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Calcification, a physiological process to be considered in the context of the whole organism

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Abstract

Marine organisms that produce calcium carbonate structures are predicted to be most vulnerable to a decline in oceanic pH (ocean acidification) based on the understanding that calcification rates will decrease as a result of changes in the seawater carbonate chemistry thereby reducing carbonate ion concentration (and associated saturation states). Coastal seas are critical components of the global carbon cycle yet little research has been conducted on acidification impacts on coastal benthic organisms. Here, a critical appraisal of calcification in six benthic species showed, contrary to popular predictions, calcification can increase, and not decrease, in acidified seawater. Measuring the changes in calcium in isolated calcium carbonate structure as well as structures from live animals exposed to acidified seawater allowed a comparison between a species' ability to calcify and the dissolution affects across decreasing levels of pH. Calcium carbonate production is dependant on the ability to increase calcification thus counteracting an increase in dissolution. Comparison with paleoecological studies of past high carbon dioxide (CO_2) events presents a similar picture. This conclusion implies that calcification may not be the critical process impacted by ocean acidification; particularly as all species investigated displayed physiological trade offs including reduced metabolism, health, and behavioural responses, in association with this calcification upregulation, which possess as great a threat to survival as an inability to calcify.

1 Introduction

Calcifying marine organisms (molluscs and foraminifera, crustacean, echinoderms and corals, coccolithophores – reviewed in Fabry et al., 2008) are predicted to be most vulnerable to decreasing oceanic pH (ocean acidification) because calcification rates may decrease as a result of reduced carbonate ion availability. However, the possibility for increased or maintained calcification under high carbon dioxide (CO_2) conditions

originates from evidence that calcifying organisms are not reliant on carbonate ions to calcify. Investigations principally of molluscs (Wilbur, 1964) but also of corals (Al-Horani et al., 2003) barnacles (Bubel, 1975) and echinoderms (Decker and Lennarz, 1988) show that bicarbonate (HCO_3^-) or $CO_{2(aq)}$ and not carbonate (CO_3^{2-}) is the origin of the carbon used in calcification. Additionally, many of these organisms produce calcium carbonate ($CaCO_3$) at a crystallisation site isolated from the surrounding seawater (Wilbur, 1964; Hart and Podolsky, 2004).

Molluscan shell calcification takes place away from the surrounding ambient seawater, at a crystallisation site in the extrapallial space (Wilbur and Yonge, 1964). Detailed investigations of shell-forming cells indicated that calcium transport and secretion may in part be dependant on metabolic energy derived from the generation of ATP. This has also been shown to be true in corals (review by Cohan and McConnaughey, 2003). Additionally an increasing amount of glycogen has been found to be present in these shell-forming cells and this may provide a source of CO_2, which can be converted to CO_3^{2-} by the enzyme carbonic anhydrase (Wilbur and Jodrey, 1955) and used to form $CaCO_3$. In barnacles, calcification takes place in the mantle cavity and again, examination of the structure of shell-secreting cells reveals a large presence of glycogen and mitochondria (Bubel, 1975). Ophiuroids possess a mesodermal skeleton, yet the epithelium is very thin and the internal barrier separating coelomic fluid from the test is not well developed. This structure can therefore be exposed to some degree to changing seawater chemistry. The skeletal structure of echinoderms is made of magnesium calcite and is therefore highly susceptible to dissolution at lowered pH. Current understanding of the calcification process in echinoderms is mainly based on echinoid studies, with little known of the process in ophiuroids (Hart and Podolsky, 2005).

When either HCO_3^- or CO_2 is the substrate for biogenic $CaCO_3$, the formation of $CaCO_3$structures (calcification) should not be inhibited directly by decreasing CO_3^{2-} concentrations (via ocean acidification). Although not new, this information often seems to be overlooked when explaining decreases in net calcification.

