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BIOCHEMICAL, METABOLIC AND MORPHOLOGICAL RESPONSES OF THE INTERTIDAL GASTROPOD *LITTORINA LITTOREA* TO OCEAN ACIDIFICATION AND INCREASED TEMPERATURE

By

SEDERCOR MELATUNAN

A thesis submitted to Plymouth University
in partial fulfillment for the degree of

DOCTOR OF PHILOSOPHY

School of Marine Science and Technology
Faculty of Science and Technology

March 2012

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Sedercor Melatunan

March 2012

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Abstract

Future changes to the pH and temperature of the oceans are predicted to impact the biodiversity of marine ecosystems, particularly those animals that rely on the process of calcification. The marine intertidal gastropod *Littorina littorea* can be used as a model of intertidal organism for investigating the effects of ocean acidification and high temperature, alone and in combination because its ability to be quickly adapt against environmental stressor. In the first study a single species population of *L. littorea* was used to test for physiological and biochemical effects underpinning organismal responses to climate change and ocean acidification. Compared with control conditions, snails decreased metabolic rates by 31% in response to elevated pCO_2 while by 15% in response to combined pCO_2 and temperature. Decreased metabolic rates were associated with metabolic depression, a strategy to match oxygen demand and availability, and an increase in end-product metabolites in the tissue under acidified treatments, indicating an increased reliance on anaerobic metabolism. This study also showed that anthropogenic alteration of CO_2 and temperature may also lead to plastic responses, a fundamental mechanism of many marine gastropods to cope environmental variability. At low pH and elevated temperature in isolation or combined showing lower shell growth than individuals kept under control conditions. Percentage change in shell length and thicknesses was also lower under acidified and temperature in isolation or combined than control condition, making shells were more globular and desiccation

rates were higher. Further studies to broader latitudinal ranges for six populations of *L. littorea* showed that shell growth decreased in all six populations under elevated pCO_2 compared to control snails particularly those at range edges. Elevated pCO_2 also affected to the reduction of shell length and width that causing shell aspect ratio to increase across latitudinal gradients except individuals from Millport, UK. Percentage changes of aperture width and aperture area were also decrease under elevated pCO_2 with greater reduction of aperture area were found at populations in the mid-ranges which is assumed this response might be linked to local adaptation of the individual to microclimatic conditions. This study also showed that metabolic rates were negatively affected by high pCO_2 and show non-linear trend across latitudinal gradients in compared to individual kept under normal pCO_2 conditions. Metabolomic analysis showed that two northern populations of Trondheim and Tromsø were distinct from other populations when exposed to low temperature (15 °C) with elevated pCO_2 due to, in part, high concentrations of thymine, uracil, valine and lysine. A similar separation also occurred under medium (25 °C) and high (35 °C) temperature exposure in which one of northern population (Trondheim) was distinct from other populations and had lower concentrations of alanine, betaine and taurine while higher of valine. These results suggest that populations at northern latitudes may apply different ionic transport mechanisms under elevated pCO_2 and elevated temperatures and those populations are likely to vary in terms of their physiological responses to this environmental challenge.

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Figure 5.4. PCA plots derived from metabolomic fingerprints of 356 *Littorina littorea* individuals. PC1 axis (variance explained 12.6%) and 2 (var. 4.0%). Individual plots are coded to show samples from different: A) populations (1: Vigo, 2: Ile de Re, 3: Roscoff, 4: Millport, 5: Trondheim and 6: Tromsø); B) temperatures (1: 15, 3: 25, and 5: 35 °C); and C) CO_2 treatments (current and future pCO_2 levels).

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Figure 5.7. Histogram of PC scores (after the removal of low temperature treatments from the two northern samples) of *Littorina littorea* from six populations under medium-term exposure to different levels of pCO_2 (normal and high) and short term exposure to three temperatures for; A) PC1 axis and B) PC2 axis. Values are means (± SE). Populations are arranged in order from south to north ranges of: 42° N (Vigo, Spain); 45° N (Ile de Re, France); 49° N (Roscoff, France); 56° N (Millport, UK); 63° N (Trondheim, Norway); and 70° N (Tromsø, Norway). Significant different means ($P \leq 0.05$) between temperature levels indicated by capital letter (15 °C), lower case (25 °C), and number (35 °C) on each population and asterisk (*) indicated significant different exist between population according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

Figure 6.1. The relationship between $\log_{10}$ ATP content and A) percentage change in shell weight [$Y = 11.854x + 0.1235$, df = 63, $R^2 = 0.3552$, $p < 0.0001$], B) percentage change in shell 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 individual snails kept for 30 d under different temperature and pCO_2 conditions.

TABLES

Table 2.1. Physicochemical parameters of the seawater in the mesocosm unit. Mean (± SE) for seawater physico-chemical parameters measured or calculated during the duration of the experiment: oxygen concentration (O_2), salinity, temperature (°C), pH (NBS scale), dissolved inorganic carbon, total alkalinity (TA), using 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 CO_2SYSs program (Pierrot et al. 2006), using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987). Different letters in the brackets indicate significant differences among treatments using Post Hoc Bonferroni test of one way ANOVA ($p < 0.05$), according to 95% Confidence Interval test for Estimate Marginal Means (EMM) with Bonferroni correction.

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Table 5.6. The percentage contribution of key metabolites (i.e. those with a > 2% contribution) to PC axes 1 and 2 after the removal of low temperature treatments from the two northern samples.

APPENDICES

Appendix 2A. Mean physiological and biochemical parameters.

Appendix 3A. Biometrics of shells of the common periwinkle *Littorina littorea* shells measured using images taken after and before 30 d exposure to different combinations of pCO_2 temperature. The measurements included shell length (SL), width (SW), shell aperture length (ApL), aperture width (ApW), thickness-1 (T_1) and thickness-2 (T_2).

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Appendix 5.1. NMR spectroscopy and spectral processing methods.

Appendix 5.2A. Mean (± SE) percentage changes of shell weight and respiration rate of *L. littorea* after 14 d exposure periods under normal and high pCO_2. The O_2 uptake was measured after exposure to different air temperatures (15, 20, 25, 30, and 35ºC) for 3h.

Appendix 5.2B. O_2 uptake of *Littorina littorea* (L.) measured after exposure to different pCO_2 for 14 d and air temperatures (given values) for 3h.

Appendix 6.1. Melatunan S., P. Calosi, S.D. Rundle, A.J. Moody, and S. Widdicombe. 2011. Exposure to elevated temperature and pCO_2reduces respiration rate and energy status in the periwinkle *Littorina littorea*. Physiological and Biochemical Zoology 84(6):583–594.

Appendix 6.2. Melatunan S., P. Calosi, S.D. Rundle, S. Widdicombe, and J.A. Moody. (Manuscript to MEPS - under review) Marine gastropod plastic responses to the combined effects of ocean acidification and elevated temperature.

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other university award without prior agreement of the Graduate Committee.

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A course of study in molecular and cellular marine biology was undertaken in the first year, included a skill development in biochemistry particularly in the enzymatic assay protocol and metabolomics analysis in collaboration with Natural Environmental Research Council and Biomolecular Analysis Facility at the University of Birmingham.

Relevant scientific seminars and conferences host by Marine Biology and Ecology Research Centre at the University of Plymouth were regularly attended, external institutions were visited for consultation purposes and several manuscripts prepared for publication.

Publication:

- Melatunan S., P. Calosi, S.D. Rundle, A.J. Moody, and S. Widdicombe. 2011. Exposure to elevated temperature and pCO2reduces respiration rate and energy status in the periwinkle *Littorina littorea*. Physiological and Biochemical Zoology 84(6):583–594. (See appendix 6.1 of this thesis)

- Melatunan S., P. Calosi, S.D. Rundle, S. Widdicombe, and J.A. Moody. (Draft Manuscript) Marine gastropod plastic responses to the combined effects of ocean acidification and elevated temperature (See appendix 6.2 of this thesis)

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CHAPTER I

Introduction

OCEAN ACIDIFICATION AND CLIMATE CHANGE

General Background

Ocean acidification (OA) is the result of an ongoing process caused by an excessive increase of carbon dioxide (CO_2) into the world's oceans (Caldeira and Wickett 2003, Kleypas et al. 2006). This chemical change to seawater is driven by increased levels of atmospheric CO_2. For the past 650,000 years and prior to the Industrial Revolution, the concentration of atmospheric CO_2 was in the range of 180 to 300 ppm by volume (ppmv) (Augustin et al. 2004, Siegenthaler et al. 2005). However, Houghton et al. (2001) reported that CO_2 levels in the atmosphere had reached 367 ppm in 1999 and would continue to increase by 1% per year over the next few decades. Current atmospheric CO_2 levels have increased to 380 ppm (Raven et al. 2005, Kleypas et al. 2006) with a resulting increased atmospheric partial pressure (pCO_2) of the gas (Jacobson 2005) (Fig. 1.1).

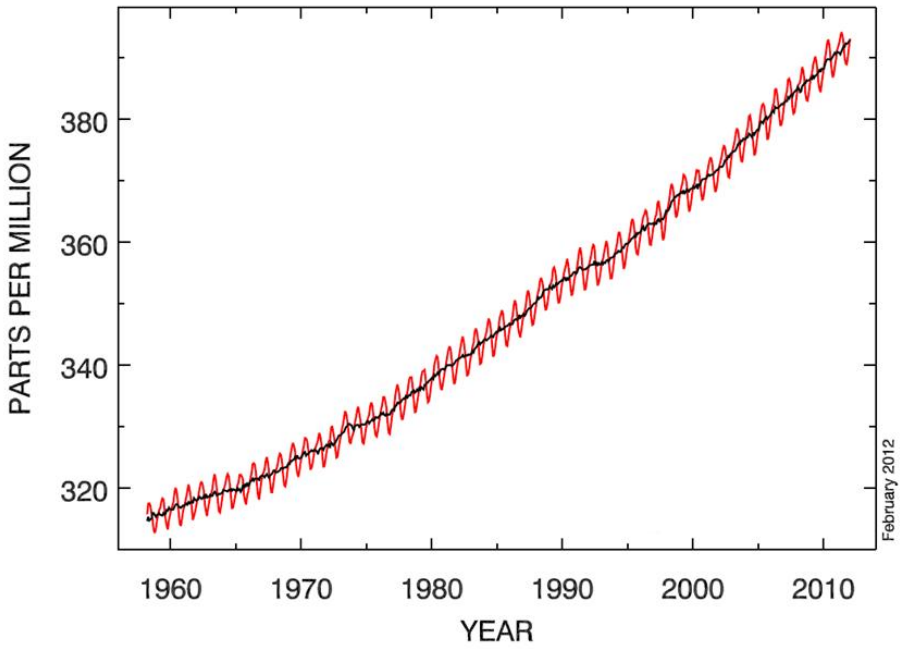

Figure 1.1. The increase of mean atmospheric carbon dioxide (CO_2) at Mauna Loa Observatory, Hawaii. The CO_2 data constitutes the longest record of direct measurements of CO_2 in the atmosphere. The red line represents monthly mean values. The black curve represents seasonally corrected data. (Source: http://www.esrl.noaa.gov/gmd/ccgg/trends/)

If CO_2 concentrations continue to increase in line with the current trend (Fig. 1.1) then it is predicted that atmospheric [CO_2] will increase by about 50% on the current concentrations by 2030 (McNeil and Matear 2008), exceeding 1000 ppm by 2100 (Raven et al. 2005).

In general, the increase of CO_2 concentration in the atmosphere is caused by an imbalance of CO_2 exchange (input and output), for example, through the burning of fossil fuels, land-use changes and the industrial production of cement (Orr et al. 2005). Although CO_2 is also absorbed by terrestrial plants (Metaphytes), more than 40% of atmospheric CO_2 enters the oceans (Falkowski et al. 2000). There are several pathways in the carbon cycle which are particularly important for this process (Fig. 1.2). Figures for the global carbon cycle (Fig.1.2) show that there is a net flux of CO_2 into the oceans of 1.7×10^{15} g C y^{-1}.

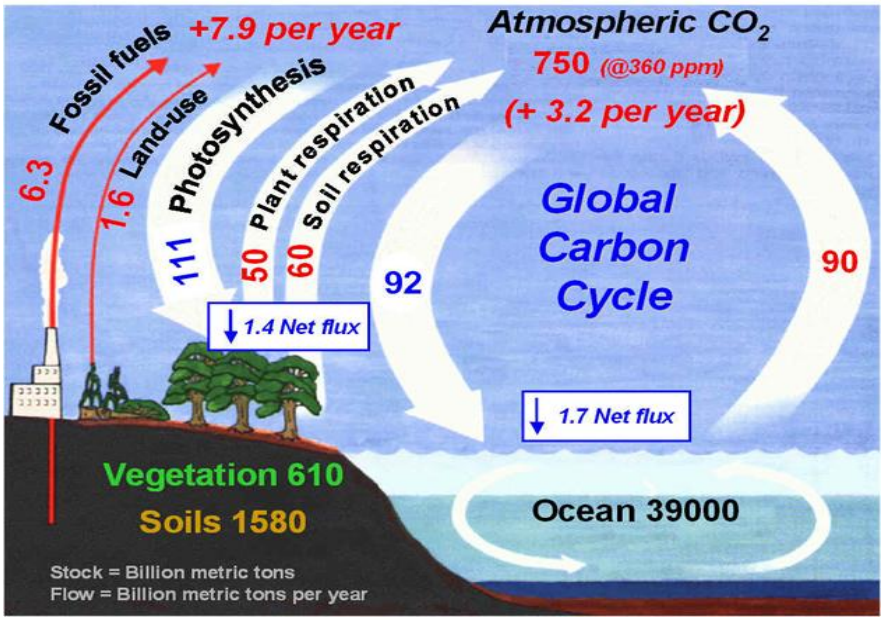

Figure 1.2. The global carbon cycle pathways, pools and fluxes. About 3.2×10^{15} g C y^{-1} of world production CO_2 enters the atmosphere. (Source: Kling et al. 2003).

Although CO_2 is not a chemically reactive gas in the atmosphere, its dissolution in seawater leads to several complex chemical, physical and biological changes (Raven et al. 2005). The initial reaction of CO_2 and water (H_2O) forms carbonic acid (H_2CO_3) which, in turn, forms bicarbonate (HCO_3^-) and, by dissociating H^+ ions, drives pH to lower values (Raven et al. 2005) (Fig. 1.3).

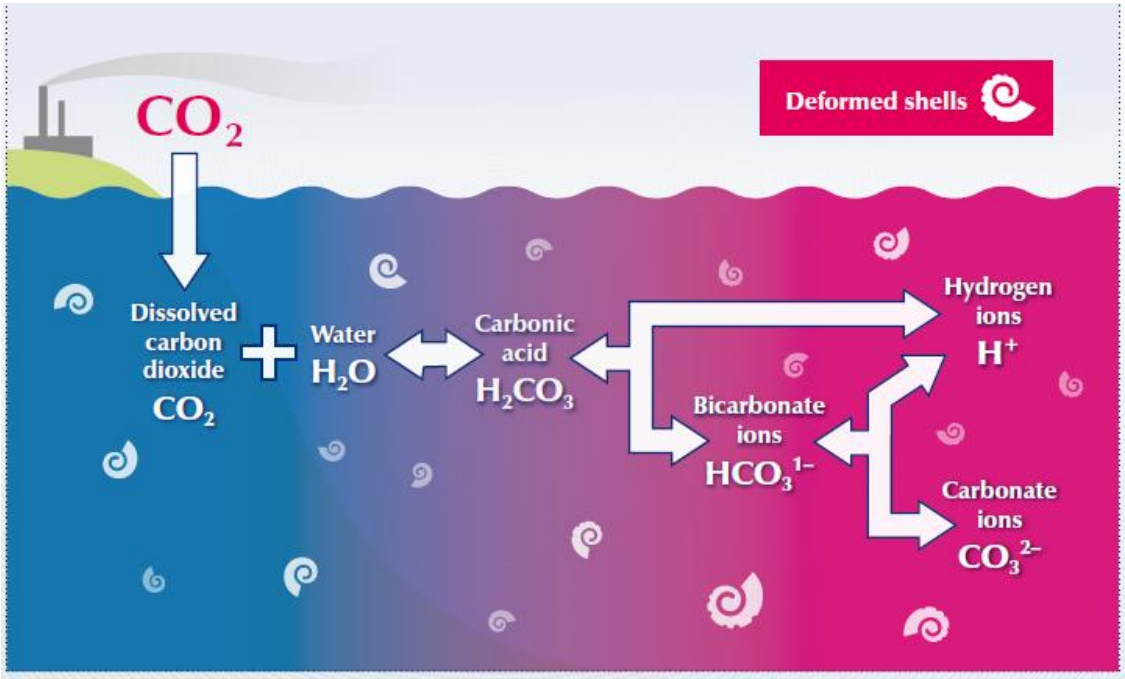

Figure 1.3. Increased CO_2 in the atmosphere is being taken up by the ocean resulting in the sea water becoming less alkaline. As CO_2 dissolves in seawater, carbonate ions neutralise free hydrogen ions, causing carbonate levels to drop. (Source: University of Maryland in Andiman et al. 2012).

Therefore, increasing [CO_2] ultimately leads to an increase in the concentration of carbonic acid (H_2CO_3) which will dissolve rapidly to bicarbonate (HCO_3^-), H^+ and carbonate ions (CO_3^{2-}) (Caldeira and Wickett 2003, Fabry et al. 2008). At present, ocean pH has been reduced by 0.1 units from ambient conditions, equivalent to a 30% increase in hydrogen ions (Sabine et al. 2004, Raven et al. 2005, Dashfield et al. 2008). Future predictions suggest that increased CO_2 levels will reduce seawater pH by 0.3 – 0.5 units

by 2100 (Caldeira and Wickett 2003, 2005) and 0.77 units by 2300s' if CO_2 release into the atmosphere continues to rise according to 'business as usual' scenario (IPCC 2007). In addition, Feely et al. (2008) have shown that corrosive water with lower aragonite saturation (Ω_{ara}) from the deep sea upwells to the continental shelf and coastal waters where it can affect intertidal regions. These coastal waters may already experience low aragonite saturation states (Feely et al. 2008, Wootton et al. 2008).

As well as altering the pH of the oceans, increased CO_2 has been suggested to be responsible for a 55% increase in radiative forcing (change in net solar radiance between different layers of the atmosphere in unit Wm^{-2}) (Jenkinson et al. 1991) and subsequent increases in air temperatures (McMullen 2009). Increased radiative forcing ultimately alters the incoming and outgoing energy balance between the Earth and its atmospheric system. When radiative forcing is positive, the Earth's surface tends to warm (IPCC 2007). In 2005 the mean value of radiative forcing was +1.6 indicating a rate of warming of +1.6 Wm^{-2} (IPCC 2007), which could increase to 8.0 Wm^{-2} by 2100 (Sokolov et al. 2009) (Fig. 1.4a).

Jenkinson et al. (1991) also proposed that increased air temperatures would increase emissions of CO_2 gas from soils, through an increase in the turnover of organic matter (Jenkinson et al. 1991). Warming caused by increased atmospheric CO_2 levels will also increase ocean temperatures (Kleypas et al. 2006, Allison et al. 2008). At present, seawater temperature has warmed by approximately 0.7 ºC compared with the pre-industrial era (Kleypas et al. 2006) and is predicted to rise by a further 2 ºC by 2050 (Huesemann 2006, Guldberg et al. 2007).

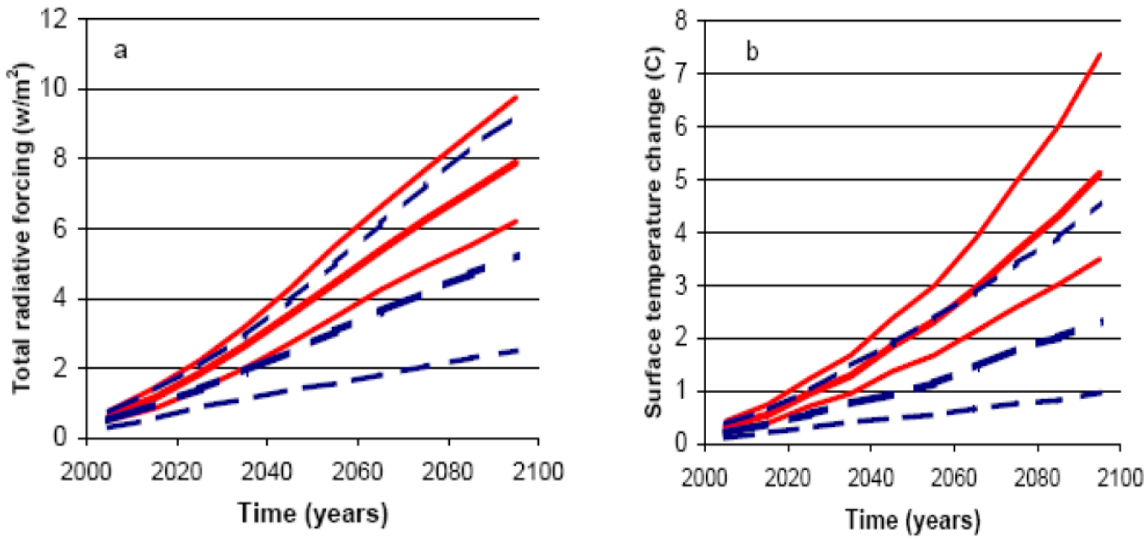

Figure 1.4. Projected changes in climatic parameters between 2000 and 2100: a) decadal mean radiative force; b) sea surface temperature change. Red solid lines show median, 5% and 95% percentile values presented in the study by Sokolov et al. (2009) and dashed blue lines show median, 5% and 95% percentile predicted by Webster et al. (2003). (Source: Sokolov et al. 2009).

Jacobson (2005) developed a model with uncertainty input parameters that predicted an increase of CO_2 to 375 ppmv there would be an increase in temperature of 3 ºC. However this model has been corrected by Sokolov et al. (2009) with certainty input parameters (e.g. world volcanic eruptions and gross domestic product (DGP) growth) which predicts that sea surface temperature (SST) will increase between 3.5 and 7.4 ºC with a median increase of 5.1 ºC by 2100 (Fig. 1.4b).

Impact of Ocean Acidification and Temperature on Calcifying Organisms

Organisms that rely on calcified structures are thought to be particularly vulnerable to OA (Kleypas et al. 1999, Orr et al. 2005, Kleypas et al. 2006, Hendriks et al. 2010, Kroeker et al. 2010, Hale et al. 2011). The increased acidity of seawater reduces

calcium carbonate saturation (Ω) and carbonate ion concentration (CO_3^{2-}) according to the following reaction:

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \qquad (1)$$

Therefore, increased H^+ causes carbonate ions (CO_3^{2-}) to react and form bicarbonate ions (HCO_3^-). Decreases in carbonate ions will decrease the precipitation of $CaCO_3$ and the carbonate saturation state calculated as:

$$\Omega = \frac{[Ca^{2+}]_{sw} \text{ x } [CO_3^{2-}]_{sw}}{[Ca^{2+}]_{sat} \text{ x } [CO_3^{2-}]_{sat}} \qquad (2)$$

In which the solution of saturation state of $[Ca^{2+}]$ and $[CO_3^{2-}]$ is calculated as:

$$k_{sp}^{'} = [Ca^{2+}]_{sat} \text{ x } [CO_3^{2-}]_{sat} \qquad (3)$$

where $k_{sp}^{'}$ is the stoichiometric solubility product for a particular mineral phase of $CaCO_3$ (i.e. calcite, aragonite, or high-magnesium calcite) (Kleypas et al. 1999, McNeil and Matear 2007, Gazeau et al. 2007, Boesch et al 2010). Reduced calcium carbonate saturation can make the process of calcification more difficult (Sigler et al. 2008). Also with a carbonate saturation state less than 1 ($\Omega < 1$) $CaCO_3$ will start to dissolve, whereas at a value greater than 1 ($\Omega > 1$) $CaCO_3$ will spontaneously precipitate. Calcium carbonate is represented in seawater in two forms, aragonite and calcite (Raven et al. 2005, Gazeau et al. 2007), which are used in the construction of skeletons and shells in all calcifying organisms (e.g. Bowen and Tang 1996, Thompson et al. 2000,

Schmidt 2005, Addadi et al. 2006, Gazeau et al. 2007). Both are soluble in acidified seawater and, although calcite is 35% less soluble than aragonite, under acidic conditions both minerals displayed no consistent pattern of dissolution rate; in other words, calcite does not have a lower dissolution rate than aragonite when exposed to acidified seawater at pH 7.4 (McClintock et al. 2009).

The alteration of CO_2 in seawater has been shown to cause a reduction in calcification rates, induce malformations, and impair growth of shells (Riebesell et al. 2000, O'Donnel et al. 2009, Nienhuis et al. 2010, Findlay et al. 2010a and b, Gaylord et al. 2011, Pistevos et al. 2011). Coccolithophores exhibited a reduction in the mineral content of their shells by about 10 - 30% when exposed to low pH condition (Muller et al. 2010) and reduced calcification rates by 66% when exposed to a CO_2 level three times that of pre-industrial levels (Zondervan et al. 2001). Coralline algae also showed an increased dissolution rate in seawater enriched by CO_2 (pH 7.7) (Martin et al. 2008). The destruction of the protective outer shell layer (periostracum) in gastropods has also been found to occur in the top shell *Osilinus turbinata* exposed to pH 7.2 and in the black foot limpet *Pattela caerulea* exposed to pH 7.4 in the volcanic CO_2 vents (Hall-Spencer et al. 2008). Pacific Oyster (*Crassostrea gigas*) and edible mussel (*Mytilus edulis*) also reduced calcification between 10 and 25% after incubation in a mesocosm for a month under CO_2 levels ranging between 700 to 2000 ppmv (Gazeau et al. 2007). It has also been found that reef building organisms reduce calcification between 11-46% (Langdon 2002). However, calcification rate can also increase under extreme CO_2 conditions as demonstrated in the mussel *M. edulis* in the Kiel Fjord (Thomsen et al. 2010).

Effects of OA on traits other than calcified structures have also been found in calcifying organisms. Larvae of the mussel *M. californianus* showed lower tissue growth when it was exposed to CO_2 of 900 ppmv for 8 d (Gaylord et al. 2011). The brittle star *Amphiura filiformis* showed muscle degeneration after being exposed to pH 6.8 in 40 d (Wood et al. 2008). Even more exposure to pH 7.7 after 8 d caused 50% mortality of a brittle star larvae *Ophiothrix fragilis* and after 25 d caused 100% mortality (Dupont et al. 2008). However, adult individuals of the velvet swimming crab *Necora puber* reached 100% mortality within 4 – 5 d when expose to 6040 ppm CO_2 (pH 7.1) as a result of the ability to compensate for the changes in haemolymph pH (Spicer et al. 2007), contrasting with adult *N. puber* that can survive 30 d expose to pH 6.69 or 21500 ppmv CO_2 (Small et al. 2010). An indirect effect of OA on the common periwinkle *Littorina littorea* was also reported by Bibby et al (2007); when exposed to low pH conditions (pH 6.6 for 15 d) this species had its ability to exhibit induced shell defences (shell thickness) disrupted.

As well there being clear evidence that OA affects calcifying organisms, temperature has also been shown to affect the process of calcification in marine organisms and can cause dramatic changes in shell plasticity. For example, Trussell and Smith (2000) demonstrated that shell thickness of the snail *Littorina obtusata* was positively correlated with temperature. The phenotypic variation of shell morphology in different temperatures may be related to the chemical properties of the different calcium carbonate shell materials (aragonite and calcite). For example decreased calcium carbonate availability at low temperatures is caused by increased solubility that makes shell deposition more difficult (Graus 1974, Vermeij 1978, Trussell and Etter 2001). Irie and Fischer (2009) also reported that temperature influenced shell size in the cowry

Monetaria annulus, with this species demonstrating a reduced size during warm season compared with the cold season. In contrast, shell thickness in *Cypraea annulus* increased linearly with increasing temperature (Irie 2006), and in the marine mussel *M. edulis* (Nielsen 1988) increased temperature resulted in increased shell size (length).

Given that increased temperature enhances calcium carbonate precipitation (Kleypas et al. 1999) the physiological mechanism for reduced calcification rates at low temperatures may be linked to reduced metabolic rates and energy production (Whiteley and Faulkner 2005). In addition, increase temperature can also create systemic hypoxia, leading to an increase in ventilation rate and reduce energy production (Pörtner 2001). An overall, increase in temperature may disrupt energy production (Chapter 2 of this thesis) and a concomitant increases in pCO_2 would lead to the disruption in oxygen transport proteins (Seibel and Walsh 2003), which in turn would disrupt calcification.

