

# Sea Hare *Aplysia punctata* (Mollusca: Gastropoda) Can Maintain Shell Calcification under Extreme Ocean Acidification

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**Abstract.** Ocean acidification is expected to cause energetic constraints upon marine calcifying organisms such as molluscs and echinoderms, because of the increased costs of building or maintaining shell material in lower pH. We examined metabolic rate, shell morphometry, and calcification in the sea hare *Aplysia punctata* under short-term exposure (19 days) to an extreme ocean acidification scenario (pH 7.3, ~2800  $\mu\text{atm } p\text{CO}_2$ ), along with a group held in control conditions (pH 8.1, ~344  $\mu\text{atm } p\text{CO}_2$ ). This gastropod and its congeners are broadly distributed and locally abundant grazers, and have an internal shell that protects the internal organs. Specimens were examined for metabolic rate *via* closed-chamber respirometry, followed by removal and examination of the shell under confocal microscopy. Staining using calcein determined the amount of new calcification that occurred over 6 days at the end of the acclimation period. The width of new, pre-calcified shell on the distal shell margin was also quantified as a proxy for overall shell growth. *Aplysia punctata* showed a 30% reduction in metabolic rate under low pH, but calcification was not affected. This species is apparently able to maintain calcification rate even under extreme low pH, and even when under the energetic constraints of lower metabolism.

This finding adds to the evidence that calcification is a largely autonomous process of crystallization that occurs as long as suitable haemocoel conditions are preserved. There was, however, evidence that the accretion of new, non-calcified shell material may have been reduced, which would lead to overall reduced shell growth under longer-term exposures to low pH independent of calcification. Our findings highlight that the chief impact of ocean acidification upon the ability of marine invertebrates to maintain their shell under low pH may be energetic constraints that hinder growth of supporting structure, rather than maintenance of calcification.

## Introduction

Ocean acidification (OA), or the lowering of seawater pH by increased addition of dissolved atmospheric  $\text{CO}_2$ , is the subject of much recent research. The potential effects of lower pH on calcifying marine species are perhaps the most prominent hypothesized impact. Lower pH and undersaturated calcium carbonate states may cause the shells of a host of marine species (molluscs, echinoderms, corals, coccolithophores, foraminiferans, calcifying algae) to undergo increased dissolution (Orr *et al.*, 2005), or, at minimum, may increase the energetic costs of maintaining and growing such structures (Dupont *et al.*, 2010).

In many invertebrates, the integrity of calcium carbonate structures directly impacts upon survival, as these provide physical protection from predators and environmental stressors such as wave action (Gaylord *et al.*, 2011). For intertidal invertebrates such as bivalves and gastropods, these

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*Abbreviations:* M, dry tissue mass;  $\text{MO}_2$ , mass-specific oxygen uptake rate; OA, ocean acidification;  $\text{pH}_T$ , total scale pH; R, respiration rate; S, salinity;  $\text{S}_B$ , shell border width;  $\text{S}_L$ , shell length;  $\text{S}_C$ , shell calcification width; T, temperature; TA, total alkalinity;  $\mu\text{atm } p\text{CO}_2$ , seawater partial pressure of  $\text{CO}_2$ .

structures also allow the body to be sealed within an enclosed space, protecting against desiccation during periods of emersion in air. Compromising these abilities, therefore, could have ongoing effects upon populations of these species and the wider community for which they provide food, habitat, and services such as water filtration.

Compromised shell strength and composition under experimental OA has been demonstrated in numerous calcifying marine invertebrates (reviewed in Harvey *et al.*, 2013). Compromised structural integrity under lower pH has also been shown in marine algae, including at a site of “natural OA,” an area of volcanic outgassing of CO<sub>2</sub> that is thought to closely mimic future OA conditions (Newcomb *et al.*, 2015). Studies on the invertebrates in this and similar “natural OA” sites show much decreased structural integrity of calcified parts (Rodolfo-Metalpa *et al.*, 2011), with consequences for survival and community dynamics (Hall-Spencer *et al.*, 2008; Kroeker *et al.*, 2011).

The additional energetic burden of maintaining calcified structures in lower pH could be an important factor for ongoing survival and reproduction of populations. The costs of maintenance metabolism are the baseline required for survival; important processes such as reproduction and growth require fuel above this baseline. In the absence of energetic trade-offs, or acquisition of additional energy, the added burden of maintaining net calcification under OA will impact upon this portion of the energetic budget. Even without this added burden, OA is frequently accompanied by physiological changes and altered metabolic rates, both decreases (Christensen *et al.*, 2011) and increases (Carey *et al.*, 2016), and so the energetic consequences of OA may be important beyond those upon the calcification pathway. In addition, altered energetics are closely coupled to feeding rates, and, therefore, OA may have ongoing ecological effects (Burnell *et al.*, 2013).

Sea hares are anaspidean gastropods or sea slugs that are globally distributed in tropical and temperate shallow waters and the intertidal. The biology of this group is well known from *Aplysia californica* (Dionísio *et al.*, 2013), a model organism for neurobiology (*e.g.*, Sattelle and Buckingham, 2006; Kandel *et al.*, 2014). Its congener *Aplysia punctata* (Cuvier, 1803) is common in the north Atlantic in shallow waters and the intertidal. Its distribution is broader than many other Northeastern Atlantic shallow water molluscs—from the Mediterranean to as far north as Greenland—and it is the largest anaspidean species in polar waters. *Aplysia* species are voracious feeders, consuming up to a third of their body weight of algae each day (Carefoot, 1987).