Here biogenic calcification is defined as the formation of calcium carbonate by ma-

rine organisms, which is a process independent of dissolution of $CaCO_3$. Most current techniques used for investigating changes in biogenic calcification are proxies for a change in the calcium carbonate concentration of calcified structures. Methods such as the alkalinity anomaly technique, quantifying calcium concentration in the calcified material (either by radioactive labelled calcium (Ca^{45}) or by spectrophotometer measurements), or measuring changes in morphological parameters of a calcified structure (e.g. shell length and mass) all indicate a net change in calcium carbonate, i.e. the overall product of calcification and dissolution. This is often correctly termed net calcification but is sometimes wrongly interpreted as the animals' ability to produce calcium carbonate. There have been no studies measuring in vivo dissolution, as far as the authors are aware, as there have been no successful methods designed to isolate the dissolution process without impacting the animal itself. Hence impacts from ocean acidification on shell growth, mineralogy or water chemistry cannot be assigned solely to a decrease in calcification but may result from expected increases in dissolution or changes in the innately-linked physiological processes. All physiological processes are closely interlinked and all of which are equally relevant for organism survival. In calcifying organisms calcification is integral in the control of other processes such as growth, metabolism and regulation of internal body pH (Pörtner, 2008).

Six different calcifying organisms were used to assess the impacts of ocean acidification on aspects of whole animal physiology and calcification in this study: three mollusc species, a gastropod limpet (*Patella vulgata*), a gastropod snail (*Littorina littorea*), and a bivalve mussel (*Mytilus edulis*); two crustaceans, both barnacle species (*Semibalanus balanoides* and *Elminius modestus*); and one echinoderm, a brittlestar (*Amphiura filiformis*). We measured either the calcium (Ca^{2+}) concentration in the calcified structures or shell morphological parameters as a proxy for a net change in calcium carbonate in live individuals exposed to lowered pH. In order to gain a basic understanding of the rates at which some of these organisms' calcium carbonate structures dissolve, we also measured the Ca^{2+} concentration in isolated shells and arms exposed to lowered pH. This measurement allowed us to quantify the change in cal-

cium carbonate when biogenic calcification was absent, which enabled us to determine a species' ability to calcify compared to dissolution rates across decreasing levels of pH and thus also across calcite and aragonite saturation states.

2 Methods

The *Amphiura filiformis*, *Mytilus edulis*, *Littorina littorea*, *Semibalanus balanoides* and *Elminius modestus* experiments were initially carried out during studies with different aims to this investigation, focusing on other physiological, histological, and ecological impacts of ocean acidification, and hence the experiments were not all conducted at the same pH levels. The calcium and metabolism data for *A. filiformis* were previously published in Wood et al. (2008) and some morphometric measurement and metabolism data for *L. littorea* have been published in Bibby et al. (2007), however the data presented on *M. edulis*, *P. vulgata*, *S. balanoides* and *E. modestus* are novel to this study and the data from Wood et al. (2008) and Bibby et al. (2007) have been reanalysed. We also bring together information on other physiological impacts, examples from the studies mentioned above and other literature, as well as paleoecological examples.

2.1 Experimental set ups

The *Amphiura filiformis*, *Patella vulgata*, *Mytilus edulis* and *Littorina littorea* experiments were carried out using acidified seawater by means of pH adjustment through bubbling of CO_2 into header tanks, and drawing water from these header tanks into the experimental containers as described in Widdicombe and Needham (2007). For details of the *A. filiformis* experiment see Wood et al. (2008); the *P. vulgata* experiment was run alongside the *A. filiformis* experiment. 10 *P. vulgata* individuals were placed in replicate 5 l containers at each pH condition; briefly the pH levels for these two experiments were 8.0, 7.7, 7.3 and 6.8. The *M. edulis* experiment is detailed in Beesley et al. (2008) with pH levels set at 8.0, 7.8, 7.6 and 6.8. The *L. littorea* experiment is de-

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tailed in Bibby et al. (2007), where only two pH conditions were examined: pH 8.0 and 6.45. The barnacle (*Semibalanus balanoides* and *Elminius modestus*) experiments were carried out in a tidal microcosm systems containing high CO_2 – air, detailed in Findlay et al. (2008), with two pH conditions: pH 8.0 and 7.7. In all experiments pH (NBS scale, Mettler-Toledo pH meter), dissolved inorganic carbon (DIC) (Ciba-Corning 965D Total CO_2 Analyser, Olympic Analytical Service), temperature and salinity (WTW LF197 combination temperature and salinity probe) were recorded throughout the experimental periods. Total alkalinity, bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and the saturation states for aragonite and calcite were all calculated from pH and DIC using CO2sys (Pierrot et al., 2006) with dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 using Dickson (1990).

