Sub-lethal predatory shell damage does not affect physiology under high CO_2 in the intertidal gastropod *Tritia reticulata*

Leonardo Querobim Yokoyama[1,2]*, Alexander Turra[1], Coleen Suckling[3,4], Gabriela Torres[3,5], Andrew Davies[3,6], Ian McCarthy[3]

[1] Instituto Oceanográfico, Universidade de São Paulo, Laboratório de Manejo, Ecologia e Gestão Costeira, Departamento de Oceanografia Biológica (Praça do Oceanográfico, 191 - Butantã - SP - 05508-120 - Brazil)

[2] Universidade Federal de São Paulo, Laboratório de Ecologia e Gestão Costeira, Instituto do Mar (Rua Carvalho de Mendonça, n° 144 - Santos - SP - 11070-100 - Brazil)

[3] Bangor University, School of Ocean Sciences (Menai Bridge, Anglesey, LL59 5AB, United Kingdom)

[4] University of Rhode Island, Department of Fisheries, Animal and Veterinary Sciences (129 Woodward Hall, 9 East Alumni Avenue, Kingston, RI 02881 - United States)

[5] Alfred-Wegener-Institute, Helmholtz Centre for Polar and Marine Research (Biologische Anstalt Helgoland Postfach 180 27483-Helgoland - Germany)

[6] University of Rhode Island, Department of Biological Sciences, (Kingston - RI 02881 - United States)

*Corresponding author: lqyokoyama@gmail.com

ABSTRACT

Ocean acidification (OA) poses a major threat to marine animals, especially marine shelled invertebrates such as molluscs. Although many organisms are capable of compensating for the effects of OA, this can impose physiological costs and impact performance (e.g. through increased metabolism and decreased growth). Sublethal injuries on shells may provoke changes in energy allocation. Under acidified conditions, organisms would spend less energy on reproduction and somatic growth to repair the damage. Therefore, we analysed the physiological responses of the intertidal gastropod *Tritia reticulata* during shell regeneration under OA conditions. We simulated a sub-lethal predation event (a notch in the outer lip of the shell) and individuals were exposed to control (pH 8.08) and low pH scenarios (pH 7.88 and 7.65). After two months exposure, all individuals showed shell repair, with a full repair rate observed in 75% of individuals. Contrary to expectations, shell repair following sub-lethal damage and OA had no apparent impact on physiological state in terms of energy reserves (as measured by whole-animal Carbon/Nitrogen) or growth potential (as measured by whole-animal RNA:Protein and RNA:DNA ratios). As an intertidal organism, *T. reticulata* could be resilient to future global environmental change because of compensatory mechanisms that are inherent in intertidal animals, and may represent a robust species with which to study future scenarios of OA in temperate coastal ecosystems. However, unrestricted food availability during experiment could have played a role in the results and therefore food limitation should be considered in future studies regarding shell repair and metabolism under the effects of OA.

Descriptors: Ocean acidification, Metabolism, Intertidal, Gastropod, Shell repair.

INTRODUCTION

One of the biggest threats of ocean acidification (OA) is the need for marine organisms to expend more energy on buffering their acid-base relationship (Sokolova et al., 2012). However, OA can also affect biological processes such as growth (Harris et al., 1999), reproduction (Suckling et al., 2015), development (Dupont et al., 2008; Brennand et al., 2010) and calcification of hard skeleton structures (Courtney et al., 2013). Molluscs are one of the taxa with the greatest potential to be affected by acidification since molluscs shells are composed of calcium carbonate (calcite and/or aragonite; Addadi et al.,

2006). Acidification alters the saturation state of calcium carbonate ($CaCO_3$), which hinders $CaCO_3$ extraction from the water and elevates the dissolution of skeletal $CaCO_3$ (Watson et al., 2012). Amongst marine Gastropoda, the responses to increased pCO_2 (causing pH reduction) have varied depending on species and duration of exposure with no consensus on organismal response (see review by Parker et al., 2013). Reduction in growth and calcification under acidified environments is expected (Shirayama and Thorton, 2005), but some species are capable of raising their calcification rates (Langer et al., 2014) whereas other species maintain the deposition of $CaCO_3$, although at the cost of their metabolic rates (Findlay et al., 2011). Similarly, physiological responses to increased pCO_2 varies in gastropods with negative (Bibby et al., 2007; Harvey et al., 2016), positive (Harvey et al., 2016) and absence of effects (Marchant et al., 2010) depending on the study species and parameters measured.

In an acidified environment, reduction in calcification may reduce shell strength and/or thickness (Bibby et al., 2007) making species more vulnerable to predation (Gazeau et al., 2013). For coastal molluscs, the magnitude and consequences of OA on predator-prey interactions at the population, community and ecosystem levels are still unknown because of the varying range of possible outcomes (Kroeker et al., 2014). Sub-lethal events from predator-prey interactions normally leaves the molluscan prey with a damaged shell (Blundon and Vermeij, 1983). This injury is common in many gastropods (Turra et al., 2005; Dietl and Alexander, 2009; Stafford et al., 2015) and may increase the risk of death because of impaired movement due to regeneration and the potential loss of body fluids (Blundon and Vermeij, 1983). The available evidence indicates that regrowth scars are not a weak point in the shell (Blundon and Vermeij, 1983) and gastropods with damaged shells can have comparable, or even faster, growth rates compared to non-damaged individuals (Geller, 1990). Under OA conditions, sub-lethal predation can force individuals to divert energy to maintain calcification or even change behaviour (Bibby et al., 2007; Beniash et al., 2010). Therefore, species that depend on protection by thicker shells or hard structures against predators could be severely affected by OA.

The netted dogwhelk *Tritia reticulata* (formerly known as *Nassarius reticulatus*; Galindo et al., 2016)

is a common gastropod species from European coastal waters (Barroso et al., 2005a), occurring on shallow sandy bottoms and in intertidal rock pools (Tallmark, 1980). As a scavenger benthic species, it is responsible for diagenetic processes in surface sediments, because of its movements in the surface layer, where organic matter is more abundant (Donazzolo et al., 1989). The biology and ecology of *T. reticulata* has been well studied (Tallmark, 1980; Barroso et al., 2005a, 2005b; Chatzinikolaou and Richardson, 2007, 2008) but the effects of OA are not known although they have been studied for closely-related *Nassarius* spp. (Zhang et al., 2014, 2015, 2016). *T. reticulata* can vary shell growth and thickening depending on location and in areas protected from wave action and with high abundance of predators it tends to produce thicker shells (Chatzinikolaou and Richardson, 2007). Therefore, in populations of *T. reticulata*, shell dissolution by acidification could pose a potential challenge through increased predation risk. Regeneration of parts of the shell lost by sub-lethal predation is a costly process and, even in natural conditions, animals will be required to divert energy from other sources such as somatic growth or reproduction (Geller, 1990).