Most sea hares, including *A. punctata*, possess an internal shell. While the shell is thin and small in comparison to other gastropods, it serves to protect the heart and other internal organs (Beesley *et al.*, 1998). In larger specimens, the shell may reach over 6 cm in length (Hunt, 1877), and

shows accretionary growth bands analogous to those seen on the shelves of bivalves or other molluscs (*e.g.*, Fig. 1B) (Usuki, 1981). The shell in *Aplysia* is formed primarily from aragonite deposited within a protein matrix (Pedrozo *et al.*, 1997; Tonejc *et al.*, 2014). Aragonite is a form of calcium carbonate that is particularly susceptible to dissolution under lower seawater pH (Mucci, 1983).

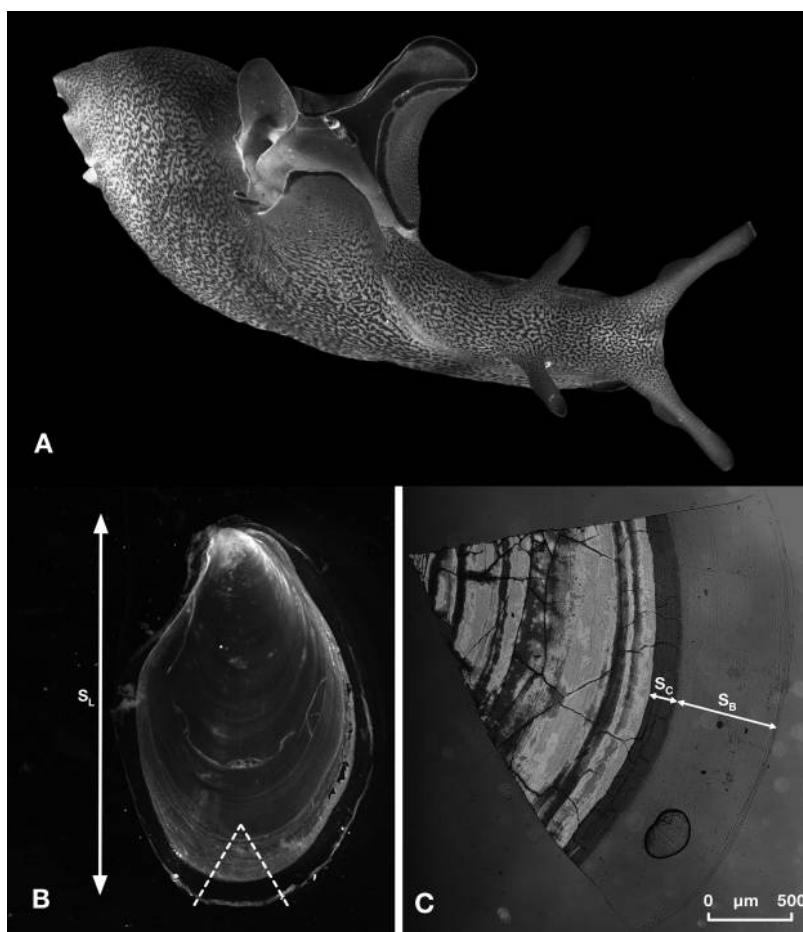
In a short-term experiment, we exposed *Aplysia punctata* to a reduction in seawater pH from ambient conditions (~8.1) to 7.3. This pH represents an extreme scenario for the end of this century, under the more pessimistic emissions scenarios (IPCC, 2014); that is, a predicted decrease in mean seawater pH of around 0.4, plus an added decrease, which would be expected due to natural pH variability in shallow or intertidal environments. In tidepools, short-term variation in pH can be considerable (up to 1.78 units) due to diurnal variations in calcification, net primary production, and temperature (Kwiatkowski *et al.*, 2016). In upwelling areas, pH can vary diurnally by up to 0.36 (Frieder *et al.*, 2012). In a Baltic fjord close to where the present study was conducted, the seasonal variability was ~0.6, with considerable changes of 0.3–0.4 occurring over timescales of days (Thomsen *et al.*, 2010; Melzner *et al.*, 2013). Another study in the same region documented, over 3 weeks, a mean diurnal amplitude in pH of 0.34, but with extreme diurnal fluctuations of up to 0.6, and a minimum pH of 7.26 (Saderne *et al.*, 2013). That study was conducted in a shallow algal and seagrass habitat, typical of that in which *A. punctata* is found (Carefoot, 1987; Ouisse *et al.*, 2012). Therefore, the chosen low pH treatment of 7.3 is likely close to the minimum pH experienced by this species in this region, albeit periodically. Ongoing maintenance at this pH represents an extreme but plausible near-future scenario, with the compounded changes of decreased mean pH plus that due to natural variability.

This is the first study to examine the responses of *Aplysia* to ocean acidification. We expected that *A. punctata* would show the typical responses of shallow water and intertidal organisms to short-term experimental exposure to lowered pH: a decrease in metabolic rate. Given this response, along with the aragonitic composition and extremely thin and fragile nature of the *A. punctata* shell, we hypothesized that low pH would also have substantial impacts upon shell growth and calcification.

