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Effects of ocean acidification and elevated temperature on shell plasticity and its energetic basis in an intertidal gastropod

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ABSTRACT: Phenotypic plasticity is a mechanism by which organisms can alter their morphology, life history or behaviour in response to environmental change. Here, we investigate shell plasticity in the intertidal gastropod *Littorina littorea* in response to the ocean acidification and elevated temperature values predicted for 2100, focusing on shell traits known to relate to protection from predators (size, shape and thickness) and resistance to desiccation (aperture shape). We also measured and desiccation rates (measured as percentage water loss). Ocean acidification was simulated by bubbling carbon dioxide into closed-circuit tanks at concentrations of 380 and 1000 ppm, giving respective pH levels of 8.0 and 7.7; temperatures were set at 15 or 20°C. Both low pH and elevated temperature disrupted the overall investment in shell material; snails in acidified seawater and elevated temperature in isolation or in combination had lower shell growth rates than control individuals. The percentage increase in shell length was also lower for individuals kept under combined acidified seawater and elevated temperature, and the percentage of shell thickness increase at the growing edge was lower under acidified and combined conditions. Shells were also more globular (i.e. had lower aspect ratios) under elevated temperature and lower pH. Desiccation rates were lower at low pH and high temperature. Counter to predictions, water loss did not relate to shell biometric measures but was negatively correlated with adenosine triphosphate (ATP) concentrations. Finally, ATP concentration was positively correlated with shell thickening and weight, confirming the idea that negative effects of exposure to elevated pCO_2/low pH and elevated temperature on shell morphology may occur (at least in part) through metabolic disruption.

KEY WORDS: Climate change · Ocean acidification · Phenotypic plasticity · Morphology · Growth · Shell thickness · Aspect ratio · Water loss · *Littorina littorea*

INTRODUCTION

Phenotypic plasticity is an important mechanism by which organisms alter their morphology, life history or behaviour in response to fluctuations in the prevailing environmental conditions (Pigliucci 2001, DeWitt & Scheiner 2004). Given the effects that anthropogenic activities are now having on environmental conditions in many ecosystems, those organisms that are more able to exhibit plastic responses may be more likely to adjust to, cope with and eventually adapt to broad scale disturbances, such as climate change (Pigliucci et al. 2006, Charmantier et al. 2008). Hence, there is a pressing need for studies that

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explicitly examine the extent to which species can exhibit plastic responses to predicted levels of environmental change (e.g. see Irie 2006, Terblanche et al. 2006) as well as the potential costs of such plasticity (DeWitt et al. 1998, Auld et al. 2010).

Marine intertidal habitats are highly heterogeneous and have been shown to contain numerous species that exhibit plasticity in their responses, for example, in response to variation in wave action (e.g. Trussell 1997, Gaylord 2000, Trussell & Etter 2001), temperature (e.g. Kenny 1983, Irie 2006) and predation pressure (e.g. Boulding & Van Alstyne 1993, Cotton et al. 2004). At the same time, as a result of increased atmospheric carbon dioxide (CO_2) levels, marine environments are predicted to experience additional environmental change with a shift toward lower pH and [CO_3^{2-}] (due to an increase in aqueous [CO_2]) and increased temperatures (Caldeira & Wickett 2003, Sokolov et al. 2009). These conditions are predicted to cause severe alterations to marine organisms' physiology, development and behaviour (e.g. Bibby et al. 2007, Munday et al. 2009, Todgham & Hofmann 2009), culminating in changes to community structure and ecosystem function (e.g. Fabry et al. 2008, Hall-Spencer et al. 2008, Wootton et al. 2008, Widdicombe et al. 2009, Hale et al. 2011, Christen et al. 2012). Given the potential for many intertidal organisms to exhibit plastic responses (Chelazzi & Vannini 1987, Trussell & Etter 2001), it might be predicted that they will be better able to respond to these environmental drivers. At the same time, this propensity for plasticity makes intertidal taxa ideal models for studying such plastic responses and associated trade-offs.

Current evidence suggests that calcifying organisms (e.g. molluscs, echinoderms and corals) are likely to be among the most susceptible to changes in seawater carbonate chemistry. Both biomineralization and $CaCO_3$ dissolution can be affected negatively by reduced pH and saturation state of $CaCO_3$ (e.g. Kleypas et al. 1999, 2006, Ries et al. 2009, Nienhuis et al. 2010, Findlay et al. 2011). At the same time, temperature increases may also lead to the disruption of calcification in marine ectotherms (e.g. Irie 2006). In fact, given that elevated temperature and reduced seawater pH are both induced by elevated atmospheric CO_2 levels, marine organisms will be exposed simultaneously to these 2 global climate drivers. Consequently, the true impacts of elevated partial pressures of CO_2 (pCO_2) or elevated temperature in isolation on the functions of marine calcifiers are likely to be greater than previously thought (e.g. Feng et al. 2009), with both factors likely to cause changes to the form and mechanical properties of shells (Gaylord et al. 2011, Dickinson et al. 2012).

Given the importance of shells for protection from predation, wave exposure, overheating and desiccation, any such disruption may have major consequences for marine calcifiers. Yet it is unlikely that effects on calcification will occur in isolation. Elevated pCO_2 has also been shown to impact the physiological and homeostatic function of marine calcifiers, for example, by causing metabolic depression (Pörtner et al. 1998, Lannig et al. 2010, Melatunan et al. 2011, Dickinson et al. 2012, but see for example Wood et al. 2008, Gutowska et al. 2010), acid-base disruption (Barry et al. 2003, Spicer et al. 2007, Stumpp et al. 2012, but see also Small et al. 2010, Donohue et al. 2012) and reduced levels of adenosine triphosphate (ATP) (Lannig et al. 2010, Melatunan et al. 2011, but see Beniash et al. 2010, Dickinson et al. 2012). Therefore, it is highly likely that with any plastic responses in shell morphology, there will be associated trade-offs caused by the reallocation of energy away from vital biological processes, such as growth (e.g. Lischka et al. 2011), reproduction (e.g. Pistevos et al. 2011) and development (e.g. Dupont et al. 2008). More specifically, in order to maintain calcification rates under ocean acidification conditions, calcifiers may require higher energy levels for the deposition of $CaCO_3$ (Bak 1983, Palmer 1983, 1992, Geller 1990, Day et al. 2000, Wood et al. 2008, 2010, Findlay et al. 2010a, 2011).