Impact of Ocean Acidification and Temperature on Ecosystems

As well as affecting single organisms, it is now clear that OA (and temperature) can have significant effects at higher ecological levels. For example, OA has serious negative effects on several fundamental biogeochemical and ecosystem processes including key elemental cycles and biodiversity (Widdicombe and Spicer 2008, Blackford 2010) and nutrient fluxes (Widdicombe and Needham 2007). These processes included decalcification of planktonic organisms, carbon and nutrient assimilation, primary production and acid-base balance, all of which potentially affect the composition, size structure and successional processes of ecosystems and may lead to a modification of energy flow and resources (Blackford 2010). Although Blackford and Gilbert (2007) suggested that the alteration of ecological function by OA is still unclear

with the potential for populations acclimating to altered ocean carbonate chemistry, Hall-Spencer et al. (2008) have showed that a shallow water benthic community in the vicinity of natural CO_2 seepage might change due to the vulnerability of important groups of organisms, with no evidence for adaptation.

In addition, raised ocean temperatures (IPCC 2007, Sokolov et al 2009) may disrupt the stratification of the upper-ocean, the availability of nutrients for phytoplankton growth and reduce primary production (Behrenfeld et al. 2006). Several studies have also revealed that climate warming has reduced ocean productivity in the past decade with potential implications for marine food webs (Kleypas et al. 2006, Behrenfeld et al. 2006, Guinotte et al. 2006, Guinotte and Fabry 2008). In addition, climate change has been predicted to cause phenological changes (Koeller et al. 2009) and shifts in species ranges and distributional patterns (Easterling et al. 2000, Roy et al. 2001, Thomas et al. 2004, Perry et al. 2005, Sekercioglu et al. 2008)

Impacts of Ocean Acidification and Temperature on Physiological Functions

Several studies have revealed negative effects of climate change and OA on the physiological function of various marine calcifying taxa (e.g. Pörtner et al. 2000, 2004, 2005, Michaelidis et al. 2005, Wood et al. 2008, 2010, Small et al. 2010). For example increased levels of pCO_2 in seawater leads to hypercapnia that causes metabolic rate depression in *Sipunculus nudus* (Reipschläger and Pörtner 1996, Pörtner et al. 1998) and brings about 31% metabolic rate reduction in jumbo squid, *Dosidicus gigas* (Rosa and Seibel 2008). Increased CO_2 levels has also been shown to decrease aerobic scope (Metzger et al. 2007, Walther et al. 2010, 2011), increase induced acidosis in extracellular fluid (Burnett 1997, Miles et al. 2006, Pane and Barry 2007, Spicer et al.

2007). Metabolic acidosis can also reduce protein synthesis, increase respiratory stress and induce metabolic depression (Seibel and Walsh 2002) which may be lethal to organisms unable to compensate for haemolymph acidosis (Burnett 1997, Spicer et al. 2007). Such lethal effects of high pCO_2 were shown in *Necora puber* exposed to highest levels of hypercapnia at 6040 ppm (pH 6.74) (Spicer et al. 2007).

Lannig et al. (2010) reported that OA affects energy metabolism in the oyster *Crassostrea gigas*. Under high CO_2 conditions a substantial reduction of ATP was found in gill tissue following an increase of succinate at the same gill tissue. In addition Cumming et al. (2011) demonstrated that high CO_2 (735 ppm equal to pH 7.78) caused low expression of chitin synthase (a key enzyme involved in synthesis of bivalve shells) indicating that the bivalve *Laternula elliptica* is working harder to calcify in acidified seawater conditions.

Rates of calcification under low pH are not only determined by environmental conditions (e.g. Ω_{ara} and Ω_{calc}) but are also determined by physiological capacity and functional performance in terms of the regulation of extracellular fluid acid-base balance and maintenance of metabolic homeostasis (Michaelidis et al. 2005, Cumming et al. 2011, Findlay et al. 2011, Gaylord et al. 2011, Whiteley 2011). The interaction between high pCO_2 and elevated temperature could lead to oxygen limitations (Pörtner and Farrell 2008, Pörtner 2010) which will add to the metabolic acidosis caused by lowering of the environmental pH which is known to disrupt oxygen transport and reduce energy production (Seibel and Walsh 2003). Such effects could be critical for intertidal organisms that may experience hypoxia during emersion – an effect that may occur concomitantly with high temperatures (Sokolova and Pörtner 2003, Larade and

Storey 2009). Species populations are likely to have different physiological strategies e.g. shifting metabolic pathways and decreasing energy budget allocation (Sokolova and Pörtner 2001, 2003, Findlay et al. 2010a and b), that would allow them to cope with local environmental conditions such as thermal stress. Such metabolic and energetic shifts are likely to have associated energetic costs. For example, the alteration of metabolism under anaerobic scope leads to a reduction in energy production and mitochondrial density (Pörtner 2002). Under such conditions, time-limited, passive survival is supported by increased synthesis of heat shock proteins (Hsp) as a cellular defence mechanism (Feder and Hofmann 1999) although prolonged exposure is likely to cause lethal effects (Gehring and Wehner 1995).

Increased temperature has also been shown to affect metabolic rates and energy metabolism of marine gastropods (Sokolova and Pörtner 2001, 2003), cause cardiac failure and reduce thermal windows (Stillman 2003, Tomanek and Helmut 2002, Denny et al. 2006). Pörtner (2001, 2002) and Frederich and Pörtner (2000) demonstrated that increasing temperature caused increased ventilation and heart rates of aerobic capacity. Progressive increases in temperature may cause excessive oxygen demand and decrease aerobic capacity (Pörtner 2001). In addition, temperature can also affect biochemical reactions for physiological homeostatis (Hochachka and Somero 1973, Prosser and Nelson 1981, Hoffman and Dionne 1983).

Initially, organisms could adjust to such temperature extremes by shifting their thermal tolerance or narrowing thermal windows via the adjustment of mitochondrial densities (Pörtner 2002). However, Pörtner (2002) also suggested that the crucial process in

shifting thermal tolerance is that the organisms should face unidirectional (higher or lower) thermal conditions which may disrupt molecular function.

As an alternative to coping with extreme temperature conditions, animals may also shift metabolic scope under anaerobic conditions or shift to hypometabolic physiology (Storey and Storey 2004). This mechanism requires shifting metabolic pathways and biochemical mechanisms for regulatory reversible transition to and from anaerobic physiology (Storey and Storey 2004, Hochachka and Somero 2005).

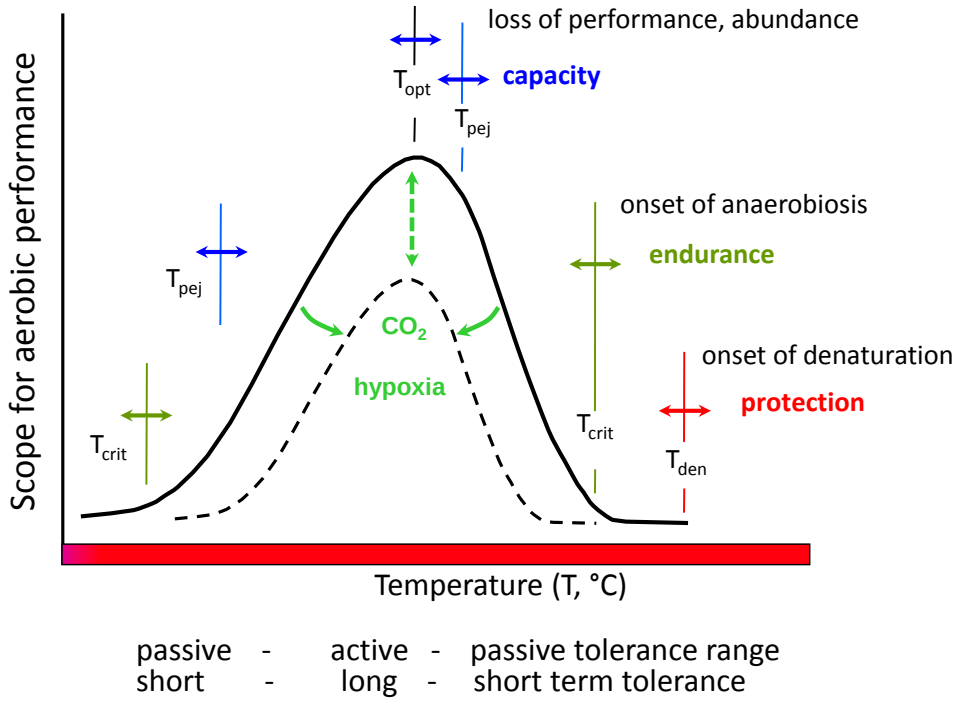

Figure 1.5. A model indicating the hierarchies of functional limitation (beyond pejus temperatures, T_{pej}), hypoxemia, anaerobic metabolism and protection through metabolic depression (below and beyond critical temperatures, T_{crit}) and denaturation as well as repair (beyond denaturation temperatures, T_{den}), (Source: Pörtner and Farrell 2008).

Pörtner and Farrell (2008) suggested that under the optimum temperature conditions (T_{opt}), aerobic performance was high but an increase or decrease in temperature beyond this optimum (T_{pej}) will lead to lowering aerobic performance. Further progress on to

the critical temperature (T_{crit}) will lead to a loss in aerobic scope and a transition to an anaerobic mode of mitochondrial metabolism (Fig. 1.5). In this stage a progressive insufficiency of cellular energy levels occurs. At more extreme temperatures (T_{den}), only time limited passive survival is supported by anaerobic metabolism or the protection of molecular functions by heat shock proteins and antioxidative defence (Pörtner 2002, Pörtner and Farrell 2008).

Pörtner and Knust (2007) have also suggested that increased temperature causes a mismatch between the demand for oxygen and the capacity of oxygen supply to tissues. Such a constraint could affect higher functions such as muscular activity, behaviour, growth, and reproduction, ultimately leading to changes in species biogeography (abundance, occupancy, position of range edges and size of the geographical range of distribution).

It is highly likely that marine organisms will be subject to the combined effects of ocean acidification and temperature and recent studies have addressed these combined effects on physiological function in various organisms for example in jumbo squid *Dosidicus gigas* (Rosa and Seibel 2008), in the decapod *Metapenaeus joyneri* (Dissnayake and Ishimatsu 2011) and in brittle star *Ophiura ophiura* (Wood et al. 2010). Rosa and Seibel (2008) found that metabolic rate of jumbo squid *D. gigas* was depressed by 31% and activity levels by 45% under combined high CO_2 and temperature. Dissanayake and Ishimatsu (2011) demonstrated that in the decapod *M. joyneri*, reduced aerobic scope and swimming ability was reduced by 30% under high CO_2 (1000 ppm = pH 6.9) and temperature (20 ˚C). Even though Wood et al. (2010) found increased in brittle star *O. ophiura* under interaction of high CO_2 and temperature, there was an apparent trade off

with increased arm degeneration. Also, increased metabolic rates in oysters *C. gigas* exposed to high CO_2 and temperature (25 ˚C) was associated with high accumulations of metabolic-end products (Lanning et al. 2010). Finally, Donohue et al. (in press) also found synergistic effects of OA and temperature on metabolic activity and heat tolerance in the intertidal crab *Porcellana platycheles*. These parameters were positively affected by temperature but exoskeleton calcification was negatively affected by high CO_2 of 2707 ppm. Clearly, more studies are needed if we are to understand more fully the synergistic effects of ocean acidification and warming on marine organisms.

Geographical variation in the impacts of Ocean Acidification and Climate Change

Impacts of ocean acidification are also suggested to vary across geographical and latitudinal boundaries (Walther et al. 2010, 2011, Findlay et al. 2010a, b). These differences may be linked to variations in interactive effects between CO_2 at each location. For example, annual sea surface temperatures (SSTs) and land surface temperatures (LSTs) in lower latitudes (southern ranges) are significantly different to those observed at higher latitudes (northern ranges) (e.g. Rastrick and Whiteley 2011, Whiteley et al. 2011 see also Whiteley et al. 1997). Such differences in responses to high CO_2 by organisms from different latitudes have been sugessted in previous studies (e.g. Findlay et al. 2010a, b, Walther et al. 2010, 2011, Cumming et al. 2011). These studies are limited to comparisons between two populations.

Studies of the effects of elevated temperature caused by climate change in species populations and in different species at different latitudinal gradients have also been documented in various taxa (e.g. Sokolova and Pörtner 2001, 2003, Stillman 2002, 2003, Compton et al. 2007, Pörtner and Knust 2007, Rastrick and Whiteley 2011,

Whiteley et al. 2011). Morphological variations in various taxa of calcified marine organisms were also found, for example, in the shallow water gastropod (Graus 1974), cowry the *Cypraea annulus* (Irie 2006), and in the zebra coral *Oulastrea crispata* (Chen et al. 2011). In general they found that morphological variations of these organisms were mainly caused by a change in $CaCO_3$ saturation state linked to temperature, however, causative factor such as salinity should also be considered.

Chemical effects of OA linked to carbonate saturation state (Ω) are likely to be linked to local seawater temperature regimes as the solubility of carbonate is temperature-dependent (Hill et al. 1999). Since the availability of carbonate depends on the saturation state of aragonite and calcite, which is temperature-dependent (see equations 2 and 3) (Kleypas et al. 1999, Azetsu-Scott et al. 2010), it does vary across latitudinal gradients (Bates et al. 2009, Yamamoto et al. 2009, Fabry et al. 2009, see also Riegl 2003 and Orr et al. 2005).

Burton and Walter (1987) showed that the precipitation of aragonite increased rapidly with temperature. Aragonite precipitated four times faster than calcite at 25 and 37 °C respectively, whilst at 5 °C precipitation of aragonite decreased relative to calcite. This trend has also been shown in the ratio of calcite and aragonite in the shell of mussel *M. edulis*, which increased with decreasing water temperature along latitudinal gradient (Lowenstam 1954, Dodd 1963). Variation in biogenic calcification across latitudinal gradients also occurs with most calcified organisms in northern latitudes tending to be thinner shelled due to low temperature (Kuklinski and Taylor 2009, Trussell and Smith 2000, Findlay et al. 2010a). Carter (1980) revealed that such mineralogical differences may have functional consequences as calcite is softer and less strong than aragonite.

Increased dissolution rates may also vary along latitudinal gradients. For example Findlay et al. (2010a and b) showed that a population of the barnacle *S. balanoides* living at the northern range limit for this species was more sensitive to high CO_2 conditions, suggesting that the potential risk of climate change is likely to be greater in populations living at higher latitudes. Latitudinal gradients in pH, $[CO_3^{2-}]$ and Ω_{ara} and Ω_{calc} have been suggested to exist in both the Northern and Southern hemisphere (Orr et al 2005).

THESIS AIMS

Background

Our understanding of the potential impacts of ocean acidification and climate change in marine ecosystems is still limited. In particular the interactive effects of increased acidity and seawater temperature are poorly understood. Low saturation levels of calcium carbonate in seawater could lead to the impairment of growth in calcified organisms due to decalcification. Interference to calcification rates may disrupt the production of defences but fundamental differences in physiology among species may lead to different impacts of climate change in shelled invertebrates. In addition, our knowledge of the effects of ocean acidification and temperature at physiological and biochemical levels are also still limited, as are variations in such responses among populations.

The overall aim of this thesis is to address some of these gaps in our knowledge of the interactive effects of ocean acidification and warming predicted to occur by 2100 (Caldeira and Wickett 2003, Orr et al. 2005, IPCC 2007) on the physiology and morphology of calcified organisms. The common periwinkle *Littorina littorea* has been

chosen as a model for assessing these gaps since recent work undertaken on this species (Bibby et al. 2007) has shown a complex pattern of physiological, morphological and behavioural responses to ocean acidification including increasing metabolic rates under low pH. The first objective of this thesis was to investigate whether such disruptions were associated with whole-animal physiological and biochemical responses, as well as to explore the link between the contexts of global climate change (GCC) in organism's physiological functions and morphological traits. The second objective of this thesis was to investigate whether different populations living along a natural thermal latitudinal gradient might respond differently in terms of their shell growth, physiology, and metabolic organization (metabolomics) when exposed to ocean acidification and elevated temperature conditions. Six populations of marine periwinkle *L. littorea* across its distributional range along the coast of the Western Atlantic in Europe were used in this study. My approach centred on a series of laboratory experiments using a mesocosm unit set up based on a modification version of the equilibration flow-through systems used by Widdicombe and Needham (2007), Dashfield et al. (2008), and Widdicombe et al. (2009).

The aims of individual chapters were as follows:

Chapter 2

The main aims of this chapter were to characterise the physiological (O_2 uptake) and biochemical (concentration determination of adenosine triphosphate – ATP, adenosine diphosphate – ADP, adenosine monophosphate – AMP, succinate and lactate include adenosine nucleotide index of adenylate energy charge – AEC and total adenylate nucleotide – TAN) responses of a single species population of *Littorina littorea* exposed to different level of pH and temperature and the combination of these two variables.

These variables were choosen based on previous studies conducted by Sokolova and Portner (2001, 2003) to describe physiological basis of intertidal gastropods in response to environmental stressor (e.g. temperature). Four treatment conditions were used, two pHs (8.0 pH unit = current condition and 7.6 pH unit = forecasted pH for 2100) and two temperatures (15 ºC = room temperature set as mean summer temperature and 20 ºC = forecasted temperature for 2100) for 30 days in a fully factorial design. Physiological measurements were undertaken at the end of exposure time.

Chapter 3

The aim of this chapter was to investigate how elevated CO_2 and temperature as well as the combination of these factors may affect phenotypic plasticity of *L. littorea*, as a fundamental mechanism in which organisms can alter their morphological traits, physiology, life-history or behaviour changes. This chapter used individuals from the same trials as in Chapter 2 in which morphometric measurements were carried out before and at the end of the exposure to high CO_2/temperature. The investigations will provide information on whether there were knock-on effects through any effect on shell shape on water loss during emersion.

Chapter 4

At present, there is a lack of understanding of how different populations of marine intertidal animals might vary in response morphologically to ocean acidification. Hence, the main aim of this chapter was to compare the shell growth responses of populations of *L. Littorea* from different latitudes exposed to ocean acidification. Animals were exposed to two different pH levels (8.0 unit = current condition and 7.6 unit = forecast

pH for 2100) for 14 days and morphometric measurement were taken before and at the end of exposure period.

Chapter 5

The main aim of this chapter was to characterise the level of physiological variations of populations of *L. littorea* from six different latitudinal gradients. Snails were exposed to ocean acidification conditions followed by a short-term exposure to different air temperatures that mimicked the conditions snails will experience in future when in intertidal zone exposed to air temperature during emersion (low tide). The characterization of physiological variations among populations was conducted through the measurement of rates of O_2 uptake and metabolic fingerprints. Snails were exposed to two different pH levels (8.0 unit = current condition and 7.6 pH unit = forecast pH for 2100) at 15 °C. Prior to measurements of O_2 uptake snails were exposed to one of five different air temperatures (15, 20, 25, 30, and 35 °C) for three hours at the same [CO_2] of acclimation in seawater (either 380 or 1000 ppmv). Tissue extractions for the determination of metabolic fingerprint were taken immediately after metabolic rates were measured. Metabolic analysis was through proton nuclear magnetic resonance (P-NMR) conducted at metabolomic facilities of School of Bioscience, University of Birmingham, UK.

Chapter 6

This final chapter presents a critical evaluation and summary of these experimental observations. Here, the discussion of the importance factors that has to be considered in developing further research on the impact of ocean acidification and elevated temperature on marine organisms. I also produce an analysis that focuses on

vulnerability levels among populations living along latitudinal gradients, based on data on growth and metabolism from Chapters 4 and 5.

STUDY SPECIES

Littorina littorea (Linnaeus, 1758) (Figure 1.6.) commonly named the edible winkle, is a gastropod snail member of the family Littorinidae (Cummins et al. 2002). Its geographical distribution ranges from the White Sea in the North to Southern Portugal in the eastern Atlantic and from Labrador to New Jersey in the western Atlantic (Reid 1996). This is the largest littorinid species in the UK, reaching up to 25 – 30 mm (Reid 1996, Gibson et al. 2001). Jackson (2005) reported that its life span can be between 5–10 years and it can reach a maximum size of 52 mm. Male and females mature in 2–3 years at a size of 10–12 mm with growth rates of 0.065-0.097 mm d^{-1}. The body whorl (width) is about 80–85% and aperture width is about 60–70% of the total height (Fretter and Graham 1962). The shell is dark brown in colour, appearing almost black when wet but can occasionally occur in pale cream or orange (Cummins et al 2002). The mature male winkle is easily recognised through the presence of a penis on the right hand side, while the female can be distinguished by a whitish ovipositor in the equivalent area (Cummins et al 2002).

In general, *L. littorea* is found on rocky shores, from high water neap to low water spring tide levels (Moore 1937). Occasionally it can also occur in the sub-littoral to depths of approximately 60m (Fretter and Graham 1962). The vertical level at which periwinkles may be found on the shore is variable and depends on factors such as exposure and weed cover (Lubchenco 1983). Food scarcity appears to set the upper limit to the vertical distribution of *L. littorea* (Yamada 1987). While it is found mainly

on semi-exposed to sheltered coasts, it is also tolerant of estuarine conditions and high exposure (Williams 1964, Fish 1972, Boulding and Van Alstyne 1993). *L. littorea* is also resistant to elevated temperature in air showing little change in oxygen usage over wide range of thermal regimes (Newell and Northcroft 1967, McMahon and Russell-Hunter 1973). Heat coma in air was at 32 °C and in water at 31 °C and heat death at 42 °C in air 40 °C in water (Sandison 1967).

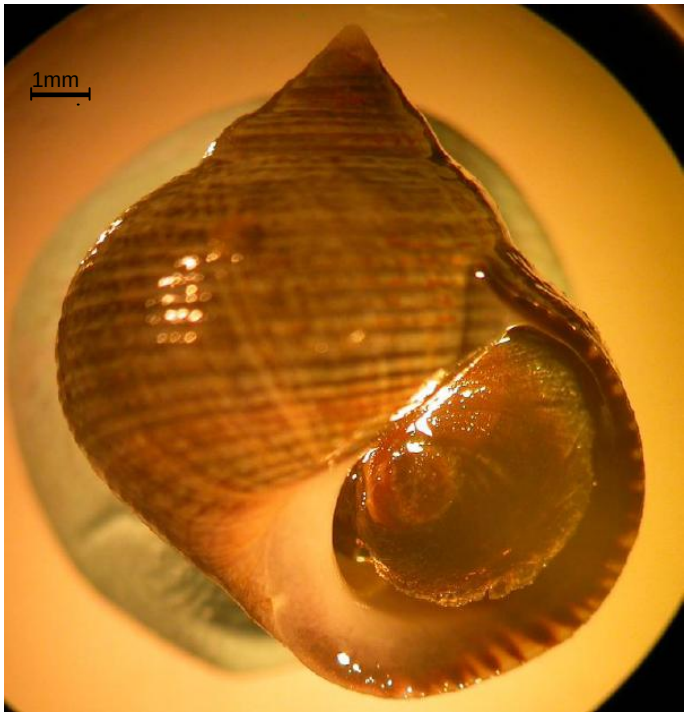

Figure 1.6. Edible periwinkle *Littorina littorea* (Linnaeus 1758). Scale bar = 1 mm.

CHAPTER 2

Exposure to elevated temperature and pCO_2 reduces respiration rate and energy status in the periwinkle *Littorina littorea*

SUMMARY

In the future, marine organisms will face the challenge of coping with multiple environmental changes associated with increased levels of atmospheric carbon dioxide partial pressure ($pCO_{2)}$, such as ocean warming and acidification. In order to predict how organisms may, or may not, meet these challenges an in-depth understanding of the physiological and biochemical mechanisms underpinning organismal responses to climate change is needed. Here, the effects of elevated pCO_2 temperature on the whole-organism and cellular physiology of the edible periwinkle *Littorina littorea* were investigated. Metabolic rates (measured as respiration rates), adenylate energy nucleotide concentrations and indexes, and end-product metabolite concentrations were measured. Compared with control conditions, snails decreased their respiration rate by 31% in response to elevated pCO_2 and by 15% in response to a combination of increased pCO_2 temperature. Decreased respiration rates were associated with metabolic depression and an increase in end-product metabolites in acidified treatments, indicating an increased reliance on anaerobic metabolism. There was also an interactive effect of elevated pCO_2 and temperature on total adenylate nucleotides, which was apparently compensated for by the maintenance of adenylate energy charge probably *via* AMP deaminase activity. Our findings suggest that marine intertidal organisms are likely to exhibit complex physiological responses to future environmental drivers, with likely negative effects on growth, population dynamics, and ultimately ecosystem processes.

INTRODUCTION

A significant increase in the burning of fossil fuels, industrial emissions and levels of deforestation over the past two hundred years have resulted in an increase in atmospheric pCO_2 (Petit et al. 1999, Augustin et al. 2004, Siegenthaler et al. 2005). This increase is predicted to have profound implications for the global climate. In addition, around 30% of the CO_2 emitted in the past two hundred and fifty years has been taken up by the oceans, leading to substantial changes to the physicochemical conditions of seawater (Caldeira and Wickett 2003, Feely et al. 2004, Orr et al. 2005), such as significant alterations to pH, $[CO_3^{2-}]$ (Feely et al. 2004), and aragonite and calcite saturation states (Ω_{ara} and Ω_{cal} respectively) (Morse et al. 2006). This phenomenon has been defined as ocean acidification (OA) (Caldeira and Wickett 2003). Current models predict that this situation will worsen, with an increase of atmospheric pCO_2 to 730-1020 ppmv by 2100 (IPCC 2007) resulting in a further reduction in oceanic pH of 0.3-0.4 with a further consequent drop in $[CO_3^{2-}]$, Ω_{ara} and Ω_{cal} (Caldeira and Wickett 2005, IPCC 2007, Gangstø et al. 2008). At the same time, global surface ocean temperatures are predicted to increase globally by 3-5 ºC over the same period (IPCC 2007, Sokolov et al. 2009).

The predicted changes to seawater pCO_2 temperature are expected to exert a negative impact on the developmental, ecological and physiological functions of marine organisms (e.g. Bibby et al. 2007, Rosa and Seibel 2008, Dupont and Thorndyke 2009, Todgham and Hofmann 2009, Findlay et al. 2009a, O'Donnell et al. 2010, Small et al. 2010, Hale et al. 2011, Pistevos et al. 2011, Whiteley 2011). In particular, exposure to OA has been shown to alter metabolic rates (e.g. Rosa and Seibel 2008), haemolymph pO_2 levels (Metzger et al. 2007) and net calcification (Findlay et al. 2009b, Ries et al.

2009). In general, it is suggested that some marine animals may respond to exposure to elevated pCO_2 by reducing their metabolic rates (e.g. Pörtner et al. 1998, Langenbuch and Pörtner 2002, Seibel and Walsh 2003, Rosa and Seibel 2008, Small et al. 2010), although Wood et al. (2010) have shown that the ophiuroid brittlestar, *Ophiura ophiura* upregulated its metabolic rate in response to exposure to low pH, which may incurring into a possible energy deficit.

Temperature is also a primary driver for physiological, life-history and ecological functions of ectotherms (Cossins and Bowler 1987), and therefore global warming is likely to affect physiological function, growth and the distribution of species (Southward et al. 1995, Stillman 2003, Pörtner and Knust 2007, Koeller et al. 2009). Temperature has been shown to greatly affect respiration rates and energy metabolism in ectotherms (Prosser 1991, Angilletta 2009). For example, in the periwinkle *Littorina saxatilis* it was shown that, during emersion, increased acclimation temperature leads to: (i) an increase in oxygen consumption rates; (ii) alteration of the activation energy of aerobic metabolism (*E*a); and (iii) the onset of anaerobiosis (as a response to the insufficient oxygen supply to tissues at elevated temperatures) (Sokolova and Pörtner 2003). These effects accounted for a significant depletion of 'high-energy' phosphates and accumulation of end products of fermentation (Sokolova and Pörtner 2001).

The combined exposure to elevated pCO_2 and temperature may exert different effects, also by acting antagonistically, additively, or synergistically, on different functions. This makes it difficult to predict the direction and intensity of organisms' responses to environmental changes (Rosa and Seibel 2008, Wood et al 2010). In addition, there have been no in-depth investigations of the interactive actions that OA and elevated

temperature will likely exert on the fundamental whole-organism and intracellular physiological responses of marine ectotherms. Here, we investigate such responses using whole-organism metabolic rates, adenylate energy nucleotide (ATP, ADP, AMP) concentrations, total adenylate nucleotides (TAN), adenylate energy charge (AEC), and the concentrations of metabolic-end products (succinate and lactate) in the edible periwinkle, *Littorina littorea*, an abundant, widespread species living within intertidal ecosystems of northwest Europe and the Atlantic coast of North America (Fretter and Graham 1962, Brawley et al. 2009).