## Materials and Methods

### Experimental set-up

Specimens of *Aplysia punctata*, ranging in size from 0.5 cm to 5 cm extended body length, were collected in Gullmar Fjord, Sweden, in January 2014, and held in natural flow-through seawater at the Sven Lovén Centre for Marine Sciences, Kristineberg, University of Gothenburg. They were maintained in these conditions for several weeks and



**Figure 1.** Sea hare *Aplysia punctata*. (A) Live specimen of *A. punctata* from Flamborough, England. The shell can be partially seen through the small oval opening in the mantle under the dosal parapodia. (Photo: Ian F. Smith) (B) A whole shell from a specimen held in control pH, as photographed under an optical microscope. Dashed lines show approximate location of sections that were subsequently removed for mounting and examination *via* confocal microscopy. Shell length ( $S_L$ ) was measured from these photographs as the maximum longitudinal diameter, using ImageJ. In this example,  $S_L = 8.26$  mm. (C) A mounted section from a specimen held in low pH, as photographed under confocal microscopy. Two images have been combined: an optical image and an image under UV light. The light gray region (originally fluorescing green under confocal microscopy images) indicates calcein-stained calcium carbonate. Shell calcification width ( $S_C$ ) represents new calcification in the period after staining, and shell border width ( $S_B$ ) is the width of the non-calcified shell border protein matrix. Three measurements of each were taken perpendicular to the shell curvature, then averaged to give a single value of each per specimen.

fed *ad libitum* on fresh, locally collected *Ulva lactuca* and *Palmaria palmata* algae. The specimens were split into two groups with the total size range represented in each; 16 were assigned to the low pH treatment, and 16 to control pH. They were transferred to 30-l glass tanks supplied with flowthrough seawater (7 °C, 32.4‰ salinity) at a rate of 1 liter  $\text{min}^{-1}$ , held in a constant temperature room with a 10-h/14-h light/dark regime approximating local conditions, and fed *ad libitum* on *P. palmata*. Treatment tank water was kept at 100% dissolved oxygen concentrations at all times *via* an air supply bubbled through a ceramic diffuser. There was a single tank per treatment; therefore, scope for statistical inference of any treatment effects must be considered

with caution. Without treatment replication, results are prone to type I errors, where a discrepancy between groups may be falsely attributed to treatment effect (Heffner *et al.*, 1996). However, lack of treatment replication does not belie the veracity of observations within that experimental group (Oksanen, 2001).

The low pH treatment was gradually adjusted from control pH (~8.1) to the low pH setpoint (7.3) over 2 days. After 9 subsequent days' maintenance in these conditions, specimens were transferred to 100-ml, clear glass jars containing the appropriate treatment water in groups while keeping biomass relatively equal in each to avoid hypoxic conditions (*i.e.*, only one large specimen per jar, up to 4

Table 1

Carbonate system parameters ( $\pm SD$ )

Treatment	T ( $^{\circ}\text{C}$ )	$\text{pH}_{\text{T}}$	S	TA	$\text{pCO}_2$ ( $\mu\text{atm}$ )	TC ( $\mu\text{mol/kg}$ )	$\Omega_{\text{Ca}}$	$\Omega_{\text{Ar}}$
Control pH	$7.1 \pm 0.2$	$8.12 \pm .03$	$32.8 \pm 0.2$	$2372 \pm 38$	$344 \pm 29$	$2185 \pm 29$	$3.35 \pm 0.27$	$2.12 \pm 0.17$
Low pH	$7.2 \pm 0.2$	$7.27 \pm .04$	$32.8 \pm 0.2$	$2418 \pm 42$	$2803 \pm 440$	$2520 \pm 54$	$0.57 \pm 0.1$	$0.36 \pm 0.06$

Total dissolved inorganic carbon (TC),  $\text{pCO}_2$ , and saturation state for calcite and aragonite ( $\Omega_{\text{Ca}}$  and  $\Omega_{\text{Ar}}$ ) were calculated from total scale pH ( $\text{pH}_{\text{T}}$ ) and total alkalinity (TA), using CO2calc ver. 1.0 for Mac OS X.

S, salinity; T, temperature;  $\mu\text{atm}$   $\text{pCO}_2$ , seawater partial pressure of  $\text{CO}_2$ .

small specimens per jar, and 6 jars in total). Calcein stock solution was added to each to a concentration of  $5 \text{ mg l}^{-1}$ , and the jars were sealed and placed back in treatment tanks. Calcein is a fluorescent chromophore that binds to calcium and has been used extensively to visualize skeletal structures and calculate growth rates in a range of vertebrates and invertebrates (Du *et al.*, 2001; Russell and Urbaniak, 2004). It is non-toxic and does not interfere with normal growth (Russell and Urbaniak, 2004). Water was changed 4 times daily, calcein staining was repeated as above with each replacement, and animals were kept in these jars for 48 hours. Jars were agitated regularly to avoid stratification. Oxygen concentration and pH were monitored in jars routinely and found to be close to tank maintenance conditions at all times. After removal from the sealed jars, animals were returned to the open treatment tanks and held for a further 6 days. Specimens were thus held for a total of 19 days: 11 days of maintenance (including 2 days to reach setpoints for low pH treatment), 2 days of staining, and 6 days of further maintenance. From available data on the congener *Aplysia californica* (Peretz and Adkins, 1982), this regime was judged long enough in which to observe substantial shell growth, even given the smaller size of *A. punctata* and lower environmental temperature.