In marine ecology, various biochemical indicators such as nucleic acid ratios (RNA:DNA and RNA:Protein) and proximate composition (proteins, lipids, elemental analysis) have been used as correlates of condition and growth rate (reviewed in Houlihan et al., 1993; Fraser and Rogers, 2007). The RNA:DNA ratio provides a measure of protein synthesis and recent growth since the amount of DNA in somatic cells remains relatively constant whilst the amount of RNA involved in protein synthesis will vary with nutritional state, age, life-stage, size and changing environmental conditions (Buckley et al., 1999; Chícharo and Chícharo, 2008). In addition, the RNA:Protein ratio is an indication of cellular 'capacity for protein synthesis' (Houlihan et al., 1993; Fraser and Rogers, 2007). Thus, nucleic acid ratios are recognised as useful indicators of *in situ* physiological and nutritional state and have been used in a range of marine taxa such as microbes, invertebrates and fishes (Ferron and Leggett, 1994; Dahlhoff, 2004; Chícharo and Chícharo, 2008), including gastropods (Wo et al., 1999; Kim et al., 2011). Proximate composition analyses (i.e., percent lipid and protein) have been

determined using measures of elemental bulk tissue carbon (C) and nitrogen (N) and the C:N ratio and these data have been used as a measure of condition in many marine taxa. This is based on the assumption that lipid contains mostly carbon whilst the majority of tissue nitrogen is found in proteins and therefore C and N will reflect tissue lipid and protein concentrations respectively and the C:N ratio will represent the changing relative amounts of lipid and protein indicating better body condition as a result of higher tissue total lipid concentrations (Fagan et al., 2011).

In this context, we evaluate the physiological status [i.e. changes in body composition (elemental C/N) and nucleic acid indicators of growth (RNA:DNA, RNA:Protein)] of the netted dogwhelk *T. reticulata* exposed to different pCO$_2$ concentrations (resulting in pH levels of 8.08, 7.88 and 7.65), during a shell regeneration process (a notch in the outer lip of the shell) following damage to simulate a sub-lethal predation event.

MATERIAL AND METHODS

ANIMAL COLLECTION AND HOLDING CONDITIONS

Individuals of *Tritia reticulata* were sampled in May 2013 at low tide from the intertidal lagoons on the rocky shore at Rhosneigr (Anglesey, North Wales, UK) following the methodology of Chatzinikolaou and Richardson (2007). After capture, specimens of *T. reticulata* were transferred to plastic buckets containing seawater and transported to marine aquaria in the School of Ocean Sciences, Bangor University. The whelks were left to acclimate for 20 days (temperature range: 9° to 11° C), with no tide simulation, and fed *ad libitum* every 2 days with live mussels (2-3 individuals) before experimentation. Abiotic ambient data (mean ± standard deviation) measured for March 2013 was seawater temperature (6.2 ± 0.7), pH (8.07 ± 0.06), and salinity (35 ± 0).

PHYSIOLOGICAL RESPONSE EXPERIMENT

Seventy individuals of *T. reticulata* (shell length 22 to 33mm) were individually identified with plastic tags (9mm²) using cyanoacrylate glue. At the start of the experiment, an initial sample of 10 individuals (mean=23.37 ± 1.59mm) were randomly selected and frozen at -80 °C as a baseline of shell size and physiological measures for the end of experiment

(hereafter referred to as the initial baseline). The remaining 60 individuals were intentionally damaged by creating a triangular notch into the external lip of the shell aperture (approximately 4mm²) and then randomly assigned to one of three pH treatments (pH 8.08, 7.88 and 7.65). The pH of the treatments were not gradually increased before the start of the experiment. Each individual was housed in an individual container (approximately 50ml) closed by a lid and perforated with holes to allow flow through seawater. Individuals were fed *ad libitum* with small pieces of mussel flesh (around 4cm² of soft tissue) every two days for 12 hours after which all remaining food items and faeces were removed by siphoning. After 60 days exposure to treatments, surviving *T. reticulata* were snap-frozen in liquid nitrogen and stored at -80 °C until subsequent laboratory analysis. Before the procedures of physiological status, all individuals were photographed. This allowed the measurement of shell length and assessment of level of shell repair. Shell length was measured with the ImageJ software (Image processing and Analysis in Java; Schneider et al., 2012). The sublethal injury provoked on *T. reticulata*, as well as in other gastropods, leaves a scar after the shell is recovered (or partially recovered). The identification of the shell breakage limit and the newly repair section is clear, such as Coleman et al. (2014) observed on two other intertidal gastropods, *Austrocochlea porcata* and *Subninella undulata*. Prior to our study, pilot tests with shell repair of *T. reticulata* under different pHs, showed the same scar in the shells of individuals that we registered in our study. Therefore, after the 60 days of exposure, it was always possible to see the notch and the extent of shell repair. In order to assess the level of shell repair, individuals were classified into three categories: no-repair, partial and full-repair. Full-repair individuals were identified when the notch was completely covered by new shell. Partial-repair was identified when individuals showed some sign of shell repair in the notch, but not sufficient to cover the whole notch. The no-repair condition was considered when the notch did not present any traces of new shell.

SEAWATER PARAMETERS

Treatments used in this study were based on the year 2100 predictions from the IPCC 'business as usual' scenario (Caldeira and Wickett, 2003; IPCC, 2013).

In this study, pH 8.08 represented pH conditions of present day (i.e. control); pH 7.88 represented moderate acidification with a 0.2 pH units reduction and pH 7.65 a high acidification with 0.4 pH units reduction (Table 1). The animals were exposed to the pH treatments for 60 days. No gradual increase in pH was applied to organisms exposed to pHs 7.88 and 7.65. To achieve low pH, CO_2 gas was bubbled into the 130L header tanks using a ceramic diffuser via a solenoid valve controlled by an Aquamedic pH-controlled computer and Aquamedic electrode system (adapted from Suckling et al., 2014, 2015). Seawater and gas were mixed via a small header pump and then gravity-fed at a rate of $0.57 \pm 0.03L$ min^{-1}.

Temperature and pH were monitored daily using a Mettler Toledo SevenGo™ SG2 with pH electrodes calibrated using NIST-certified buffers. Salinity (hand-held refractometer) was measured once every 5-7 days, TCO_2 (Ciba Corning TCO_2 Analyzer 965, Olympic Analytical) and water samples were taken every 10-14 days. Nutrient analysis (phosphate and silicate) was carried out commercially by the Scottish Association for Marine Sciences Research Services (Oban, UK) using a flow injector analyser technique. Carbonate seawater parameters such as partial pressure of CO_2 (pCO_2) and saturations states with respect to calcite and aragonite were calculated using the program CO2SYS (Lewis and Wallace, 1998).