2.2 Measurement of calcium content

All shells and arms were taken at the end of each experiment and frozen at −20°C for further analysis. Concentrations of calcium (Ca) was measured using methods described in Spicer and Eriksson (2003); briefly this involved dissolving the shells and arms in 10% nitric acid after drying and weighing and the total calcium concentration determined using atomic absorption spectrophotometer (Varian SpectrAA 50). The proportion of Ca in the shell or arm (mg Ca L^{-1}/mg shell L^{-1}) was calculated from the known total mass of the shell or arm (mg) and the volume of acid used in the digest (L).

3 Results

All six species showed a response to acidified conditions with perhaps the most surprising result being that four of these six had increased levels of calcium in low pH conditions (Fig. 1).

Over the respective experimental exposures (ca. 40 days), the Ca^{2+} concentration

of shells of live limpets and the arms of live brittlestars either remained constant or increased significantly compared to the control as the treatment pHs decreased (Fig. 1a). The Ca^{2+} concentration in the shells of live mussels (Fig. 1a) and barnacles (Fig. 1d) did not differ significantly compared to the controls as pH decreased. These changes occurred despite the seawater in the low pH treatments having lower calcite and aragonite saturation states (Table 1) due to a reduction in carbonate ions. In some cases, treatments were completely undersaturated for $CaCO_3$, with calcite becoming undersaturated at ~pH 7.3 and aragonite becoming undersaturated at ~pH 7.6.

The Ca^{2+} concentration in isolated shells of limpets and mussels and arms of brittlestars decreased over the exposure period (7 d) compared to the controls (Fig. 1b). The percent change in Ca^{2+} concentration (overall increase or decrease) relative to the control showed that Ca^{2+} concentration in isolated mussel shells decreased by up to 1.5% per day while live shells did not differ from the control (Fig. 2). A similar pattern was exhibited by limpets and brittlestars (Fig. 2).

All the morphological shell parameters in *L. littorea* (width, height, area, perimeter, aperture area, aperture perimeter and shell thickness) increased in low pH treatments compared to the control (Fig. 1c): there was ~67% more growth in shell height, ~30% more growth in shell width and ~40% more growth in shell thickness under low pH conditions compared to the control. This increased growth implies that acidification was not preventing the animals from producing their shells and hence formation of $CaCO_3$ was possible at lowered pH. We do not have measures of the mineral structure of the shell and therefore cannot ascertain if there was any impact on shell structure however both calcite and aragonite were undersaturated in the low pH treatment, indicating that dissolution is likely to have been occurring in the low pH treatment.

These results indicated that there was a large amount of dissolution taking place on isolated shells and arms while the presence of a live animal within its calcium carbonate structure prevented this high rate of dissolution; a rate much greater than might be expected to occur in vivo. In vivo dissolution remains an expected response to lowered pH primarily due to both the continued external exposure of $CaCO_3$ structures to the

lowered pH water in the shell bearing species (*Mytilus edulis, Littorina littorea, Patella vulgata, Semibalanus balanoides* and *Elminius modestus*) and the poor internal regulatory capacity of both *Amphiura filiformis* and the aforementioned species. This results in the internal fluids having similar chemical composition to the surrounding seawater, therefore the endoskeleton and inner shell surface respectively are also bathed in lowered pH fluid. Our results showing continued presence and in some cases growth of calcified structures may be interpreted as the animals still being able to produce $CaCO_3$, i.e. calcify, thus replacing the $CaCO_3$ lost through dissolution. This supports the hypothesis that calcification in molluscs, crustaceans and echinoderms relies on either HCO_3^- or CO_2 and is not dependent on the CO_3^{2-} concentration or calcite/aragonite saturation states but may be related to metabolism (Lewis and Cerrato, 1997). Perhaps more importantly it demonstrates that there is a great degree of biological control on dissolution with complex links to other physiological processes (e.g. Pörtner, 2008). In some instances organisms are able to completely overcome dissolution to increase their levels of calcium carbonate, while in other organisms levels are maintained or even slowly decline.