#### Seawater chemistry

Temperature (T), salinity (S), and pH (total scale ( $\text{pH}_{\text{T}}$ )) were measured daily throughout maintenance. Total alkalinity (TA) was determined every 3 days. Seawater carbonate chemistry was maintained using a computerized feedback system (AquaMedic, Bissendorf, Germany) that regulates pH by addition of pure  $\text{CO}_2$  gas directly into the seawater ( $\pm 0.02$  pH units). Total alkalinity was determined on filtered water samples with a titration system (TitroLine alpha plus; SI Analytics GmbH, Mainz, Germany). Total scale pH was measured using a Metrohm (827 pH lab) electrode and adjusted for pH measurements on the total scale using Tris (Tris/HCl) and AMP (2-aminopyridine/HCl) buffer solutions with a salinity of 32, provided by Unité d'Océanographie Chimique, Université de Liège, Belgium. Seawater carbonate system speciation (Table 1) was calculated from  $\text{pH}_{\text{T}}$ , and TA was determined by CO2calc

ver. 1.0 for Mac OS X, using the dissociation constants of Mehrbach *et al.* (1973), as refitted by Dickson and Millero (1987). Seawater chemistry parameters were stable throughout the maintenance period, and in the low pH treatment the saturation states for calcite and aragonite were highly undersaturated (Table 1).

#### Respirometry experiments

Oxygen consumption was determined *via* closed-chamber respirometry, as previously used on marine invertebrates (Carey *et al.*, 2014; Carey and Sigwart, 2014). Due to the low metabolic rates of invertebrates, particularly in smaller specimens and under low temperatures, closed-chamber techniques are considered the most reliable method of determining oxygen uptake rates (Lighton, 2008). The food source was removed 24 h before experiments. To avoid interference with the oxygen probe, and to prevent specimens from attaching to the inner surface of the respirometry chamber and sustaining possible injury during removal at the end of the experiments, specimens were placed individually in a nylon mesh envelope, allowed to become accustomed to this for one hour, then placed into cylindrical respirometry chambers that were then sealed while under water within the treatment tanks. An oxygen probe (FOXY Systems; Ocean Optics, Dunedin, FL), which was connected to a PC laptop that recorded oxygen readings at intervals of 1 second, was inserted. The chambers were placed in a temperature-controlled tank situated on a laboratory "rocker" platform that rotated approximately once every 2 s. Oxygen recordings showed that this action was sufficient to keep chambers internally mixed. Chambers ranged in volume from approximately 100 ml to 400 ml. Six trials were run simultaneously for up to 3 h, or until an approximate 10% decrease from the initial concentrations of 100% oxygen saturation was observed. Six control runs, which were identical except for the absence of a specimen, were conducted to determine microbial oxygen consumption and a correction based on this applied to specimen runs.

After each trial, chambers were dried externally and weighed. The specimen was removed and blotted dry, and its wet mass determined. Dry chamber mass and specimen wet mass were subtracted from the total chamber mass to



determine internal water mass; this figure was converted to volume using the seawater density for the appropriate temperature and salinity (Fofonoff and Millard, 1983). Each individual trace was examined and a stable representative section selected to determine routine oxygen consumption ( $R$ ,  $\text{mg h}^{-1}$ ) for each individual. From these data, the mass-specific oxygen uptake rate ( $\text{MO}_2$ ,  $\text{mg h}^{-1} \text{g}^{-1}$ ) was determined for each individual as  $R$  divided by dry mass ( $M$ ).

### Shell morphometry

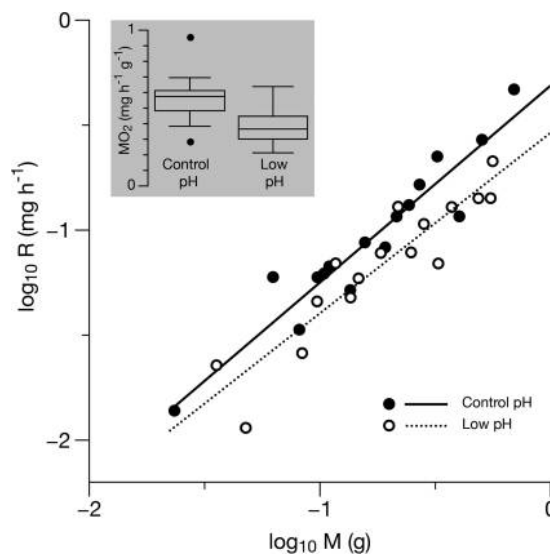
After respirometry, the internal shell of each specimen was dissected out. The remainder of the specimen was dried for 24 h at 60 °C and weighed to obtain dry tissue mass ( $M$ , g). The shell was photographed under an optical microscope (e.g., Fig. 1B). A small section from the anterior most part of the shell was then removed (Fig. 1C); this region is the area of maximum shell growth and least dorsoventral inflation, allowing the sampled section to be mounted flat on a glass microscope slide for examination. These sections were examined under a confocal laser-scanning microscope (Leica TCS SP5; Leica, Wetzlar, Germany) to determine the extent of calcein staining, and photographed under both full-spectrum and UV lights.

Three distinct regions of the shell were identifiable: the central part of the shell, already calcified during the calcein staining treatment; newly crystallized calcium (unstained); and the uncalcified growing shell margin (Fig. 1C). Quantitative measurements were determined from digital images via ImageJ. Using images captured with confocal microscopy, we measured the widths of uncalcified shell border ( $S_B$ ) and new calcification ( $S_C$ ), taking the mean of three radii to give a single reported value of  $S_B$  and  $S_C$  for each specimen. Total shell length ( $S_L$ ) was measured as the maximum longitudinal diameter from photographs of whole shells.