MATERIALS AND METHODS

Animal collection

Adult individuals of *L. littorea* (shell width 13-15 mm) were collected during May 2009 from the rocky intertidal at Hannafore Point in Looe Bay (Cornwall, UK - 50º 20´ 36.87´´ N, 4º 27´ 16.83´´ W). Snails were transported to the laboratory within two hours and were maintained for ten days in aerated filtered seawater (pH 8.01, salinity 35.0 ppt at 15 ºC in two plastic aquaria (56 l, 130 individuals in each aquarium). Animals were fed every second day *ad libitum* on *Ulva lactuca* and *Fucus serratus* throughout this period.

Mecososm setup

A factorial design incorporating seawater with two levels of both pCO_2 and temperature representing current conditions (385 ppmv/pH 8.0 and 15 ºC) and future predicted conditions for the year 2100 (1000 ppmv/pH 7.6 and 20 ºC) (Caldeira and Wickett 2005, IPCC. 2007, Sokolov et al. 2009) were used. Individual snails were haphazardly allocated to one of these four combinations of pCO_2 and temperature treatment levels

(64 individuals per treatment) and exposed for 30 days. A CO_2/air-equilibration microcosm was set up for each treatment in a temperature-controlled room maintained at 15 ºC (12 h light and 12 h dark), as a modified version of the equilibration flow-through systems used by Widdicombe and Needham (2007), Dashfield et al. (2008) and Widdicombe et al. (2009). Briefly, each CO_2 equilibration system consisted of a header tank (80 l) in which the seawater was either aerated by bubbling normal air or acidified by bubbling pure CO_2 gas. From each of the four header tanks seawater was gravity fed (600 ml min^{-1}) continuously to one of four exposure tanks (23×15×14 cm, 5 l), which were held in larger holding tanks (60×35×15 cm, 32 l). Each exposure tank contained sixteen plastic pots (45 ml) with 25 holes (diam. 3 mm), each containing a single individual snail (mean shell width 12.9 ± 5.3 mm, SD). The excess water from the exposure tanks flowed into the holding tanks and was transferred into a large plastic container (45×36×35 cm, 56 l), aerated, and recirculated via a submersible pump (EP68, Hengtong Aquarium Co. Ltd., Hengtong, Taiwan) to the header tanks. Fifty per cent of seawater in each header tank and sump was replaced weekly, debris removed every two days and deionized water added as needed to maintain stable salinity levels and guarantee good water quality (i.e. minimize ammonia accumulation). CO_2 gas was released into the header tank using a multi-stage CO_2 regulator (EN ISO 7291, GCE, Worksop, UK) connected to a flip-flop control solenoid valve (ORIFICE 3/16 Closed System, Peter Paul Electronics Co. Inc., New Britain, USA) controlled by a calibrated pH controller (pH-201 Digital, Dream Reef, Humberston, UK).

Seawater temperature in two of the holding tanks was increased to 20 ºC using aquarium heaters (Rio 1700, Aqua Vital™, Bristol, UK); the other two holding tanks were maintained at 15 ºC by the ambient conditions in the temperature-controlled room

where the experiment was conducted. Seawater temperature, salinity, pH, total Dissolved Inorganic Carbon (DIC) and dissolved oxygen in exposure tanks were measured daily. Temperature and salinity were measured using a handheld multimeter (YSI 85, YSI Inc., Yellow Springs, USA), pH using an Inlab 413SG pH electrode and Sevengo pH meter (Mettler-Toledo GmbH, Sonnenbergstrase, Switzerland) employing the National Bureau of Standards (NBS) pH standards. Total DIC was measured using a CO_2 analyser (965D, Corning Ltd., Cambridge, UK). Carbonate system parameters that were not directly measured were calculated using CO_2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO_4 dissociation constant from Dickson (1990).

Metabolic rates

Metabolic rates of individual snails were measured using oxygen uptake as a proxy following the method developed by Spicer and Erickson (2003). Six individual snails were selected haphazardly from each aquarium (in total 96 individuals), placed individually in a blacked out glass jar (70 ml) to rest (following preliminary tests) for 30 min. O_2 levels were measured right before closing the jars and after 85 min using calibrated O_2 meter (Model 781, Strathkelvin Instruments, Glasgow, UK) equipped with an O_2 electrode (1302 electrode, Strathkelvin Instruments, Glasgow, UK). Oxygen uptake was calculated as the delta of the O_2 levels at the beginning and at the end of the incubation period, and was expressed as μmol O_2 g^{-1} wet weight h^{-1}. No snail was exposed to hypoxic conditions (i.e. below 80% O_2 saturation).

Intracellular metabolites and metabolic-end products

For the preparation of tissue, four snails were pooled haphazardly from each aquarium and inspected for possible infections. Individual foot muscles (approx. 0.2 g) were quickly dissected, cleaned in fresh seawater, frozen immediately in liquid nitrogen and stored at -80 ºC. For extraction, frozen tissue was powdered using a pre-cooled pestle and mortar. In order to remove protein, the powdered tissue was extracted in perchloric acid with the ratio of one part of tissue in two parts of perchloric acid (0.9 M). The extracted tissues were centrifuged using a microcentrifuge (Sorvall Legend Micro 17, PLS Ltd., (Surrey, UK) for 10 minutes at 3000 gav. at 4 ºC. The supernatant was transferred to another microcentrifuge tube, and the precipitate was extracted again in perchloric acid (0.2 M) using one part of precipitate to three parts of perchloric acid. The supernatants from both extractions were then pooled together and the pH of the solution was adjusted to 6.0-6.5 using 2.0 M potassium hydroxide, after which it was left resting for 1 h in an ice-bath. After rapid centrifugation for 3 min at 10,000 gav; the extracts were stored at -80 ºC.

All reagents were purchased from Sigma Aldrich (Poole, UK) except for enzymes which were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Concentrations of energy metabolites adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and end-products accumulation (succinate, D-Lactate and L-Lactate) were determined spectrophotometrically in the perchloric acid extracts (Bergmeyer 1985b). The assay procedures for measurement of adenylate nucleotides are described in protocols provided by Calzyme Lab. Inc. that were modified based on the reaction described by Strominger et al. (1956), while the end products were assayed using commercial kits (Megazyme International Ireland Ltd.,

Wicklow, Ireland; succinate, K-SUCC II/5; D-lactate, K-DATE 03/06; and L-lactate, K-LATE 03/06). However, using this method, L-Lactate was not detected.

Adenylate energy charge index (AEC), a biomarker used to assess the disruption of energy status due to environment challenges (Luca-Abbott et al. 2000), was calculated using the formula:

$$AEC = \frac{[ATP] + ½\,[ADP]}{[ATP] + [ADP] + [AMP]} \qquad (4)$$

Total adenylate nucleotide (TAN) has been used as a proxy to gauge the physiological condition of the organism following environmental anaerobiosis associated with cyclical variations in temperature, dissolved oxygen and pH (Dehn 1992, Luca-Abbott et al. 2000, Suska and Scotnicka 2010) and is employed here to assess the effect of the interaction between elevated pCO_2 and temperature on the pool of nucleotides available for ATP synthesis.

$$TAN = [ATP] + [ADP] + [AMP] \qquad (5)$$

Statistical analyses

The effect of pCO_2, temperature and their interaction on O_2 uptake, energy metabolism and metabolic-end products were analysed using a two-way ANCOVA, with tank as a random factor nested within pCO_2 and temperature and weight of total body tissue as a covariate. All data met assumptions for normality as untransformed data or following $\log_{10}$ transformation (maximum $Z_{64} = 1.181$, $p = 0.123$). Variances were homogeneous

for O_2 uptake and other metabolic parameters (maximum $F_{3,60} = 1.721$, $p = 0.172$), but not for [ADP], [succinate] and [D-Lactate] (minimum $F_{3,60} = 3.223$, $p = 0.029$). In light of the fact that our experimental design included four treatments with a minimum of 16 replicates per treatment per measurement, we assumed that the ANCOVA design employed should be tolerant of deviation from the assumption of normality and heteroscedasticity (Sokal and Rohlf 1995, Underwood 1997). The tank tested had a significant effect on respiration rate, [ATP] and TAN measured in this study (minimum $F_{12,48} = 1.966$, $p < 0.039$), but not on [ADP], [AMP], AEC, [succinate] and [D-Lactate] (maximum $F_{12,48} = 1.506$, $p = 0.156$). However, in the cases where the term 'tank' was found significant, removing this factor (tank effect) from the calculation caused no change to the significance of the main factors, and thus tank effect is considered marginal. All analyses were conducted using SPSS 19.

RESULTS

Microcosm parameters

Seawater physiochemical parameters were stable over the experimental period (see Table 2.1). Mean pCO_2 values were 428 ± 17 ppmv in the control treatments and 998 ± 30 ppmv in the acidified treatments, resulting in mean pHs of 8.04 ± 0.005 and 7.66 ± 0.003 in control and acidified treatments, respectively. Mean temperatures under control and elevated conditions were 15.1 ± 0.1 and 20.3 ± 0.1 ºC, respectively.

Table 2.1. Physicochemical parameters of the seawater in the mesocosm unit. Mean (± SE) for seawater physico-chemical parameters measured or calculated during the duration of the experiment: oxygen concentration (O_2), salinity, temperature (°C), pH (NBS scale), dissolved inorganic carbon, total alkalinity (TA), using 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 CO_2SYSs program (Pierrot et al. 2006), using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987). Different letters in the brackets indicate significant differences among treatments using Post Hoc Bonferroni test of one way ANOVA ($p < 0.05$), according to 95% Confidence Interval test for Estimate Marginal Means (EMM) with Bonferroni correction.

Parameter	Current temperature.	Elevated temperature	Elevated pCO_2	Elevated pCO_2* temperature
[O2] (mg l^{-1})	6.75 ± 0.01 (a)	7.09 ± 0.15 (a)	6.68 ± 0.17(a)	6.85 ± 0.2(a)
Salinity (ppt)	34.77 ± 0.03 (a)	35.12 ± 0.1(a)	35.06 ± 0.04(a)	35.12 ± 0.1(b)
Temperature (°C)	14.43 ± 0.06 (a)	20.91 ± 0.1(b)	14.89 ± 0.1(a)	20.68 ± 0.1(b)
pH	8.03 ± 0.01 (a)	8.04 ± 0.01 (a)	7.67 ± 0.01(b)	7.65 ± 0.0(b)
DIC (µmol Kg^{-1})	1596 ± 50 (a)	1573 ± 40 (a)	1596 ± 40(a)	1723 ± 46(a)
TA (µequiv kg^{-1})*	1726 ± 52 (a)	1743 ± 44(a)	1627 ± 40(a)	1767 ± 48(a)
pCO_2(µatm) *	428 ± 17 (a)	428 ± 13(a)	998 ± 30(b)	1185 ± 33(c)
[HCO_3^-] (µmol kg^{-1}) *	1489 ± 47 (a, b)	1444 ± 36(b)	1518 ± 38(a, b)	1633 ± 44(a, c)
[CO_3^{2-}] (µmol kg^{-1}) *	89.79 ± 3.5 (a)	114.3 ± 5 (b)	40.58 ± 1.3(c)	51.50 ± 1.8(c)
Ωcal *	2.14 ± 0.08 (a)	2.73 ± 0.12 (b)	0.97 ± 0.03(c)	1.23 ± 0.04(c)
Ωara *	1.37 ± 0.05 (a)	1.78 ± 0.1(b)	0.62 ± 0.02(c)	0.80 ± 0.03(c)

Metabolic rates

Metabolic rates of individual *Littorina littorea*, here measured as O_2 uptake, were significantly lower by an average of 31% under elevated pCO_2 when compared to control snails after the 30 d exposure period (Table 2.2, Fig. 2.1 and Appendix A). In addition, elevated temperature caused no significant changes in metabolic rates in comparison to control pCO_2 conditions. However, under elevated pCO_2, temperature appears to override CO_2 leading to a significant increase in metabolic rate (+18%) (Fig.

2.1) as indicated by the presence of a significant interaction between pCO_2 and temperature (Table 2.2). Finally, there was also a significant negative relationship between tissue weight and O_2 uptake (Table 2.2).

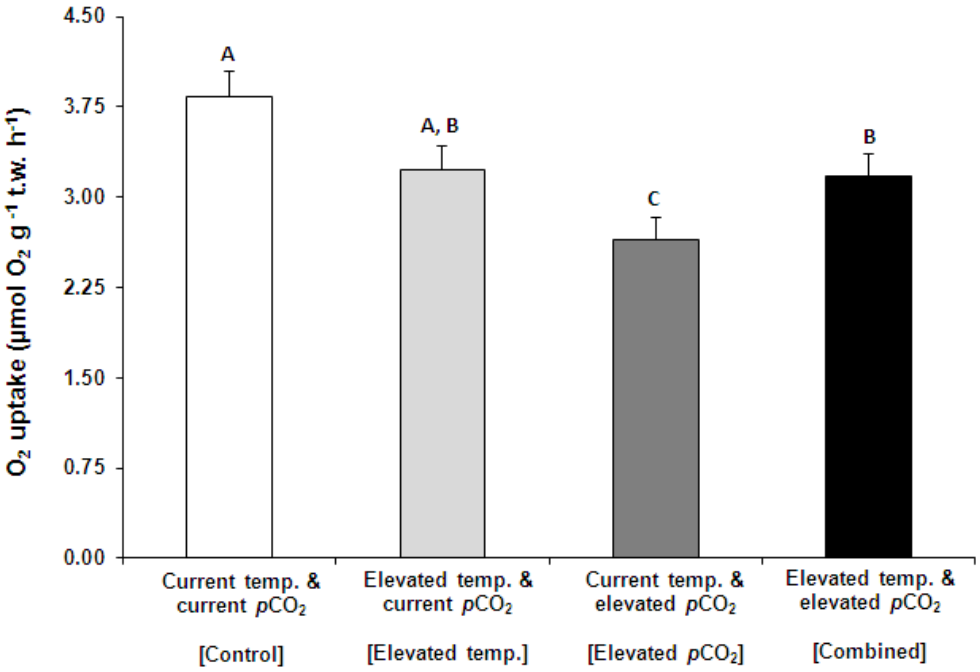

Figure 2.1. The rates of O_2 uptake of the snail *Littorina littorea* exposed to different combinations of pCO_2 (428 and 998 µatm) and temperature (15 or 20 °C) on: control (white), elevated temperature (light grey), elevated pCO_2 (dark grey) and elevated pCO_2 and temperature (dark). Values are means (± SE). Significantly different treatments ($p \leq 0.05$) are indicated by different letters according to the Estimate Marginal Mean test (EMM) with Bonferroni correction.

Table 2.2. Results of multiple ANCOVA testing for the effect of elevated temperature and pCO_2 and their interaction on respiration rate, metabolic energy and end products of metabolism in the periwinkle *Littorina littorea*. Degrees of freedom (df), mean of square (MS), F-ratio (F), probability level (*p*). [1] This is the wet weight of snails minus their shells.

Trait	Source	df	MS	F	*p*
O_2 uptake	pCO_2	1	.231	8.553	**0.013**
(µmol mg^{-1} t.w. h^{-1})	temperature	1	.018	.707	0.415
	interaction	1	.206	7.574	**0.018**
	weight of animal[1]	1	.191	13.878	**< 0.0001**
	tank	12	.027	1.966	**0.039**
ATP	pCO_2	1	.764	34.789	**< 0.0001**
(µmol g^{-1} t.w.)	temperature	1	.474	27.584	**< 0.0001**
	interaction	1	.228	10.357	**0.007**
	tank	12	.022	2.546	**0.011**
ADP	pCO_2	1	.759	18.021	**< 0.0001**
(µmol g^{-1} t.w.)	temperature	1	.449	10.655	**0.007**
	interaction	1	.002	.037	0.848
AMP	pCO_2	1	.687	4.563	**0.037**
(µmol g^{-1} t.w.)	temperature	1	1.205	8.004	**0.006**
	interaction	1	.002	.015	0.902
AEC ratio	pCO_2	1	.00002	.006	0.939
	temperature	1	.004	1.145	0.289
	interaction	1	.044	11.958	**0.001**
TAN	pCO_2	1	16.110	29.711	**< 0.0001**
(µmol g^{-1} t.w.)	temperature	1	16.616	30.644	**< 0.0001**
	interaction	1	3.915	7.221	**0.020**
	tank	12	.542	3.381	**0.001**
Succinate	pCO_2	1	1.376	7.996	**0.006**
(µmol g^{-1} t.w.)	temperature	1	3.119	18.129	**< 0.0001**
	interaction	1	6.661	38.424	**< 0.0001**
D-Lactate	pCO_2	1	6.7540	16.410	**< 0.0001**
(µmol g^{-1} t.w.)	temperature	1	.0751	1.825	0.182
	weight of animal[1]		3.4500	7.859	**0.007**
	interaction	1	17.465	42.437	**< 0.0001**

Energy metabolites

Mean [ATP] ranged from 2.15 μmol g^{-1} t.w. under control conditions to 1.01 μmol g^{-1} t.w. under the high pCO_2 and temperature conditions, with levels under control pCO_2 condition at 15 ºC comparable to those to reported previously for *Mytilus edulis* (Beis and Newholmes 1975) and *L. saxatilis* (Sokolova and Pörtner 2003) (Appendix A). Mean [ATP] was significantly lower after 30 d exposure to elevated CO_2 and temperature in isolation (-53% in both cases) and even more so under the combined condition (-63%), compared with control conditions (Tables 2.2, Fig. 2.2A and Appendix A). A similar pattern was found for [ADP], although the elevated pCO_2 treatments did not show any significant difference (Table 2.2, Fig. 2.2B). Mean [AMP] was highest and significantly different from any other of the treatments under control conditions, lowest under combined elevated pCO_2 temperature conditions, and intermediate at elevated pCO_2 temperature in isolation, with these two treatments showing no significant difference between each other (Tables 2.2, Fig. 2.2C and Appendix A). In general, elevated pCO_2 led to a comparable decrease in mean [AMP] at both temperatures tested here, as no significant interaction was found (Table 2.2). Mean total adenylate nucleotides (TAN) showed the same basic pattern of changes as [ATP] across the different treatments, with a significant decrease in mean TAN of 48% under both elevated pCO_2or elevated temperature conditions, and a further reduction by 64% under combined conditions (Table 2.2, Fig. 2.2D and Appendix 2A). Mean adenylate energy charge (AEC) also showed a pattern comparable to that observed for [ATP] with the exception that the mean level measured under combined conditions was similar to that found in the control; thus elevated temperature exerts a different effect on mean AEC under control and elevated pCO_2 conditions, as indicated by the existence of a significant interaction (Table 2.2, Fig. 2.2E and Appendix 2A).

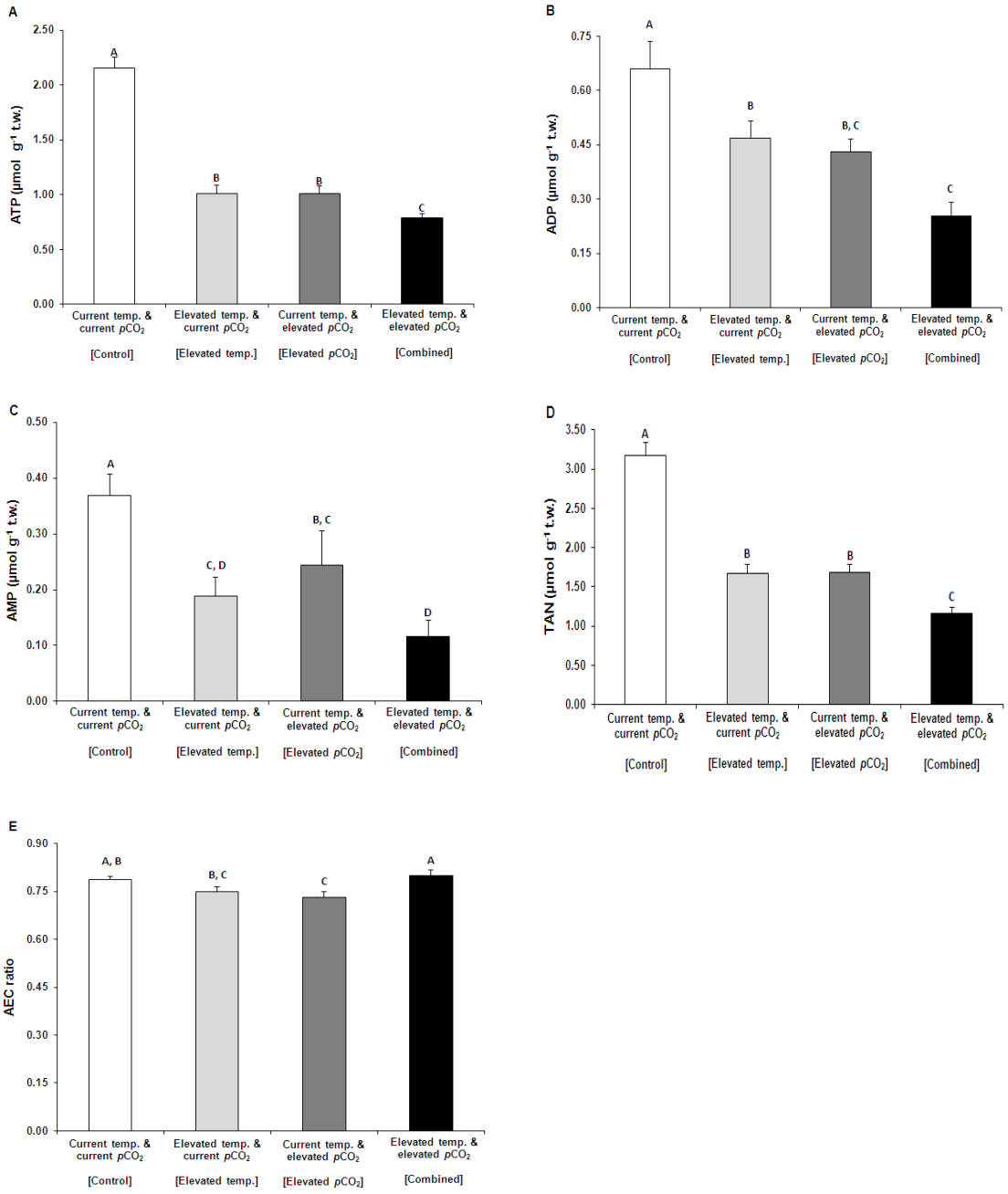

Figure 2.2. The cellular nucleotide energy of the snail *L. littorea* exposed to different combinations of pCO_2 (428 and 998 µatm) and temperature (15 or 20 °C) on: (A) [ATP], (B) [ADP], (C) [AMP], (D) [TAN], and (E) AEC ratio of the snail *L. littorea*. Histogramms represent mean values (± SE) determined for the four employed treatments. Significantly different means ($p \leq 0.05$) are indicated by different letters according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

Metabolic-end products

Levels of succinate found in the tissues of *L. littorea* were similar after 30 d exposure to control, elevated temperature and combined conditions, but under high pCO_2 conditions significantly higher levels were found (Fig. 2.3A). Hence, the effect of hypercapnia is dependent on temperature as indicated by a statistically significant interaction (Table 2.2). When compared to control snails, mean [D-lactate] was higher in snails exposed to either elevated pCO_2 or temperature, and these groups were comparable with each other (Fig. 2.3B). However, D-lactate in animals exposed to the combined conditions was lower and not significantly different to levels in control animals, giving rise to a significant interaction between pCO_2 temperature (Fig. 2.3B, Table 2.2). A significant negative correlation was also found between individuals soft tissue wet weight and [D-lactate], indicating that smaller individuals accumulated more lactate than large ones. No relationship between individual weight and [succinate] was found.

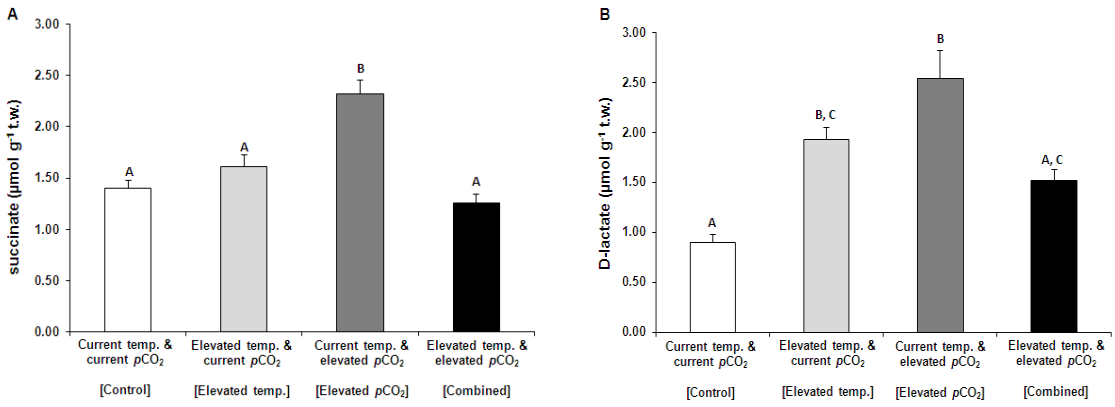

Figure 2.3. The cellular nucleotide energy of the snail *L. littorea* exposed to different combinations of pCO_2 (428 or 998 ppmv) and temperature (15 or 20 °C) on (a) [succinate] and (b) [D-lactate]. Histogramms represent mean values (± SE) determined for the four employed teatments. Significantly different means ($p \leq 0.05$) are indicated by different letters according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

DISCUSSION

The study shows that the marine intertidal invertebrate *Littorina littorea* undergoes a significant reduction in metabolic rate, as indicated by a depression in respiration rate, when exposed for a prolonged period to either elevated pCO_2 in isolation or, to a lesser extent, to combined elevated pCO_2 temperature. Most importantly, we show, for the first time, that the metabolic reduction caused by prolonged exposure to elevated pCO_2 is combined with a significant disruption of aerobic metabolism.

Metabolic rates

It has been widely reported that exposure to environmental hypercapnia, leading to changes in extracellular acid-base balance, affects metabolic mode and respiration rate in a variety of marine invertebrates, including: the sipunculid worm *Sipunculus nudus* (Reipschläger and Pörtner 1996, Pörtner et al. 1998a), the bivalve mollusc, *Mytilus galloprovincialis* (Michaelidis et al. 2005); the Humboldt squid *Dosidicus gigas* (Rosa and Seibel 2008); the edible crab *Cancer pagurus* (Metzger et al. 2007); and the velvet swimming crab *Necora puber* (Small et al. 2010). In all these cases a decrease in O_2 uptake was reported at low pH induced by hypercapnia. In contrast, in the brittle star *Ophiura ophiura* an increase in O_2 uptake at low pH was observed (Wood et al. 2010), as was no significant change in standard metabolic rates in the cuttlefish *Sepia officinalis* (Gutowska et al. 2008). This data demonstrates that littorinid snails experience a reduction of metabolic activity (-31%) comparable to that reported for most marine invertebrates exposed to hypercapnic conditions. The capacity for metabolic reduction in molluscs as a response to environmental challenges (e.g. hypoxia) is high (Guppy and Withers 1999). Substantial depression in metabolism are used as a strategy to ensure survival by matching ATP supply and demand (Bishop and Brand 2000, Seibel and Walsh 2003). However, the reduction seen here was relatively

modest compared to fully depressed, hypometabolic states in molluscs (≥ 80%; Guppy and Withers 1999), and there was no behavioural evidence that the animals had entered a fully hypometabolic state. Thus we proposed that the term 'depression' is used more cautiously within the context of metabolic rate reduction reported in most marine invertebrates as a response to the exposure to OA.

The observed hypercapnia-induced metabolic reduction, which was similar to that found in *S. nudus* by Reipschläger and Pörtner (1996) and Pörtner et al. (2000), could be explained by either a decrease in ATP demand or a decrease in ATP production, or both. Lowering of pH leads to an initial acid-base imbalance, requiring a variety of compensatory mechanisms, including ionic exchange, to maintain cellular pH homeostasis, with a concomitant requirement for ATP (Seibel and Walsh 2003). However, it has been suggested that changes in the method of cellular pH homeostasis, e.g. a switch to the use of Na^+/H^+ and Na^+-dependent Cl^-/HCO_3^- exchange from use of H^+-ATPase, leads to a reduction in the rate of hydrolysis of ATP required for this process (Pörtner et al. 2000). An alternative possibility could be hypercapnia-induced disruption to the supply of oxygen, e.g. *via* effects on oxygen transport proteins such as haemocyanin, and hence disruption to the supply of ATP generated via aerobic metabolism (Seibel and Walsh 2003).

When elevated CO_2 and temperature were combined their negative effect on O_2 uptake was much less pronounced, although there was still a significant reduction in metabolic rates compared to the control. Under this combined condition there was also a significant decline in TAN compared to the control, and compared to either elevated temperature or to elevated CO_2 in isolation. As discussed in more detail below this may

indicate an increased demand for ATP, and hence may explain why an additional increase in temperature led to an increase in the O_2 uptake rate by snails. A similar interactive effect between elevated pCO_2 (1 kPa $\approx$ 1000 µatm) and temperature (20 ºC) has also been shown in the Penaeidae crustacean *Metapenaeus joyneri* (Dissanayake and Ishimatsu 2011).