4 Discussion

Understanding how biological processes such as calcification influence the oceans' natural feedback mechanisms is fundamental when attempting to predict how the oceans' carbonate system will change in the future. Knowledge of the rates at which such processes take place is also vital in making such predictions. Models indicate that under ocean acidification $CaCO_3$ saturation states will become undersaturated (Caldeira and Wickett, 2003) leading to increased $CaCO_3$ dissolution rates. We have shown, however, that biogenic calcium carbonate formation may increase or remain constant despite falling carbonate saturation levels and associated increasing dissolution rates (Andersson et al., 2006). Future rates of net carbonate production will represent a trade off between the antagonistic processes of calcification and dissolution.

Dissolution may exert a cost, physically or energetically on organisms and additional impacts of hypercapnia and acidosis on metabolism and physiology may also interfere with an animal's homeostatic function (Pörtner, 2008).

Recent experiments focusing on a single physiological process, such as growth of calcifying organisms under hypercapnia, potentially overlook the possibility that increased calcification may have counteracted some, or all, shell dissolution that was occurring at the same time as the animals were growing (e.g. Michaelidis et al., 2005; Gazeau et al., 2007; Cooper et al., 2008). Shell growth or net calcification may appear to be slower or reduced under hypercapnic conditions compared to the control, yet this may be a result of increased dissolution rates or impairment to other physiological processes, not necessarily a reduction in the animals' ability to calcify.

While the six species presented in this study are all benthic calcifiers, they vary greatly in lifestyle, and therefore it needs to be considered whether the abiotic environments differ in their natural pH conditions. The most notable exception is the brittlestar *Amphiura filiformis* which lives within the sediment which is naturally lower in pH (Widdicombe unpublished data). However it has been shown (Zhu et al., 2006) that burrow irrigation results in porewater pH reflecting the overlying water rather than that of the sediment; it can be assumed this is the case for *A. filiformis* which continually ventilates its burrow. The remaining species investigated in this study were all intertidal, and studied under immersed conditions, thus the altered seawater pH reflects the conditions these species experienced. Under natural conditions these species typically, with the exception of *Littorina littorea*, shut down during emersion. Therefore their internal pH may decrease for short term periods due to the build up of respiratory CO_2, however this does not mirror these experiments due to the short term nature of these episodes, and because these current experiments result in the total immersion, both internally and externally, of the animal in lowered pH seawater.

Our findings also have implications for our understanding of past episodes of CO_2 rise, ocean acidification and biodiversity crisis, and find support in recent paleoecological studies. The fossil record is an archive of global-level experimental data on the

response of the biosphere to climatic and environmental change, and understanding past changes allows us to place the present-day crisis in its historical and scientific context. The geochemical and paleontological proxies that are used to estimate past levels of atmospheric CO_2, such as the stomatal index of fossil leaves (McElwain et al., 1999) and the carbon isotope signature of ancient soil carbonates (Cerling, 1991), demonstrate that CO_2 has fluctuated over the Phanerozoic and at times in the past has greatly exceeded present-day levels and the maximum predictions for the coming century (Royer et al., 2004), albeit on very different timescales to the present-day crisis. All of the major mass extinction events of the past 500 million years show evidence of associated climate change, including CO_2 rise and global warming (Twitchett, 2006). The Late Triassic mass extinction event, for example, occurred during a relatively fast 400% rise in atmospheric CO_2 levels from ca. 600 to 2400 ppm (e.g. McElwain et al., 1999; Beerling and Berner, 2002) and increased dissolution may have had a leading role to play in the extinctions of marine invertebrates (Hautmann, 2004). Measurements of bivalve size and shell thickness through this event demonstrated a temporary reduction in size but increase in shell thickness (Mander et al., 2008), which would be a predicted response to increasing acidification based on our laboratory studies. The timescale of present day climate change is faster than the events recorded in the fossil record, where changes are more likely to result from evolutionary adaptation. However such evidence does support the survival and continued calcification potential of benthic invertebrates in a high CO_2 world. In addition, the metabolic change seen in paleoecological data (Hautmann, 2006) is consistent with the results of some recent ocean acidification studies highlighted here (e.g. Wood et al., 2008; Bibby et al., 2007) which also found increased calcification and metabolism in species today under ocean acidification conditions.