### Data analysis

Analysis of covariance (ANCOVA) was used to examine differences between metabolic rate, measured as respiration rate ( $R$ ), across pH treatments with mass ( $M$ ) as covariate. Multiple regression was used to examine the effects upon shell calcification ( $S_C$ ) of the other metrics (pH, dry tissue mass, mass-specific oxygen uptake rate, shell length, and shell border width). Pairwise linear dependence between morphological and physiological parameters (dry tissue mass, shell length, shell border width, shell calcification width, and mass-specific oxygen uptake rate) was assessed using Pearson correlations.

Statistical analyses were conducted in R (R Core Development Team, 2015). All data met the normality and homogeneity of variance assumptions of ANCOVA and ANOVA tests (Shapiro-Wilk and Bartlett tests, respectively). Measurements of variability are Standard Error (SE), unless otherwise noted.



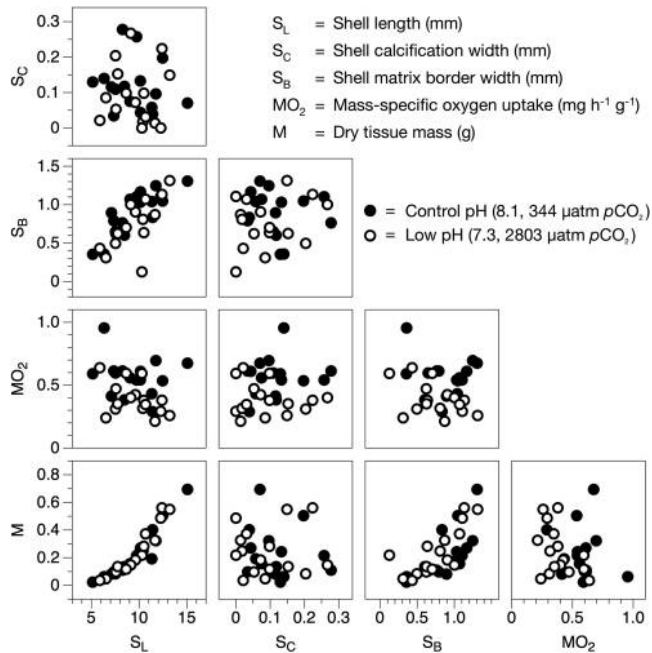
**Figure 2.** Oxygen uptake in *Aplysia punctata* under control and low pH. Scatter plot shows the relationship between mass and metabolic rate through linear regressions on  $\log_{10}$ -transformed data for individual oxygen uptake rate ( $R$ ,  $\text{mg h}^{-1}$ ) against dry tissue mass ( $M$ , g). The black line and solid circles are control pH, the dashed line and open circles are low pH. Regression equations: control pH,  $y = 0.94x - 0.31$ ,  $R^2 = 0.90$ ; low pH,  $y = 0.86x - 0.54$ ,  $R^2 = 0.85$ . Inset shows the same data expressed as summary boxplots of mass-specific oxygen uptake rates ( $\text{MO}_2$ ,  $\text{mg h}^{-1} \text{g}^{-1}$ ): medians (lines), interquartile ranges (boxes),  $1.5\times$  extended interquartile ranges (whiskers), and outliers.

## Results

Specimens ranged in mass (dry tissue mass ( $M$ )) from 0.023 g to 0.694 g. Sizes in the experimental groups did not significantly differ in  $M$  (one-way ANOVA,  $F_{1,30} = 0.08$ ,  $P = 0.78$ ) or shell length (one-way ANOVA,  $F_{1,30} = 0.04$ ,  $P = 0.84$ ).

Oxygen uptake rates ( $R$ ) were significantly reduced under low pH;  $R$  differed between control and acidified treatments (ANCOVA,  $F_{1,28} = 13.23$ ,  $P = 0.001$ ). Regression slope values (i.e., the metabolic scaling exponent, “ $b$ ”) were not significantly different between pH levels ( $F_{1,28} = 0.37$ ,  $P = 0.55$ ), the actual values being 0.94 in control pH and 0.86 in low pH (Fig. 2). At the  $\log_{10}$ -midpoint of the mass range (0.127 g), the uptake ( $\text{mg h}^{-1}$ ) predicted from the models was  $0.071 (\pm 0.011)$  in control pH and  $0.050 (\pm 0.009)$  in low pH, a decrease of 30%.

Figure 3 shows a correlation matrix of all measured parameters. Because pH had no effect on any metric except the mass-specific oxygen uptake rate, correlation tests were conducted on the entire dataset. Shell calcification ( $S_C$ ) was not significantly correlated with total shell length (Pearson correlation,  $r = -0.10$ ,  $P = 0.59$ ), shell border width ( $r = 0.12$ ,  $P = 0.51$ ), mass-specific oxygen uptake rate ( $r = 0.09$ ,  $P = 0.62$ ), or dry-tissue mass ( $r = -0.06$ ,  $P = 0.74$ ). The only significant correlations were between the morphological parameters of mass,  $S_L$ , and  $S_B$  ( $S_L:S_B$ ,  $r = 0.73$ ,  $P <$



**Figure 3.** Correlation matrix for morphological and physiological measurements of *Aplysia punctata*. Solid circles are control pH, open circles are low pH. M, dry tissue mass (g);  $MO_2$ , mass-specific oxygen uptake ( $mg\ h^{-1}\ g^{-1}$ );  $S_B$ , shell matrix border width (mm);  $S_C$ , shell calcification width (mm);  $S_L$ , total shell length (mm);  $\mu atm\ pCO_2$ , seawater partial pressure of  $CO_2$ .