Here, we investigate the extent to which shells of the intertidal gastropod *Littorina littorea* are affected by elevated-pCO_2-induced acidified seawater, elevated temperature and these factors in combination. In particular, we focus on shell traits that relate to the ecology of this species in terms of protection from predators (mass, shell size and shape as well as thickness) and desiccation (shell aperture size and shape). Finally, for the first time, we formally test if energy limitations (i.e. ATP levels) previously reported for this species when exposed to elevated temperature and pCO_2 (Melatunan et al. 2011) are associated with plasticity in shell morphology (as predicted by Pörtner 2008 and Findlay et al. 2009, 2011).

MATERIALS AND METHODS

Experimental design

We used a multi-factorial nested design to assess the potential influence of altered seawater pH and temperature on snail growth, shell biometrics and

snail water loss. Two pH_{NBS} levels were selected, based on current (8.0) and predicted values for the year 2100 (7.7) corresponding to global ocean pCO_2 values of 380 and 1000 µatm, respectively (Caldeira & Wickett 2003). Two water temperature levels were used: 15 ± 0.1°C (mean ±1 standard error [SE]), which corresponded to the mean monthly sea surface temperature at the collection site (Joyce 2006), and 20 ± 0.1°C, which assumes an increase of +5°C in line with predicted warming trends for mean sea-surface temperatures (Sokolov et al. 2009). Local seawater temperature at the time of sample collection was 14°C (determined using a YSI 85 handheld multimeter).

Animal collection and preparation

Littorina littorea individuals (shell width: 13 to 15 mm) were collected in May 2009 from the rocky intertidal shore at Hannafore Point, Looe Bay in Cornwall (50° 20′ 36.87″ N, 4° 27′ 16.83″ W). Individuals were returned to the laboratory within 2 h. Before being introduced into the experimental set-up, snails were acclimated in 2 large plastic aquaria (capacity 56 l, 130 individuals in each aquarium) for 10 d in aerated seawater (e.g. Sokolova & Pörtner 2001, 2003, Calosi et al. 2008, 2010, Melatunan et al. 2011) at 15°C and salinity 33, measured using a handheld multi-meter and pH probe (Mettler Toledo S47 SevenMulti™ dual meter/pH conductivity). Individuals were fed ad libitum on *Ulva lactuca* and *Fucus serratus* every second day during the acclimation period.

Mesocosm setup

Four CO_2/air-equilibration mesocosms (one per treatment) were set up in a controlled-temperature room maintained at 15°C (12 h light and 12 h dark) as modified versions of the equilibration flow-through systems used by Widdicombe & Needham (2007). Briefly, mixed gas of CO_2-air was passed through the water in header tank and pumped via gravity to the experimental unit. Tanks were filled with fresh seawater (14°C, salinity 34, pH 8.04, dissolved inorganic carbon [DIC] 1600 µmol kg^{-1} and total alkalinity [TA] 1730 µequiv kg^{-1}). Seawater pH was monitored throughout the experiment using a pH controller (Aqua Digital PH-201, Reef Dreams). Excess seawater from the experimental unit was left flowing via gravity into a common sump (50 cm length, 45 cm width and 35 cm height), where seawater was degasified via vigorous air bubbling with an air pump. A submersible pump (EP68, Hengtong Aquarium) then circulated the sump seawater back to the header tanks at controlled pH, which triggered the injection of new CO_2 via the CO_2 controller (CO_2 solenoid, Peter Paul Electronic) until the required pH was reached. A submersible pump in the header tanks allowed for rapid homogenisation of the physico-chemical parameters of the seawater. In addition, 50% of the seawater in the experimental system was changed weekly, and a small quantity of distilled water was added, as needed, to avoid salinity fluctuations and ammonia build-up. Experimental units were maintained at one of 2 water temperature levels (15 and 20°C) using automatic submersible water heaters (100 W Submersible Aquarium Fish Tank Heater, Reef Dreams).

Four aquaria (23 cm length, 15 cm width and 15 cm height) with 48 holes (∅ 10 mm; 9 and 15 holes in each short and long side, respectively) were placed in each experimental unit (65 cm length, 38 cm width and 15 cm height). Sixteen plastic pots (20 ml, ∅ 3 mm and 5 cm height), each containing an individual snail, were submerged in each aquarium for the 30 d exposure period. The holes in each aquarium and pot (∅ 3 mm) ensured seawater circulation within each system. Hence, a total of 256 individuals were used in the experiment. The position of each aquarium within each tank was moved, at random, each week during the experimental period to help avoid the negative effect of pseudo-replication (Morrison & Morris 2000). However, the choice of having a single header tank per treatment was also partly dictated by the desire to increase the accuracy in producing large volumes of seawater of the required pH levels (see Hurlbert 1984).

Water physico-chemistry

Physico-chemical parameters within the mesocosm unit were measured daily during the experimental period of 30 d. Oxygen concentration (O_2), salinity and temperature (°C) were measured using a handheld multi-meter (YSI 85), pH_{NBS} was measured with a pH microprobe (Seven Easy, Mettler-Toledo) attached to a calibrated pH meter (S47 SevenMulti™, Mettler-Toledo), and DIC was measured with a total CO_2 analyser (965D, CIBA Corning Diagnostic). TA, pCO_2, bicarbonate and carbonate ion concentration ($[HCO_3^-]$ and $[CO_3^{2-}]$) and calcite and aragonite saturation states (Ω_{cal} and Ω_{ara}) were calculated at the

end of the experiment (Table 1) using CO2SYS (Pierrot & Wallace 2006).