Energy metabolism

Both elevated temperature and pCO_2 in isolation affected AEC and TAN in the foot tissue of the marine periwinkle *L. littorea*. The decreases in AEC were mainly due to decreases in [ATP], but without corresponding increases in [ADP] and [AMP]. Instead, levels of ADP and AMP were significantly lower compared to the control with either elevated temperature or pCO_2 in isolation, leading overall to significant reductions in TAN. Although elevated temperature and pCO_2 had adverse effects, the AEC in both cases was still maintained above the levels (0.5-0.7) considered to represent a threshold of severe decline in energy status (Lucca-Abbot et al. 2000). It therefore seems likely that the animals had successfully used substantial decreases in TAN in order to protect AEC, probably *via* activation of AMP deaminase (Gibbs and Bishop 1977), and hence depletion of AMP in response to an initial decrease in AEC (Chapman and Atkinson 1973). Nevertheless, although AEC was largely protected, the approximate halving of TAN might have the consequence of reducing the snails' capacity for work, perhaps making them more vulnerable to additional environmental challenges due to a further increase of energy utilization (Giesy et al. 1981). As noted above, under elevated pCO_2 conditions, *L. littorina* showed substantial metabolic reduction, which may be indicative of this energy limitation. In order to maintain homeostasis under energy limitation, such as that shown to be caused by hypoxia, many aquatic organisms reduce ATP demand to

match ATP production (Bishop and Brand 2000, Seibel and Walsh 2003) thereby entering a hypometabolic state where O_2 uptake rates are reduced to 5 – 40% of normal levels (Storey and Storey 2004). In this study, although respiration rate fell, this was not to an extent where the animals would have been expected to enter a hypometabolic state (Shick et al. 1986, De Zwaan et al. 1991, Pörtner and Grieshaber 1993), and neither was there any indication that they had, i.e. they remained active and responsive.

It is of interest that combined elevated pCO_2 temperature did not have a synergistic effect on either AEC or O_2 uptake rate. Nevertheless, the combined conditions did lead to a significant drop in TAN compared to elevated temperature or pCO_2 in isolation. This indicates, despite the normal AEC value, that combined elevated temperature and pCO_2 put further strain on the ability of snails to match ATP supply and demand, which may explain the relatively high O_2 uptake (comparable with the control). A likely consequence of this strain on energy metabolism is that the animals have lower capacity for growth, consistent with data on effects of elevated temperature and pCO_2 on growth of *L. littorea* (Chapter 3 and 5).

Metabolic-end products

Exposure of *L. littorina* to elevated temperature or pCO_2 in isolation led to a significant increase in D-lactate levels, and, in the case of pCO_2, in succinate levels. Both of these 'end products' of anaerobic metabolism, are required, for example, during both functional and environmental hypoxia (Pörtner et al. 1984, Grieshaber et al. 1994). D-lactate accumulation is more associated with short term functional hypoxia brought about by animal behaviour, in which there is rapid reoxidation of NADH by pyruvate, catalysed by D-lactate dehydrogenase, and allowing maintenance of ATP regeneration

via glycolysis. In contrast, succinate accumulation is more associated with environmental hypoxia, i.e. longer term hypoxia brought about by environmental changes, in which there is a slower reoxidation of NADH, catalysed by Krebs' cycle enzymes (Livingstone 1983). The ATP yield in the accumulation of succinate depends on the energy store used (either aspartate or glycogen) but is greater than that obtained in the accumulation of D-lactate (Larade and Storey 2002).

Because of the length of exposure used in this study (30 d), it was assumed that the levels of D-lactate and succinate were steady state levels, i.e. that the rate of accumulation in each case matches the rate of loss e.g. in the case of succinate as a consequence of further metabolism to propionate and acetate (De Zwaan et al. 1976, Zebe 1977, Schöttler 1980). Hence, differences in lactate and succinate levels probably arose from differences in accumulation rate, with the caveat that differences in steady state levels could also arise from differences in the rate of loss. Hence, it seems that elevated temperature and pCO_2 in isolation lead to an increased reliance on anaerobic metabolism by the snails, involving both cytosolic and mitochondrial compartments (Sokolova and Pörtner 2003). Levels of succinate were similar to those seen by Sokolova and Pörtner (2003) where *Littorina saxitilis* was exposed to elevated temperature in water for short periods (18 hours). However, in the same study no accumulation of lactate was found, in contrast to the substantial increases in D-lactate seen here, suggesting that littorinid species may be able to employ different forms of metabolic plasticity (Greenway and Storey 2001). The result also showed that prolonged exposure to elevated pCO_2 and temperature in combination had clear effects on energy metabolism, probably adversely affecting supply of ATP and requiring compensatory changes in ATP demand. Although the snails were able to maintain AEC

at relatively normal levels it is questionable whether they would have been able to continue to do so if faced with fluctuations in O_2 supply, such as are seen in an intertidal ecosystem. Hence, it would be interesting in future to look at the ability of *L. littorea* to cope with acute environmental challenges, e.g. by following changes in anaerobic metabolic-end products, adenylate nucleotide pool, and arginine phosphate levels during air exposure at elevated temperature (Sokolova and Pörtner 2001), following prolonged exposure of the animals to elevated pCO_2 temperature in combination.

Here there was evidence that the marine intertidal gastropod *L. littorea* undergoes physiological disruption under high pCO_2 temperature conditions in isolation, as well as combined. Metabolic reduction was characterised by decreased rates of O_2 uptake and decreased energy status (AEC and TAN). Increased levels of metabolic-end products (D-lactate and succinate), also indicated an increased reliance in anaerobic metabolism under elevated temperature and/or pCO_2 conditions. Although snails were able to survive under future global change conditions (no mortality during the study period was observed), they experience a chronic mismatch between energy supply and demand, which could make them more vulnerable to further environmental alternations often occurring in the intertidal environment (such as hypoxia, see Larade and Storey 2009). In the context of global climate change, this keystone intertidal gastropod might face difficulties in maintaining physiological homeostasis, and this could ultimately influence growth, reproduction and population dynamics. Moreover, whilst our results may well represent a worst-case-scenario, it is also possible that changes in environmental conditions may go beyond the scenario predicted for surface oceans (Thomsen et al. 2010), given the intertidal ecosystem is characterised by a complex pattern of seawater pCO_2 levels (Agnew and Taylor 1986), notwithstanding species

ability for rapid adaptation to future climatic change scenarios (Collins and Bell 2004, 2006, Findlay et al. 2011, Pistevos et al. 2011, Sunday et al. 2011).

CHAPTER 3

Marine gastropod plastic responses to the combined effects of ocean acidification and elevated temperature

SUMMARY

Phenotypic plasticity is a fundamental mechanism by which organisms can alter their morphology, physiology, life-history or behaviour in response to environmental changes. Although such plasticity can be induced by natural environmental variation it is becoming evident that anthropogenically induced environmental change may also lead to plastic responses, with ecological implications. Here in this chapter, investigation of plastic response in shell morphology in the intertidal gastropod *Littorina littorea* in response to ocean acidification and elevated temperature in line with predicted levels due to climate change, focusing on shell traits known to relate to protection from predators (size and thickness) and resistance to desiccation (shape) were conducted. Both low pH and elevated temperature disrupted the overall investment in shell material, with snails in acidified seawater and elevated temperature in isolation or combined showing lower shell growth rates than individuals kept under control conditions. Percentage change in shell length increase was also lower for individuals kept under combined acidified seawater and elevated temperature, and shell thickness at the growing edge was lower under acidified and combined conditions as well as shell shape. Shells were more globular under elevated temperature and lower pH and desiccation resistance of snails was also negatively affected by low pH and elevated temperature. Counter to predictions, however, water loss was only weakly related to shell biometric traits.

INTRODUCTION

Phenotypic plasticity is an important mechanism by which organisms alter their morphology, physiology, life-history or behaviour in response to fluctuations in the prevailing environmental conditions (Pigliucci 2001, DeWitt and Scheiner 2004). Given a drastic effect 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 adapt to broad scale disturbances such as climate change (Charmantier et al. 2008). Indeed, it has been proposed that this creation of "novel" environmental conditions may be a means by which plasticity leads to phenotypic changes defined as property of individual genotypes to produce new phenotype when exposed to different environmental conditions (Pigliucci et al. 2006). He also revealed that phenotypic plasticity is also known as an adaptive strategy of the animal to improve its survival and fitness, although Sultan (1995) revealed that phenotypic plasticity is not always adaptive due to the biochemical, physiological, or developmental biological constraints. Hence, there is a pressing need for studies that examine the extent to which species can exhibit plastic responses to predicted levels of environmental change.

Marine intertidal habitats are characterised already by high heterogenity and contain numerous species that exhibit plastic responses to natural environmental variation in, for example, wave action (e.g. Gaylord 2000) and predation pressure (e.g. Boulding and Alstyne 1993). At the same time, marine environments are exposed to anthropogenically induced environmental variation such as that associated with climate change and rising atmospheric carbon dioxide (CO_2) concentrations. Current predictions suggest that environmental conditions in marine systems are likely to undergo a shift

towards lowered pH (due to increase in aqueous [CO_2]) and increased temperatures, as a result of increased atmospheric CO_2 levels (Caldeira and Wickett 2003, Sokolov et al. 2009). These conditions are expected to cause severe alterations the developmental physiology and behaviour of marine organisms (e.g. Bibby et al. 2007, Todgham and Hofmann 2009, Munday et al. 2009a) culminating in changes to community structure and ecosystem function (e.g. Wootton et al. 2008, Feng et al. 2009, Hale et al. 2011). Calcification plays an important role in the plastic responses of many marine taxa that alter their exoskeleton size and shape in response to environmental variation (e.g. Cotton et al 2004, Irie and Fischer 2009), and this key physiological process appears also to be affected by reduced seawater pH, carbonate ion concentration (ocean acidification), and temperature (e.g. Rodolfo-Metalpa et al. 2009, Lombardi et al. 2010). Evidence suggests that calcification rates decrease with decreasing pH (e.g. Gazeau et al. 2007), but more recent experimental studies have shown that calcification rates can also increase in some taxa when they are exposed to low pH (Findlay et al. 2009, Ries et al. 2009). Similarly, temperature increases may lead to the disruption of calcification in marine ectotherms (e.g. Irie 2006). Given that elevated temperature and reduced pH are both induced by elevated atmospheric CO_2 levels, marine ecosystems will be exposed simultaneously to these two key environmental drivers. Consequently, the true impacts of elevated pCO_2 on the function of marine calcifiers are likely to be greater than previously thought (e.g. Feng et al. 2009).

Marine gastropods depend heavily on a calcareous shell for protection against predation, wave exposure and desiccation. The shell morphology within this group has been shown to vary widely in response to spatial and temporal environmental variability (Avery and Etter 2006), e.g. in temperature (Irie 2006) and predation threat (e.g. Trussell and

Nicklin 2002, Cotton et al. 2004). Here, investigations to the extent to which shells of the intertidal gastropod *Littorina littorea* are affected by elevated-pCO_2-induced acidified seawater, elevated temperature or these factors in combination were carried out. In particular, these investigations were focused on shell traits that relate to the ecology of this species in terms of protection from predators (mass, shell size and shape, and thickness) and desiccation (shell aperture size and shape).

MATERIALS AND METHODS

Experimental design

A multi-factorial design was used to assess the potential influence of altered seawater pH and temperature on snail mass, shell biometrics, and snail water loss. The two pH_{NBS} levels selected were based on current (8.0) and predicted values for the year 2100 (7.7) corresponding to the global ocean pCO_2 of 380 and 1000 ppm, respectively (Caldeira and Wickett 2003). Two water temperature levels were used, 15 ± 0.1°C, which correspond to the mean monthly sea surface temperature (SST) at the collection site at time of collection (13.1ºC) (Joyce 2006) and to the seawater temperature during collection (determined using a YSI 85 handheld multimeter, YSI Inc., Yellow Springs, USA), and 20 ± 0.1°C, which assumes an increase of +5°C in line with future predictions for global warming trend for sea surface temperatures (Sokolov et al. 2009).

Animal collection and preparation

Littorina littorea individuals (shell width 13-15 mm) were collected in May 2009 at the lowest tide possible (between 1.0 and 1.7m above *chart datum*) from the rocky intertidal shore at Hannafore Point in Looe Bay, Cornwall (50° 20’ 36.87’’ N and 4° 27’ 16.83’’

W). Individuals were returned to the laboratory within two hours and before being introduced into the experimental set-up were maintained in two large plastic aquaria (capacity 56 l, 130 individuals in each aquarium) for 10 days in aerated seawater at 15 °C salinity 33 ppt. Individuals were fed *ad libitum* on *Ulva lactuca* and *Fucus serratus* every second day throughout this period.

Mesocosm setup

Four CO_2/air-equilibration mesocosms (one per treatment) were set up in a CT-room maintained at 15 °C (12:12 L:D), as modified versions of the equilibration flow-through systems used by Widdicombe and Needham (2007). Gas of CO_2-air mixed was passed through the water in header tank and fed by gravity to the experimental unit. Seawater pH was monitored using pH controllers (Aqua Digital PH-201, Reef dreams Inc., Hampshire, UK). The acidified seawater from header tank was supplied to the experimental tanks; it was replaced by discharge seawater, that has pH level higher than required pH (set pH 7.6), from the sump (50 cm length, 45 cm width and 35 cm height) causing the pH in the header tank to increase. An increase in the pH level triggered the supply of CO_2 from the CO_2 controller (CO_2 solenoid, Peter Paul Electronic Inc., New Britain, USA) to bubble seawater until pH reached the required level.

Four aquaria (23 cm length, 15 cm width and 15 cm height) with forty-eight holes (ø 10 mm, 9 and 15 holes on each side) were placed on each experimental tank (65 cm length, 38 cm width and 15 cm height). Sixteen plastic pots (20 ml, ø 316 mm and 5 cm height) each containing an individual animal were placed in each aquarium for a 30 d exposure period. The holes on each aquarium and pot were made to ensure seawater circulation.

For further details on the CO_2 equilibration system, monitoring of physico-chemical parameters and carbonate system calculations see Chapter 2 (Table 2.1).

Biometric measurements

Shell morphological parameters known to relate to predation susceptibility were measured at the start of the experiment and after 30 d exposure. These parameters were shell length and width, aperture length and width, shell thickness of the inner lip of the shell (which lies alongside the columellar axis in the posterior aperture of the shell, referred to hereafter as thickness-1), and thickness of the outer lip (the growing tip lying along the anterior portion of the shell, hereafter thickness-2) (Cotton et al. 2004) (Appendix 3B). 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).

All measurements were carried out on images collected with a digital camera (Coolpix 4500, Nikon UK Ltd., Surrey, UK) mounted on a light microscope (SDZ-IR-P, Kyowa Optical, Ltd., Tokyo, Japan). Each image was measured using image processing UTHSCSA Image tool program for Windows 2003 (Rundle et al. 2004, Al-Mazrouai 2008) calibrated using a micrometer (1.000 ± 0.001 mm). Differences between initial and final parameters measures were expressed as percentage change.

Water loss

Water loss was measured in a sub-set of six snails from each replicate aquarium (total N = 96 individuals over all). The shells were first cleaned, by removing epibionts with a cotton bud, and excess water in the operculum (while the foot muscle was retracted) was absorbed with a cotton bud by using a gentle pressure on the shell aperture. Each

individual was then weighed using a digital balance (PF-203, Fisher Scientific, Leicestershire, UK) and placed on an aluminium tray in a programmable oven at 30 °C for 6 h. After this time, snails were re-weighed, before being returned to their individual pots in the original experimental tanks to acclimate for 2 h. Individuals were then collected and dried at constant temperature of 100 °C for 24 h and dry weight was measured. Water loss was determined as the percentage of total body weight change after this exposure (Sokolova and Pörtner 2001).

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, shell thicknesses-1 and -2) and water loss were analysed using a two-way ANCOVA, with 'tank' as a random factor nested within pCO_2 and temperature and initial weight and shell sizes were used as covariate. 'Tank' had a significant effect on most parameters measured in this study (minimum $F_{1,255} = 1.937$, $p = 0.031$) with the exception of percentage change in shell aspect, aperture length and water loss (maxim $F_{1,96} = 0.921$, $p = 0.535$). However, in those cases where the term 'tank' was found to be significant, removing it did not change the patterns of significance of factors investigated, and thus tank effect was considered marginal. Where found to be non-significant the term tank was removed from the analysis. Most data met the assumption for normality, untransformed 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 aspect where no transformation was beneficial (minimum $Z_{256} = 1.476$, $p = 0.026$). Variances were homogeneous for percentage change 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, $p = 0.041$). In light of the fact that our experimental design included four 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 assumption of normality and heteroscedasticity (Underwood 1997, Sokal and Rohlf 1995). Pairwise comparisons were conducted using the 95% Confidence Interval test calculated for Estimated Marginal Means (EMM) with Bonferroni correction. Finally, a correlation analysis was conducted to verify the existence of a possible relationship between water loss and shell aperture aspect. All analyses were conducted using SPSS 17.

RESULTS

Microcosm parameters

Seawater physico-chemical parameters were stable over the experimental period (see Chapter 2, Table 2.1). Mean pCO_2 values were 428 ± 17 ppmv in the control treatments and 998 ± 30 ppmv in the acidified treatments, resulting in mean pHs of 8.04 ± 0.005 and 7.66 ± 0.003 in control and acidified treatments, respectively. Mean temperatures under control and elevated conditions were 15.1 ± 0.1 and 20.3 ± 0.1 ºC, respectively.

Shell weight, size and shape

Mean percentage change in shell wet weight (shell with tissue); shell length and shell shape of *Littorina littorea* decreased under low pH and elevated temperature conditions in isolation (Table 3.1). The percentage change in shell weight was three and five times higher, respectively, under current temperature conditions (6.4%) compared to acidified seawater (1.6%) and acidified and elevated temperature in combination (-1.8%) (Fig. 3.1 and Appendix B). As a result, shells were significantly heavier and longer under

current temperature conditions compared with those under combined elevated pCO_2 temperature conditions; weights, lengths and shell shape for the other two treatments did not significantly differ between each other (Fig. 3.1).

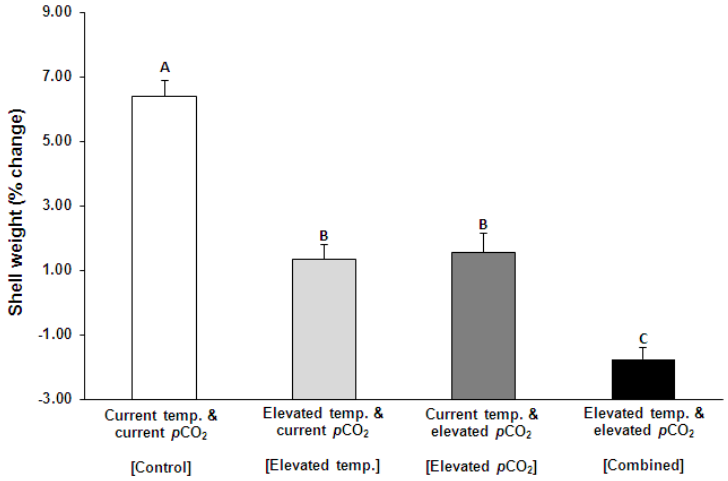

Figure 3.1. Effect of 30 d exposure to different combinations of elevated pCO_2 and temperature on the percentage change on shell wet weight of the snail *Littorina littorea* exposed at different temperatures and pCO_2 levels. Histograms represent mean values (± SE) determined for the four employed treatments: current temperature (white), elevated temperature (light grey), elevated pCO_2 (dark grey), elevated pCO_2 and temperature (black). Significantly different means ($p \leq 0.05$) are indicated by different letter based on the 95% Confidence Interval test for Estimate Marginal Means (EMM) with Bonferroni correction.

Percentage increase in shell width was not affected by elevated pCO_2, elevated temperature or their interaction (see Table 3.1).

Table 3.1. Result of multiple ANCOVA tests for the effect of elevated pCO_2 and temperature and their interaction on different shell traits and water loss in the common periwinkle *Littorina littorea*. Degrees of freedom (df), mean of square (MS), F-ratio (F), probability level (*p*) are reported.

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**
ShellThickness-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 ap. 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 ap. 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 ap. 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

Shell Thickness

Both measures of shell thickness decreased at the higher temperature under normal pH and increased with temperature under acidified conditions (Fig. 3.2A and B) giving a significant interaction between elevated pCO_2 and temperature (Table 3.1). Mean percentage change in shell thickness-1 was significantly higher under current temperature and pCO_2 conditions (38.84%), lower under acidified conditions (8.10%), and intermediate for the other two treatments (both c23%) (Fig. 3.2A). Mean percentage change in shell thickness-2 was also significantly higher (55.20%) under current temperature conditions, and was actually lower due to negative growth under acidified conditions (-27.12%), and intermediate for the other two treatments, which did significantly differ from each other (Fig. 3.2B).

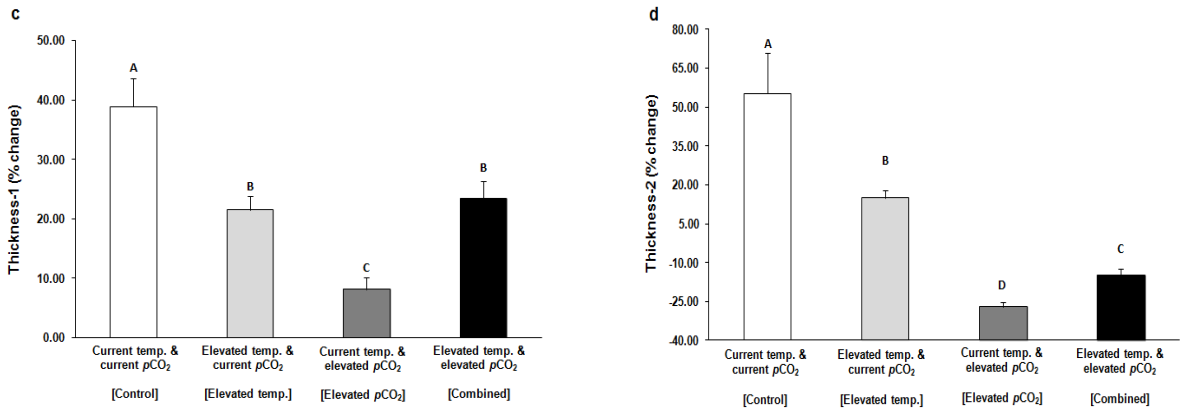

Figure 3.2. Effect of 30 d exposure to different combinations of elevated pCO_2 and temperature on the percentage change on: A) shell thickness-1 and B) shell thickness-2 of the snail *L. littorea* exposed at different temperatures and pCO_2 levels. Histograms represent mean values (± SE) determined for the four employed treatments: current temperature (white), elevated temperature (light grey), elevated pCO_2 (dark grey), elevated pCO_2 and temperature (black). Significantly different means ($p \leq 0.05$) are indicated by different letter based on the 95% Confidence Interval test for Estimate Marginal Means (EMM) with Bonferroni correction.

Shell aperture size and shape

Percentage aperture length increase was significantly affected by elevated temperature (+10.2% increase) and by elevated pCO_2 and elevated temperature in combination (+9.8%) (Table 3.1) (Fig. 3.3). The lowest value for percentage change in aperture length was recorded under acidified conditions (1.7%), whilst control conditions were found to be intermediate (7.0%) (and significantly different) to the elevated temperature treatments and the acidified treatment (Fig. 3.3). Mean percentage increase in aperture width and mean percentage changes in aperture shape (i.e. aperture ratio) were not significantly affected by elevated pCO_2 or temperature (see Table 3.1).

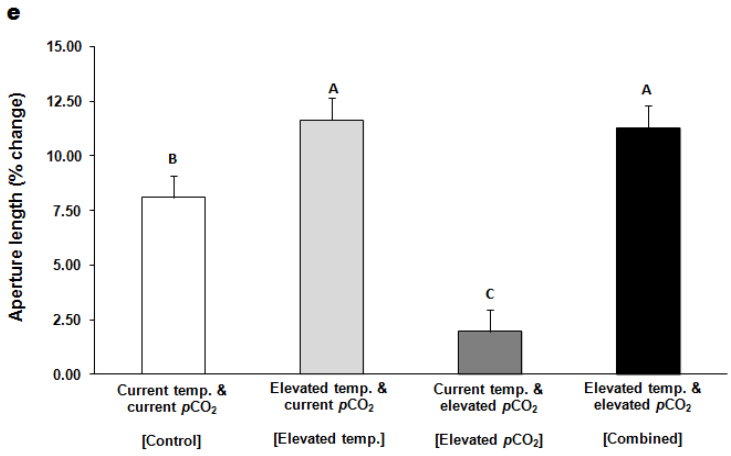

Figure 3.3. Effect of 30 d exposure to different combinations of elevated pCO_2 and temperature on the percentage change on aperture length of the snail *L. littorea* exposed at different temperatures and pCO_2 levels. Histograms represent mean values (± SE) determined for the four employed treatments: current temperature (white), elevated temperature (light grey), elevated pCO_2 (dark grey), elevated pCO_2 and temperature (black). Significantly different means ($p \leq 0.05$) are indicated by different letters based on the 95% Confidence Interval test for Estimate Marginal Means (EMM) with Bonferroni correction.

Water loss

Mean percentage water loss increased under both low pH (-31.4%) and at elevated temperature (-38.8%) conditions (Appendix C), these two factors showing a negative additive effect as both had a significant effect on water loss in isolation and no significant effect of their interaction was detected (see Table 3.1 and Fig. 3.4A). Water loss increased with mean percentage change in aperture shape, but this trend was marginally non-significant ($R^2 = 0.030$, df = 95, $p = 0.09$, Fig. 3.4B).

In addition, there was a significant positive relationship between shell aperture shape and water loss ($R^2 = 0.098$, df = 95, $p = 0.0029$).

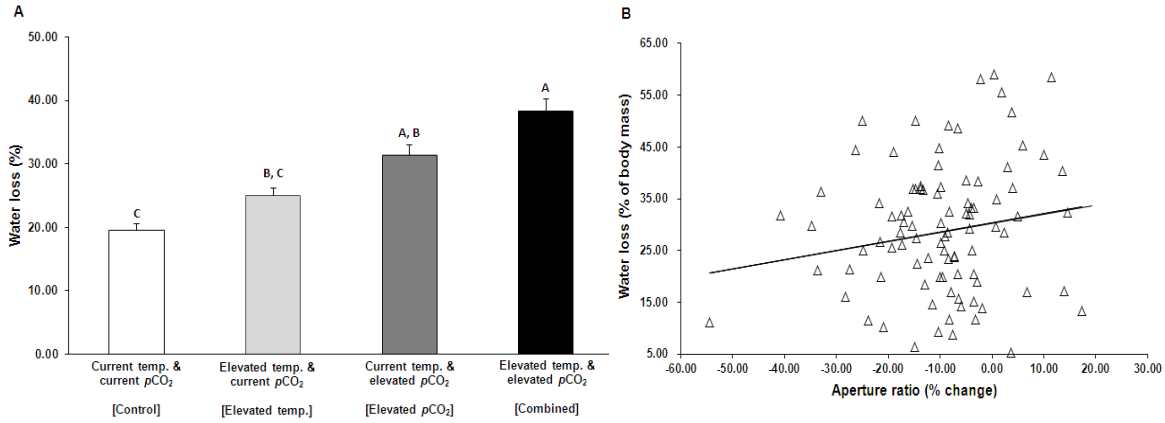

Figure 3.4. Effect of 30 d exposure to different combinations of elevated pCO_2 and temperature on the A) percentage change of water loss and B) relationship between shell percentage change in aperture ratio and water loss of the snail *L. littorea* exposed at different temperatures and pCO_2 levels. Histograms represent mean values (± SE) determined for the four employed treatments: current temperature (white), elevated temperature (light grey), elevated pCO_2 (dark grey), elevated pCO_2 andtemperature (black). Significantly different means ($p \leq 0.05$) are indicated by capital letter to the 95% Confidence Interval test for Estimate Marginal Means (EMM) with Bonferroni correction. Full line in the scatter plot data trend: equation, R^2, degree of freedom (df), a probability value (p) are provided. [Y = 0.1766x + 30.312, df = 95, $R^2 = 0.0301$, $p = 0.091$].

DISCUSSION

A complex pattern of responses to the combined exposure to ocean acidification and global warming appears to be emerging across different phyla and even closely related species (e.g. Rodolfo-Metalpa et al. 2009, Ries et al. 2009, Hale et al. 2011). Here it is shown that responses to global change within a species can also be complex when considering how ecologically-relevant traits respond differently to environmental changes. Specifically, shell morphometric traits in the marine gastropod *Littorina littorea*, respond differently to elevated pCO_2, elevated temperature and their combination, with a mixture of responses from single, additive, synergistic and no effects. As the traits investigated potentially underpin the ability of this intertidal organism to protect itself from predators and desiccation, the plastic responses shown suggest that exposure to elevated pCO_2 and temperature may alter physiological capacities and tolerances, life-history, ecology and ultimately fitness and survival.