Seawater calcite saturation states were super-saturated ($\Omega > 1$) across all treatments. Aragonite saturation states were supersaturated ($\Omega > 1$) within pH 8.08 conditions but compromised under moderately low pH conditions (pH 7.88) and fully under-saturated within the lowest pH conditions (pH 7.65; $\Omega < 1$; Table 1). Mean temperature and salinity were similar across all treatments (Salinity: $F_{2,131}=0.84$,

$p=0.43$; temperature: $F_{2,173}=0.06$, $p=0.94$). Seawater pH within treatment showed little variation around the set values throughout the experimental period and were significantly different amongst treatments (pH: $H_2=153.84$, $p<0.001$; pH 8.08> pH 7.88 > pH 7.65; Table 1).

PHYSIOLOGICAL STATUS

In the laboratory, frozen *Tritia reticulata* were rapidly removed from their shells by gently breaking the shells using a hammer and transferred into a mortar containing liquid nitrogen where the sample was ground to a fine powder by pestle whilst remaining immersed in liquid nitrogen. Approximately 10mg of the sample was transferred to a pre-cooled, pre-weighed microcentrifuge tube and dried for 48 hours in a freeze drier (Super Modulyo freeze dryer, Edwards, West Sussex, Kent). The remaining sample were quickly transferred to a second pre-cooled, pre-weighed microcentrifuge tubes and snap frozen in liquid nitrogen and stored at -80 °C until subsequent biochemical analysis.

For each *T. reticulata* individual, triplicate dried samples were transferred to pre-weighed tin cartridges (Elemental Microanalysis, Okehampton, Devon) and weighed using a micro scale (Mettler Toledo, Leicester, UK). The average sample mass was $0.742 \pm 0.113mg$ (range 0.504-0.997mg). Elemental analysis of the whole-animal samples was conducted using a Flash EA 1112 CHNS-O Analyser (Thermo Scientific, MA, USA) to determine the % carbon and % nitrogen content for each sample.

In order to measure whole-animal protein, and RNA and DNA content, duplicate samples (*ca.* 100mg) from each *T. reticulata* were homogenised on ice in 2ml 0.2M perchloric acid (PCA) and centrifuged

Table 1. Seawater parameters for *Tritia reticulata* exposed to pH 8.08, pH 7.88 and pH 7.65 for 60 days. Values for pCO_2 and carbonate saturation states of calcite (Ω calcite) and aragonite (Ω aragonite) were calculated using CO2SYS (Pierrot et al., 2006) with refitted constants (Mehrbach, et al., 1973; Dickson and Millero, 1987). Data are presented as mean values ± standard error.

Seawater parameter	pH 8.08	pH 7.88	pH 7.65
Temperature (°C)	13.4 ± 0.5	13.5 ± 0.4	13.5 ± 0.4
Salinity	35 ± 0	35 ± 0	35 ± 0
pH_{NIST}	8.04 ± 0.01	7.88 ± 0.01	7.68 ± 0.01
pCO_2 (µatm)	427 ± 14	602 ± 15	767 ± 22
Ω calcite	2.19 ± 0.04	1.59 ± 0.05	1.37 ± 0.04
Ω aragonite	1.40 ± 0.02	1.02 ± 0.03	0.88 ± 0.03

(6000g, 4 °C, 15 minutes). The precipitated pellet was solubilised in 0.3M NaOH and the protein content was measured using the Folin-phenol method of Lowry et al. (1951) as modified by Schacter and Pollack (1973). Bovine serum albumin (SIGMA Aldrich) was used as a standard. To each solubilised protein sample, 20% PCA was added, the samples centrifuged (6000g, 4 °C, 15 minutes) and RNA content in the supernatant from each sample measured using the Orcinol assay (Mejbaum, 1939). Type IV calf liver RNA (SIGMA Aldrich) was used as a standard. The remaining pellet was washed with 2% PCA, centrifuged (6000 g, 4°C, 15 minutes) and the process repeated. To each pellet, 0.6N PCA was added and the samples incubated at 70°C for 30 minutes. Samples were cooled on ice and centrifuged (6000g, 4 °C, 30 minutes). DNA content in the supernatant was measured by spectropho-tometry at 260nm (Amaral et al., 2009). Nucleic acid concentrations were calculated as the RNA:Protein ratio (μg RNA mg^{-1} protein) and as the RNA:DNA ratio, (μg DNA μg^{-1} RNA). These biochemical ratios have been used as growth biomarkers in marine inverte-brates providing indicators of nutritional condition, the capacity for protein synthesis and growth rates in marine invertebrates (Dahlhoff, 2004; Chícharo and Chícharo, 2008).

STATISTICAL ANALYSIS

All data are presented as mean values $\pm$ SD and were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) prior to statistical analysis. Differences in shell length of the individuals after exposure to the pH treatments were compared using a one-way ANOVA. A Chi-Square test was used to evaluate possible differences in the repair ratio (total repair: partial repair) between pH treatments (8.08, 7.88 and 7.65). Biochemical and elemental analysis data for the 4 treatment groups (Initial Baseline, pH 8.08, pH 7.88 and pH 7.65) were compared using a one-way ANOVA with percentage data arcsine-transformed prior to analysis. Where the ANOVA was significant, *post-hoc* pairwise compari-sons were conducted using Scheffe's test (since sam-ples sizes were unequal). Pearson correlation was ap-plied to assess for relationships between shell length and all biochemical parameters. For the RNA:Protein ratio data, where a significant correlation was ob-served, shell length was included as a covariate in

an ANCOVA to control for the size effect between treatments. Statistical analyses were conducted us-ing SPSS for Windows v20 and PAST software using a significance value of $\alpha=0.05$.

RESULTS

During the experiment, mortality was very low with only one individual from the pH 7.65 treatment dying. Shell lengths of *T. reticulata* at the end of the experiment ranged from 24.7 to 33.7mm (Mean$_{8.08}$=28.2$\pm$2.8; Mean$_{7.88}$=27.1$\pm$2.1; Mean$_{7.65}$=27.5$\pm$2.5) with no significant difference in size after 60 days of exposure (ANOVA, $F_{2,58}$=0.78; p=0.46).

All surviving individuals demonstrated shell re-pair after 60 days of exposure (Figure 1), with the exception of two individuals, one from pH 8.08 and other from pH 7.88, that suffered damage to the shell lip during handling and their repair rate could not be assessed. More than 75% of the individuals in each treatment had fully repaired their shells, with the high-est frequency registered at pH 7.88 (80%), followed by pH 7.65 and 8.08 (78.9% and 75%, respectively). There was no significant difference in the repair ratio patterns (total repair:partial repair) among pH treat-ments (χ^2_2=0.23; p=0.89). Four individuals in each treatment showed partial repair (Frequency$_{8.08}$=20%; Frequency$_{7.88}$=20%; Frequency$_{7.65}$=21.1%).