Biometric measurements

We measured shell morphological parameters known to relate to susceptibility to predation and water loss, i.e. shell length and width, aperture length and width, shell thickness of the inner lip of the shell (i.e. the point alongside the columellar axis in the posterior aperture of the shell, referred to hereafter as thickness-1) and of the outer lip (i.e. the growing tip along the anterior portion of the shell, hereafter thickness-2) as well as the total weight of the intact individual (Cotton et al. 2004) (Appendix 1). These parameters were also used to calculate measures related to shell shape, including aspect ratio (shell length:shell width) and aperture ratio (shell aperture length:shell aperture width). Values for these measures were calculated for each individual as the proportional difference between values at the start and end of the exposure period.

All measurements were carried out on images collected with a digital camera (Coolpix 4500, Nikon UK) mounted on a light microscope (SDZ-IR-P, Kyoma Optical). Each image was measured using the UTHSCSA Image tool program for Windows 2003 calibrated using a micrometer (1.000 ± 0.001 mm).

Water loss

Percentage water loss (WL) was measured in a subset of 6 snails from each replicate aquarium (total n = 96 individuals) using Eq. (1) (Sokolova & Pörtner 2001):

$$WL = \frac{W_{in} - W_{exp}}{W_{in} - W_{dry}} \times 100\% \quad (1)$$

where W_{in}, W_{exp} and W_{dry} are the initial wet weight, weight after the exposure period and final dry weight of a snail (mg) respectively. Briefly, W_{in} and W_{exp} were determined with a digital scale (PF-203, Fisher Scientific) before and after exposure in air to 30°C for 6 h on an aluminium tray in a programmable oven (Sokolova & Pörtner 2001). After exposure, snails were returned to their individual pots in the original experimental tanks to acclimate for 2 h and then were dried at constant temperature of 100°C for 24 h to determine W_{dry}. Because the snails never surfaced, they did not experience any form of desiccation prior to the experimental trials.

Table 1. Mean (±1 SE) values of seawater physico-chemical parameters measured or calculated during the duration of the experiment: oxygen concentration (O_2), salinity, temperature (°C), pH (NBS scale, Mettler-Toledo pH meter), dissolved inorganic carbon (DIC, Total CO_2 Analyser, CIBA Corning 965D) total alkalinity (TA, Alkalinity Titrator, AS-ALK2, Apollo SciTech) using the method developed by Dickson et al. (2007), carbon dioxide partial pressure (pCO_2), bicarbonate and carbonate ion concentration ($[HCO_3^-]$ and $[CO_3^{2-}]$), calcite and aragonite saturation state (Ω_{cal} and Ω_{ara}). *indicates parameters that were calculated using the CO2SYS program (Pierrot & Wallace 2006), using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson & Millero (1987). Different letters after values indicate significant differences among treatments based on estimated marginal mean tests with Bonferroni correction (α = 0.05). Treatments: Control = current temperature (temp.) & current pCO_2; Elevated temp. = elevated temp. & current pCO_2; Elevated pCO_2 = current temp. & elevated pCO_2; Combined = elevated temp. & elevated pCO_2

Treatment	$[O_2]$ (mg l^{-1})	Salinity	Temperature (°C)	pH	DIC (µmol kg^{-1})	TA (µequiv kg^{-1})*	pCO_2 (µatm)*	$[HCO_3^-]$ (µmol kg^{-1})*	$[CO_3^{2-}]$ (µmol kg^{-1})*	Ω_{cal}*	Ω_{ara}*
Control	6.75 ± 0.01^a	34.77 ± 0.03^a	14.43 ± 0.06^a	8.03 ± 0.01^a	1596 ± 50^a	1726 ± 52^a	428 ± 17^a	1489 ± 47^{ab}	89.79 ± 3.5^a	2.14 ± 0.08^a	1.37 ± 0.05^a
Elevated temp.	7.09 ± 0.15^a	35.12 ± 0.05^a	20.91 ± 0.09^b	8.04 ± 0.01^a	1573 ± 40^a	1743 ± 44^a	428 ± 13^a	1444 ± 36^b	114.31 ± 5.0^b	2.73 ± 0.12^b	1.78 ± 0.08^b
Elevated pCO_2	6.68 ± 0.17^a	35.06 ± 0.04^a	14.89 ± 0.05^a	7.67 ± 0.01^b	1596 ± 40^a	1627 ± 40^a	998 ± 30^b	1518 ± 38^{ab}	40.58 ± 1.3^c	0.97 ± 0.03^c	0.62 ± 0.02^c
Combined	6.85 ± 0.17^a	35.12 ± 0.07^b	20.68 ± 0.08^b	7.65 ± 0.01^b	1723 ± 46^a	1767 ± 48^a	1185 ± 33^c	1633 ± 44^{ac}	51.50 ± 1.8^c	1.23 ± 0.04^c	0.80 ± 0.03^c

Statistical analyses

The effects of elevated pCO_2, temperature and their interaction on total wet weight, biometric shell characteristics (shell length and aspect ratio, aperture length, width and aperture ratio and shell thicknesses-1 and -2) and water loss were analysed using a 2-way analysis of covariance (ANCOVA), with aquarium as a random factor nested (Bennington & Thayne 1994) within pCO_2 × Temperature. In addition, for shell parameters, the value at the beginning of the incubation of the metric under the present study was employed as a covariate to control for potential difference in the plastic responses of a specific trait (e.g. initial shell length for % change in shell length; Appendix 2). The factor 'Aquaria' had a significant effect on most parameters measured in the present study (minimum $F_{1,255}$ = 1.937, p = 0.031), with the exception of percentage change in shell aspect, aperture length and water loss (maximum $F_{1,96}$ = 0.921, p = 0.535). However, in those cases where the factor Aquaria was significant, removing this factor did not change the patterns of significance of the main factors, and thus, the aquaria effect is considered marginal. When found not significant, the term Aquaria was removed from the analysis. Most data met the assumption for normality as untransformed data or following $\log_{10}$ transformation (maximum Z_{256} = 1.306, p = 0.066), with the exception of percentage change in shell length, aperture length and aperture ratio, for which no transformation was beneficial (minimum Z_{256} = 1.476, p = 0.026). Variances were homogeneous for percentage changes of aspect ratio and water loss (maximum $F_{15,240}$ = 1.420, p = 0.158) but not for the other variables (minimum $F_{15,240}$ = 1.758, p = 0.041). Because our experimental design included 4 treatments with a minimum of 16 replicates per treatment per measurement, we assumed that the ANOVA design employed should be tolerant to deviation from the assumptions of normality and heteroscedasticity (Sokal & Rohlf 1995). Pairwise comparisons were conducted using the 95% confidence interval test calculated for estimated marginal means. Finally, we tested for a relationship between the water loss and shell aperture ratio and $\log_{10}$ [ATP] (snail foot muscle) as well as between the percentage change in shell metrics and $\log_{10}$ [ATP] using Pearson's correlation. Data for $\log_{10}$ [ATP] of *Littorina littorea* individuals under elevated temperature and pCO_2 were taken from Melatunan et al. (2011) as [ATP] has been already used as proxy for the energy status of periwinkle snails (see Sokolova & Pörtner 2001, 2003). All analyses were conducted using SPSS 17.