Shell growth, thickness and shape plasticity

Under control temperatures conditions, the shells of *L. littorea* were heavier and longer than those produced under low pH and elevated temperature, a result that is in agreement with that for the mussel *Mytilus galloprovincialis*, for which increased seawater acidity by 7.3 pH unit (509 ppm) caused a reduction in shell growth (measured as shell length) and soft-body weight (Michaelidis et al. 2005). Elevated temperatures have also been reported to induce smaller metamorphic size in the gold-ringed cowry *Monetaria annulus* (Irie and Fisher 2009), and to cause disruptions of metabolic rates, growth and fitness in the periwinkle *Littorina saxatilis* (Sokolova and Pörtner 2001). Not surprisingly, 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 on exposure to combined elevation in pCO_2 and temperature (e.g. Rodolfo-Metalpa et al. 2009, Pistevos et al. 2011).

Littorina littorea also showed a reduced shell thickness under acidified conditions; this reduction in shell thickness occurred mainly at the growing tip (thickness-2) rather than more centrally on the body whorl (thickness-1). Dissolution at the growing tip of the shell under acidified seawater conditions (CO_2 = 788 ppm) has previously been reported in the planktonic pteropod *Clio pyramidata* (Orr et al. 2005) and *Nucella lamellose* (Nienhuis et al. 2010). Such reductions in shell thickness in marine calcifiers have been considered to be the result of either (or both) dissolution of calcium carbonate structures exposed to acidified seawaters (e.g. Orr et al. 2005, Michaelidis et al. 2005, Nienhuis et al. 2010), or insufficient uptake of calcium carbonate and secretion of calcified material (see Findlay et al. 2009 for a review). Qualitative measurements of shell dissolution rates undertaken in empty shells of *L.littorina* kept under the experimental condtions as has described in this chapter, show that under elevated temperature and CO_2 conditions empty shells show considerable dissolution rates (-3 to 15%) when compared to those recorded in snails (-1.7 to 4%). This evidence may indicate that snails are still able to calcify under the experimental conditions tested here, despite dissolution taking place. The idea that marine organisms can still calcify under low pH conditions, or even hyper-calcify; have now been documented in a variety of marine calfiers (see Ries et al. 2008, Findlay et al. 2011). Here, it shows that elevated pCO_2 exerts a different effect on shell thickness at different temperatures. In fact in the acidified treatments, shell thickness decrease at control temperature but increased under elevated temperature. This may be due to increased calcite and aragonite saturation states with increasing

temperature and thus a decrease in the rate of passive dissolution, with Ω calcite being just below and just above values of 1 under acidified and combined acidified and elevated temperature conditions, respectively. However under high pCO_2 conditions, higher temperatures have been also shown to increase metabolic rates and AEC (see Chapter 2), thus helping to maintain a certain degree of calcification (see Chapter 6).

A reduced shell thickness in marine gastropods may increase their susceptibility to predation (Boulding and Alstyne 1993, Trussell and Etter 2001) as thicker apertural lips provide better defence against shell crushing predators (Vermeij 1978). Hence the reduced thickness of *L. littorea* shells observed is likely to have fitness implications. The proportional change in shell shape in *L. littorea* was also affected significantly by elevated temperature, elevated pCO_2 and the combined effects of these factors. Shells kept under current temperature conditions were more elongated, whilst under other treatment conditions, shells had a more globular shape. Previous studies show that shell shape in *L. littorea* varies with growth rate, animals showing rapid growth with more globose, thinner shells than slower growing animals (Kemp and Bertness 1984). These differences in growth rate were for snails growing under either high (low growth rate) or low levels of competition (fast growth rate). Our finding that faster growing snails at lower temperatures had a more elongate shape suggest that under conditions of elevated acidity and temperature, normal growth trajectories will likely be disrupted, thus may affect other shell plastic responses.

Shell shape in aquatic gastropods is also thought to play an important role in defence. A globular shell shape in the freshwater snail *Physa spp*. has more resistance to crushing by predators, such as crayfish, than an elongated shape (DeWitt et al. 2000). Shell shape

was an important correlate with crab predation rates in marine intertidal gastropods (Cotton et al. 2004). In particular, Cotton et al. (2004) found that species with a larger aspect ratio (i.e. with a more elongated shape) were more vulnerable to crab predation and hypothesised that this was due to reduced handling efficiency in shells with a flatter more discoid shape. The findings seem to suggest that, in terms of shell shape, shell growth in more acidic warmer conditions produced a shell shape that may be less susceptible to crab predation. However, as shell thickness is reduced under low pH and elevated temperature, acquiring a more globose shape may enable snails to compensate for a possible reduction in shell strength.

Shell aperture plasticity and water loss

The marine gastropod *Thais lapillus* increases operculum size (Gibson 1970) and *Patella spp*. decreases aperture size (Cabral 2007) in order to maintain constant body temperature and reduce desiccation. Overall, shells of *L. littorea* exposed to elevated temperature conditions show greater shell aperture length and proportional increase of these two parameters, when compared to low temperature, whilst shell aperture width did not vary following exposure to elevated pCO_2 and temperature. As a consequence, the shapes of the aperture become more elongated under elevated temperature conditions, and more rotund under current temperature conditions, with likely negative consequence for desiccation rates. Most shelled molluscs and barnacles reduce desiccation by completely closing the open aperture area with the operculum (Shick et al. 1988). However, this strategy may reduce the rate of O_2 uptake, thus impairing the productions of energy for metabolism (Sokolova and Pörtner 2001). Consequently, it can be concluded that under a future global change scenario *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 and Tewksbury 2009), yet at the expense of fundamental activities such as feeding and reproduction, and thus not without a cost in fitness.

In general, phenotypic plasticity enables organisms to respond to environmental variability (West-Eberhard 2003), and can be defined as a measure of 'organismal malleability' (Huey and Berrigan 1996). Here we report the first in-depth investigation of plastic responses for a marine invertebrate to the combined exposure of elevated pCO_2 and temperature that will likely occur in the near future; according to projected pCO_2-pH and SST levels (IPCC. 2007, Sokolov et al. 2009). In particular, we have shown that the plastic responses of different morphometric traits of the calcified structure (shell) of invertebrate species are differently (but almost always negatively) affected by prolonged exposure to either low pH, elevated temperature or their interaction. Hence, under future global climate scenarios, individuals of the periwinkle snail *L. littorea* might be predicted to be smaller in size, with thinner, more rotund shells. Observed changes in shell aperture shape and sizes, with shell aperture more elongated under acidified conditions, will likely cause an increase in individuals' water loss. The emerging pattern of responses observed here is rather complex but suggest that global climate change will likely have far reaching consequences for the ecology of marine organisms.

Chapter 4

The influence of ocean acidification on the shell morphology of *Littorina littorea* (L) populations across a latitudinal gradient

SUMMARY

At present, there is a lack of understanding of how different populations of marine intertidal animals might vary in their responses to ocean acidification (OA). In this chapter, the effects of OA on wet weight and shell morphology were compared in six populations of the intertidal gastropod *Littorina littorea* from a latitudinal range of 3,780 km along the west coast of Europe. Overall, OA had a significant effect on the percentage change in wet weight and shell growth in animals exposed for 14 d to increased pCO_2 by 1294 µatm. Wet weight decreased in all six populations under increased pCO_2 compared to control snails which showed between 1.5 - 4.5% increases in growth. However, there were differences between populations in the degree to which they were affected. Populations at range edges experienced the greatest reduction in wet weight, which was broadly reflected in shell width and thickness. There were also effects of increased pCO_2 on the shape and size of the shell aperture, with mid-range populations showing a decrease in the width and size (area) of this opening to the shell. Together these findings suggest that future pCO_2 may negatively affect the ability of snails to construct their shells and that different populations may be more susceptible than others, which may have implications for their ecology such as their susceptibility to predators and water loss, as well as population dynamics and gene flow, this ultimately affecting their biogeography.

INTRODUCTION

Increasing levels of CO_2 in the ocean has led to a decrease in pH, and a decrease in $[CO_3^{2-}]$ and saturation states for calcite and aragonite (Ω_{ara} and Ω_{calc}), a phenomenon known as ocean acidification (OA) (Caldeira and Wickett 2003, Meehl et al. 2007). Mounting evidence from both laboratory experiments and field observations strongly suggests that many marine species may be negatively affected by OA (Doney et al. 2009). Calcifying organisms such as coccolithophores, foraminifera, corals, molluscs, and echinoderms are predicted to be most vulnerable because they possess a limited ability for physiological compensation which may affect the process of calcification (see Ries et al. 2008, Pörtner et al. 2008, Findlay et al. 2011 and chapter 3 of this thesis) and limit organism ability to counteract the positive dissolution of calcium carbonate structures (Fabry et al. 2008). Negative effects of OA on marine molluscan shell growth and dissolution rates have been reported for species such as *Mytilus galloprovincialis* (Michaelidis et al. 2005) and *Nucella lamellosa* (Nienhuis et al. 2010). Bibby et al. (2007) also found a reduction in the ability of *Littorina littorea* to thicken their shell in response to a predator cue when exposed to low pH conditions, although Findlay et al. (2009b, 2011) found a slight increase in shell thickness and changes in other morphological features (width and height) for the same species.

Shell growth in calcifying organisms is dependent on the availability of $CaCO_3$ (Milliman1974). Two common forms of calcium carbonate crystals in the organic matrix deposited in most marine calcified animals' skeleton are calcite and aragonite (Bowen and Tang 1996, Burman and Schmitz 2005, Fabry et al. 2009). Aragonite is a crystal structure of $CaCO_3$ similar to calcite but plays different functions in shell structures; aragonite is present mainly in the inner, nacreous layer, while calcite is found

mixed with protein on the outer shell (Bandel 1990, Burman and Schmitz 2005). In addition, aragonite is stronger but more soluble in acidic water than calcite (Kleypas et al. 1999). Whenever the carbonate ion [CO_3^{2-}] in the ocean declines, calcified organisms may struggle to takeup sufficient aragonite (Ω_{ara}) or calcite (Ω_{cal}) for skeleton construction (Kleypas et al. 2006, Fabry et al. 2008, Cohen and Holcomb 2009). When Ω_{ara} is > 1, this makes it easier for organisms to takeup sufficient carbonate ions [CO_3^{2-}] for the formation of $CaCO_3$ while if Ω_{ara} is < 1, calcium ion starts dissolve (Nienhuis et al. 2010).

To date, studies focusing on shell dissolution rate and calcification in marine organisms have been documented for single populations such as the mussels *Mytilus galloprovincialis* (Michaelidis et al. 2005) and *M. californianus* (Gaylord et al. 2011), the Pacific oyster *Crassostrea gigas* (Gazeau et al. 2007), and the dogwhelk *Nucella lamellosa* (Nienhuis et al. 2010). All of these studies showed decreased shell calcification rates, but little information exists on how this effect might vary across latitudinal gradients. Rates of calcification are also dependent on carbonate saturation states, which are temperature dependent (Kleypas et al. 1999). At cold-temperatures, reduced calcification rates may be more prominent than at warmer temperatures due to lower stoichiometric solubility products for aragonite or calcite, as well reductions in metabolic activity levels in colder environments (Pörtner 2008, Findlay 2011).
Given the lack of information of how the effects of OA may vary among populations of marine calcifiers, the main aim of this chapter was to investigate whether the effects of OA on the growth of the widespread intertidal periwinkle *Littorina littorea* (L.) varied among populations from across a latitudinal gradient. Six populations of *L. littorea*

living along the thermo-latitudinal gradient in the eastern Atlantic, from the Iberian Peninsula to Scandinavia were investigated.

MATERIALS AND METHODS

Study site

Six populations of the edible periwinkle *Littorina littorea* covering almost the entire range of this species in the northeastern Atlantic were used in this series of experiments (see Figure 4.1). Collection points were spaced fairly evenly between Vigo in northern Spain and Tromsø in northern Norway), giving a latitudinal range of 28° (42 – 70° N), equating to about 3,780 km (calculated using Google Earth © 2011 software, Google) (Figure 4.1).

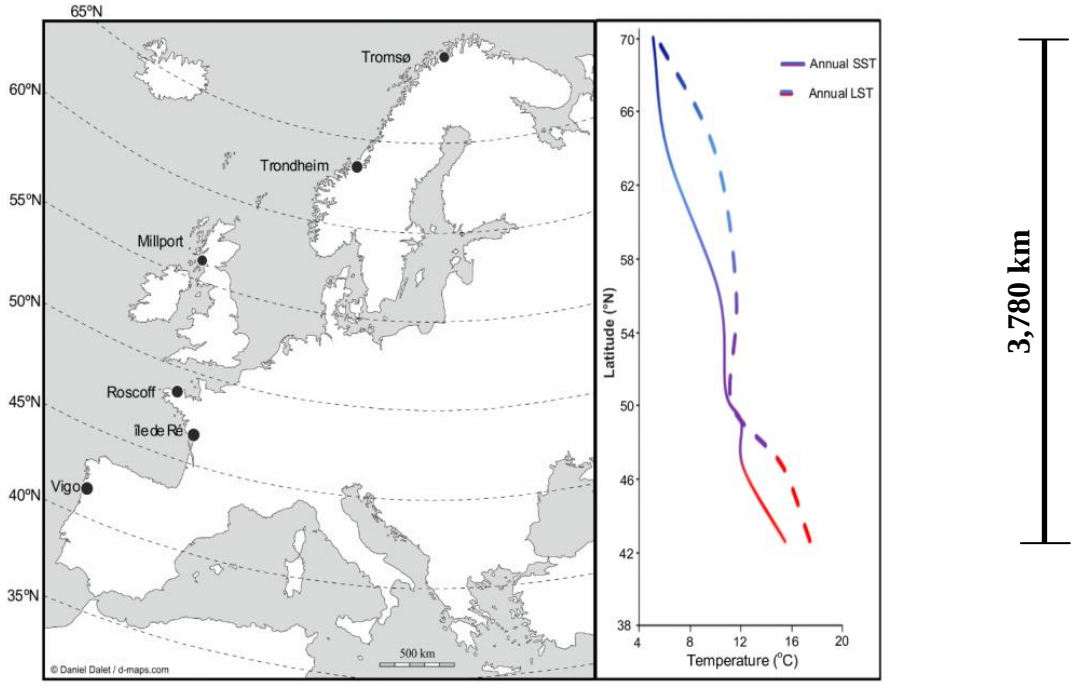

Figure 4.1. Map indicating the locations where populations of the edible periwinkle *Littorina littorea* (L.) were collected along the Western coastline of Europe. The sample sites were 1 = Vigo in Spain, 2 = Ile de Re and 3 = Roscoff in France, 4 = Millport in UK and 5 = Trondheim and 6 = Tromsø in Norway. The graph on the right hand side shows average sea-surface (SST: continuous line) and land-surface temperatures (LST: dash line) for this range.

Mean annual sea surface temperatures (SSTs) for 2005–2009, across this latitudinal range were obtained from the National Oceanic and Atmospheric Administration/National Aeronautics and Space Administration, Advanced Very High Resolution Radiometer (NOAA/NASA, AVHRR Pathfinder 5, http://poet.jpl.nasa.gov/). Spot maximum and minimum SSTs measurements were also taken at each collection location on the date of sampling (Table 4.1). A minimum, maximum and average annual land surface temperature (LST) for each sampling location was also collected from the World Climate web site (http://www.worldclimate.com/). In general there was no significant difference among mean SST across latitudes during summer except main LSTs. Annual SSTs and LSTs decreased with latitude. For additional details on the temperature profile of the sampling locations see Appendix 4.1.

Table 4.1. Spot measurements of sea and land-surface temperature at collection sites on sampling dates include annual temperature of sea- and land-surface on each location. Maximum (max), minimum (min) and mean temperature indicate maximum, minimum and mean temperature level of the month when sample were collected in each collection.

Locality (Latitude - ºN)	Sea-surface temperature (ºC)				Land-surface temperature (ºC)				Month
	Max	Min	Mean	Annual	Max	Min	Mean	Annual	
Vigo (42.13º)	21.8	14.2	18.00	15.30	22.00	14.80	18.40	17.43	Sept
Ile de Re (46.20º)	16.6	16.35	16.48	14.40	23.00	16.00	20.00	15.33	July
Roscoff (48.73º)	15.95	15.87	15.91	12.90	17.00	16.00	16.39	10.25	July
Millport (55.73º)	12.50	12.20	11.6	12.40	14.29	9.29	15.30	11.64	June
Trondheim (63.45º)	15.40	14.60	15.00	7.00	19.80	11.90	15.85	9.93	August
Tromsø (69.62º)	14.65	13.15	13.9	5.80	14.00	7.00	10.50	5.33	August

Animal collection

During summer 2010 (late June to early September), adult *Littorina littorea* (shell width 12 – 15 mm) were collected by hand from the intertidal zones of rocky shores at the sampling localities. Snails were collected during low tide (from as slow as possible in the tidal zone) from beds of macroalgae (*Fucus serratus* and *Ascophyllum nodosum*). Approximately 250 adult individuals of *L. littorea* (shell width 12 - 15 mm) were collected from each site and were transported to the Marine Biology and Ecology Research Centre at the University of Plymouth (Plymouth, UK) within 48 h of collection. Prior to transport, live snails were placed into rectangular plastic containers (20 x 20 x 12 cm) with a perforated lid (25 holes, ø 10 mm) to allow air-circulation and reduce overheating during transport. About 50 - 75 individual snails were placed in each container with damp *Fucus serratus* to prevent desiccation and mechanical damage (see Calosi et al. 2008, 2010). No mortality was recorded during transport and snails were in good condition and showed normal levels of activity upon arrival at the laboratory. Once in the laboratory, snails and algae were transferred to aerated filtered seawater (pH 8.01, salinity 35.0 ppt, 15 ºC) and maintained for 14 d in four plastic aquaria (56 l, max. 75 ind. *per* aquarium) prior to transfer to the experimental set up. This pre-acclimation period was considered sufficient to standardise snail physiological conditions following stress from collection and transportation, as well as to remove as much as possible any 'environmental signature' due to the exposure to local conditions (see Sokolova and Pörtner 2003, Terblanche et al. 2007, Calosi et al 2008). During the pre-acclimation, and later, snails were fed every second day *ad libitum* on *Fucus serratus* and 50% seawater volume was replaced every four days to maintain stable salinity and pH conditions, and eliminate the accumulation of ammonia. All specimens were kept in a temperature-controlled environment under a12:12 h Light:Dark regime).

Experimental design and mesocosm setup

A factorial design incorporating seawater with two levels of pCO_2 representing current conditions (385 µatm, pH 8.0) and future predicted levels for the year 2100 (1000 µatm, pH 7.6) (Caldeira and Wickett 2005, IPCC. 2007) and temperatures at 15 ºC was used. A CO_2/air-equilibration microcosm was set up for each treatment in a CT-room maintained at 15 ºC (12 h : 12 h, L : D), as a modified version of the equilibration flow-through systems used by Widdicombe and Needham (2007) and that described in Chapter 4. Briefly, each CO_2 equilibration system consisted of a header tank (80 l) in which the sea water was either aerated by bubbling normal air or acidified by bubbling pure CO_2 gas. From each of the header tanks seawater was gravity fed (600 ml min^{-1}) continuously to a larger holding tank (60×35×15 cm, 32 l) containing 30 plastic pots (80 ml) each having 30 holes (diam. 4 mm) to ensure good water circulation, and containing a single snail (shell width 13.67 ± 1.40 mm, mean ± SD). The excess water from the holding tanks flowed into a sump (a large plastic container size 45×36×35 cm, 56 l), was aerated vigorously to ensure CO_2 degasification, and was recirculated *via* a submersible pump (EP68, Hengtong Aquarium Co. Ltd., Hengtong, Taiwan) to the header tanks. CO_2 gas was released into the header tank using a multi-stage CO_2 regulator (EN ISO 7291, GCE, Worksop, UK) connected to a flip-flop solenoid valve (ORIFICE 3/16 Closed System, Peter Paul Electronics Co. Inc., New Britain, USA) controlled by a calibrated pH controller (pH-201 Digital, Dream Reef, Humberston, UK).

Seawater temperature in the holding tanks was maintained at 15 ºC by ambient conditions in the CT room where the experiment was conducted. Seawater temperature, salinity, pH, total Dissolved Inorganic Carbon (DIC) and dissolved oxygen in exposure

tanks were measured daily. Temperature and salinity were measured using a handheld multimeter (YSI 85, YSI Inc., Yellow Springs, USA), and pH using an Inlab 413SG pH electrode and Sevengo pH meter (Mettler-Toledo GmbH, Sonnenbergstrase, Switzerland) employing the US National Bureau of Standards (NBS) pH standards. Total CO_2 was measured using a CO_2 analyser (965D, Corning Ltd., Cambridge, UK). Carbonate system parameters that were not directly measured were calculated using CO2SYS (Pierrot et al. 2006), using constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO_4 dissociation constant from Dickson (1990).

Measuring shell morphology and growth

Shell morphological parameters were calculated as the proportional changes between measurements at the start of the experiment and after 14 d exposure. These parameters were shell length and width, shell shape, measured as aspect ratio (shell length:shell width), aperture shape measured by the aperture ratio (aperture length:aperture width) and aperture area, and shell thickness measured at two places: alongside the columellar axis in the posterior aperture of the shell (thickness 1), and at the growing tip lying along the anterior portion of the shell (thickness 2). All measurements were carried using image processing (as described in Chapter 3). Images of individual snails were taken using a digital camera (Nikon Coolpix 4500, Nikon Corp, Essex, UK) attached to a high performance stereomicroscope (Leica MZ12$_5$, Leica Microsystems Ltd., Heerbrugg, Switzerland) at low magnification (4x). Each individual was placed with the shell aperture perpendicular to the microscope field of view. A scale bar was placed alongside the snail and was used to calibrate shell measurements. All images were analysed using a digital image analysis tool (UTHSCA Image Tool, Vers.3.0).

The percentage change in total wet weight over the experimental exposure was also measured, as a proxy for growth, as the difference between wet weight at the beginning and the end of the exposure time. The shell was blotted using dry tissue paper and animals were placed aperture down for 10 min (Sokolova and Pörtner 2001, Al-Mazrouai 2008, see also Chapter 3) to allow any remaining water to be expelled *via gravity* and absorbed with the use of a cotton bud by pressing down gently on the operculum. Each individual was then placed into a plastic vial and weighed using an analytical digital balance (Sartorius 1201 MP2, DWS Inc., Bohemia, USA).

Statistical analyses

A two way ANCOVA was performed to assess significant effects of elevated pCO_2, latitudinal position/population and their interaction on the percentage change in shell biometric parameters (wet weight, shell width, shell length, shell shape, aperture width, aperture length, aperture ratio, aperture area, and shell thickness on the inner and outer lips). In this test, tank was set as a random factor nested within pCO_2. The initial size for each parameter measured here was used as covariate. Estimated margin mean with Bonferroni correction was used to assess pairwise differences for significant differences shown by the ANCOVAs. Data for aperture shape met assumptions for normality following arcsine transformation ($Z_{1080} = 0.738$, $p = 0.648$) but this was not the case for the other morphological parameters (minimum $Z_{1080} = 2.459$, $p = 0.00012$). Variances were homogeneous for percentage change of shell width and aperture shape (maximum $F_{11, 1068} = 1.78$, $p = 0.0534$), but not for other morphological parameters (minimum $F_{11, 1068} = 3.990$, $p < 0.0001$). However, it is assumed that the ANCOVA design employed should be fairly tolerant of deviation from the assumption of normality of distribution and homogeneity of the variances given that we employed an orthogonal experimental

design with twelve treatments (minimum number of replicates per treatment was thirty) (Sokal and Rohlf 1995, Underwood 1997), and a high number of replicates was used (1080 individual snails). 'Tank' had a significant effect on aperture width, aperture length, aperture shape, thickness-1 and thickness-2 (minimum $F_{4, 1079} = 2.94$, $p = 0.02$), and was thus kept in the analysis. However, the 'tank' effect was also accompanied by strong significant effect of pCO_2 or its interaction which means that 'tank' was not affecting the biometric changes in isolation. In addition, in the cases where the term 'tank' was found to have a significant effect on the morphometric traits investigated here, removing this factor from the analysis caused no change to the significance of the main factors (pCO_2 and population), and thus tank effect is considered marginal. All analyses were conducted using SPSS 19.

RESULTS

Microcosm parameters

Seawater physico-chemistry was stable over the experimental period (14 d) with little variation around the mean for all parameters (Table 2). Mean dissolved inorganic carbon (DIC) values were 1540 ± 19 µmol kg^{-1} in the control treatments and 1841 ± 19 µmol kg^{-1} in the acidified treatments. Mean CO_2 values were 343 ± 6 µatm (mean ± SE) in the control treatments and 1294 ± 17 µatm in the acidified treatments, resulting in mean pHs of 8.1 ± <0.01 and 7.6 ± <0.01 in control and acidified treatments, respectively. Mean temperatures under normal and high CO_2 conditions were 14.96 ± 0.05 and 14.65 ± 0.02 ºC, respectively.

Table 4.2. Mean seawater physico-chemical parameters for four treatments over 14 days of the experiment. *Calculated using CO_2SYSs (Pierrot et al. 2006) using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

Parameter	Normal pCO_2	High pCO_2
O_2 (mg l^{-1})	6.78 ± 0.03	6.64 ± 0.03
Salinity (ppt)	35.12 ± 0.06	35.57 ± 0.03
Temperature (°C)	14.96 ±0.05	14.65 ± 0.02
pH	8.11 ± 0.004	7.61 ± 0.003
DIC (µmol kg^{-1})	1539.67 ± 18.59	1841.13 ± 18.95
TA (µequiv kg^{-1}) *	1701.02 ± 19.20	1441.13 ± 18.95
pCO_2 (µatm) *	343.03 ± 6.05	1293.73 ± 16.85
$[HCO_3^-]$ (µmol kg^{-1}) *	1421.92 ± 17.47	1750.87 ± 18.03
$[CO_3^{2-}]$ (µmol kg^{-1}) *	104.95 ± 1.25	41.62 ± 0.50
Ωcal *	2.50 ± 0.03	0.99 ± 0.01
Ωara *	1.61 ± 0.02	0.64 ± 0.01

Wet weight

Exposure to elevated pCO_2 led to a decrease in percentage change in snail total wet weight but the degree of the effect varied between populations, as indicated by a significant interaction between the terms 'population' and 'pCO_2' (Table 4.3). Range edge populations showed a 3-4 times greater percentage decrease than centre range populations (Fig. 4.2A and Appendix 4.2). Under high pCO_2 conditions percentage change in wet weight was always negative for all populations tested, mean percentage change in wet weight ranging between -3.59 and -0.45% (Fig. 4.2A).

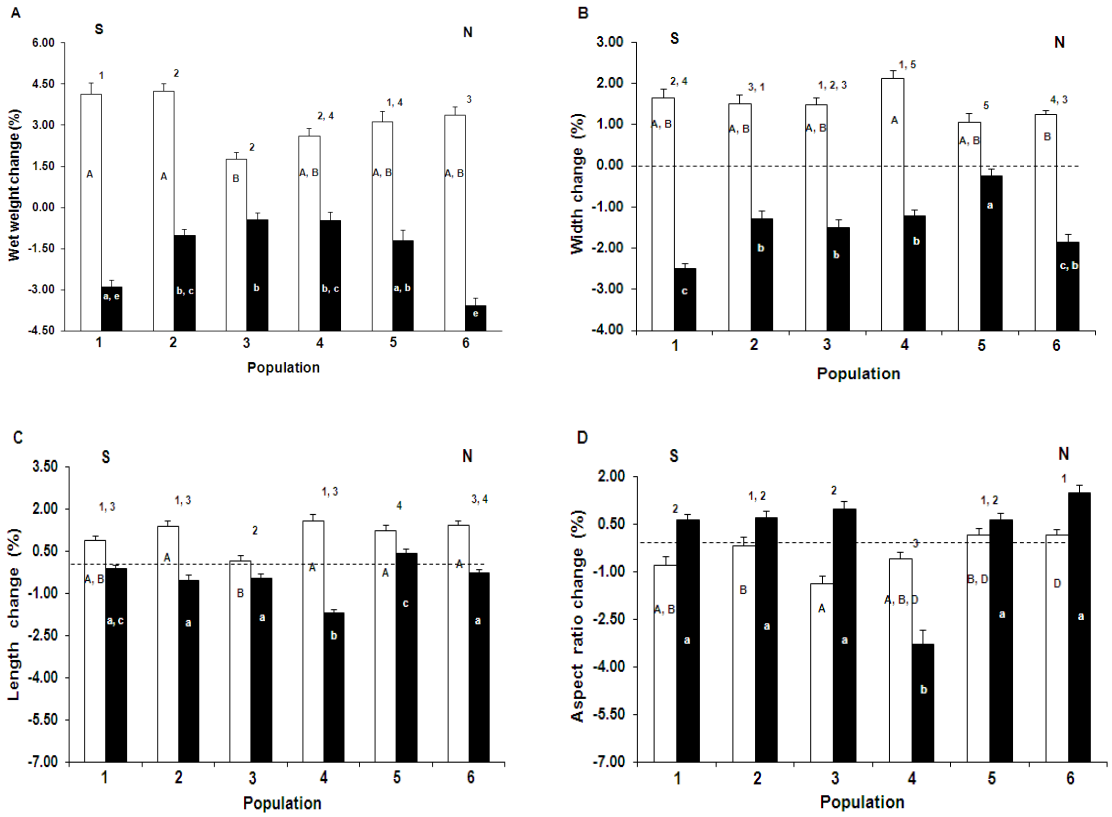

Figure 4.2. Mean (± SE) percentage change of shell parameters of *L. littorea* exposed to conditions of current (white) and future pCO_2 (black): A) wet weight, B) shell width, C) shell length, and D) aspect ratio. Significantly different means ($p \leq 0.05$) within a pCO_2 treatment are indicated by capital letters for current pCO_2 conditions and by lower case letters for future pCO_2 conditions. S and N indicate southern and northern latitudes respectively. Different numbers indicate that a significant difference exists between the overall mean percentage of a trait among different populations (combining both current and future pCO_2), according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

This contrasted with growth under current pCO_2 conditions with wet weight increase positive for all populations (Fig. 4.2A).