No differences in elemental body composition were observed after 60 days in the three experimen-tal treatments. Whole-animal mean nitrogen and car-bon content of *T. reticulata* ranged between 9.5 and 10.5% of the dry weight and 41.3 and 43.4% of the dry weight respectively (Figure 2A) with calculated C:N ratios ranging between 4.2 and 4.5 (Figure 2B). ANOVA analysis indicated no significant differences in nitrogen content (ANOVA, $F_{3,65}$=2.53, p=0.07), car-bon content (ANOVA, $F_{3,65}$=2.07, p=0.11) or C:N ratio (ANOVA, $F_{3,65}$=2.18, p=0.10) between the initial base-line and the three experimental treatments.

Mean RNA:Protein ratios ranged between 25.1 and 28.5μg RNA mg^{-1} protein (Figure 2C) and there were no significant differences between treatments (ANCOVA, $F_{3,63}$=2.54, p=0.06; Figure 2C). However, RNA:DNA ratios ranged between 4.5 and 6.6μg RNA μg^{-1} DNA (Figure 2D) and were significantly differ-ent (ANOVA, $F_{3,64}$=5.02, p=0.004; Figure 2D) RNA:DNA ratios were significantly higher in the initial baseline
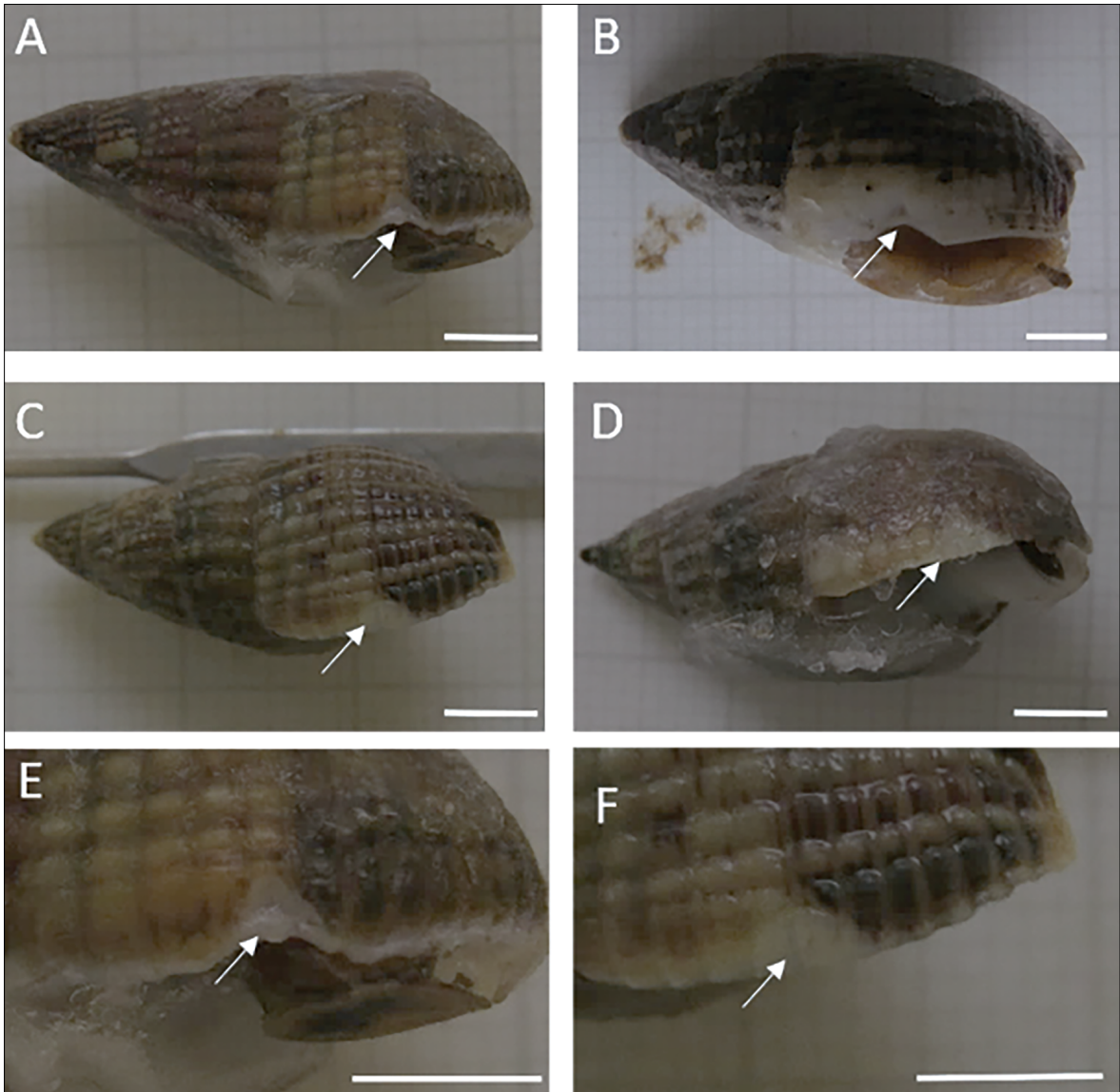
Figure 1. Examples of shell damage applied on the netted dogwhelk *Tritia reticulata* and status of repair after 60 days of exposure to pHs 8.08 (A, E), 7.88 (C, F) and 7.65 (B, D). Few individuals (less than 5 per treatment) showed partial repair (A-B and E) and mostly showed total repair of the shell (C-D and F). The white arrows indicate the damage and, when the case, the repair. Scale bar: 5mm. Photographs were lightened to increase clarity.

samples compared to the three pH groups (Scheffe's *post-hoc* multiple comparisons, all $p<0.03$). No differences were observed between the three pH groups (Scheffe's *post-hoc* multiple comparisons, all $p>0.90$).

DISCUSSION

The results of this study show that seawater acidification did not impair rates of shell repair of *Tritia reticulata* as most individuals had fully repaired their shells after two-months' exposure to OA.