RESULTS

Shell weight, size and shape

Snails exposed to lower pH and elevated temperature exhibited significantly lower increases in weight than those under current levels. Snails in low pH and elevated temperature showed respective increases in weight of 1.6% and 1.4% compared with 6.4% in current conditions; those exposed to both factors in combinations exhibited a decrease in weight of 1.8% (Fig. 1a, Appendix 3). The percentage change in shell length was also lower under elevated temperature (5.8%) and both factors in combination (4.4%), but not in acidified seawater (8.4%), compared with those kept in current conditions (10.3%) (Fig. 1b, Appendix 3). In addition, the percentage change in aspect ratio was lower under elevated temperature (–2.4%), elevated pCO_2 (–2.51%) and both factors combined (–3.31 ± 0.01%) compare with current conditions (3.1%) (Fig. 1c, Appendix 3).

Shell thickness

Both measures of shell thickness decreased under conditions of elevated temperature under normal pH conditions but increased with temperature under acidified conditions (Fig. 1d,e), giving a significant interaction between pCO_2 and temperature (Table 2). Mean percentage change in shell thickness-1 (inner lip) was significantly higher under current temperature conditions (39%), lower under acidified conditions (8.1%) and intermediate for the other 2 treatments (range between 21.5 and 23.5%) (Fig. 1d, Appendix 3). Mean percentage change in shell thickness-2 (outer lip) differed significantly between all treatments and was highest and positive under current (55.2%) and elevated temperature (15.0%) conditions and lowest and negative under acidified (–27.1%) and combined (–14.7 ± <0.1%) conditions (Fig. 1e, Appendix 3).

Shell aperture size and shape

Mean percentage aperture length increase was significantly affected by elevated temperature (11.6% increase) and by pCO_2 and elevated temperature in combination (11.3%) (Fig. 1f, Table 2); these 2 treatments were comparable. The lowest value for mean percentage change in aperture length was recorded under acidified conditions (2.0%), whilst control con-