At ocean acidification levels predicted to occur within the next 100–300 years, a pH decrease by 0.30–0.77 units (IS92a carbon dioxide emissions scenario, IPCC, 2007), there is evidence that increasing calcification comes at a cost. Investigations of whole-animal physiology and behavioural measures, such as general health (using lysoso-

mal leakage as a proxy), reproduction (assessment of gonad state), muscle mass, metabolism and predation response, have shown that several are impacted as a consequence of the up regulation of calcification and metabolism: for example, there was increased muscle degradation in *Amphiura filiformis* (Wood et al., 2008), a lowered predation avoidance response in *Littorina littorea* (Bibby et al., 2007), and reduced health in *Mytilus edulis* (Beesley et al., 2008). Other investigations, with similarly small changes in pH, show that acid-base balance cannot be maintained in other mollusc and echinoderm species under acidified conditions (Michaeladis et al., 2005; Miles et al., 2007). A longer term (6 month) sea urchin acidification experiment (Shirayama and Thornton, 2005) appears to provide evidence that some species are not able to maintain a high rate of calcification in order to overcome an increased rate of dissolution. The decrease seen in test thickness (Shirayama and Thornton, 2005) did not account for total mass loss of *Hemicentrotus pulcherrimus* and *Echinometra mathaei* indicating a loss of soft tissue, as seen in *A. filiformis* (Wood et al., 2008). Ocean acidification therefore may not directly result in a reduced ability to calcify, but it does appear to cause negative impacts on all tested organisms. This highlights the importance of bringing together the current literature to gain a holistic insight when evaluating parameters such as calcification but also the need to investigate other processes in both calcifying and non-calcifying species.

Paleoecological studies of past episodes of CO_2 rise provide some data concerning longer term changes. One characteristic of extinction episodes, especially those associated with CO_2 rise such as the Late Permian and Late Triassic events, is a dramatic decline in the size of marine organisms (the Lilliput effect) (Hautmann, 2006; Twitchett, 2007; Mander et al., 2008). The costs associated with the need for increased calcification may have a role to play in this phenomenon. Changes in shell mineralogy, from aragonite to calcite, have also been observed in Triassic-Jurassic bivalves and interpreted as reflecting a need to conserve energy as metabolic rates increased (Hautmann, 2006). This change in mineral structure, which may also be an adaptation to ocean acidification by benthic calcifiers today, reduces metabolic costs of calcification

indirectly because calcite is less prone to dissolution and hence the rate at which the structure needs to be replenished in low pH conditions is reduced. There are apparent differences in the deep-sea coral ecosystems between the North Atlantic and the North Pacific, the latter of which has much a shallower aragonite saturation horizon (ASH). In the North Pacific six of the seven stylasterid species of coral (Cairns and Macintyre, 1992), used calcite to form their spicules and skeletons, yet only 10% of all known stylasterid species produce calcite instead of the more soluble aragonite. Cold-water corals have also been found living close to the ASH, suggesting they have mechanisms to cope with high rates of dissolution, yet they do not flourish or form large structure, as in the North Atlantic (Guinotte et al., 2006). At marine volcanic CO_2 vent sites, although the abundance of calcifying animals' decreases with increasing pH, these organisms are nonetheless found under acidified seawater conditions (Hall-Spencer et al., 2008).

5 Conclusions

Results from both the laboratory and from paleoecological records suggest that animals are capable of altering their biology to be able to cope with a decrease in pH. As research now homes in on realistic scenarios, we may find that within the predicted pH ranges, at least in terms of producing calcium carbonate, animals are able to compensate. Other physiological processes are more likely to be impacted as a cost to increased energy expenditure of producing calcified material in a more acid ocean, therefore organisms may grow less (i.e. become smaller on average, as is evident from experimental and paleoecological data) and/or over longer timescales they may change their mineralogical structures. While work to date (see Fabry et al., 2008 for review) has made some steps in determining physiological responses to high levels of CO_2, research should focus on whole animal physiology in both non-calcifying and calcifying organisms as well as investigate the possibility of mineral and size changes over longer time-scales.

