exhibited the greatest growth, also possessed the weakest newly formed shell. Although there were no significant differences between infection categories at pH 7.6, in the most extreme treatment (pH 7.4), *M. novaezealandensis*-infected snails again exhibited the weakest newly formed shell. These results, in combination with the shell growth and dissolution data, indicate that *M. novaezealandensis* has the greatest effect on the calcifying ability of *Z. subcarinatus* and that these effects increase the vulnerability of snails infected with *M. novaezealandensis* to the stressors associated with OA.

The tensile strength of shell formed prior to exposure to acidified seawater, interpreted as a measure of the vulnerability to the dissolution of shell formed in natural conditions, was significantly reduced in all infected individuals following exposure to acidified seawater. The only observable difference between infection categories was a significantly greater strength in the shell formed by all infected snails and maintained at pH 8.1 (Fig. 5B). This pattern is the opposite of what we found in the newly formed shell data, which suggests that there may be differences in the process of calcification that occur after the formation of $CaCO_3$ structures at the growing edge of shell, e.g. a further thickening or strengthening of shell. Additionally, as the age of all snails at the time of infection is unknown, changes in the tensile strength of older shell may not represent an interaction of infection stress and the formation of $CaCO_3$ structures.

The data presented here clearly shows that reduced pH negatively affects the quality of shell produced in, or exposed to, acidified seawater. Differences in the calcification ability of hosts caused by particular species of infecting parasite are also demonstrated by the data and indicate that infection can increase the rate of shell growth and result in stronger shell but may increase the vulnerability of shell to dissolution in acidified seawater. The pH treatments chosen for this experiment correspond to current average surface ocean conditions (~pH 8.1) and predicted conditions for the year 2100 (pH 7.6) and 2300 (pH 7.4) (IPCC 2014). Consequently, we can use the data from this study to better understand the current effects of parasitic infection on the calcifying ability of marine gastropods and as a predictor of how the interaction of trematode infection and acidified seawater may change over time due to OA. At the current average surface oceanic pH of 8.1, the effects of parasitic infection vary significantly between snails infected with different species of parasite and between infected and uninfected individuals. Infection can increase rates of shell growth (*M. novaezealandensis*) and dissolution (*M. novaezealandensis, Philophthalmus* sp. and *Acanthoparyphium* sp.), reduce the strength of newly formed calcified structures (*Philophthalmus* sp.), or result in greater strength of older shell structures (*Philophthalmus* sp. and *Acanthoparyphium* sp.). As pH is reduced to 7.6, there are shifts in the relationship between infection categories. Firstly, there are greater differences between snails in different infection categories in terms of growth rates, with the highest growth rates observed in infected snails. Secondly, all snails exhibited greater dissolution, although there are significant differences between infection categories, with infected individuals experiencing the greatest dissolution. In old and newly formed

shell, there are no longer differences in tensile strength between any infection categories. When pH is reduced to 7.4, most differences between infection categories disappear. Significant differences are only found in dissolution rates, with infected snails again experiencing the greatest dissolution, and reductions in tensile strength of newly formed $CaCO_3$ structures, with infected snails again exhibiting the weakest shell.

These results suggest that parasitic infection will alter the calcifying ability of marine gastropods in the short- to mid-term, i.e. the next 50 to 100 yr, but not in the long-term, i.e. 200 to 300 yr. Any reductions in shell strength, either through parasitic infection or exposure to acidified seawater, may significantly affect the population of *Z. subcarinatus* at LPB, as up to 80% of individuals are infected with trematode parasites (Fredensborg et al. 2005), and shell strength is a key factor protecting *Z. subcarinatus* from predation by shell crushing crabs (Kamiya & Poulin 2012). Any abiotic or biotic factors that disproportionately affect infected individuals could have dramatic consequences for established intra-specific competitive dynamics between infected and uninfected snails.

Of course, when predicting changes on the ecosystem scale over such a long time period, other factors may modify the effects of OA, e.g. increased temperature can increase seawater pH (Hunter 1998). However, the data presented here strongly suggests that OA has the potential to disrupt the current relationship between trematode parasites and their gastropod hosts. Any such disruptions on a regional or global scale could have unforeseen and dramatic effects on the role of host and parasite organisms in marine ecosystems.

The simulated OA conditions in this experiment were expected to reduce shell growth and strength due to the reduced availability of carbonate ions. However, some results confound these expectations, suggesting that the interaction of parasitic infection and ocean acidification will be unpredictable and depend upon the species of infecting parasite. These findings emphasise the importance of assessing the extent of parasitic abundance in populations of marine organisms and the species-specific interactive effects of infection and acidified seawater. Parasites are a ubiquitous component of all marine systems (Sousa 1991, Mouritsen & Poulin 2002), and previous studies of the responses of many invertebrate species to stressors associated with OA may have been confounded by the unrecorded effects of marine parasites.

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