Furthermore, any increased calcification and repair costs did not affect physiological state and growth as both body composition (as indicated by elemental C:N) and nucleic acid biomarkers of protein synthesis and recent growth (RNA:DNA and RNA:Protein) were similar amongst the three treatments (pH 8.08, 7.88 and 7.65). Although these results highlight a possible compensatory ability of *T. reticulata*, it is necessary to consider the implications of food availability. For example, the bivalve *M. edulis* has shown that
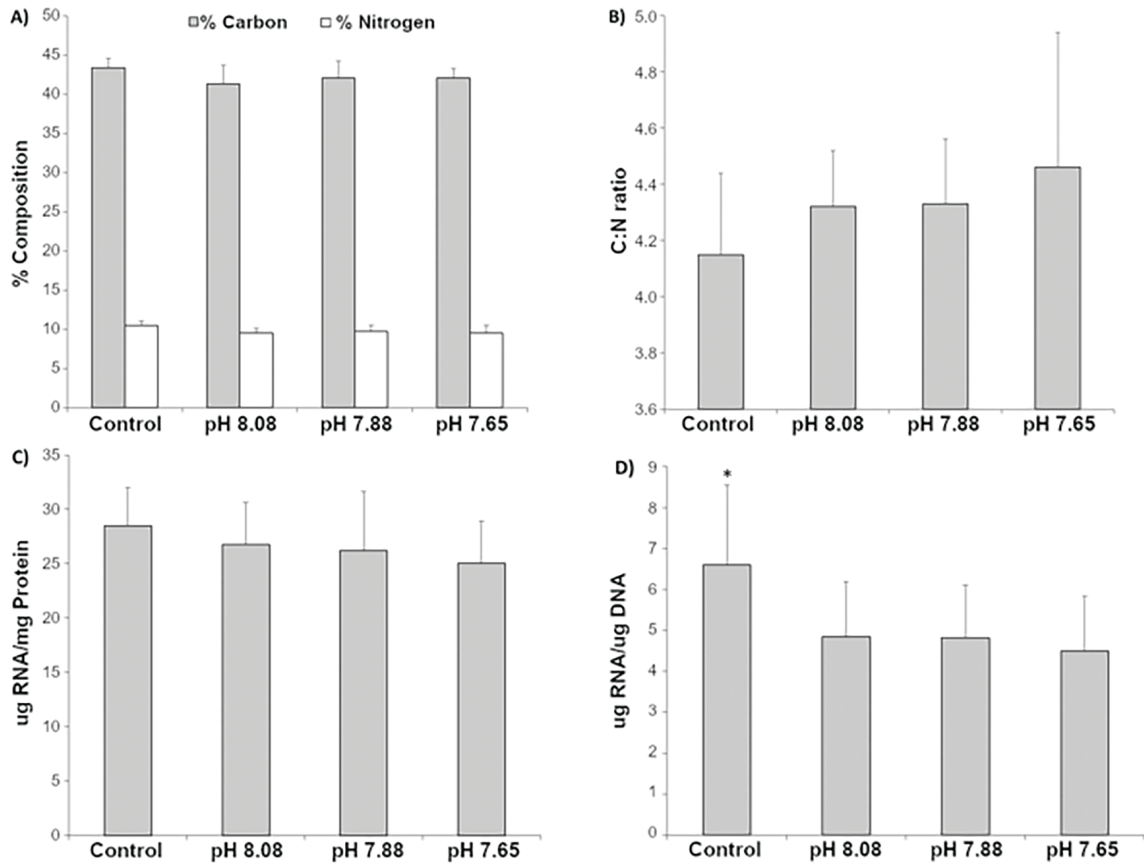
Figure 2. Biochemical indicators of body composition and growth of the netted dogwhelk *Tritia reticulata* across all treatments (Initial baseline, pH 8.08, pH 7.88 and pH 7.65). Data are presented (mean ± standard deviation) for: (A) % Carbon and % Nitrogen composition; (B) C:N ratio; (C) RNA:Protein ratio; and (D) RNA:DNA ratio. Histograms indicate the mean values and bars above each histogram the standard deviation. * indicates significant differences against other treatments ($p<0.05$).

juveniles and adults are less negatively impacted by low pH scenarios when food resources are not limited (Melzner et al., 2011; Thomsen et al., 2013). In our experiment, food was not limiting and, as a result, rates of calcification and growth, and physiological state could have been maintained during exposure to OA. Thus, both food availability and compensatory ability of *T. reticulata* under hypercapnia could be equally responsible for our observations.

Under OA scenarios, shell production would be expected to exhibit increased energetic costs, reducing the energy available for somatic growth and reproduction (Sokolova et al., 2012). Our results show that under medium-term exposure to OA, *T. reticulata* possesses the capacity to maintain calcification with no measurable impact on physiological status. However, other species may possess different strategies to counter the effects of OA, for example, when exposed to low pH the estuarine gastropod *Indothais gradata* regulates its physiological responses by either extending feeding and energy uptake or conserving energy by depressing metabolism (Proum et al., 2017). These authors also observed changes in behaviour such as isolation and escape from the acidic water. Intertidal gastropods can maintain function near optimal conditions because they can regulate intracellular ion and pH under a great range of environmental conditions (Proum et al., 2017). *T. reticulata* is likely to be resilient to the effects of OA because of the compensatory mechanisms that are inherent in intertidal animals, inhabiting an environment where highly fluctuating conditions can occur naturally over a tidal cycle (Wootton et al., 2008; Whiteley et al., 2018). As highlighted by Maas et

al. (2012), the ambient environmental chemistry that a species will naturally encounter can influence its resilience to ocean acidification. Intertidal species may be more resilient to future changes in H$^+$ concentrations, as natural oscillations in pH can occur in rocky shore tidal pools (Wootton et al. 2008; Whiteley et al., 2018).

Food availability could correlate with the ability to tolerate adverse stressors, such as OA. When exposed to different acidified scenarios and food concentrations, the bivalve *M. edulis* exhibited less internal less internal shell corrosion at high food concentrations (Melzner et al., 2011). In this bivalve, these authors showed that internal energy budget allocation is related to shell corrosion. In the gastropods *Patella granatina* and *P. argenvillei*, shell lost by erosion are continuously replaced and the cost of erosion increases with the age (Day et al., 2000). Therefore, considering the necessity of molluscs to maintain shell integrity, nutritional state will be a major factor in determining the tolerance of a species to OA. In this regard, we cannot exclude the possibility that the *ad-libitum* feeding regime applied in our study provided *T. reticulata* with enough energy to maintain calcification and physiological state during OA exposure. How *T. reticulata* would react in terms of calcification and physiological performance in the face of OA under a limited food resource scenario is an important question for future research. The ability to compensate and maintain calcification or suffer shell damage under OA scenarios appears to be species-specific and dependent on the pH to which the species is exposed and the timescale of exposure (McClintock et al., 2009; Coleman et al., 2014; Chatzinikolaou et al., 2017).