0.0001;  $S_B:M$ ,  $r = 0.69$ ,  $P < 0.0001$ ;  $S_L:M$ ,  $r = 0.94$ ,  $P < 0.0001$ ). This last relationship of M to  $S_L$  was best described by an allometric power law:  $M = 15.61 S_L^{0.30}$ ,  $r^2 = 0.96$ . Unlike the case with R (Fig. 2),  $MO_2$  and M were not significantly correlated, most likely due to the mass-scaling exponent values (0.94 and 0.86) being close to isometric.

Shell calcification width ( $S_C$ ) was not significantly different between control and low pH treatments (one-way ANOVA,  $F_{1,30} = 0.83$ ,  $P = 0.37$ ). The average length of new calcification (over 6 days) was  $S_C = 0.12 (\pm 0.02)$  mm in control pH and  $S_C = 0.09 (\pm 0.02)$  mm in low pH. Only two specimens showed no detectable calcification, both in the low pH treatment. Visual inspection through dissection and brightness of fluorescence showed no obvious qualitative differences between the two treatment groups in overall shell calcification. Shell border ( $S_B$ ), or the width of uncalcified material on the shell margin, and the combined total growth ( $S_C + S_B$ ) did not differ between pH treatments under ANOVA analysis (one-way ANOVAs;  $S_B$ ,  $F_{1,30} = 1.46$ ,  $P = 0.24$ ;  $S_C + S_B$ ,  $F_{1,30} = 1.85$ ,  $P = 0.18$ ).

Under multiple regression analysis,  $S_C$  was not associated with any significant interactions with the other variables ( $MO_2$ , M,  $S_L$ ,  $S_B$ , or pH) (Table 2; Fig. 3). Alternative models were assessed using the *step* function in R (R Core Development Team, 2015) to perform stepwise regression backwards from the full model. The minimal adequate

**Table 2**

Multiple regression model of shell calcification ( $S_C$ , mm) against other physiological and morphological factors

	Intercept	SE	SS	df	F	P
$S_C$	0.199	0.147				
pH	-0.010	0.037	0.00517	1,26	0.784	0.384
M	0.117	0.242	0.00054	1,26	0.082	0.777
$MO_2$	0.004	0.111	0.00003	1,26	0.004	0.949
$S_L$	-0.021	0.019	0.00306	1,26	0.464	0.502
$S_B$	0.100	0.075	0.01088	1,26	1.651	0.210
$S_C$	0.148	0.059				
$S_L$	-0.014	0.009	0.00187	1,29	0.312	0.581
$S_B$	0.106	0.066	0.01548	1,29	2.585	0.119

Top six rows = full model; bottom three rows = minimal adequate model after stepwise regression backwards from the full model, using Akaike information criterion (AIC).

M, dry tissue mass (g);  $MO_2$ , mass-specific oxygen uptake ( $mg\ h^{-1}\ g^{-1}$ );  $S_L$ , total shell length (mm);  $S_B$ , shell border width (mm); SE, standard error; SS, sum of squares; df, degrees of freedom; F, F-statistic; P, probability.

model ( $S_C \sim S_L + S_B$ ) did not contain any significant interactions (Table 2). Shell border width as a proportion of total shell length ( $S_L$ ) was consistent across body size (around 8% to 9%), and while the  $S_B:S_L$  proportion in low pH ( $7.8 \pm 0.6\%$ ) was less than in control pH ( $9.4 \pm 0.5\%$ ), this was marginally not significant (Kruskal-Wallis H test = 3.41,  $P = 0.06$ ). Similarly, under multiple regression analysis, pH as an effect on  $S_B$  was marginally non-significant ( $F_{1,28} = 3.41$ ,  $P = 0.075$ ).

## Discussion

*Aplysia punctata* showed no decrease in calcification of new shell material, although there was a significantly reduced metabolic rate under low pH. This is despite both the calcite and aragonite saturation states being highly undersaturated in experimental water chemistry conditions (Table 1), which might be expected to result in active dissolution of unprotected calcified material. The low pH treatment of 7.3 that we chose was purposely extreme, but represents a plausible level that may be sustained for periods in this marine habitat in the near future, given the predicted decreases in mean pH of around 0.4 under pessimistic emissions scenarios (IPCC, 2014), and the substantial short-term natural variability in pH in other Baltic fjords of 0.4 to 0.6 (Thomsen *et al.*, 2010; Melzner *et al.*, 2013; Saderne *et al.*, 2013). Our expectation was that increased costs of acid-base regulation in low pH would impact upon the calcification of the shell of *A. punctata*. In contrast, under this extreme low pH regime, and even when metabolic functioning was reduced, *A. punctata* maintained normal shell calcification.

To our knowledge, only one other study has examined

responses under ocean acidification of *Aplysia* or any other sea hare species (the clade Anaspidea); low pH was shown to increase mortality in the embryos of a tropical sea hare (Davis *et al.*, 2013). One additional study found increased respiratory pumping in *Aplysia californica* in seawater into which CO<sub>2</sub> was bubbled, causing extreme and rapid hypercapnia (a decrease to 5.9 pH over 10 min; Croll, 1985). While the respiratory pumping rate is a proxy for metabolic rate, it is likely that the reported effect was a stress response to extreme low pH, or the animal attempting to expel noxious water from its mantle cavity.