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Table 1. System data (mean ±95% confidence interval) for the control and the most extreme CO_2 conditions used in each of the six experiments. For all experiments salinity, temperature, pH and DIC data were measured, all other data (A_T=total alkalinity; CO_3^{2-}=carbonate ion concentration; $\Omega_{calcite}$=calcite saturation state; $\Omega_{aragonite}$=aragonite saturation state) were calculated from pH and DIC using CO2sys with the solubility constant of Mehrbach et al. (1973) refit by Dickson and Millero (1989).

	Treatment	Salinity (psu)	Temp (°C)	pH	DIC (μmol kg^{-1})	A_T (μEq kg^{-1})	CO_3^{2-} (μmol kg^{-1})	$\Omega_{calcite}$	$\Omega_{aragonite}$
Control	*Mytilus*	35.1±0.47	17.96±0.78	8.08±0.09	1880±650	2160±697	189±58.0	4.37±1.34	2.83±0.87
	Patella	35.8±0.26	16.98±0.23	7.89±0.05	1940±110	2099±120	117±16.1	2.79±0.38	1.80±0.24
	Littorina	35.0±0.0	15.00±0.0	7.96±0.04	1235±217	1363±213	76±5.1	1.76±0.12	1.13±0.07
	Amphiura	36.0±0.14	15.06±0.26	7.90±0.27	1916±71	2069±79	111±10.2	2.50±0.22	1.61 ±0.14
	Semibalanus	35.7±0.30	14.39±0.25	8.05±0.03	2185±110	2417±133	169±18.9	3.84±0.46	2.42±0.23
	Elminius	34.5±0.33	14.65±0.23	7.96±0.02	2003±58	2192±69	136±9.0	3.22±0.22	2.06±0.14
HighestCO_2	*Mytilus*	35.1±0.52	17.41±0.92	6.41±0.22	2940±970	2057±620	4.20±3.5	0.10±0.08	0.06±0.05
	Patella	36.2±0.36	15.88±0.27	6.60±0.06	2390±246	1981±232	6.5±1.6	0.16±0.04	0.10±0.02
	Littorina	35.0±0.0	15.00±0.0	6.64±0.06	2537±239	2116±155	6.7±0.9	0.15±0.02	0.10±0.01
	Amphiura	36.0±0.0	14.41±0.17	6.81±0.04	2219±191	1963±160	9.6±1.2	0.22±0.03	0.13±0.02
	Semibalanus	35.6±0.32	14.70±0.28	7.72±0.02	2517±113	2613±124	95.0±9.1	2.15±0.20	1.36±0.13
	Elminius	34.5±0.30	14.95±0.20	7.73±0.05	2652±302	2753±323	101.0±23.7	2.40±0.55	1.54±0.35