Small, sub-lethal shell damage can result from predation encounters (Blundon and Vermeij, 1983) and gastropods will need to divert energy to repair it in order to maintain shell integrity as mortality rates are higher for shell-damaged individuals compared to gastropods with undamaged shells (Geller, 1990). In our study, all individuals showed signs of shell repair (with >75% showing full repair in 60 days) and, in these situations, rapid response/repair rates to shell damage would also be expected in order to reduce predation vulnerability (Geller, 1990) and increase protection from wave impact. *T. reticulata* appears to compensate for OA effects, at least during a 60 day exposure period, by maintaining both shell production

and not restraining its physiological state (i.e. changes in RNA:DNA, RNA:Protein or C:N). These findings have also been reported for RNA:DNA ratios in sea urchins (*Paracentrotus lividus*; Catarino et al., 2012) and scallops (*Pecten maximus;* Sanders et al., 2013). Similarly, reduced pH has no effect on the physiological responses of other nassarids. Zhang et al. (2016) report no change in rates of ingestion, absorption, respiration and excretion in *N. festivus* after 31 days exposure to acidified conditions. After 3 days of exposure to acidification, *Nassarius conoidalis* had a reduction in ingestion, absorption, respiration and scope for growth, however it recovered from this metabolic depression within 30 days (Zhang et al., 2015). Two possible explanations were suggested: (1) an initial effect of acidification on the ionic availability in water, which interferes with the transport of oxygen in the blood in the respiration of organisms and (2) acclimation to acidified conditions as the exposure period increased. Therefore, a strong resilience and/or an acclimation to acidification stress appears to be a common feature among scavenger nassarids (Zhang et al., 2015, 2016; this study). The lack of influence of pH on the ratios of RNA:DNA, RNA:Protein and C:N within the current study indicate that *T. reticulata* could be resilient within the medium-term to OA conditions when food is not limiting, because of the compensatory mechanisms that are inherent in intertidal taxa (Wootton et al., 2008; Whiteley et al., 2018).

The up-regulation of calcification (Gutowska et al., 2008; Rodolfo-Metalpa et al., 2011) and metabolism (Thomsen and Melzner, 2010) under OA can be difficult to maintain in the long term as these increased energetic costs may result in the impairment of other processes, such as reproduction, growth and locomotion (Anderson et al., 2011). Further evaluations of the resilience of *T. reticulata* and other intertidal species to OA and other global climate change factors are important to identify robust species (Tate et al., 2017). If resilient characteristics pass from adults to their progenies, the species could be at an advantage under a future global climate change scenario. In the case of *T. reticulata*, future studies could focus on long-term exposure to OA and the relation between food availability, shell repair (juveniles and adults) and physiological state.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

L.Q.Y.: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing.

A.T.: Conceptualization, Funding acquisition, Methodology, Writing – review & editing.

C.S.: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Writing – review & editing.

G.T.: Resources, Writing – review & editing.

A.D.: Conceptualization, Methodology, Resources, Writing – review & editing.

I.M.: Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Writing – review & editing.

REFERENCES

ADDADI, L., JOESTER, D., NUDELMAN, F. & WEINER, S. 2006. Mollusk shell formation: a source of new concepts for understanding biomineralization processes. *Chemistry*, 12, 4, 981-987.

AMARAL, V., PENHA-LOPES, G. & PAULA, J. 2009. RNA/DNA ratio of crabs as an indicator of mangrove habitat quality. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 19, S1, S56-S62.

ANDERSON, A. J., MACKENZIE, F. T. & GATTUSO, J. P. 2011. Effects of ocean acidification on benthic processes, organisms, and ecosystems. In: GATTUSO, J. P. & HANSSON, L. (eds.), *Ocean Acidification*. New York: Oxford University Press Inc.

BARROSO, C. M., MOREIRA, M. H. & RICHARDSON, C. A. 2005a. Age and growth of *Nassarius reticulatus* in the Ria de Aveiro, north-west Portugal. *Journal of the Marine Biological Association of the United Kingdom*, 85, 151-156.

BARROSO, C. M., NUNES, M., RICHARDSON, C. A. & MOREIRA, M. H. 2005b. The gastropod statolith: a tool for determining the age of *Nassarius reticulatus*. *Marine Biology*, 146, 6, 1139-1144.

BENIASH, E., IVANINA, A., LIEB, N. S., KUROCHKIN, I. & SOKOLOVA, I. M. 2010. Elevated level of carbon dioxide affects metabolism and shell formation in oysters *Crassostrea virginica*. *Marine Ecology Progress Series,* 419, 95-108.

BIBBY, R., CLEALL-HARDING, P., RUNDLE, S., WIDDICOMBE, S. & SPICER, J. 2007. Ocean acidification disrupts induced defences in the intertidal gastropod *Littorina littorea*. *Biology Letters*, 2, 2, 67-74.

BLUNDON, J. A. & VERMEIJ, G. J. 1983. Effect of shell repair on shell strength in the gastropod *Littorina irrorata*. *Marine Biology*, 76, 41-45.

BUCKLEY, L. J., CALDARONE, E. M. & ONG, T. L. 1999. RNA-DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. *Hydrobiologia*, 401, 265-277.

BRENNAND, H. S., SOARS, N., DWORJANYN, S. A., DAVIS, A. R. & BYRNE, M. 2010. Impact of ocean warming and ocean acidification on larval development and calcification in the sea urchin *Tripneustes gratilla*. *PLoS ONE*, 5, 6, e11372. DOI: https://doi.org/10.1371/journal.pone.0011372

CALDEIRA, K. & WICKETT, M. E. 2003. Anthropogenic carbon and ocean pH. *Nature*, 425, 365.

CATARINO, A. I., BAUWENS, M. & DUBOIS, P. 2012. Acid-base balance and metabolic response of the sea urchin *Paracentrotus lividus* to different seawater pH and temperatures. *Environmental Science and Pollution Research*, 19, 6, 2344-2353.

CHATZINIKOLAOU, E., GRIGORIOU, P., KEKLIKOGLOU, K., FAULWETTER, S. & PAPAGEORGIOU, N. 2017. The combined effects of reduced pH and elevated temperature on the shell density of two gastropod species measured using micro-CT imaging. *ICES Journal of Marine Science*, 74, 1135-1149.

CHATZINIKOLAOU, E. & RICHARDSON, C. A. 2007. Evaluating growth and age of netted whelk *Nassarius reticulatus* (Gastropoda: Nassariidae) using statolith growth rings. *Marine Ecology Progress Series*, 342, 163-176.

CHATZINIKOLAOU, E. & RICHARDSON, C. A. 2008. Population dynamics and growth of *Nassarius reticulatus* (Gastropoda: Nassariidae) in Rhosneigr (Anglesey, UK). *Marine Biology*, 153, 4, 605-619.

CHÍCHARO, M.A. & CHÍCHARO, L. 2008. RNA:DNA ratio and other nucleic acid derived indices in marine ecology. *International Journal of Molecular* Sciences, 9, 8, 1453-1471.

COLEMAN, D. W., BYRNE, M. & DAVIS, A. R. 2014. Molluscs on acid: gastropod shell repair and strength in acidifying oceans. *Marine Ecology Progress Series*, 509, 203-211.

COURTNEY, T., WESTFIELD, I. & RIES, J. B. 2013. CO_2-induced ocean acidification impairs calcification in the tropical urchin *Echinometra viridis*. *Journal of Experimental Marine Biology and Ecology*, 440, 169-175.

DAHLHOFF, E. P. 2004. Biochemical indicators of stress and metabolism: applications for marine ecological studies. *Annual Review of Physiology*, 66, 1, 183-207.