Table 4.3. Results of multiple ANCOVAs testing for the effects of elevated pCO_2 on morphological traits in six populations of the periwinkle *Littorina littorea*. Degrees of freedom (df), mean of square (MS), F-ratio (F), probability level (*p*) are shown.

Trait	Source	df	MS	F	*p*
Growth (%)	pCO_2	1	11.79	33.9	**0.002**
	population	5	0.36	1.12	0.451
	pCO_2*population	5	0.34	22.5	< **0.0001**
	initial weight (covar)	1	0.61	40.57	< **0.0001**
Width (%)	pCO_2	1	2372.72	202.14	< **0.0001**
	population	5	41.23	3.62	0.09
	pCO_2* population	5	11.68	4.73	< **0.0001**
	initial width (covar)	1	51.44	20.85	< **0.0001**
Length (%)	pCO_2	1	674.21	17.43	**0.009**
	population	5	21.04	0.57	0.723
	pCO_2* population	5	38.28	15.95	< **0.0001**
	initial length (covar)	1	12.09	5.04	**0.025**
Shape (%)	pCO_2	1	120.65	0.96	0.371
	population	5	114.55	0.93	0.532
	pCO_2* population	5	124.91	24.39	< **0.0001**
	initial shell ratio (covar)	1	483.06	94.31	< **0.0001**
Aperture width (%)	pCO_2	1	1048.68	8.42	**0.002**
	population	5	125.04	1.69	0.288
	tank (pCO_2)	4	57.6	7.96	< **0.0001**
	pCO_2* population	5	75.8	10.48	< **0.0001**
	initial Ap.width (covar)	1	177.53	24.54	< **0.0001**
Aperture length (%)	pCO_2	1	5768.43	4.79	0.072
	population	5	537.3	0.53	0.75
	tank (pCO_2)	4	142.04	4.81	**0.001**
	pCO_2* population	5	1070.45	36.23	< **0.0001**
	initial Ap. length (covar)	1	3108.07	105.2	< **0.0001**
Aperture shape (%)	pCO_2	1	1728.42	1.65	0.239
	population	5	1379.62	1.83	0.261
	tank (pCO_2)	4	293.53	6.74	< **0.0001**
	pCO_2* population	5	795.65	18.27	< **0.0001**
	initial Ap. Ratio (covar)	1	9704.2	222.79	< **0.0001**
Aperture area (%)	pCO_2	1	2078.26	2.39	0.183
	population	5	264.07	0.32	0.884
	pCO_2* population	5	863.66	21.27	< **0.0001**
	initial Ap.cross-sec (covar)	1	2626.52	64.7	< **0.0001**
Thickness-1 (%)	pCO_2	1	44828.27	23.53	**0.003**
	population	5	3134.56	1.77	0.273
	tank (pCO_2)	4	210.9	2.94	**0.02**
	pCO_2* population	5	1836.95	25.57	< **0.0001**
	initial T-1 (covar)	1	9165	127.59	< **0.0001**
Thickness-2 (%)	pCO_2	1	305651.1	206.19	< **0.0001**
	population	5	9938.91	9.79	**0.012**
	tank (pCO_2)	4	970.89	4.95	**0.001**
	pCO_2* population	5	1062.08	5.41	< **0.0001**
	initial T-2 (covar)	1	62219.74	317.02	< **0.0001**

Shell size and aspect ratio

Exposure to elevated pCO_2 led to a decrease in percentage change in shell width with a greater reduction found in populations at the range edges when compared to those from the centre of the range (Fig. 4.2B and Appendix 4.2), as indicated by a significant interaction between 'population' and 'pCO_2' (Table 4.3). Under high pCO_2 conditions, the mean percentage change in width was always negative for all populations tested, with percentage change ranging between -2.50 and -0.25 % (Fig. 4.2B and Appendix 4.2) and positive for all populations when snails were kept under current pCO_2 conditions, with mean percentage change ranging between +1.05 and +2.11% (Fig. 4.2B and Appendix 4.2). Mean percentage change in shell width was found to be significantly lower for snails kept under elevated pCO_2 conditions when compared with those kept under control conditions across and within populations. There was no significant difference in shell width increase between populations (Table. 4.3).

Exposure to elevated pCO_2 also led to a decrease in snails' mean percentage change in shell length for all populations tested, but with different intensities among populations as indicated by a significant effect of interaction of population and pCO_2 (Table 4.3). Unlike shell width, however, a greater reduction in shell length was found in only one population, Roscoff (Fig. 4.2C and Appendix 4.2). Mean percentage change in shell length in snails kept under high CO_2 conditions was negative for most populations and ranged between -1.71and -0.12 % compared with that under current pCO_2 conditions, which was positive for all populations ranging between +0.15 and +1.56% (Appendix 4.2). Mean percentage change in shell length was significantly lower for snails kept under elevated pCO_2 conditions across and within all populations (see Fig. 42C and Table 4.3).There was no significant difference in shell length change between

populations (Table. 4.3). There was a significant interaction between 'pCO_2' and 'population' for aspect ratio change. All populations except population 4 showed an increase in percentage change in aspect ratio (i.e. became longer and thinner) under elevated pCO_2 (Table 4.3).

Aperture width and aperture area

The mean percentage change in aperture width was negative for all populations kept under elevated pCO_2 (range -3.80 and -0.85 %) but positive for most populations under current pCO_2 conditions (+0.08 to +0.86%) (Fig. 4.3A). Exposure to elevated pCO_2 led to a general decrease in percentage change in shell aperture width, with populations living at the centre of the range showing the greatest reduction (Roscoff and Millport, 1.5 to 2.3 fold) compared to those at the range edges (Fig. 4.3A and Appendix 4.2), as indicated by the significant interaction between 'population' and 'pCO_2' (Table 4.3). Mean percentage change in aperture width was found to be significantly lower for snails kept under elevated pCO_2 conditions across and within populations (Fig. 4.3A and Table 4.3).

Percentage change of aperture area also showed a greater decreased in central range populations (Ile de Re, Roscoff and Millport) when compared to that measured at the extreme range edges (Fig. 4.3B) under high pCO_2, (app. 6-fold), indicated by significant effect of interaction between 'pCO_2' and 'population' (Fig. 4.3B).

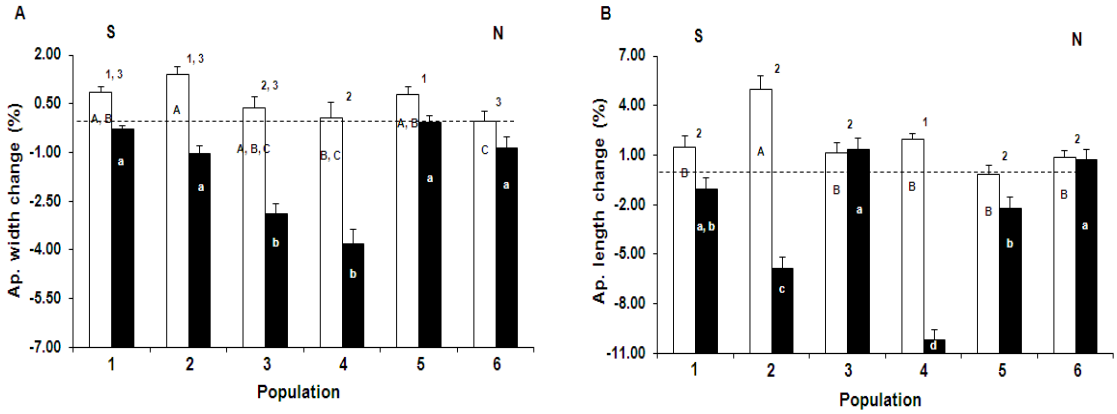

Figure 4.3. Mean (± SE) percentage change of shell parameters of *L. littorea* exposed to conditions of current (white) and future pCO_2 (black): A) aperture width and B) aperture area. Significantly different means ($p \leq 0.05$) within a pCO_2 treatment are indicated by capital letters for current pCO_2 conditions and by lower case letters for future pCO_2 conditions. S and N indicate southern and northern latitudes respectively. Different numbers indicate that a significant difference exists between the overall mean percentage shell aperture width, aperture area and thickness-2of different populations (combining both current and future pCO_2), according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

Thickness

Exposure to elevated pCO_2 led to a decrease in thickness-1 and thickness-2 in all populations tested, but this decrease varied among populations, as indicated by a significant interaction between the terms 'population' and 'pCO_2' (Table 4.3 and Fig. 4.4A and B). Under high pCO_2 conditions percentage change in shell thickness 1 was negative for all populations tested (range -24 and -3%) (Fig. 4.4A and Appendix 4.2), but positive for all populations under current pCO_2 (range +1.44 and +9.0%) (Fig. 4.4A and Appendix 4.2). There was no significant effect of population on thickness-1 (Table. 4.3).

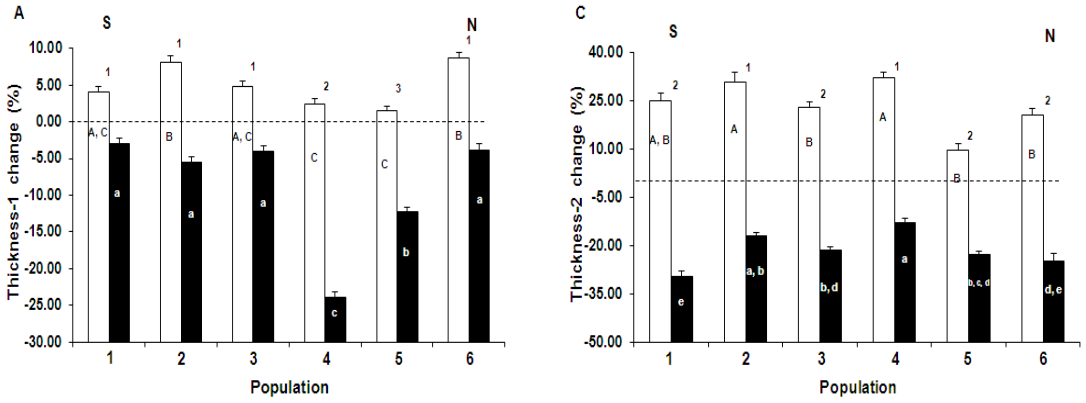

Figure 4.4. Mean (± SE) percentage change of shell parameters of *L.littorea* exposed to conditions of current (white) and future pCO_2 (black): A) shell thickness-1 and B) shell thickness-2. Significantly different means ($p \leq 0.05$) within a pCO_2 treatment are indicated by capital letters for current pCO_2 conditions and by lower case letters for future pCO_2 conditions. S and N indicate southern and northern latitudes respectively. Different numbers indicate that a significant difference exists between the overall mean percentage shell width, length and shape of different populations (combining both current and future pCO_2), according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

Mean percentage change in thickness-2 was also found to be significantly lower for all populations under elevated pCO_2 conditions when compared to those at control conditions (Table 4.3).A greater response was found in thickness-2. Under elevated pCO_2 the percentage change in shell thickness-2, in general, decreased from central ranges to range edges with the reduction at range edges (particularly Vigo) (Fig. 4.4B). Under high pCO_2 conditions percentage change in shell thickness-2 was always negative for all populations tested (range -30 and -13%) (Fig. 4.4B and Appendix 4.2) whilst under current pCO_2 conditions shell thickness-2 was found to be positive for all populations (range +10 and +32%) (Fig. 4.4B and Appendix 4.2).

DISCUSSION

The main aim of this chapter was to investigate whether the potential effects of OA varied among populations of the intertidal gastropod *Littorina littorea* collected from across this species range in the North Eastern Atlantic. There was a clear effect of increased pCO_2, with all populations showing a loss in wet weight over the duration of the experiment, which seemed to reflect a significant decrease in the width and thickness of the shell. This effect was most pronounced in populations from the northern and southern range edges. At the same time, there was a significant effect of pCO_2 on the shape and size of the shell aperture, this time with the mid-range populations showing the greatest effect from ocean acidification, with significantly narrower and smaller apertures.

Reduced shell wet weight termed 'shell growth' under high CO_2 has been demonstrated in the marine mussel *Mytilus galloprovincialis* (Michaelidis et al. 2005). This species reduced its shell growth by about 11% after 18 d and by over 30% after 90 d of exposure. Decreased shell length under high CO_2 has also been found in *M. edulis* (Berge et al. 2006), shell diameter or shell width of foraminifera species (Fujita et al. 2011), and shell thickness in the larval of the mussel *M. californianus* (Gaylord et al. 2011). Such shell reductions under high CO_2 conditions were thought to be caused by the dissolution of $CaCO_3$ structures made of aragonite or calcite or both (Kleypas et al. 1999, 2006, Berge et al. 2010, Nienhuis et al. 2010, Gaylord et al. 2011, Pistevos et al. 2011). Even though calcification still occurs under high CO_2 conditions, it occurs to the same or lower rates than normal but clearly not sufficiently fast to compesante for the ongoing passive dissolution of the shell (S. Melatunan et al. *pers. obs.*, see page 80). Overall effects of OA can cause reduced shell integrity and weakened shell structure

(Gaylord et al. 2011) and may increase susceptibility of snails to predators (e.g. DeWitt et al. 1998, Trussell and Etter 2001, Trussell and Nicklin 2002, Mikolajewsky and Johansson 2004, Bibby et al. 2007, Gaylord et al. 2011). There is some evidence that marine gastropods may also use shell shape as a way to reduce their vulnerability to predators. For example, more globular or discoid shaped shells, with a lower aspect (i.e. length to width) ratio may be more difficult for crabs to handle (Cotton et al. 2004). Although, the shell shape of *Littorina littorea* did change as a result of ocean acidification in this study, this seemed to relate more to a decrease in shell width rather than an overall change in shell shape.

Populations at the southern- (Vigo) and northern-most (Tromsø) edges of the range were affected most by high pCO_2 than central range populations, and had a reductions in shell growth between 2.5 and 4.5-fold, and in shell thickness between 1.5 and 2 fold, respectively, greater than central range populations. These similar responses of range edge populations could reflect similar physiological mechanisms or similar $CaCO_3$ saturation states in these two locations. However, current evidence shows that the Southern Atlantic Ocean (32^o N) (e.g. Bates et al. 2009 see also Riegl 2003) and northern latitude in the northern hemisphere (Orr et al. 2005, Yamamoto et al. 2009, Bates et al. 2009, Fabry et al. 2009) may already experience lower aragonite saturation. Feely et al. (2008) and Wootton et al. (2008) also reported that several coastal areas have experienced lower aragonite saturation levels due to upwelling of corrosive water to Surface Ocean. Hence, decreasing similar patterns of shell weight and thickness cannot be related to (at least solely) to current aragonite saturation state at the southern and north edges of the geographical range of distribution of *L. littorea*. Michaelidis et al. (2005) proposed that rather than dissolution rate, lower metabolic rate caused

reduction in growth rate of *M. galloprovincialis*. Findlay et al. (2011) also proposed that changes in growth following the exposure to high CO_2 in *L. littorea* can be linked to the alteration of energy metabolism. Findlay et al. (2010a and b) demonstrated that calcification rate in barnacle *Semibalanus balanoides* was lower at northern compared with southern latitudes, however my results are likely to be the product of the interplay between direct shell dissolution and indirect alteration of calcification patterns due to metabolic depression (Portner et al. 1998, Michaelidis et al. 2005) and/or energy budget reallocation (Sibley and Calow 1986, Findlay et al. 2010a and b, see also Wood et al. 2008, 2010).

Differences in shell aperture shape between populations were marked with three of the populations (Ile de Re, Roscoff and Millport) having much narrower apertures with smaller areas. Smaller apertures may reduce the chance of desiccation stress (Goodfriend 1986, Perrott et al. 2007) in these populations; variation of air temperature in central range areas around Northern France and British Isles can reach up to 50 ºC (Whiteley et al. 1997). At the same time aperture area could be influenced by other local pressures, such as the presence of shell entry predators.

It is likely that future anthropogenic emissions of CO_2 and resulting ocean acidification may have severe consequences for marine calcifying organisms and ecosystems. Changes of ocean carbonate chemistry as a product of increased [CO_2] into the ocean have displayed extensively direct effect on organismal function and indirect effects on their habitats. One of the most important repercussions of increasing ocean acidity relates to the production of shells out of calcium carbonate ($CaCO_3$). Decalcification is a direct consequence that affects morphological traits of marine organisms particularly

those that produce $CaCO_3$ for growth. Although the magnitude of this effect depends on species identity and the structural composition of shells (mainly of aragonite and/or calcite), all six populations of the periwinkle snail *Littorina littorea* used in this study showed shell dissolution. This dissolution rate led to changes in shell growth and shape and caused vulnerability increase in particular to morphological features that function as defences in snails.

This result indicates that future ocean acidification produces complex plastic responses of morphological traits that would have far reaching ecological consequences for the marine periwinkle *L. littorea*. The complexities of morphological changes observed in different populations of periwinkles exposed to elevated pCO_2 is indicates that future predictions of the responses of marine organism to ocean acidification might be very challenging (Doney et al. 2009, Fabry et al. 2009, Kleypas and Yates 2009).

Chapter 5

Latitudinal variation in the physiological and metabolomic responses of *Littorina littorea* to high CO_2

SUMMARY

Future ocean acidification has the potential to adversely affect many marine organisms with growing evidence suggesting that many species could suffer through reduced calcification and growth rates and physiological disruption under predicted future levels of ocean pH. To date, however, research on the effects of ocean acidification and elevated temperatures among species and populations has been scarce or partial. In this chapter, an investigation of the effects of medium-term exposure to elevated pCO_2 on the metabolic rate and metabolic profiles of six populations of the edible periwinkle *Littorina littorea* across a latitudinal gradient in the Eastern Atlantic were carried out. In addition, snails were exposed to different short-term temperatures are meant to assess how pre-exposure to pCO_2 influenced their metabolic responses under different temperature regimes during emersion. Metabolic rate measured as rates of O_2 uptake after medium-term exposure to high pCO_2 conditions varied among populations: southern populations showed a decreased rate, centre-range populations an increased rate, and northern populations showed no significant changes (population 5 decreased and population 6 increased) compared with those exposed in normal pCO_2. There was also an overall negative relationship between metabolic rates and latitude under control conditions, while under high pCO_2 metabolic rates showed a non-linear trend across latitudinal gradients. Metabolomic analysis using proton nuclear magnetic resonance (P-NMR) showed that the two northern populations were distinct from other populations when exposed to medium-term elevated pCO_2 at short-term low temperature (15 ºC) due, in part, to high levels of thymine, uracil, valine and lysine. A similar cluster also occurred under high temperature exposures in which one of the northern most populations was distinct from other populations and had lower concentrations of alanine, betaine and taurine but higher concentrations of valine. Overall these results

suggest that populations at southern and central range may apply different ionic transport mechanisms in response to medium-term elevated pCO_2 and short-term elevated temperatures and those populations are likely to vary in terms of their physiological responses to this environmental challenge.

INTRODUCTION

Increasing atmospheric concentrations of CO_2 as a consequence of steadily growing anthropogenic emissions (Petit et al. 1999, Augustin et al. 2004, Siegenthaler et al. 2005) will not only lead to global warming but also to an acidification of the oceans (Caldeira and Wickett 2003, Feely et al. 2004, Orr et al. 2005). During the last century the global mean surface ocean pH value has dropped by 0.1 unit (from 8.2 to 8.1) (Caldeira and Wickett 2003), and it is projected to continue decreasing by a further 0.70 units by year 2300 if emissions of anthropogenic CO_2 are not reduced (Caldeira and Wickett 2003, Hoffman and Schellnhuber 2010). This phenomenon is called Ocean Acidification (OA) and combined with increasing sea surface temperatures, will represent a unprecedented challenge to marine ecosystems, with calcifying marine organisms generally predicted to be most at risk to declining ocean pH (Pörtner et al. 2004, Michaelidis et al. 2005, Nienhuis et al. 2010, Gaylord et al. 2011). Indeed, increasing OA and temperature have been shown to alter the physiology of calcified organisms by reducing their metabolic rates (Rosa and Seibel 2008, Melatunan et al. 2011, but see Wood et al. 2008) and haemolymph pO_2 levels (Metzger et al. 2007), causing a shift in metabolic pathways (Lanning et al. 2010, Melatunan et al. 2011 and Chapter 3, Zhang et al. 2011a) and calcification rates (Ries et al. 2009, Findlay et al. 2011).

Even though we are acquiring a relatively good understanding of the physiological consequences of the exposure of marine organisms to OA, studies looking at the level of physiological variation under exposure to high CO_2 in different species and populations are scarce (but see for example Walther et al. 2010, 2011). In addition, Findlay et al. (2010a and 2010b) demonstrated that populations of the barnacle *Semibalanus*

balanoides living at the north and southernmost limit of their distribution differed in their response to low pH. At its southern range (Southwest coast of England) barnacles exhibited positive growth when sea surface temperature (SSTs) was above 13 ºC compared with the northern population in Svalbard that show a negative impact of exposure to OA conditions on growth and larval survival when temperatures were maintained at 4.8 ºC. Growth was not affected when temperature was increased by +4 ºC.

In general, animals able to inhabit a broad range of environmental conditions are predicted to possess broader physiological windows, a greater physiological plasticity to cope with environmental fluctuations (Calosi et al. 2008, 2010, Morley et al. 2009, Bozinovic et al 2011) and to occur over greater latitudinal ranges (Pörtner 2002, Calosi et al. 2008, 2010, Whiteley et al. 2011, Rastrick and Whiteley 2011). At the same time, populations living at the edges of latitudinal ranges of distribution of a species may vary in their physiological responses. Warm-adapted species and populations living at lower latitudes may tolerate increased temperature by adjusting their aerobic metabolic rate to be lower (Pörtner 2002, Pörtner and Knust 2007), although evidence exists for the opposite trend (e.g. Rastrick and Whiteley 2011) but range edge populations may also possess lower phenotypic plasticity (see Stillman 2003, Deutsch et al. 2008, Tewksbury et al. 2008, Tomanek 2008). Taxa at high latitudes may increase their metabolic rate to compensate for the fact they live in a cold environment (Krog 1954, Pörtner 2002, Schaefer and Walters 2010, Sorte et al. 2011), although some cold-adapted organisms do not show such responses (Rastrick and Whiteley 2011, Bozinovic et al. 2011). These differences in populations' responses may mean that populations from different

positions within the latitudinal range of a species may differ in their abilities to cope with OA (Walther et al. 2010, 2011).

Exposure to elevated CO_2 conditions leads to internal hypercapnia, which causes decreased metabolic rates, reduced energy budgets and disrupted physiological functions (Pörtner et al. 1998, Melatunan et al. 2011 and Chapter 3 of this thesis). The ability to maintain cellular homeostasis will largely depend on organisms' capacity for osmo-ionic and acid-base balance (Pörtner et al. 1998a, 2000, Whiteley 1999, 2011). In addition, the maintainance of homeostatic physiology in order to maintain biogeographic distribution can be also done through molecular-level adaptations to thermal conditions (Dong and Somero 2009), while some organisms possess physiological plasticity in response to temperature variability (Stillman 2003, Calosi et al 2008, Tomanek 2008, Bozinovic et al. 2011 Whiteley et al. 2011). Both of these strategies may also vary between species populations from different latitudes.

Studies characterizing physiological variations among populations living across thermo-latitudinal gradients using selected metabolites have been conducted in various organisms for example in the limpet *Lotia spp.* (Dong and Somero 2009), in the killifish *Fundulus heteroclitus* (Powers et al. 1991), and western sandpipers *Calidris mauri* (Williams et al. 2007). Metabolomics is a rapid holistic approach for characterising multiple and complex metabolic responses of various organisms in response to various environmental conditions (Viant 2007). This approach is able to detect small metabolites with low molecular weights and can be used to examine the physiological conditions of a cell or organism and associate the metabolic changes to genetic or environmental modulation (Viant 2003, Lin et al. 2006, Wu et al. 2008, Tyagi

et al. 2010). To date, proton-nuclear magnetic resonance (P-NMR) has been widely used in toxicology (e.g. Tjeerdema 2008), ecotoxicology (e.g. Rosen et al. 2011), physiology of fishes (e.g. Viant 2003), pharmaceutics and human health (e.g. Tian et al. 2005), but it has never being used for the in-depth characterisation of the metabolic responses of a marine organism to the exposure to elevated pCO_2 and temperature

Hence, the main aim of this chapter was to assess whether different populations of the edible periwinkle *Littorina littorea* varied in their metabolic response to increased pCO_2. As the populations collected spanned nearly the entire latitudinal range of this species in the Eastern Atlantic, this can provide better understanding to assess whether any variation between populations was related to their position across this latitudinal range (i.e. acclimatisation/adaptation to different thermal regimes). At the same time, the effect of the interaction between medium-term pre-acclimation to elevated pCO_2 in sea water and short term exposure in air were also carried out. This was intended to describe how the influence of intertidal rocky shore with two different conditions of high and low tides in combination with high pCO_2 and temperature may influence the snails' metabolism. A general characterization of metabolism was generated by measuring rates of O_2 uptake, whilst a more detailed view of their metabolic responses was gained by using NMR to measure the metabolic fingerprinting of individual snails.

MATERIALS AND METHODS

Study sites and animal collection

Snails of the edible periwinkle *Littorina littorea* were sampled in summer (June-September) 2010 from six intertidal locations in the north-eastern Atlantic between 42 –

70º N latitude, from Vigo in Northern Spain to Trømso in Northern Norway. *L. littorea* individuals (shell width 13 - 15 mm) were collected at the lowest tide possible (between 1.0 to 1.7 m above chart datum). Details of the study sites, sample collection and snail maintenance are given in Chapter 4.

Exposure to reduced pH

As described in Chapter 4, snails were acclimated in seawater to two levels of pCO_2 representing current conditions (385 µatm, pH 8.0) and those predicted for 2100 (1000 µatm, pH 7.6) (Caldeira and Wickett 2005, IPCC. 2007). A CO_2/air-equilibration microcosm was set up for each treatment in a CT-room maintained at 15 ºC (12 h light and 12 h dark), as a modified version of the equilibration flow-through systems used by Widdicombe and Needham (2007), Dashfield et al. (2008) and Widdicombe et al. (2009). Briefly, each CO_2 equilibration system consisted of a header tank (80 l) in which the sea water was either aerated by bubbling normal air or acidified by bubbling pure CO_2 gas. Further details of this set up are given in Chapter 4.