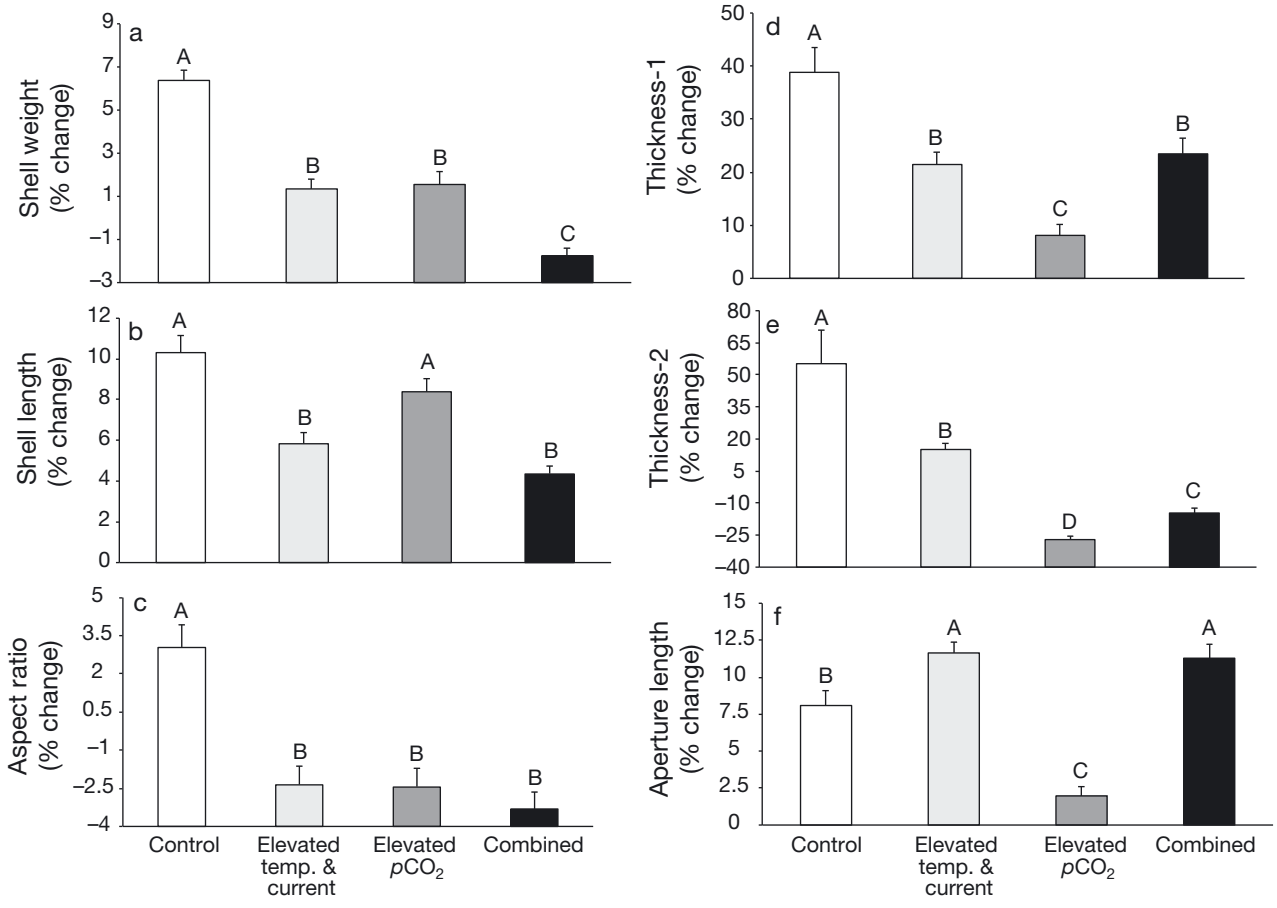

Fig. 1. Response (% change) of *Littorina littorea* shell traits to 30 d exposure to different combinations of pCO_2 and temperature: (a) shell wet weight, (b) shell length, (c) aspect ratio, (d) shell thickness-1, (e) shell thickness-2, (f) aperture length of snails exposed at different temperatures under elevated and current pCO_2 levels. Histograms represent means (±1 SE) for the 4 treatments: current temperature and pCO_2 (control), elevated temperature, elevated pCO_2 and elevated pCO_2 and temperature combined. Significantly different means ($p \leq 0.05$) are indicated by different capital letters (A, B, C and D), according to estimated marginal means (EMM) tests with Bonferroni correction

ditions were intermediate (8.1%) and significantly different from all other treatments (Fig. 1f, Appendix 3). Mean percentage increase in aperture width and mean percentage changes in aperture shape (i.e. aperture ratio) were not significantly affected by pCO_2 or temperature (see Table 2, Appendix 3).

Water loss

Mean percentage water loss increased progressively from current conditions (19.5%) through those of elevated temperature and low pH to both factors in combination (38.3%) (Fig. 2, Appendix 3), although this trend was due to primary effects of pH and temperature rather than a significant interaction between these factors. A positive non-significant relationship between percentage water loss and mean percentage change in aperture ratio was found (R^2 = 0.03, df = 95, p = 0.09).

Energy levels and shell plastic responses and water loss

There were significant positive relationships between $\log_{10}$ [ATP] and percentage change in shell weight (Fig. 3a; R^2 = 0.355, df = 63, $p < 0.0001$) and thickness-2 (R^2 = 0.224, df = 63, $p < 0.0001$; Fig. 3b). In contrast, there was a significant negative relationship between water loss and $\log_{10}$ [ATP] (Fig. 3c; R^2 = 0.230, df = 63, $p < 0.0001$). No other significant relationships between $\log_{10}$ [ATP] and shell changes (%) were found ($p > 0.05$).

Table 2. Results of 2-way ANCOVAs investigating the effect of elevated pCO_2 and temperature on shell traits and water loss in the common periwinkle *Littorina littorea*. cov: covariate, df: degrees of freedom, MS: mean of square, *F*: *F*-ratio, p: probability level. **Bold:** significant

Trait	Source	df	MS	*F*	p
Shell wet weight (% change)	pCO_2	1	893.4	26.2	**<0.0001**
	Temperature	1	1531.4	46.4	**<0.0001**
	Interaction	1	84.8	2.5	0.141
	Aquaria	12	34.2	3.7	**<0.0001**
	Initial wet weight (cov)	1	886.6	96.3	**<0.0001**
Shell length (% change)	pCO_2	1	176	5.6	**0.036**
	Temperature	1	868.7	27.4	**<0.0001**
	Interaction	1	65	2.1	0.178
	Aquaria	12	31.7	2.7	**0.002**
	Initial shell length (cov)	1	1374.6	118	**<0.0001**
Shell width (% change)	pCO_2	1	34.7	0.8	0.396
	Temperature	1	138.3	3.2	0.097
	Interaction	1	1.5	0.04	0.852
	Aquaria	12	44.4	2.7	**0.002**
	Initial shell width (cov)	1	824.2	50.5	**<0.0001**
Shell aspect (% change)	pCO_2	1	237.7	8.8	**0.012**
	Temperature	1	116.4	4.3	0.059
	Interaction	1	0.8	0.03	0.865
	Aquaria	12	27.3	2.1	**0.016**
	Initial aspect ratio (cov)	1	5264.3	409.3	**<0.0001**
Shell thickness-1 (% change)	pCO_2	1	3823.6	31.3	**<0.0001**
	Temperature	1	60.3	0.5	0.483
	Interaction	1	1643.3	13.5	**<0.0001**
	Initial shell thick-1 (cov)	1	27788.7	227.7	**<0.0001**
Shell thickness-2 (% change)	pCO_2	1	15413	12.6	**0.003**
	Temperature	1	1995.8	1	0.329
	Interaction	1	4914.4	2.7	0.123
	Aquaria	12	1962	5.6	**<0.0001**
	Initial shell thick-2 (cov)	1	50019.1	142.7	**<0.0001**
Aperture length (% change)	pCO_2	1	296.4	8.2	**0.014**
	Temperature	1	409.8	12.1	**0.004**
	Interaction	1	191.2	5.3	**0.041**
	Aquaria	12	36.4	2	**0.028**
	Initial aperture length (cov)	1	2837.3	153	**<0.0001**
Aperture width (% change)	pCO_2	1	3319.4	12.3	**0.004**
	Temperature	1	588.8	2	0.187
	Interaction	1	1062	3.5	0.088
	Aquaria	12	315.4	10	**<0.0001**
	Initial aperture width (cov)	1	4167.8	131.1	**<0.0001**
Shell aperture aspect (% change)	pCO_2	1	6240.1	11.2	**0.005**
	Temperature	1	252.4	0.4	0.551
	Interaction	1	2351.7	3.7	**0.079**
	Aquaria	12	664.2	7.4	**<0.0001**
	Initial aperture ratio (cov)	1	17282.6	192.8	**<0.0001**
Water loss (%)	pCO_2	1	4075.8	29.7	**<0.0001**
	Temperature	1	1085.4	7.7	**0.007**
	Interaction	1	1.1	0.01	0.928
	Initial weight (cov)	1	48.5	0.4	0.554

DISCUSSION

A complex pattern of responses following exposure to ocean acidification and global warming appears to be emerging across phylogenetically diverse taxa (e.g. Martin et al. 2008, Ries et al. 2009, Rodolfo-Metalpa et al. 2009, Cigliano et al. 2010, Hale et al. 2011). Here, we show that plastic responses under predicted climate change scenarios can also be complex within a species. Shell morphometric traits in