DAY, E. G., BRANCH, G. M. & VILJOEN, C. 2000. How costly is molluscan shell erosion? A comparison of two patellid limpets with contrasting shell structures. *Journal of Experimental Marine Biology and Ecology*, 243, 2, 185-208.

DICKSON, A. G. & MILLERO, F. J. 1987. A comparison of the equilibrium-constants for the dissociation of carbonic-acid in seawater media. *Deep-Sea Research*, 34, 10, 1733-1743.

DIETL, G. P. & ALEXANDER, R. R. 2009. Patterns of unsuccessful shell-crushing predation along a tidal gradient in two geographically separated salt marshes. *Marine Ecology*, 30, 116-124.

DONAZZOLO, R., DEGOBBIS, D., SFRISO, A., PAVONI, B. & ORIO, A. A. 1989. Influence of Venice lagoon macrofauna on nutrient exchange at the sediment-water interface. *The Science of the Total Environment*, 86, 3, 223-238.

DUPONT, S., HAVENHAND, J., THORNDYKE, W., PECK, L. & THORNDYKE, M. 2008. Near-future level of CO_2-driven ocean acidification radically affects larval survival and development in the brittlestar *Ophiothrix fragilis*. *Marine Ecology Progress Serie*s, 373, 285-294.

FAGAN, K. A., KOOPS, M. A., ARTS, M. T. & POWER, M. M. 2011. Assessing the utility of C:N ratios for predicting lipid content in fishes. *Canadian Journal of Fisheries and Aquatic Sciences*, 68, 2, 374-385.

FERRON, A. & LEGGETT, W. C. 1994. An appraisal of condition measures for marine fish larvae. *Advances in Marine Biology*, 30, 217-303.

FINDLAY, H. S., WOOD, H. L., KENDALL, M. A., SPICER, J. I., TWITCHETT, R. J. & WIDDICOMBE, S. 2011. Comparing the impact of high CO_2 on calcium carbonate structures in different marine organisms. *Marine Biology Research*, 7, 6, 565-575.

FRASER, K. P. P. & ROGERS, A. D. 2007. Protein metabolism in marine animals: the underlying mechanism of growth. *Advances in Marine Biology*, 52, 267-362.

GALINDO, L. A., PUILLANDRE, N., UTGE, J., LOZOUET, P. & BOUCHET, P. 2016. The phylogeny and systematics of the Nassariidae revisited (Gastropoda, Buccinoidea). *Molecular Phylogenetics and Evolution*, 99, 337-353.

GAZEAU, F., PARKER, L. M., COMEAU, S., GATTUSO, J. P., O'CONNOR, W. A., MARTIN, S., PÖRTNER, H. O. & ROSS, P. M. 2013. Impacts of ocean acidification on marine shelled molluscs. *Marine Biology*, 160, 2207-2245.

GELLER, J. B. 1990. Reproductive responses to shell damage by the gastropod *Nucella emarginata* (Deshayes). *Journal of Experimental Marine Biology and Ecology*, 136, 77-87.

GUTOWSKA, M. A., PÖRTNER, H. O. & MELZNER, F. 2008. Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO_2. *Marine Ecology Progress Series*, 373, 303-309.

HARRIS, J. O. MAGUIRE, G. B., EDWARDS, S. J. & HINDRUM, S. M. 1999. Effect of pH on growth rate, oxygen consumption rate, and histopathology of gill and kidney tissue for juvenile greenlip abalone, *Haliotis laevigata* Donovan and blacklip abalone, *Haliotis rubra* Leach. *Journal of Shellfish Research*, 18, 611-619.

HARVEY, B. P., MCKEOWN, N. J., RASTRICK, S. P. S. BERTOLINI, C., FOGGO, A., GRAHAM, H., HALL-SPENCER, J. M., MILAZZO, M., SHAW, P. W., SMALL, D. P. & MOORE, P. J. 2016. Individual and population-level responses to ocean acidification. *Scientific Reports,* 6, 20194. DOI: https://doi.org/10.1038/srep20194

HOULIHAN, D. F., MATHERS, E. M. & FOSTER, A. R. 1993. Biochemical correlates of growth rate in fish. In: RANKIN, J. C. & JENSEN, F. B. (eds.), *Fish Ecophysiology*. London: Chapman and Hall.

IPCC (Intergovernmental Panel on Climate Change). 2013. Climate change 2013: The physical science basis. Summary for policymakers. *Working Group I Contribution to the IPCC Fifth Assessment Report*, viewed 27 Jun 2019, <http://www.ipcc.ch>.

KIM, D. H., BAEK, J. M., LEE, J. H., KIM, B. R., YOON, S. J. & KIM, J. H. 2011. Food effect on the diel variations and starvation of the melania snail *Semisulcospira gottschei* using RNA/DNA ratios. *Fisheries and Aquatic Sciences*, 14, 4, 411-416.

KROEKER, K. J., SANFORD, E., JELLISON, B. M. & GAYLORD, B. 2014. Predicting the effects of ocean acidification on predator-prey interactions: a conceptual framework based on coastal molluscs. *Biological Bulletin*, 226, 3, 211-222.

LANGER, G., NEHRKE, G., BAGGINI, C., RODOLFO-METALPA, R., HALL-SPENCER, J. M. & BIJMA, J. 2014. Limpets counteract ocean acidification induced shell corrosion by thickening of aragonitic shell layers. *Biogeosciences*, 11, 24, 7363-7368.

LEWIS, E. & WALLACE, D. W. R. 1998. *Program developed for CO_2 system calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory.* Oak Ridge, Tennessee: U.S. Department of Energy, viewed Dia Mês ANO, <https://cdiac.esd.ornl.gov/oceans/co2rprt.html>.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193, 1, 265-275.

MAAS, A. E., WISHNER, K. F. & SEIBEL, B. A. 2012. The metabolic response of pteropods to acidification reflects natural CO_2-exposure in oxygen minimum zones. *Biogeosciences*, 9, 747-757.

MARCHANT, H. K., CALOSI, P. & SPICER, J. I. 2010. Short-term exposure to hypercapnia does not compromise feeding, acid–base balance or respiration of *Patella vulgata* but surprisingly is accompanied by radula damage. *Journal of the Marine Biological Association of the United Kingdom*, 90, 1379-1384.

MCCLINTOCK, J. B., ANGUS, R. A., MCDONALD, M. R., AMSLER, C. D., CATLEDGE, S. A. & VOHRA, Y. K. 2009. Rapid dissolution of shells of weakly calcified Antarctic benthic macro-organisms indicates high vulnerability to ocean acidification. *Antarctic Science*, 21, 5, 449-456.

MEHRBACH, C., CULBERSON, C. H., HAWLEY, J. E. & PYTKOWlCXZ, R. M. 1973. Measurement of apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnology and Oceanography*, 18, 897-907.