Oxygen consumption is considered a proxy for metabolic rate because animals typically do not store substantial amounts of oxygen; they meet their oxygen requirements by taking it from the surrounding environment. Oxygen consumption may not entirely represent metabolic rate because it does not account for anaerobic processes, but in most animals anaerobic metabolism is not sustained for prolonged periods and does not make up a substantial portion of standard or resting metabolic rates; it is also minimal at times when oxygen is plentiful. Therefore, oxygen uptake rate in near-saturated conditions of oxygen is considered an excellent proxy for metabolic rate in aquatic breathers (Lighton, 2008). In our experiment, low pH caused an approximate 30% decrease in metabolism in *Aplysia punctata*. Given the very high feeding rates of sea hares (Carefoot, 1987; Dionísio *et al.*, 2013), any ongoing impact upon metabolic demand has the potential to alter feeding patterns and, speculatively, have cascading ecological effects. Metabolic depression is a common effect of exposure to low pH in intertidal animals, and may be related to the need to conserve energy during periodic hypercapnic conditions (Christensen *et al.*, 2011). Interestingly, under two other common intertidal stressors, hypoxia and air-exposure, *Aplysia californica* will decrease its heart rate, and presumably its metabolic rate, in order to conserve energy (Dieringer *et al.*, 1978; Carefoot, 1987). The result that we saw here may therefore represent a typical short-term response, and exposure over longer timescales may result in different metabolic responses and different conclusions as to the overall effect of OA upon metabolic rate. In another gastropod, a 14-month acclimation to OA resulted in consistent metabolic reductions (Queirós *et al.*, 2015), but under similarly long-term acclimation no ongoing metabolic response was observed in several echinoderms (Appelhans *et al.*, 2014; Moulin *et al.*, 2015; Suckling *et al.*, 2015).

The metabolic scaling exponent ( $b$ ) represents the increasing metabolic efficiency with body size; the values that we found for  $b$  (0.94 and 0.86) are somewhat higher than the previously reported value of 0.76 for *A. punctata* (Marsden *et al.*, 2012). That study, however, used a narrow size range, covering less than one order of magnitude (0.25 to 0.81 g dry mass, estimated from fig. 3 of Marsden *et al.*, 2012) compared to a broader size range that was examined here

(0.023 g to 0.694 g). Estimates of  $b$  are highly sensitive to limited size ranges (Sokal and Rohlf, 1995), as reflected in the large difference in  $r^2$  values ( $r^2 = 0.53$  in Marsden *et al.*, 2012 vs. 0.90 and 0.85 in the present study). In addition,  $b$  is known to be somewhat plastic to environmental and biological factors (Glazier, 2009; Carey and Sigwart, 2014), so it may vary intraspecifically for other reasons. Similarly high scaling exponents have been reported in other *Aplysia* species; in *A. dactylovela*,  $b = 0.93$  (Carefoot, 1988), and in *A. brasiliana*,  $b = 0.89$  to 0.91 (Donovan *et al.*, 2006), and may be related to their high physical activity and metabolic rates (Glazier, 2010; Carey *et al.*, 2013). The present study, with a wider size range, provides the most robust estimate of metabolic scaling in *A. punctata*, or similar species, to date. However, the maximum size that we examined was around 7 g wet mass, while this species can reach 130 g (Carefoot, 1987), so there is ample scope for improved estimates.

Few studies have quantified metabolic rate in *Aplysia*. Those that have done so utilized specimens of different sizes and examined them in different temperatures, making direct comparisons of rates difficult. The one other study that has examined *A. punctata* (Marsden *et al.*, 2012), utilizing data from 8 specimens collected in 1977, documented rates approximately half those that we observed here when converted to common body mass and temperature (conversions not shown). The disparity could be connected to seasonal, regional, feeding state, or experimental differences, all of which can introduce interspecific variability in metabolic rate. Two other studies examined metabolic rates in different *Aplysia* species, reporting rates of approximately 25% (Carefoot, 1988) and 75% (Donovan *et al.*, 2006) of those we report here in *A. punctata* after conversion to common mass and temperature (conversions not shown). These were, however, much larger species, up to 0.5 kg live mass in Donovan *et al.*, and examined at tropical temperatures of 25 °C, so differences would be expected, even after conversion to common mass and temperature.

*Aplysia* are relatively large animals, and their main predator avoidance strategies are unpalatability or ejection of an ink that contains a toxin (Carefoot, 1987). The internal shell is small and not particularly robust, but does provide protection for internal organs (Beesley *et al.*, 1998). The importance of this function and the structure of the shell in general in sea hares has not been well studied. Shell size among our sampled specimens was closely correlated with body mass, according to the power law,  $S_L = 15.61 M^{0.30}$ . Similar scaling relationships have been reported in *Aplysia californica* ( $S_L = 5.1 M^{0.33}$ , Peretz and Adkins, 1982) and *Aplysia fasciata* ( $S_L = 9.23 M^{0.27}$ , Krakauer, 1974), with the difference in intercepts best explained by different relative proportions of shell to tissue in different species. Shell calcification rate was here found to be size-independent, which agrees with shell growth patterns in *A. californica*



(Peretz and Adkins, 1982). That study observed growth of  $0.23 \text{ mm day}^{-1}$ , but the much lower rate observed here ( $0.02 \text{ mm day}^{-1}$ ) could be largely explained by the temperature difference ( $7 \text{ }^{\circ}\text{C}$  vs.  $16 \text{ }^{\circ}\text{C}$ ).