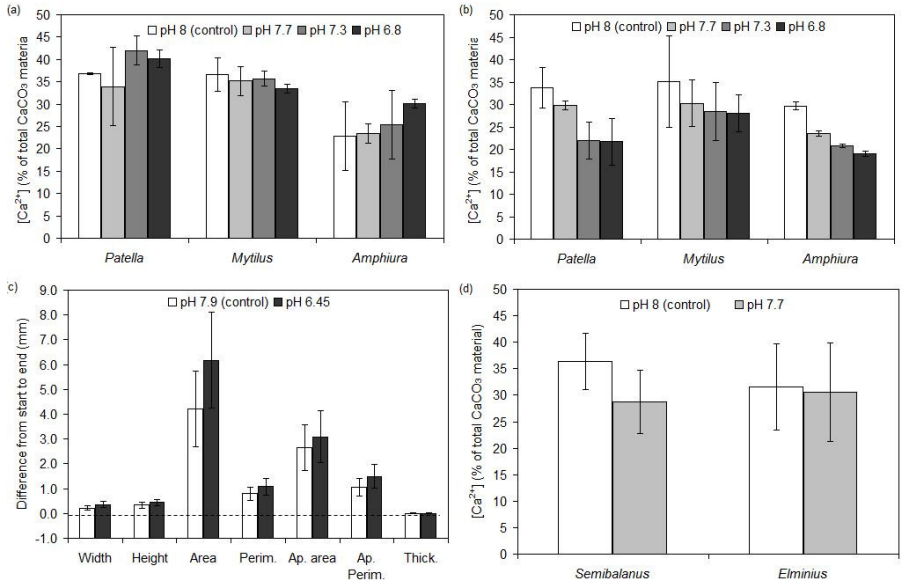

Fig. 1. **(a)** Calcium ion concentration (percentage of total structure) in the shells of live *Patella vulgata*, *Mytilus edulis* and arms of *Amphiura filiformis* (from Wood et al., 2008) in control pH 8 (white bars), pH 7.7 (light grey bars), pH 7.3 (dark grey bars), pH 6.8); **(b)** Calcium ion concentration (percentage of total structure) in the shells of dead *Patella vulgata*, *Mytilus edulis* and arms of *Amphiura filiformis* in control pH 8 (white bars), pH 7.7 (light grey bars), pH 7.3 (dark grey bars), pH 6.8 mussels (grey bars) and arms of brittlestars (black bars); **(c)** Mean difference (measurement at end – measurement at start of experiment) in shell parameters of *Littorina littorea* (shell width, height, area, perimeter (Perim.), aperture area (Ap.area), aperture perimeter (Ap. Perim.) and shell thickness (Thick)) in the control pH 7.9 (white bars) and the treatment pH 6.45 (black bars), where values above zero represent an increase (mm); **(d)** Calcium ion concentration (percentage of total structure) in post-metamorph barnacle shells for each species (*Elminius*=*Elminius modestus*, *Semibalanus*=*Semibalanus balanoides*) in the control pH 8 (white bars) and pH 7.7 (grey bars). Error bars represent 95% confidence intervals.

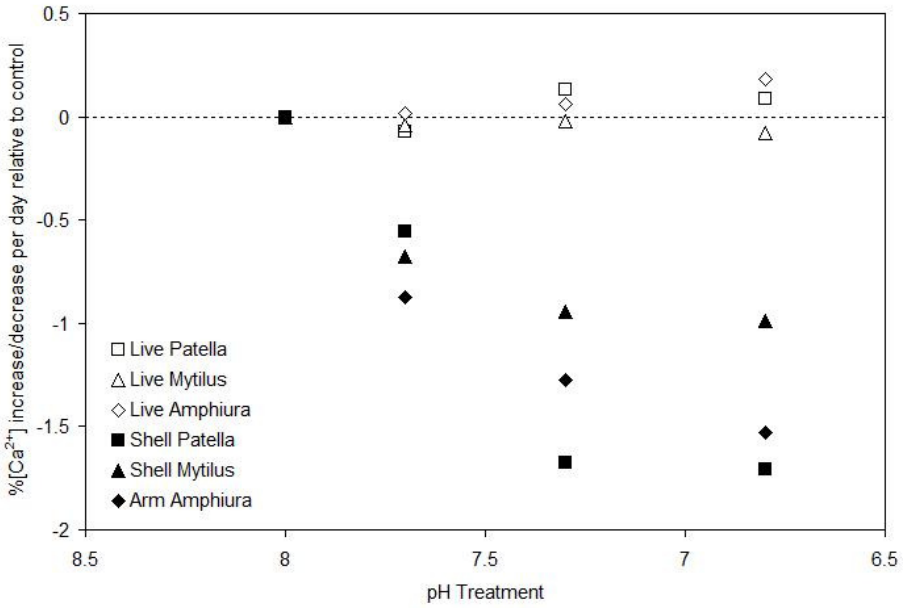

Fig. 2. The increase or decrease in calcium ion concentration (percentage total structure) at each pH relative to the control of live (open squares) and dead *Patella vulgata* shells (closed squares), live (open diamonds) and dead *Amphiura filiformis* arms (closed diamonds) and live (open triangles) and dead *Mytilus edulis* shells (closed triangles). These means are standardised to an increase or decrease per day, assuming that there was a linear change in dissolution or calcification over the experimental time period.

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