MEJBAUM, W. 1939. Über die bestimmung kleiner pentosemengen insbesondere in derivaten der adenylsaüre. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie*, 258, 117-120.

MELZNER, F., STANGE, P., TRÜBENBACH, K., THOMSEN, J., CASTIES, I., PANKNIN, U., GORB, S. N. & GUTOWSKA, M. A. 2011. Food supply and seawater pCO_2 impact calcification and internal shell dissolution in the blue mussel *Mytilus edulis*. *PLoS One*, 6, 9, e24223. DOI: https://doi.org/10.1371/journal.pone.0024223

PARKER, L. M., ROSS, P. M., O'CONNOR, W. A., PÖRTNER, H. O., SCANES, E. & WRIGHT, J. M. 2013. Predicting the response

of molluscs to the impact of ocean acidification. *Biology,* 2, 2, 651-692.

PIERROT, D., LEWIS, E. & WALLACE, D. W. R. 2006. *MS Excel Program Developed for CO₂ System Calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory.* Oak Ridge, Tennessee: U.S. Department of Energy. DOI: https://doi.org/10.3334/CDIAC/otg.CO2SYS_XLS_CDIAC105a

PROUM, S., HARLEY, C. D., STEELE, M. & MARSHALL, D. J. 2017. Aerobic and behavioral flexibility allow estuarine gastropods to flourish in rapidly changing and extreme pH conditions. *Marine Biology*, 164, 1-14.

RODOLFO-METALPA, R., HOULBRÈQUE, F., TAMBUTTÉ, É., BOISSON, F., BAGGINI, C., PATTI, F. P., JEFFREE, R., FINE, M., FOGGO, A., GATTUSO, J. P. & HALL-SPENCER, J. M. 2011. Coral and mollusc resistance to ocean acidification adversely affected by warming. *Nature Climate Change*, 1, 308-312.

SANDERS, M. B., BEAN, T. P., HUTCHINSON, T. H. & LE QUESNE, W. J. F. 2013. Juvenile king scallop, *Pecten maximus*, is potentially tolerant to low levels of ocean acidification when food is unrestricted. *PLoS ONE*, 8, 9, e74118. DOI: https://doi.org/10.1371/journal.pone.0074118

SCHACTER, G. R. & POLLACK, R. I. 1973. Simplified method for quantitative assay of small amounts of protein in biologic material. *Analytical Biochemistry*, 51, 2, 654-655.

SCHNEIDER, C.A., RASBAND, W.S. & ELICEIRI, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9, 671-675.

SHIRAYAMA, Y. & THORNTON, H. 2005. Effect of increased atmospheric CO₂ on shallow water marine benthos. *Journal of Geophysical Research: Oceans*, 110, C9, CO9S08. DOI: https://doi.org/10.1029/2004JCO02618

SOKOLOVA, I. M., FREDERICH, M., BAGWE, R., LANNIG, G. & SUKHOTIN, A. A. 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research*, 79, 1-15.

STAFFORD, E. S., TYLER, C. L. & LEIGHTON, L. R. 2015. Gastropod shell repair tracks predator abundance. *Marine Ecology*, 36, 4, 1176-1184.

SUCKLING, C. C., CLARK, M. S., BEVERIDGE, C., BRUNNER, L., HUGHES, A. D., HARPER, E. M., COOK, E. J., DAVIES, A. J. & PECK, L. S. 2014. Experimental influence of pH on the early life-stages of sea urchins II: increasing parental exposure gives rise to different responses. *Invertebrate Reproduction and Development*, 58, 3, 161-175.

SUCKLING, C. C., CLARK, M. S., RICHARD, J., MORLEY, S. A., THORNE, M. A. S., HARPER, E. M. & PECK, L. S. 2015. Adult acclimation to combined temperature and pH stressors significantly enhances reproductive outcomes compared to short-term exposures. *Journal of Animal Ecology*, 84, 3, 773-784.

TALLMARK, B. 1980. Population dynamics of *Nassarius reticulatus* (Gastropods, Prosobranchia) in Gullmar Fjord, Sweden. *Marine Ecology Progress Series*, 3, 51-62.

TATE, R. D., BENKENDORFF, K., LAH, R. A. & KELAHER, B. P. 2017. Ocean acidification and warming impacts the nutritional properties of the predatory whelk, *Dicathais orbita*. *Journal of Experimental Marine Biology and Ecology*, 493, 7-13.

THOMSEN, J, CASTIES, I., PANSCH, C., KÖRTZINGER, A. & MELZNER, F. 2013. Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. *Global Change Biology*, 19, 4, 1017-1027.

THOMSEN, J. & MELZNER, F. 2010. Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis. Marine Biology*, 157, 12, 2667-2676.

TURRA, A., DENADAI, M. R. & LEITE, F. P. P. 2005. Predation on gastropods by shell-breaking crabs: effects on shell availability to hermit crabs. *Marine Ecology Progress Series*, 286, 1, 279-291.

WATSON, S. A., PECK, L. S., TYLER, P. A., SOUTHGATE, P. C., TAN, K. S., DAY, R. W. & MORLEY, S. A. 2012. Marine invertebrate skeleton size varies with latitude, temperature, and carbonate saturation: implications for global change and ocean acidification. *Global Change Biology*, 18, 10, 3026-3038.

WHITELEY, N. M., SUCKLING, C. C., CIOTTI, B. J., BROWN, J., MCCARTHY, I. D., GIMENEZ, L., & HAUTON, C. 2018. Sensitivity to near-future CO₂ conditions in marine crabs depends on their compensatory capacities for salinity change. *Scientific Reports*, 8, 1, 15639.

WO, K. T., LAM, P. K. S. & WU, R. S. S. 1999. A comparison of growth biomarkers for assessing sublethal effects of cadmium on a marine gastropod, *Nassarius festivus*. *Marine Pollution Bulletin*, 39, 1-12, 165-173.

WOOTTON, J. T., PFISTER, C. A. & FORESTER, J. D. 2008. Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. *Proceedings of the National Academy of Sciences*, 105, 48, 18848-18853.

ZHANG, H., CHEUNG, S. G. & SHIN, P. K. S. 2014. The larvae of congeneric gastropods showed differential responses to the combined effects of ocean acidification, temperature and salinity. *Marine Pollution Bulletin*, 79, 1-2, 39-46.

ZHANG, H., SHIN, P. K. & CHEUNG, S. G. 2015. Physiological responses and scope for growth upon medium-term exposure to the combined effects of ocean acidification and temperature in a subtidal scavenger *Nassarius conoidalis. Marine Environmental Research*, 106, 1, 51-60.

ZHANG, H., SHIN, P. K. S. & CHEUNG, S. G. 2016. Physiological responses and scope for growth in a marine scavenging gastropod, *Nassarius festivus* (Powys, 1835), are affected by salinity and temperature but not by ocean acidification. *ICES Journal of Marine Science*, 73, 3, 814-824.