The constant rate of calcification across both animal size and pH treatment, despite differences in shell margin widths and metabolism, suggests that calcification in *Aplysia* may be a largely autonomous process of crystallization that occurs as long as suitable conditions are present, or that the process is prioritized within the energy budget. During growth, shell is accreted as a proteinaceous matrix that includes dolomite particles that are the crystallization nuclei for calcification (Pedrozo *et al.*, 1997; Tonejc *et al.*, 2014). Crystallization occurs as long as appropriate cellular pH is maintained around these nuclei and, from our data, it appears that *Aplysia punctata* can adequately control cellular acid-base balance even in the presence of substantial decreases in environmental pH. *Aplysia californica* has been shown to have strong control of tissue pH, utilizing carbonic anhydrase and urease to tightly control acid-base balance and thus aragonite deposition in the statocyst (Pedrozo *et al.*, 1997). Our results suggest that *A. punctata* can similarly regulate shell formation, perhaps utilizing the same enzymes; and, under even this extreme OA scenario, calcification was not compromised. In molluscs, the tissue fluid around calcification sites is commonly at a much lower pH than that of the surrounding seawater, yet lower seawater pH can still cause reduced shell integrity (Melzner *et al.*, 2011). Likely it is not simply a case of pH or  $p\text{CO}_2$  value *per se* that causes reduced calcification; overall energy budget and food limitation may be the critical factor in the energy required to maintain calcification (Melzner *et al.*, 2011).

The shell of *A. punctata* is small in comparison to the size of the animal, and so shell growth and calcification are likely to comprise a minor part of an individual's overall energy budget. However, even under the extreme pH conditions of our study, reduced calcification was not observed, which suggests that the process is highly controlled and important enough to be maintained despite the potential energetic costs. We could not quantify the calcium content of the shell part that had been present before exposure to the experimental OA treatment, but visual inspection through dissection and brightness of fluorescence showed no obvious distinction between the two treatment groups, and shell calcification was not correlated with any morphometric or physiological parameters (Fig. 3). Our data did suggest the possibility that growth was suppressed along with metabolism in low pH, as shown by the narrowing of the shell border matrix, although under statistical analysis this was a marginally non-significant difference (Kruskal-Wallis H test,  $P = 0.06$ ). Construction of proteinaceous "scaffolding" such as this may be the critical shell-building process that is disrupted under climate change scenarios *via* energetic trade-offs or shortfalls, rather than the disruption to calci-

fication caused by external pH changes. Disruption to calcification, however, may have been observed under longer exposures, which is typical with longer acclimation (Melzner *et al.*, 2011). Such a disruption could potentially be explained by ongoing impacts to growth of the proteinaceous matrix; calcification would eventually be disrupted because growth of the matrix is a prerequisite, and with less matrix there would be less calcification.

These patterns suggest a potential deterministic growth pattern, where shell calcification occurs at a steady pace throughout the animal's life and in variable conditions of health or environment. This increase in the relative energetic investment in shell growth under hypercapnia has been well documented in the cephalopod mollusc *Sepia officinalis* (Gutowska *et al.*, 2010). In that species, hypercapnic juveniles grow more slowly in body size, and though their cuttlebones are denser, they grow the same deterministic number of laminae within the shell structure (Sigwart *et al.*, 2015). This type of deterministic shell growth may be present in many molluscs and shell-bearing invertebrates that have the physiological capacity to maintain calcification in undersaturated conditions. Hypercalcification, or growth of a denser shell in response to experimental OA conditions, has also been observed in several other groups, including fish and crustaceans (Ries *et al.*, 2009), and attributed to the strong acid-base regulatory physiology in these animals. But such capacity may be present in a broader suite of animals.

The technical problems of achieving fully replicated experimental designs is a recognized issue in ocean acidification research, where there is concern that apparent effects may be due to variability rather than induced change (Wernberg *et al.*, 2012; Cornwall and Hurd, 2015). The results of the present study likewise did not include independent treatment replication and also involved a relatively brief acclimation duration. Tank effects could have caused the metabolic depression that we observed in the low pH treatment group; however, lower metabolism is an established typical response of marine invertebrates to short-term experimental OA (Christensen *et al.*, 2011). Similarly, any differences in shell growth could disappear over longer timescales. Other OA studies have utilized similar or shorter timescales and observed relevant biological responses (*e.g.*, Catarino *et al.*, 2012, 19 days; Zittier *et al.*, 2013, 12 days; Ferrari *et al.*, 2015, 4 days), and these brief timescales are also relevant to responses under short-term fluctuations such as under low pH brought about by upwelling, particularly given that such fluctuations are likely to be more frequent and severe in the near-future ocean. The ongoing process of calcification, which did not appear to differ across pH levels, is direct observational evidence that this process can be maintained under an extreme OA scenario during acute short-term exposures, which are long enough to observe noticeable shell growth.



Under even extreme ocean acidification scenarios, *Aplysia punctata* can maintain shell calcification despite a significant reduction in metabolic rate, although overall shell growth may be disrupted. The ongoing energetic consequences of ocean acidification in the long term to this species and other shallow coastal invertebrates remain uncertain. The capacity for invertebrates to maintain calcification under suboptimal abiotic conditions with strong internal acid-base regulation may be more widespread than has been recognized to date.

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