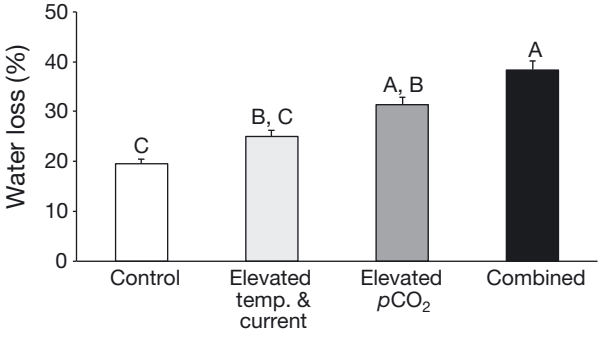

Fig. 2. The percentage change in water loss in *Littorina littorea* following 30 d exposure to different combinations of pCO_2 and temperature. Histograms represent mean values (±1 SE) determined for the 4 employed treatments: current temperature and pCO_2 (control), elevated temperature, elevated pCO_2 and elevated pCO_2 and temperature. Significantly different means ($p \leq 0.05$) are indicated by different capital letters (A, B, C and D) according to EMM tests with Bonferroni correction

the intertidal gastropod *Littorina littorea* respond differently to elevated pCO_2 and elevated temperature, with a mixture of single, additive, synergistic and no effects depending on which trait is considered. As the traits investigated potentially underpin the ability of this intertidal gastropod to protect itself from predators and avoid desiccation, our results suggest that exposures to future global change scenarios (Caldeira & Wickett 2003, Sokolov et al. 2009) may alter the tolerance of this species and, ultimately, its fitness and survival but do so via complex physiological and ecological pathways.

Shell growth, thickness and shape

Under low pH and elevated temperature in isolation, *Littorina littorea* increased less in weight and were shorter than snails grown under current conditions. Similar results have been obtained for other calcifying organisms. For example, increased seawater acidity caused a reduction in shell growth of the oysters *Crassostrea gigas* (Lannig et al. 2010) and *Crassostrea virginica* (Beniash et al. 2010), larvae of the Mediterranean pteropods *Cavolinia inflexa* (Comeau et al. 2010) and the mussels *Mytilus edulis* (Gazeau et al. 2010) and *Mytilus californianus* (Gaylord et al. 2011). Moreover, elevated temperatures have also been reported to induce smaller metamorphic size in the gold-ringed cowry *Monetaria annulus* (Irie & Fischer 2009) and to disrupt the metabolism, growth and fitness of the periwinkle *Littorina*

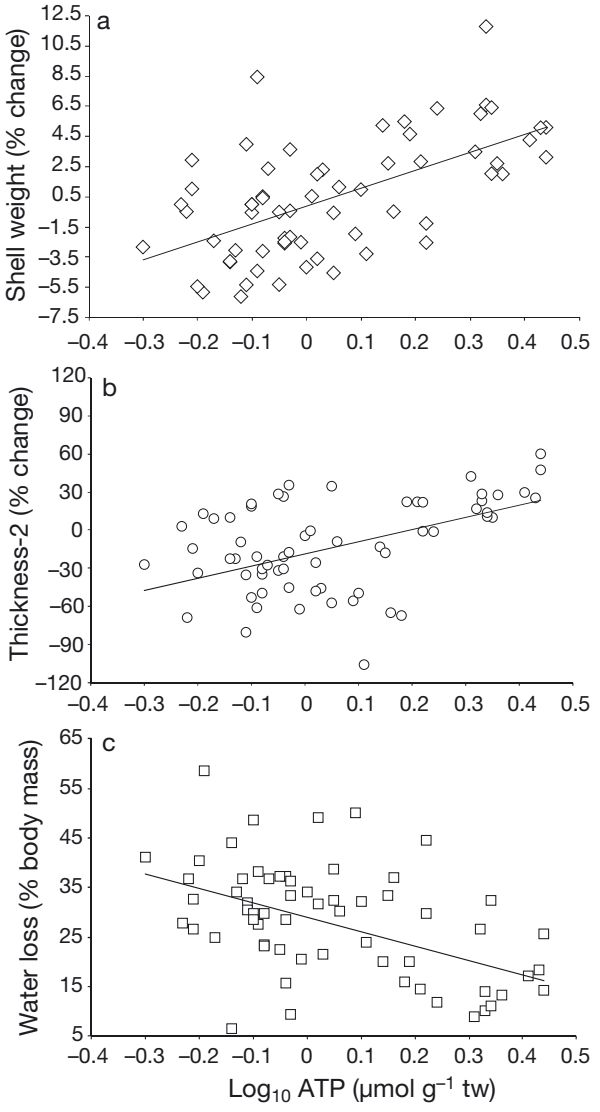

Fig. 3. Relationships between $\log_{10}$ ATP content of *Littorina littorea* and (a) percentage change of shell weight ($y = 11.854x + 0.1235$, df = 63, $R^2 = 0.3552$, $p < 0.0001$), (b) percentage change of thickness-2 ($y = 96.144x + 18.906$, df = 63, $R^2 = 0.2239$, $p < 0.0001$) and (c) water loss ($y = -29.151x + 29.043$, df = 63, $R^2 = 0.2295$, $p < 0.0001$) in snails maintained for 30 d under different temperature and pCO_2 conditions. Lines indicate linear regressions

saxatilis (Sokolova & Pörtner 2001). Not surprisingly, in *L. littorea*, the combined exposure to both low pH and elevated temperature had a greater negative effect on shell growth than either of these factors in isolation, a result in line with those for other marine calcifying organisms exposed simultaneously to elevated pCO_2 and temperature (e.g. Rodolfo-Metalpa et al. 2009).