Metabolic rates

Metabolic rates of individual snails were measured at the end of the exposure period (14 d), using rates of O_2 uptake as a proxy following the method developed by Spicer and Erickson (2003) and adapted for periwinkles by Melatunan et al. (2011) - see also Chapter 3. Here, as well as investigating how medium-term, pre-exposure to low pH affected O_2 uptake, the assessment of how short-term exposure to different temperatures immediately prior to metabolic measurements affected responses was also conducted, as snails will potentially face the greater challenges at low tide when living in high CO_2-warmer world. Hence, six individual snails were selected haphazardly from each

experimental tank (giving a total of thirty individuals per population/ pCO_2 treatment/temperature combination) and exposed in air in an incubator (Perspex cube, size 60 x 60 x 50 cm), with an automatic heater (Microclimate Advance Heating System, Net Pet Shop Limited, Manchester, UK) to one of five temperatures (15, 20, 25, 30 and 35 ºC) for 3h. The CO_2 in the incubator was adjusted to the level of pCO_2 that snails had previously been exposed to, i.e. either 380 or 1000 ppm. Regulation of the pCO_2 was controlled through a gas analyser (LI-7000 CO_2/H_2O, LI-COR Environmental Ltd, Cambridge, UK) connected to a computer. Following exposure to air, each individual snail was placed in a blacked out glass jar (70 ml) for 5 min, which was then submersed in a glass aquarium (49 x 23 x 28 cm, cap. 32 l) containing seawater at15 ºC using a close bottle respirometer (a backed out glass jar, volume 70 ml). Initial O_2 concentration was recorded just before the jar was sealed and after 1h of incubation using an O_2 meter (Model 781, Strathkelvin Instruments, Glasgow, UK) with an O_2 electrode (1302 electrode, Strathkelvin Instruments, Glasgow, UK). Rates of O_2 uptake was expressed as μmol O_2 g^{-1} wet weight h^{-1}.

Metabolic fingerprinting

In order to assess whether the metabolic fingerprinting of individual snails was influenced by medium-term exposures to pCO_2 followed by short-term exposure to aerial exposure at different temperatures at similar pCO_2 levels, ten snails from each of three temperature exposures (15, 25, 35 ºC) from each pCO_2 treatment were selected for metabolomic analysis. Immediately following oxygen consumption trials, tissue samples (0.20 – 0.25 g) of foot muscle were dissected from each individual by cracking the shell quickly using a mini grip clamp (Quick-Grip Bar Clamp Q/G5122QC, Hyquip Ltd, Lancashire, UK). This tissue was then cleaned in fresh seawater, placed in a 1.5 ml

Eppendorf tube (Sigma-Aldrich Co. LLC., Gillingham, UK), frozen in liquid nitrogen (temperature of -160 ºC) and stored in a freezer (-80 ºC).

Tissue extraction for metabolomics

Tissue extracts were prepared for use in Nuclear Magnetic Resonance (NMR) spectroscopy using a two-step extraction procedure (Lin et al. 2007, Wu et al. 2008) carried out at Natural Environment Research Council (NERC) Metabolomics Facility in the School of Biosciences at the University of Birmingham, UK. Twelve foot muscles were pooled haphazardly from sample trays (2 foot muscles per tray in which each tray contained foot tissues from one location), dissected into small fractions (60 – 100 mg), and snap frozen in liquid nitrogen in Precellys tubes containing steel balls (Precellys tube, cap. 7 ml., Bertin Technologies Corp., Saint Quentin en Yvelines Cedex, France). All these procedures were carried out in less than ten seconds. The frozen samples were then mixed with HPLC grade MeOH/H_2O at a ratio of 6:1.275 and homogenized with a Precellys-24 bead-based homogenizer (Stretton Scientific, Stratton, UK) at 6800 rpm, 2 x 20 sec. Homogenised samples were transferred to glass vials (Fisher TUL 520 006J glass vial 1.8 ml, Fisher Scientific Ltd., Loughborough, UK) and mixed with pesticide grade $CHCl_3$/H_2O at a ratio of 6:2.4. Samples were rapidly mixed by vortexing (Vortex Mixer ZX3, VELP Scientifica Srl., Usmate, Italy) for 30 s, and cooling on an ice bath for 10 minutes. Solvents (extracted solutions) were then centrifuged (Centrifuge Biofuge Primo-CFH-240-010A, Thermo Scientific Heraeus Corp., Leicestershire, UK) at 4000 *g* for 10 min at 4 ºC and biphasic polar and non-polar extractions were separated using a Hamilton syringe (Hamilton syringe, SZR-110-040M 500 µL, Fisher Scientific Ltd., Loughborough, UK) in 1.8 ml glass vials. Polar fractions of 400 µl were

dried in a rotary evaporator (SpeedVac SPD111VP115, Thermo Scientific Corp., Surrey, UK) connected to a refrigerator (Refrigerated Vapor Trap RVT4104, Thermo Scientific Corp., Surrey, UK) and a vacuum pump (Laboport Diaphragm Pump N840.3FTP, KNF Neurberger Inc., New Jersey, USA).

NMR spectroscopy and spectral processing

NMR spectroscopy and spectral processing were carried out by NERC Metabolomics staff in the School of Biosciences at the University of Birmingham, UK. Briefly, 360 dried polar extractions were analysed using proton NMR two dimensional coupling constant resolve (2-D pJRES) following methods described by Viant et al. (2003) and Parson et al. (in press). See Appendix 5.1 for details of the analytical procedures.

Statistical analyses

Oxygen uptake

The effect of elevated pCO_2 temperature on rates of O_2 uptake was analysed using a three-way ANCOVA, with tank as a random factor nested within pCO_2 tissue weight as covariate. Two-way ANCOVAs were also performed to assess differences between temperature and pCO_2 treatments within populations. Data of O_2 uptake rates both for individual populations and across populations met assumptions for normality following $\log_{10}$ transformation (minimum $Z_{176} = 1.290$, $p = 0.072$). Variances were also homogeneous for O_2 uptake rates for all populations (minimum $F_{1,178} = 0.803$, $p = 0.525$) except for snails from Millport ($F_{1,178} = 3.948$, $p < 0.004$). However, here it was assumed that the ANCOVA design employed should have been fairly tolerant of deviation from the assumption of normality of distribution and homogeneity of the variances given the high number of replicates used (1080 individual snails) and the

orthogonal experimental design with twelve treatments (min. number of replicates per treatment was 30) (Sokal and Rohlf 1995, Underwood 1997).

Tank had a significant effect on O_2 uptake, but when tested at the individual population level a significant tank effect was found only for three populations (maximum $F_{4, 176} = 16.553$, $p < 0.0001$ and minimum $F_{4, 176} = 2.459$, $p < 0.048$). In these cases, removing tank from the calculation caused no change to the significance of the main factors, and thus it was assumed the tank effect to be marginal.

Metabolite data

Principle Component Analysis (PCA) (performed using XLSTAT version 2011.5 Addinsoft Ltd., Iowa, USA) was used to gain an overall measure of the metabolic profiles of individual snails for all derived metabolites. Data for 182 metabolites were identified using NMR and were used in this analysis. Key metabolites contributing to variation along each of the first two PCA axes were identified. The assignment of key metabolites was based on the percentage contribution to the separation of samples along PC axes. There were four classes of metabloite: 1) energy storage compounds; 2) amino acids; 3) tricarboxylic acid group; and 4) organic osmolytes (Zhang et al. 2011b)

Scores on PC axes one and two were also used as dependent variables to test for differences between populations, pCO_2 treatment and temperature using a three way ANCOVA.

RESULTS

Mesocosm parameters

Mean (± SE) pCO_2 values were 343 (± 6) µatm in the control treatment and 1294 (± 17) µatm in the acidified treatment, resulting in mean pHs of 8.11 ± (<0.01) and 7.61 ± (<0.01) in control and acidified treatments, respectively. Mean pCO_2 in the incubator were 404 (±2.0) and 1212 (±13) µatm for control and elevated pCO_2 conditions, respectively; while mean air temperatures were 15.8 (±0.13), 20.2 (±0.2), 25.7 (±0.2), 30.4 (±0.2), and 35.3 (±0.3) °C respectively. Seawater physiochemical parameters measured during the experimental period are summarised in Table 4.3.

Metabolic rates

Rates of O_2 uptake of the populations characterised in this study ranged between 1.1 and 3.5 µmol O_2 g^{-1} t.w. h^{-1} in the temperature range tested (Fig. 5.1, Appendix 2B). No clear trend in mean population O_2 uptake of the snails' collected across the thermo-latitudinal range tested here, and exposed to elevated pCO_2 and temperature together or in isolation was found (Fig. 5.1 A – F) despite the presence of significant 3- and 2-way interactions (Table 5.1). Further, there was no clear trend in rates of O_2 uptake of snails within each individual population exposed to either pCO_2, temperature, or their interaction (Table 5.2).

In general, at low air temperature (15 °C) there was no significant difference in O_2 uptake rates between control and elevated pCO_2 for all populations (except population in Ile de Re and Trondheim). Rate of O_2 uptake at 15 ˚C ranged between 2.0 – 3.5 µmol O_2 g^{-1} t.w. h^{-1} (Appendix 5.2A and B). When air temperature increased by 5 ˚C (up to 35 ˚C) significant differences in O_2 uptake between control and future pCO_2 were only

found in the southernmost population (Vigo) at 30 ºC (Fig. 5.1A), the Millport population at 20 and 25 ºC, the Tromsø population at 35 ºC (Fig. 5.1F); there were no significant differences for the other populations (Fig. 5.1).

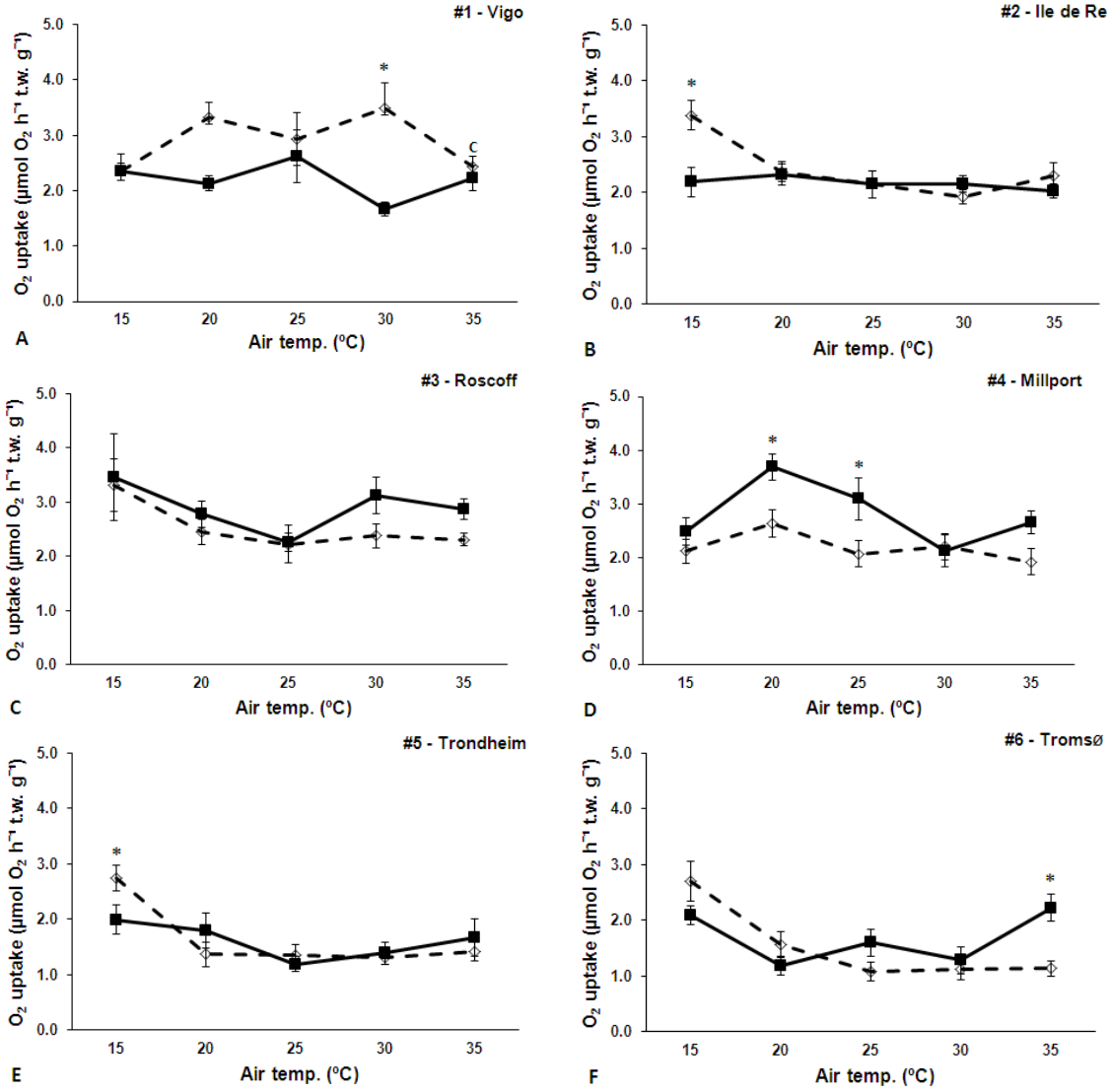

Figure 5.1. Mean (± SE) O_2 uptake rates (per unit mass) of six *Littorina littorea* populations under medium-term exposure to different levels of pCO_2 (current levels - 380 µatm – open diamond and dashed line; future levels- 1000 µatm full squares and solid line) and short-term exposure to five temperatures (15, 20, 25, 30 and 35 ºC). Populations are arranged in order from south to north. Asterisk (*) indicates significant difference between control and future pCO_2 conditions according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

Table 5.1. Result of a three-way ANCOVA test on rates of O_2 uptake of six populations of *L. littorea* exposed to two levels of pCO_2 and five temperatures. Degree of freedom (df), mean of square (MS), F-ratio (F), and probability level (*p*).

Trait	Source	df	MS	F	*p*
O_2 uptake (µmol mg^{-1} t.w. h^{-1}) of all population	pCO_2	1	0.01	0.2	0.66
	temperature	4	0.2	5.7	**0.003**
	population	5	0.9	57.4	**< 0.0001**
	pCO_2*temperature	4	0.1	1.8	0.17
	pCO_2*population	5	0.1	8.1	**< 0.0001**
	temperature*population	20	0.1	4.3	**< 0.0001**
	pCO_2*temperature*population	20	0.04	2.6	**< 0.0001**
	tank	24	0.4	2.6	**< 0.0001**
	tissue weight	1	0.7	4.4	**0.04**

Table 5.2. Results of two-way ANCOVAs on rates of O_2 uptake (µmol mg^{-1} t.w. h^{-1}) of six populations of *L. littorea* exposed to two levels of pCO_2 and five temperatures. Degree of freedom (df), mean of square (MS), F-ratio (F), and probability level (*p*) are provided.

Population	Location	Source	df	MS	F	*p*
1	Vigo, Spain	pCO_2	1	0.03	2.5	1.114
		temperature	4	0.02	1.2	0.308
		interaction	4	0.06	4.5	**0.002**
		tissue weight	1	0.7	53	**< 0.0001**
2	Ile de Re, France	pCO_2	1	0.1	2.286	0.206
		temperature	4	0.03	3.5	**0.01**
		interaction	4	0.03	3.3	**0.012**
		tissue weight	1	0.1	8.1	**0.005**
		tank	4	0.02	2.5	**0.048**
3	Roscoff, France	pCO_2	1	0.1	0.4	0.56
		temperature	4	0.03	3	**0.02**
		interaction	4	0.01	1.4	0.246
		tissue weight	1	0.9	88	**< 0.0001**
		tank	4	0.2	16	**< 0.0001**
4	Millport, UK	pCO_2	1	0.3	14.92	**< 0.0001**
		temperature	4	0.1	5	**0.001**
5	Trondheim, Norway	pCO_2	1	0.02	1.2	0.52
		temperature	4	0.2	8.6	**<0.0001**
6	Tromso, Norway	pCO_2	1	0.1	0.5	0.51
		temperature	4	0.2	13	**< 0.0001**
		interaction	4	0.1	5.5	**< 0.0001**
		tank	4	0.1	6.1	**< 0.0001**

Given the lack of any clear consistent effects of temperature, and that the main aim of this chapter is to assess how the exposure to elevated pCO_2 differs among populations living along a thermal-latitudinal gradient, a further set of analyses was carried out after pooling data from all temperature treatments. The results of this analysis showed that under normal pCO_2 conditions mean O_2 uptake decreases linearly with increasing latitude (Fig. 5.2) [$y = -0.0492x + 4.8804$, df = 5, $R^2 = 0.9538$, $p < 0.0001$]. Highest rates of mean O_2 uptake (2.43 and 2.9 µmol O_2 g^{-1} t.w. h^{-1}) were shown by southern populations (Vigo and Ile de Re) and lowest rates (1.52 and 1.65 µmol O_2 g^{-1} t.w. h^{-1}) by populations at northern range of distribution (Trondheim and Tromsø). Mid-range populations (Roscoff and Millport) showed intermediate levels of O_2 uptake rates (2.2 and 2.5µmol O_2 g^{-1} t.w. h^{-1}) (see Fig. 5.3 and Appendix 5.2A). In contrast, under high pCO_2 conditions, the relationship between mean O_2 uptake of individual populations and their latitudinal position was best described by a non-linear non-monotonic relationship (Fig. 5.2) [$y = -0.0038x^2 + 0.3933x - 7.644$, df = 5, $R^2 = 0.60$, $p = 0.02$]. Under high pCO_2 conditions, mean O_2 uptake rates of the two southern populations showed intermediate levels (2.2 and 2.18 µmol O_2 g^{-1} t.w. h^{-1} respectively) compared with the two mid-range populations which showed the highest rates of O_2 uptake (between 2.9 and 2.8 µmol O_2 g^{-1} t.w. h^{-1} respectively), and the two northern populations which showed the lowest rates of O_2 uptake (1.6 and 1.7 µmol O_2 g^{-1} t.w. h^{-1} respectively) (see Fig. 5.3 and Appendix 5.2B). A clear difference in the response of mean rates of O_2 uptake of different populations of *L. littorea* exposed to elevated pCO_2 conditions was found, with southern populations showing a significant decrease, mid-range populations a significant increase and the populations in Tromsø and Trondheim showing respectively small but statistically shows no significant differences in mean rates of O_2 uptake (Fig. 5.2 and 5.3).

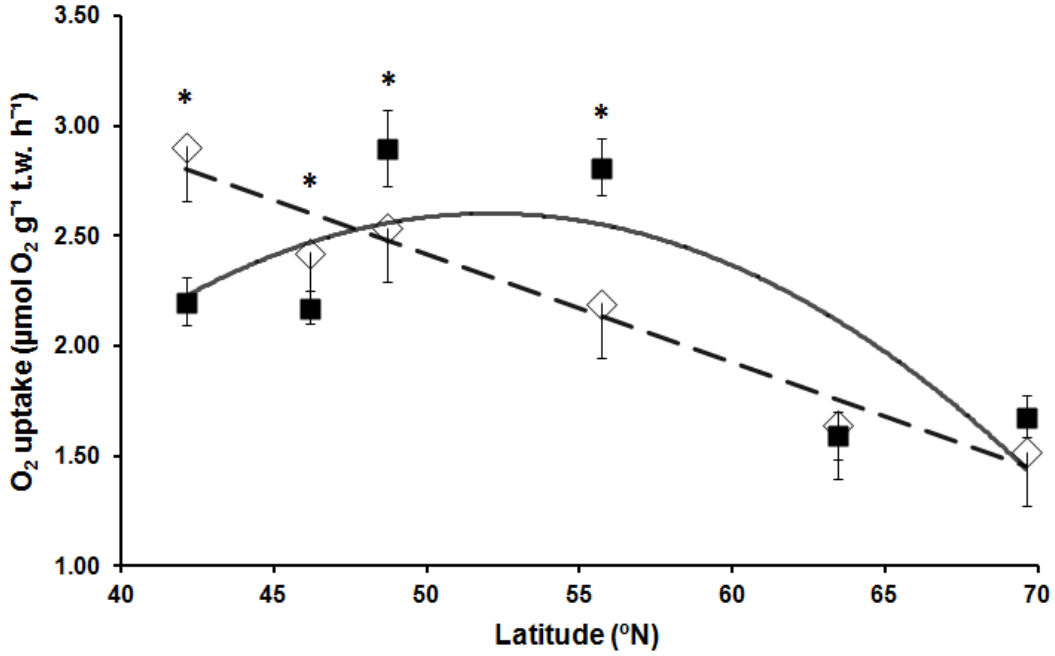

Figure 5.2. Relationship between latitudinal position and mean O_2 uptake rates under current (dashed line) and future pCO_2 (solid line) conditions in six populations of *Littorina littorea* living across the latitudinal range between 42 – 70 °N. Populations are arranged in order from south to north ranges of: 42° N (Vigo, Spain); 45° N (Ile de Re, France); 49° N (Roscoff, France); 56° N (Millport, UK); 63° N (Trondheim, Norway); and 70° N (Tromsø, Norway). Dash lines is linear regression of O_2 uptake rates under normal pCO_2 [$y = -0.0492x + 4.8804$, df = 5, $R^2 = 0.9538$, $p < 0.0001$] and solid line is first order polynomial correlation of O_2 uptake under high pCO_2 [$y = -0.0038x^2 + 0.3933x – 7.644$, df = 5, $R^2 = 0.60$, $p = 0.02$].

Metabolomic fingerprinting

Poor quality spectra were obtained for four samples and so these were omitted from the analyses giving a total of 256 samples that were used. The amount of variation explained by the first two PCA axes was low, probably due to the unequal weight of datasets typically generated by Field Identification Metabolites Analysis (FIMA), in which lower dimensional components (low metabolite variables in individual samples) are often produced (Scholz 2006). Removing these components could cause the loss of

information on metabolites that play key roles. However, by applying standardization procedures within the PCA, errors were reduced. In addition, Ludwig and Viant (2009) found low variance in metabolomics data analysed using PCA, therefore the effect of low variance in this test can be tolerated.

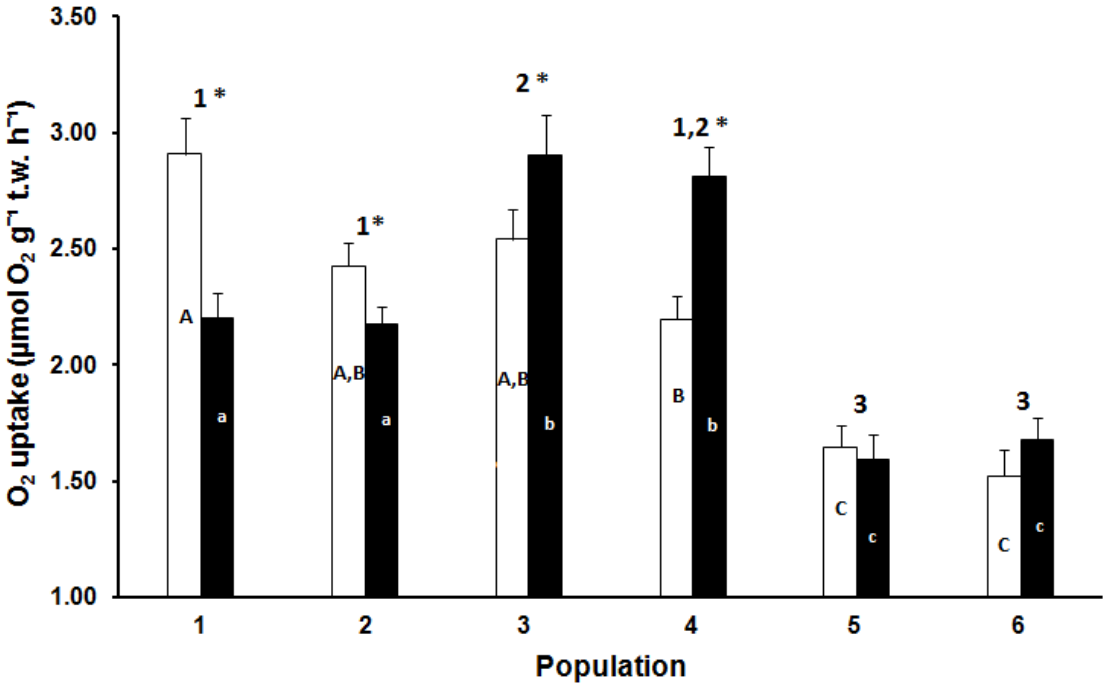

Figure 5.3. O_2 uptake rates (per unit mass) of *Littorina littorea* from six populations under medium-term exposure to different levels of pCO_2 (current – white and future – black) and short term exposure to five different air temperatures. Values are means (± SE). Populations are arranged in order from south to north ranges of: 1 (Vigo, Spain); 2 (Ile de Re, France); 3 (Roscoff, France); 4 (Millport, UK); 5 (Trondheim, Norway); and 6 (Tromsø, Norway). Significant different means ($p \leq 0.05$) between control pCO_2 are indicated by different capital letters and for future pCO_2 conditions by different lower case. Number indicates significant difference exists between populations at combined (control and future pCO_2) and asterisk (*) indicated significant different between control and future pCO_2 conditions according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

Samples formed two main clusters when plotted on the first two PC axes (Fig. 5.4A – C). One of these clusters was very tight and was centred on the origins of both axes; the other showed a much greater variation between samples and contained only samples from the two northernmost populations (Trondheim and Tromsø) exposed to low temperature (15 °C).

Mean PC1 values for the two northernmost populations measured at 15 °C under control and high CO_2 conditions were significantly higher than for any other population and temperature combination (Fig. 5.5A), and for other temperatures at these two sites (Fig. 5.5A), as indicated by the presence of a significant three-way interaction between the terms pCO_2, temperature and population (Table 5.3). In contrast, mean PC2 values showed no significant differ among populations at any temperature; however a marginal significant interaction of temperature and population was found.

When data from different temperatures were pooled, the northern populations showed significantly higher mean PC1 values under both current and future CO_2 conditions (Fig. 5.5C and Table 3), with mean PC1 at low and high CO_2 conditions from the same population differing significantly only for Tromsø; whilst for mean PC2 the only significant difference was found between the populations in Trondheim and Tromsø under control CO_2 conditions.

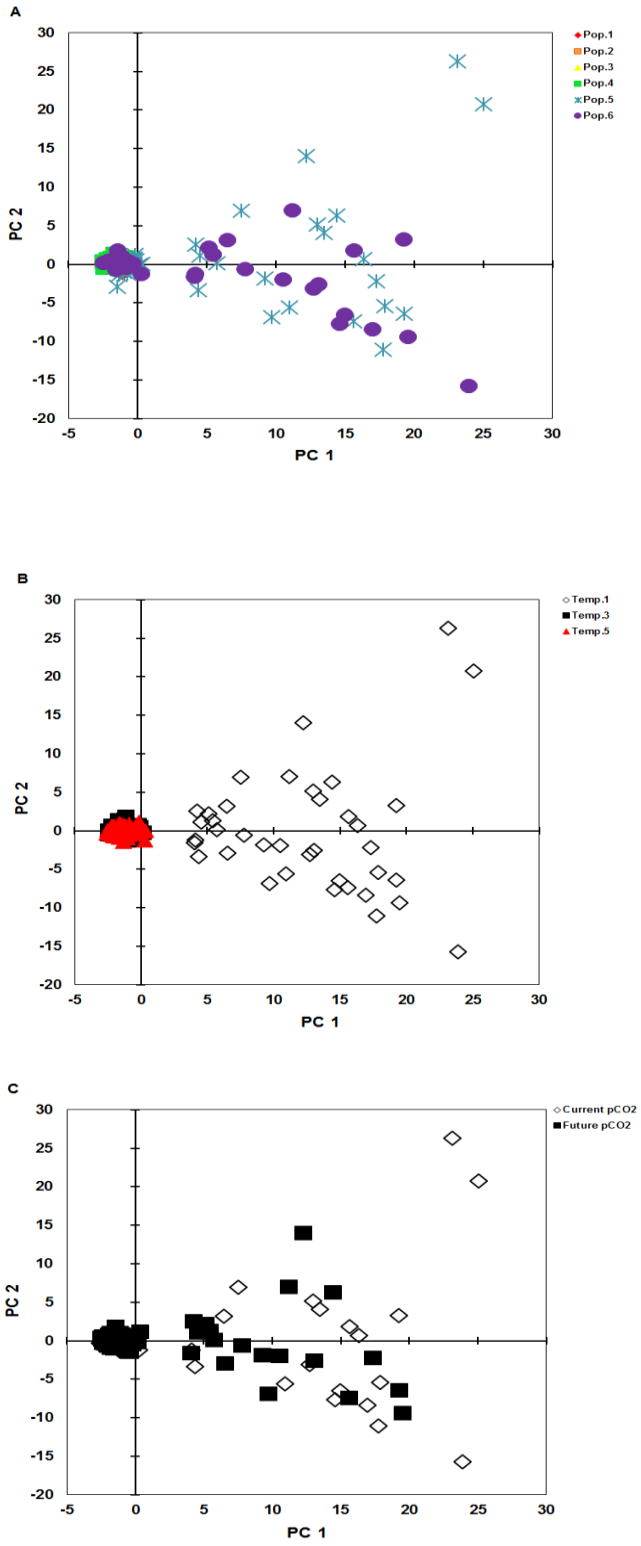

Figure 5.4. PCA plots derived from metabolomic fingerprints of 356 *Littorina littorea* individuals. PC1 axis (variance explained 12.6%) and 2 (var. 4.0%). Individual plots are coded to show samples from different: A) populations (1: Vigo, 2: Ile de Re, 3: Roscoff, 4: Millport, 5: Trondheim and 6: Tromsø); B) temperatures (1: 15, 3: 25, and 5: 35 °C); and C) CO_2 treatments (current and future pCO_2 levels).