Littorina littorea also showed reduced lower shell thickening under acidified conditions. This reduction in shell thickness occurred mainly at the growing tip (thickness-2) rather than more centrally on the body whorl. Dissolution at the growing tip of the shell under acidified seawater conditions has previously been reported in the planktonic pteropod *Limacina helicina* (Lischka et al. 2011). Such reductions in shell thickness in marine calcifiers have been considered to be the result of either or both processes of dissolution of calcium carbonate structures exposed to acidified seawaters (e.g. Michaelidis et al. 2005, Orr et al. 2005, Nienhuis et al. 2010) or insufficient deposition of calcium carbonate material (see Findlay et al. 2011). An additional trial in the same experimental unit we used showed that total shell dissolution in empty shells of *L. littorea* over a 30 d exposure to elevated pCO_2 (1293 ± 16.85 µatm, mean ± SE) was −3 to −15%. Compared with values observed in live snails (−1.7 to −4.1%), these data suggest that although shell dissolution does occur, it has a relatively small impact on changes in total shell weight. Since calcification is energetically costly (Palmer 1983), a possible explanation for our observation is that reduced energy status in *L. littorea* under elevated temperature and pCO_2 conditions (Melatunan et al. 2011) leads to a lower capacity for calcification, as observed in the juvenile oyster *Crassostrea virginica* (Dickinson et al. 2012).

Our study also shows that elevated pCO_2 exerts a different effect on shell thickness at different temperatures. The fact that snails kept under acidified and high temperature conditions had thicker shells than those at low pH and current temperatures may be due to an increase in calcite and aragonite saturation states with increasing temperature (see Dickson 2010). Hence, a decrease in the rate of passive dissolution may have occurred due to Ωcal being just below and just above values of 1 under acidified and combined acidified and elevated temperature conditions, respectively. Whilst we did not explicitly test for shell strength, a reduction in shell thickness may lead to a reduction in shell strength (e.g. see Gaylord et al. 2011, Dickinson et al. 2012). As thicker apertural lips are likely to provide better defence against shell crushing predators (Vermeij 1987, Bourdeau 2010), the morphological changes we observed in *Littorina littorea* may increase its susceptibility to predation (Boulding & Van Alstyne 1993, Trussell & Etter 2001).

Shell shape in aquatic gastropods is thought to play an important role in predator defence. A globular shell shape in the freshwater snail *Physa* spp. has more resistance to crushing predators, such as crayfish, than an elongated shape (DeWitt et al. 2000), and species of marine intertidal gastropods possessing shells with a larger aspect ratio (i.e. with a more elongated shape) have also found to be more vulnerable to crab predation, possibly due to a reduced handling efficiency of shells with a flatter, more discoid shape (Cotton et al. 2004). Here, the proportional change in shell shape in *Littorina littorea* was affected significantly by elevated temperature, elevated pCO_2 and the combined effect of these factors. Shells kept under current temperature conditions were more elongated but had a more globular shape under other treatment conditions. This finding suggests that in more acidic and warmer conditions, snails produce a shell shape that may be less susceptible to predation compared with those kept under acidified conditions. As shell thickness is reduced under low pH and elevated temperature, acquiring a more globose shape could enable snails to compensate for a possible reduction in shell strength.

Shell aperture plasticity and water loss

The size of the shell aperture and operculum in gastropods has been shown to exhibit plasticity in response to environmental conditions. For example, the marine gastropod *Thais lapillus* increases operculum size (Gibson 1970), the land snail *Cepaea* spp. reduces aperture size (Goodfriend 1986) and the common limpet *Patella* spp. decreases base-aperture size (Cabral 2007) in order to maintain constant body temperatures and reduce desiccation. Overall, the shell aperture shapes of *Littorina littorea* exposed to elevated temperature conditions were more elongate compared to the more rotund shape found under current temperature conditions. This altered shell aperture shape may have negative consequences for desiccation rates. Most shelled gastropods and barnacles reduce desiccation by completely closing the open aperture area with the operculum (Shick et al. 1988). However, this strategy could potentially affect rates of oxygen uptake (Broekhuysen 1940, Gibson 1970), thus impairing organismal production of energy metabolites (e.g. Sokolova & Pörtner 2001) and ultimately altering acid-base status (Egginton et al. 1999). Consequently, under predicted climatic conditions, *L. littorea* may be exposed to a significantly increased desiccation risk, unless thermoregulatory behavioural plastic responses can mediate this situation (as suggested for terrestrial ectotherms by Huey & Tewksbury 2009).

A metabolic basis for plasticity and water loss?

In general, increased ocean acidity causes internal acidosis, which leads to the disruption of metabolism and homeostatic functions and the reduction of energy transduction (ATP) (Pörtner et al. 1998, Lannig et al. 2010, Melatunan et al. 2011). Low pH can also alter biomineralisation, disrupt shell and soft-body growth (Beniash et al. 2010, Findlay et al. 2010a,b) and fitness (Pörtner 2008). It has been suggested that alterations to metabolic energy due to exposure to ocean acidification rather than passive shell dissolution will be more likely to impact calcification (Findlay et al. 2011). In fact, as calcium transport and secretion in shell-forming cells of molluscs and corals is partly ATP-dependent (see Findlay et al. 2011 for a review), disruption of energy metabolism could underpin the observed decreases in shell growth in various molluscs (e.g. Michaelidis et al. 2005, Beniash et al. 2010, Gaylord et al. 2011, present study). In a parallel investigation of the physiological responses of *Littorina littorea*, Melatunan et al. (2011) showed that a 30 d exposure to low pH, elevated temperature and their interaction caused a significant drop in both oxygen consumption rates and ATP levels. Here, we show that ATP levels correlate positively with shell-related plastic responses (i.e. percentage change in shell weight and shell thickness-2), thus demonstrating a potential link between organismal energy status, calcification and plastic responses under elevated pCO_2 and temperature conditions. Moreover, the observed disruption to growth (i.e. increase in mass) and shell thickness may be mediated by the alteration of homeostatic processes induced by ocean acidification and elevated temperature rather than by the decrease in saturation status (Ω) as previously thought (see discussion in Pörtner 2008, Findlay et al. 2009, 2011). As energy metabolites play a key role in energy transduction in the intracellular space, underpinning whole-organism exercise capacity, it is possible that individuals of *L. littorea* with lower levels of ATP may have a reduced ability to close their operculum tightly to prevent water loss (e.g. Greenway & Storey 2001). This constraint could explain, in part, the significant negative correlation between ATP levels and water loss. Consequently, it is likely that water loss in *L. littorea* will increase during emersion as a result of the disruption of physiological functions resulting from increased haemolymph acidosis and reduced energy transduction exerted by ocean acidification (for review, see Pörtner et al. 2004, Lannig et al. 2010), elevated temperature (Sokolova & Pörtner 2001) and their combined action (e.g. Melatunan et al. 2011).

In general, phenotypic plasticity enables organisms to respond to environmental variability (West-Eberhard 2003, Bozinovic et al. 2011) and can be defined as a measure of 'organismal malleability' (Huey & Berrigan 1996). Here, we report an in-depth investigation of the plastic responses of a marine invertebrate to the combined exposure of elevated pCO_2 and temperature that, according to projected pCO_2-pH and sea surface temperature levels, are predicted to occur in the near future (Solomon et al. 2009, Sokolov et al. 2009). Under such conditions, individuals of *Littorina littorea* might be predicted to be smaller in size, with thinner, more rotund shells, and, hence, be potentially more vulnerable to predators. Altered shell aperture shapes could also cause an increase in individuals' water loss during emersion. The pattern of responses observed here is rather complex but clearly suggests that global climate change could have far-reaching consequences for the ecology of some marine organisms. In addition, we believe that our proposed mechanistic (ATP-based) explanation for changes in marine organisms' plastic responses and water loss provides a direct link between energy metabolism, phenotypic responses and functional vulnerability to global climate change, providing a model to be tested more broadly in marine calcifiers.

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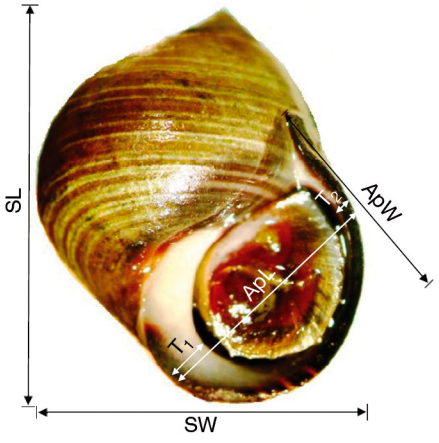
APPENDICES



Appendix 1. *Littorina littorea.* Biometric measurements made of the shell of the common periwinkle using images taken before and after 30 d exposure to different combinations of pCO_2 and temperature. SL: shell length; SW: shell width; ApL: shell aperture length; ApW: shell aperture width; T_1: thickness 1; T_2: thickness 2

Appendix 2. *Littorina littorea*. Final mean values (±1 standard error) for shell biometric traits and water loss of snails following 30 d exposure to different combinations of pCO_2 and temperature. Data are means (±1 standard deviation). For all variables at all treatment levels, the sample size was 64 individuals

Treatment	Growth (g)	Shell length (mm)	Shell width (mm)	Aspect ratio	Shell thickness-1 (mm)	Shell thickness-2 (mm)	Aperture length (mm)	Aperture width (mm)	Aperture ratio	Water loss (% body mass)
Current temp. & current pCO_2 (control)	1.91 ± 0.29	15.84 ± 0.95	13.57 ± 0.83	1.17 ± 0.06	1.88 ± 0.23	0.58 ± 0.10	8.99 ± 0.62	9.35 ± 0.80	0.97 ± 0.09	19.54 ± 11.71
Elevated temp. & current pCO_2 (elevated temp.)	1.64 ± 0.26	14.91 ± 0.87	12.77 ± 0.78	1.17 ± 0.04	1.83 ± 0.17	0.59 ± 0.10	8.84 ± 0.54	8.77 ± 0.69	1.01 ± 0.07	25.01 ± 12.22
Current temp. & elevated pCO_2 (elevated pCO_2)	1.81 ± 0.26	15.44 ± 0.85	13.32 ± 0.77	1.16 ± 0.04	1.68 ± 0.22	0.50 ± 0.08	8.79 ± 0.66	7.50 ± 0.75	1.18 ± 0.14	31.38 ± 9.18
Elevated temp. & elevated pCO_2 (combined)	1.67 ± 0.27	15.11 ± 0.95	13.15 ± 0.90	1.14 ± 0.04	1.78 ± 0.25	0.55 ± 0.10	8.81 ± 0.58	8.21 ± 1.05	1.09 ± 0.13	38.32 ± 11.48

Appendix 3. *Littorina littorea*. Percentage change in shell biometric traits and water loss of snails following 30 d exposure to different combinations of pCO_2 and temperature. Data are means (±1 standard error)

Treatment	Shell weight (% change)	Shell length (% change)	Shell width (% change)	Aspect ratio (% change)	Shell thickness-1 (% change)	Shell thickness-2 (% change)	Aperture length (% change)	Aperture width (% change)	Aperture ratio (% change)
Current temp. & current pCO_2 (control)	6.39 ± 0.48	10.31 ± 0.77	7.49 ± 0.61	3.06 ± 0.86	38.84 ± 0.04	55.16 ± 0.11	8.08 ± 0.07	19.25 ± 1.36	−15.59 ± 2.52
Elevated temp. & current pCO_2 (elevated temp.)	1.35 ± 0.43	5.83 ± 0.07	8.20 ± 0.62	−2.37 ± 0.01	21.47 ± 0.03	15.03 ± 0.01	11.64 ± 0.05	22.04 ± 0.71	−15.80 ± 1.28
Current temp. & elevated pCO_2 (elevated pCO_2)	1.56 ± 0.57	8.39 ± 0.07	9.65 ± 0.45	−2.45 ± 0.01	8.10 ± 0.03	−27.12 ± 0.02	1.97 ± 0.05	12.49 ± 0.61	−12.64 ± 0.99
Elevated temp. & elevated pCO_2 (combined)	−1.75 ± 0.34	4.35 ± 0.06	7.81 ± 0.66	−3.31 ± 0.01	23.47 ± 0.04	−14.68 ± 0.02	11.28 ± 0.07	20.30 ± 0.30	−15.09 ± 2.06

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