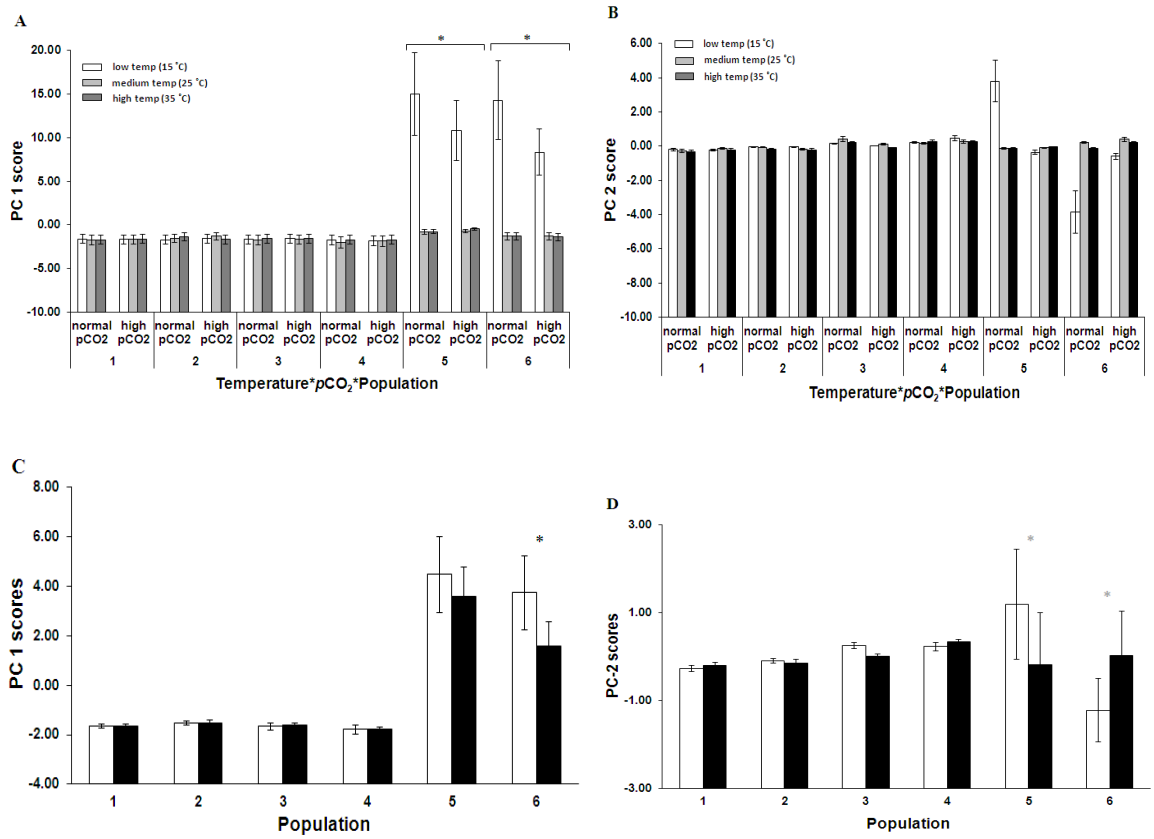

Figure 5.5. Mean (± SE) PC1 and PC2 scores of six populations of *Littorina littorea* exposed medium term to different levels of pCO_2 (normal and high) and short term to three temperatures. Significantly different means ($p \leq 0.05$) between temperature levels are only found in low temperature. Asterisks (*) indicated significant differ between normal and high pCO_2 between population (1: Vigo, 2: Ile de Re, 3: Roscoff, 4: Millport, 5: Trondheim and 6: Tromsø): A) PC1 axis and B) PC2 axis B) and significant differ of between normal (white) and high pCO_2 (black) on individual population of: C) PC1 axis and D) PC2 axis according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

Table 5.3. Results of three way ANCOVAs testing for differences in PC scores of six populations of *L. littorea* exposed to two levels of pCO_2 five temperatures. Degrees of freedom (df), mean of square (MS), F-ratio (F), and probability level (*p*).

Trait	Source	df	MS	F	*p*
PC1 scores	pCO_2	1	18.1	4.3	**0.04**
	temperature	2	768.7	183.3	**< 0.0001**
	population	5	360.4	86.0	**< 0.0001**
	pCO_2*temperature	2	27.0	6.4	**0.002**
	pCO_2*population	5	10.4	2.5	**0.03**
	temperature*population	10	304.4	72.6	**< 0.0001**
	pCO_2*temperature*population	10	9.9	2.4	**0.011**
PC2 scores	pCO_2	1	0.3	0.05	0.83
	temperature	2	0.6	0.08	0.92
	population	5	11.0	1.6	0.20
	pCO_2*temperature	2	0.5	0.1	0.93
	pCO_2*population	5	8.8	1.3	0.27
	temperature*population	10	12.9	1.9	**0.05**
PC-1 scores *vs* pCO_2	pCO_2	1	23.8	5.6	**0.03**
	population	5	368.3	74.9	**< 0.0001**
	pCO_2*population	5	13.0	3.0	**0.03**
PC-2 scores *vs* pCO_2	pCO_2	1	0.02	0.002	0.90
	population	5	10.0	1.4	0.21

Individual metabolites that contributed most significantly to the separation of sites along PC1 were thymine (app. 4%) and PC2 were acetamide (app. 7%). In general, the most important contributors to PC1 (all on the positive loading) were (in order) those associated with energy storage (thymine, uracil, ornithine, and 2,3 butanediol), branched chain amino acids (valine, lysine and methionine) and tricarboxylic acid cycles (glycolic acid, a-Ketoglutarate and formic acid). While the metabolites that were dominant along the positive loading for PC2 were tricarboxylic acid those associated with cycles (isobutyric acid, 2-Methylbutyric acid, isovaleric acid, lactic acid and 3-Methyl-2-oxovaleric acid), energy storage (cytidine, dioxycholic acid and N-Acetylasparty

Table 5.4. The percentage contribution of key metabolites (i.e. those with a > 2% contribution) to PC axes 1 and 2.

Metabolite PC1	Metabolites classes	% Contribution	Key metabolites PC2 (+ loading vector)	Metabolites classes	% Contribution
Thymine	Energy storage	+3.5	Acetamide	Organic osmolytes	+6.6
Valine	Amino acid	+3.2	Cytidine	Energy storage	+5.6
Lysine	Amino acid	+3.1	Isobutyric acid	Tricarboxylic acid	+5.1
Uracil	Energy storage	+3.1	Dioxycholic acid	Energy storage	+3.7
Ornithine	Energy storage	+3.1	N-Acetyllysine	Amino acid	+3.5
Methionine	Amino acid	+2.9	2-methylbutyric acid	Tricarboxylic acid	+3.0
Glycolic acid	Tricarboxylic acid	+2.8	Isovaleric acid	Tricarboxylic acid	+2.8
a-Ketoglutarate	Tricarboxylic acid	+2.4	Lactic acid	Tricarboxylic acid	+2.1
2,3 Butanediol	Energy storage	+2.3	N-Acetylaspartyglutamate (NAAG)	Energy storage	+2.1
Dimethylamine	Organic osmolytes	+2.0	3-Methyl-2-oxovaleric acid	Tricarboxylic acid	+2.0
Formic acid	Tricarboxylic acid	+2.0	Dimethylamine	Organic osmolytes	+2.0
			Isoleucine	Amino acid	-3.5
			Leucine	Amino acid	-3.2
			Pyroglutamic acid	Amino acid	-2.1
			Acetic acid	Organic osmolytes	-2.0

glutamate (NAAG)), organic osmolytes (acetamide and dimethylamine) and a branched chain amino acids (N-Acetyllisine) (Table 5.4). Metabolites contributing negatively to the loading for PC1 were all below 2%, while negative loading for PC2 were amino acids groups (isoleucine, leucine and pyroglutamic acid) and one organic osmolyte compound, acetic acid (Table 5.4). In order to investigate patterns within the tight cluster of points produced in the first PCA, a second PCA was carried out after removing the low temperature samples from the two northern populations (Fig. 5.6A – C). This analysis produced different clusters in which two northern populations tended to reverse their position with respect to their geographical location; populations in Trondheim remained separate. There was a significant population effect for both PC1 and PC2 axes, but not for pCO_2 temperature (Fig. 5.7A and B, Table 5.5).

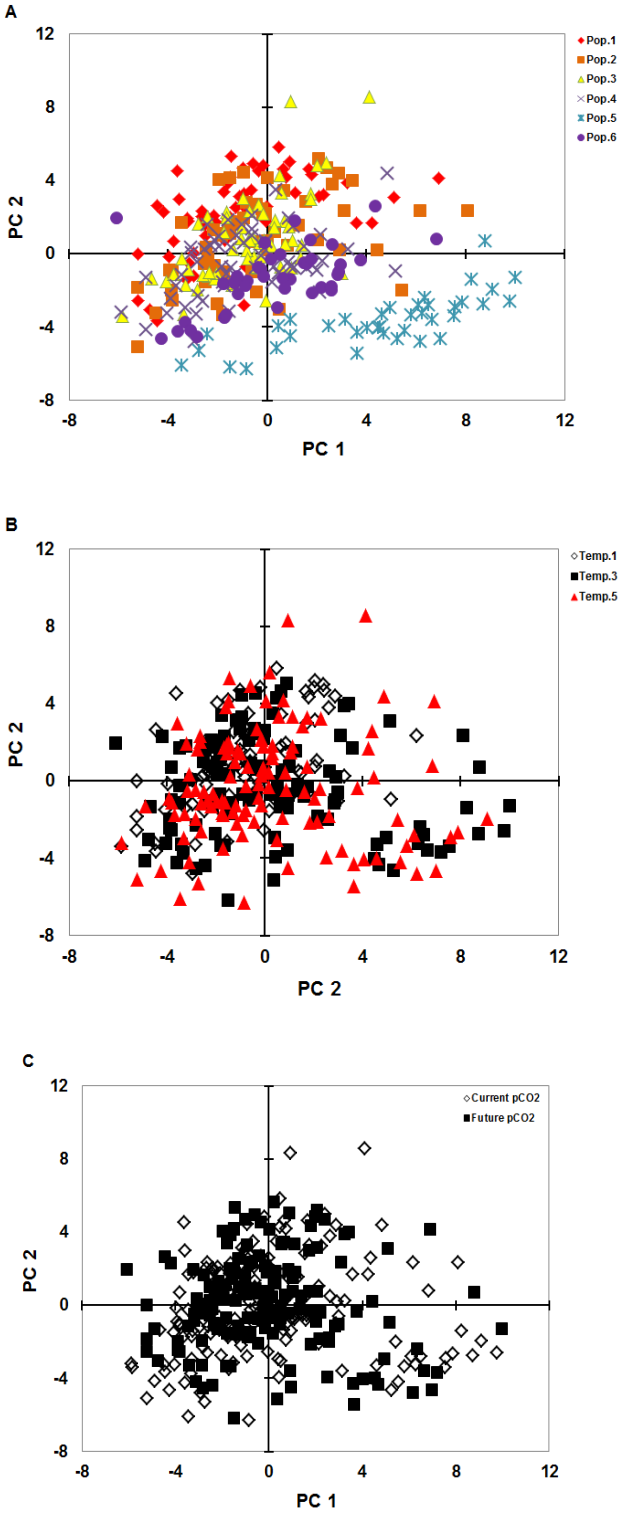

Figure 5.6. PCA plots derived from metabolomic profiles (after removed two northern samples at low temperature) of 318 *Littorina littorea* individuals on PC axes 1 (variance explained 5.7%) and 2 (var. 4.3%). Individual plots are coded to show samples from different: A) populations (1: Vigo, 2: Ile de Re, 3: Roscoff, 4: Millport, 5: Trondheim and 6: Tromsø); B) temperatures (1: 15, 3: 25, and 5: 35 ˚C); and C) CO_2 treatments (current and future pCO_2 levels).

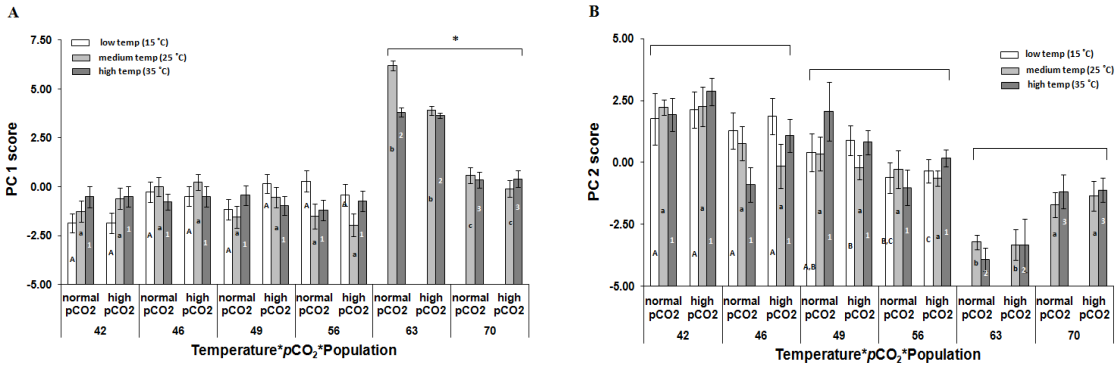

Figure 5.7. Histogram of PC scores (after the removal of low temperature treatments from the two northern samples) of *Littorina littorea* from six populations under medium-term exposure to different levels of pCO_2 (normal and high) and short term exposure to three temperatures for; A) PC1 axis and B) PC2 axis. Values are means (± SE). Populations are arranged in order from south to north ranges of: 42° N (Vigo, Spain); 45° N (Ile de Re, France); 49° N (Roscoff, France); 56° N (Millport, UK); 63° N (Trondheim, Norway); and 70° N (Tromsø, Norway). Significant different means ($P \leq 0.05$) between temperature levels indicated by capital letter (15 °C), lower case (25 °C), and number (35 °C) on each population and asterisk (*) indicated significant different exist between population according to the Estimate Marginal Means test (EMM) with Bonferroni correction.

Table 5.5. Results of three-way ANCOVAs testing for differences in PC scores (after the removal of low temperature treatments from the two northern samples) of six populations of *L. littorea* exposed to two levels of pCO_2 five temperatures. Degrees of freedom (df), mean of square (MS), F-ratio (F), and probability level (*p*) are provided.

Trait	Source	Df	MS	F	*p*
PC-1 scores	pCO_2	1	1.8	0.2	0.63
	temperature	2	5.7	0.8	0.47
	population	5	22.3	3.0	**0.012**
	pCO_2*temperature	2	1.7	0.2	0.8
	pCO_2*population	5	5.5	0.7	0.60
	temperature*population	9	8.8	1.2	0.31
PC-2 scores	pCO_2	1	4.2	0.9	0.34
	temperature	2	10.4	2.2	0.11
	population	5	41.5	9.0	< **0.0001**
	pCO_2*temperature	2	4.3	0.9	0.40
	pCO_2*population	5	2.6	0.6	0.73
	temperature*population	9	6.1	1.3	0.23
	tissue weight	1	56.0	12.0	**0.001**

Table 5.6. The percentage contribution of key metabolites (i.e. those with a > 2% contribution) to PC axes 1 and 2 after the removal of low temperature treatments from the two northern samples.

Key metabolites PC1 (+ loading vector)	Metabolites classes	% Contribution	Key metabolites PC2 (+ loading vector)	Metabolites classes	(%) Contribution
Alanine	Amino acid	+7.8	Betaine	Organic osmolytes	+8.5
Valine	Amino acid	+7.2	Taurine	Amino acid	+7.1
Isoleucine	Amino acid	+7.0	N,N-Dimethylglycine	Amino acid	+6.7
Glycine	Amino acid	+6.6	cis-Aconitic acid	Tricarboxylic acid	+3.9
Proline	Amino acid	+5.8	Nicotinamide adenine dinucleotide hydrate (NADH)	Energy storage	+3.9
Methionine	Amino acid	+5.7	trans-Aconitic acid	Tricarboxylic acid	+3.4
Glycolic acid	Energy storage	+4.3	Histamine	Energy storage	+3.4
Glycerol	Tricarboxylic acid	+3.6	Adenosine 5' diphosphate	Energy storage	+3.1
N-Acetylglutamine	Tricarboxylic acid	+3.4	N-Acetylmethionine	Tricarboxylic acid	+3.0
N-Acetylalanine	Tricarboxylic acid	+2.6	Tyrosine	Amino acid	+2.7
N-Acetillisine	Tricarboxylic acid	+2.6	Sarcosine	Tricarboxylic acid	+2.0
Thymine	Energy storage	+2.3			

The individual metabolites that contributed most to the separation along PC1 were alanine (7.8%) and on PC2 betaine (8.5%). Metabolites contributing most to variation on axis 1 were those associated with branched chain amino acids (alanine, valine, isoleucine, glycine, proline and methionine) and tricarboxylic acid cycles (Glycolic acid, glycerol, N-Acetylglutamine, N-Acetylalanine and N-Acetillisine) while only two metabolites of energy storage (glycolic acid and thymine) contributed to the separation along this axis (Table 5.6). Metabolites that contributed positively to PC2 were those associated with tricarboxylic acid cycles (cis-Aconitic acid, trans-Aconitic acid, N-Acetylmethionine and sarcosine), amino acids (taurine, N,N-Dimethylglycine and tyrosine), and energy storage (NADH, histamine and ADP) (Table 5.6).

DISCUSSION

To my knowledge, this is the first study that demonstrates differential physiological and metabolic responses in a series of populations of a marine organism from across their entire thermo-latitudinal gradient of distribution. Surprisingly no clear pattern of variation in metabolic rate in snails kept under different temperatures and acidification levels were found, but when data from different temperature exposures where pooled, significant latitudinal patterns emerged under both control and elevated pCO_2 conditions. Southern populations showed a decreased metabolic rate, centre-range populations an increased rate and northern populations only small changes compared with those exposed in normal pCO_2. There was also an overall negative relationship between metabolic rates and latitude under control conditions, while under high pCO_2 the metabolic rate showed a non-linear trend across the latitudinal gradient investigated. Metabolomic analysis using proton nuclear magnetic resonance (P-NMR) showed that the two northern populations were distinct from other populations exposed to short term low temperature (15 ºC) due, in part, to high levels of thymine, uracil, valine and lysine. A similar separation also occurred under high temperature exposures in which one of the northern populations (Trondheim) was distinct from other populations and had lower concentrations of alanine, betaine and taurine but higher concentrations of valine.

Metabolic rate

The investigation of metabolic rates of taxa living along latitudinal gradients and environmental gradients in general have been used to explore the possible evolution of thermal traits and current levels of thermal adaptation in a variety of animal groups (see for example Scholander et al. 1953, Clarke 1993, Childress 1995, Seibel and Carlini 2001, Addo-Bediako et al. 2002, Lardies et al. 2004, Jacobsen and Brodersen 2008,

Whiteley et al. 2011, Rastrick and Whiteley 2011, Sorte et al. 2011, Lardies et al. 2011). Here there is an existence of a significant linear negative pattern of metabolic rate with latitude in *L.lLittorea* under normal pCO_2 conditions. In fact, populations at the southern range (Vigo and Ile de Re) possess the highest metabolic rates, and thus potentially greater costs of living, whilst the northernmost one has the lowest metabolic rates observed along the entire thermo-latitudinal gradient investigated. This indicates that in *L.lLittorea* there is no metabolic cold adaptation (Scholander et al. 1953), thus adding another example to the list of works which provide evidence against the existence of a negative relationship between metabolic rates and ambient environmental temperatures in marine organisms (see also *Krogh's rule* in Gaston et al. 2009). Similarly, Rastrick and Whiteley (2011) have shown that in gammarid amphipods, taxa with a more southern distribution (in the northern hemisphere) had higher metabolic rates at their respective habitat temperatures.

Despite the clear relationship between metabolic rates and latitude, when exposed to high pCO_2 conditions the latitudinal pattern of metabolic rates in *L. littorea* observed under current conditions changes substantially as a result of the different responses of each population at different latitudes. In fact, the southern populations (Vigo and Ile de Re) showed metabolic depression under high pCO_2 conditions, whilst central range populations (Roscoff and Millport) upregulate metabolic rates, and finally populations from the north of the range (Trondheim and Tromsø) show subtle variations. Decreased metabolic rates under hypercapnia have been documented in various organisms (Reipschläger and Pörtner 1996, Pörtner et al. 1998, Michaelidis et al. 2005, Metzger et al. 2007, Rosa and Seibel 2008, Small et al. 2010 but see for example Gutowska et al. 2010), including previously in *L. littorea* (Melatunan et al. 2011 and Chapter 2).

Depressed metabolic rates under elevated pCO_2 in southern populations of *L. littorea* in this study may be related to the lack or limited capacity in this species for acid-base regulation (see Melzner et al. 2009, Lannig et al. 2010, Whiteley et al 2011), and could be used as a strategy to match energy production and demand, helping conserving snails energy status (Guppy and Withers 1999). Snails could already be experiencing frequent and intense oscillations in pCO_2 (e.g. Hall-Spencer et al. 2008, Veron 2008) but whether this is particularly intense on the southern range of distribution of *L. littorea* is still unclear. However, there is some recent evidence that some areas in the Arctic have already experienced low aragonite saturation due to accumulation of CO_2 (Yamamoto et al. 2009) and the Southern Atlantic Ocean (32º N) has also shown high fluxes of CO_2 (Bates et al. 2009 see also Riegl 2003).

Increase of metabolic rates under high pCO_2, as shown in mid-range populations of *L. littorea* has been already reported in other taxa such as the Antarctic bivalve *Laternula elliptica* (Cummings et al. 2011) and in echinoderms (see Wood et al. 2008, 2010, Christensen et al. 2011, but see Stumpp et al. 2011). In general, increase in metabolic rates is thought to indicate the ability of an organism to compensate for low extra- and intra-cellular pH (e.g. Pörtner et al. 1998b, Pörtner et al. 2004). No direct evidence exists that individuals from central populations have any better extracellular fluid regulatory ability than individuals from the southern range, but evidence does exist in support of the idea that regulatory abilities in invertebrates also vary with latitude and environmental gradients (see Mellanby's rule in Gaston et al. 2009). Finally, the ability of northern populations (Trondheim and Tromsø) to maintain their metabolic rates virtually unchanged under high pCO_2 conditions indicates these populations have ability for physiological compensation. Such compensation has been shown already under high

CO_2 in the cuttlefish *Sepia officinalis* (Gutowska et al. 2010), the common limpet *Patella vulgata* (Marchant et al. 2010) and the gammarid amphipod *Echinogammarus marinus* (Egilsdottir et al. 2009).

Metabolomic fingerprints

Northern populations show distinctive metabolomic patterns compared to southern and central populations. Snails from Trondheim and Tromsø possess much higher values for the primary PCA under both current and elevated pCO_2 conditions, with snails from Trondheim showing higher values for the secondary PCA under control pCO_2 conditions. Differences in metabolomic profiling (or metabolic pathways utilisation) may indicate difference in the ability of the snailsto adaptat to the different thermal regimes (Hochachka and Somero 2005). The results suggest that northern populations possess significantly different metabolic machinery compared to southern and central ones, but also that subtle differences in metabolic adaptation exist between the two northern populations themselves. Differences in the metabolite responses in population of snails inhabiting different thermal-environments have already being reported in *L. saxatilis* (Sokolova and Pörtner 2003), and differences in the myosin heavy chain (myHC) across a latitudinal gradient were shown in gammarid amphipods (Rock et al. 2009). For example *G. duebeni duebeni* in northern latitudes was able to conserve metabolic rates that related to higher MyHC isform diversity compared with intertidal species *G. oceanicus*. The separation of the two northern range populations here is mainly due to the increased production of [thymine] (0.13 – 0.17 mM - app. 1000-fold higher), [uracil] (0.41 – 0.44 mM - only present in these two populations), [valine] (2.1 – 2.74 mM - app. 13-fold higher), and [lysine] (1.5 – 3.1 mM - app. 100-fold higher) when compared with the other populations. High production of thymine and uracil

indicate the availability of energy storage and the state of DNA and RNA stability (Thureau et al. 2006) whilst increased concentrations of valine and lysine indicate osmo-ionic regulatory availability (Preston and Stevens 1982). It is likely that high levels of these metabolites in these two northern populations at 15 ºC was associated with elevated pCO_2 was not caused by elevated temperature. A supposition confirmed by these animals showing negative growth in high pCO_2 conditions (see also Chapter 4).

Finally, the Trondheim population was distinct from all other populations, as it shows a decrease in [alanine] (6.2 – 7.2 mM - app. 4-fold lower), [betaine] (13.0 – 15.7 mM) and [taurine] (6.3 – 7.8 mM - app. 1.6 –fold lower), and an increase in [valine] (0.24 – 0.31 mM - 1.5-fold higher). In general, molluscs contain high concentrations of alanine, betaine and taurine besides proline and arginine in their tissue to counteract hypertonicity caused by high osmotic fluxes (Carr et al. 1996). In addition, betaine has also been identified in maintaining osmotic balance in shrimp (Saoud and Davis 2005). To counteract hypertonicity pressure in which ionic transport of Cl^- and Na^- are not able to function alone, either Cl^- or Na^- are coupled with amino acid groups e.g. alanine, betaine or taurine (Schoffeniels 1970, Gerard and Gilles 1972, Marangos et al. 1989, Deaton 2001). Hence, reduction in the former three metabolites was likely caused by their utilization to counteract the osmo-ionic regulatory process of hyperosmolality condition when animals were exposed to extreme environmental conditions (Burg and Ferraris 2008).

An integrative view

The combined investigation of metabolic rates and metabolic fingerprints of individual snails show that different populations may be utilising different strategies in the face of

elevated pCO_2 conditions. In Chapter 4, it was observed that southern and northern populations show the highest decrease in shell growth under high CO_2 conditions; here it is shown that snails also experience greater changes in either metabolic rates or the utilization of metabolic pathways (this chapter). Thus it can be concluded that snails may have experienced a considerable reduction in energy available for growth: either *via* metabolic depression in southern populations, which lead to a general lowering of ATP production (Lannig et al. 2010, Melatunan et al. 2011), or *via* changes in metabolic pathways utilisation observed in northern populations, which lead to changes in the energy budget reallocation away from growth. ATP availability is strongly positively related to shell growth (S. Melatunan, P. Calosi, S.D. Rundle, S. Widdicombe and J.A. Moody – unpublished data), and in other organisms where changes and reallocation of the energy budget have been claimed to occur under high pCO_2, energy always seems to have been taken away from growth (e.g. in barnacles [Findlay et al. 2010a and b], in echinoderms [Wood et al. 2008, 2010, Stumpp et al. 2011). Langenbuch and Pörtner (2003) reported that metabolic acidosis caused by elevated pCO_2 inhibited 80% of cellular biosynthesis. Also, as metabolic rates are directly linked to protein synthesis (Whiteley et al. 1997, Pörtner et al. 1998a), the decrease in metabolic rates experienced by southern populations would lead to a lowering of protein synthesis, which could explain the decrease in growth shown by these snails (see Chapter 4). At the northern edge of the range, populations show only marginal changes in metabolic rates. Based on the results for metabolomic fingerprinting, however, these populations possess high energy storage capacities, indicated by the presence in high levels of thymine, uracil, ornithine, and alanine, compared to southern or central populations. For example thymine is approx. 1000-fold higher and ornithine is approx. 8-fold when compared to levels detected in southern and central populations, whilst uracil is only present in

northern populations. This mechanism may allow these populations to grow even in unfavourable environmental conditions, but given that under elevated pCO_2 the mole concentration of the metabolites are lowered significantly at 15 °C, future CO_2 conditions may impose significant costs on the life-history and ecological functions of *L. littorea*.

Significant differences in growth observed in the populations of Trondheim and Tromsø under high pCO_2 condition may also be explained by difference in metabolomic responses. For example [alanine] and [valine] were higher in snails from Trondheim, but [betaine] and [taurine] were higher in snails from Tromsø. In addition, elevated CO_2 will increase nitrogen excretion (Pörtner et al. 1998a) and could cause the accumulation of ammonia, urea and uric acid (Wright 1995), which may interfere with ionic transport processes (Mans et al. 1983). It seems that snails from Trondheim may produce elevated quantities of alanine and valine to counteract osmo-ionic disruptions under high pCO_2 conditions, whilst snails in Tromsø produce elevated quantities of betaine and taurine to carry out the same function. Al-Moghrabi et al. (1993) reported that the scleractinian coral *Galaxea fascicularis* facilitated ionic transport exchanges by coupling Na^+/valine pump which is more energy efficient than the utilisation of other amino acid groups, which could lead to up to 25-fold greater cost in energy. Additionally, the higher production of [alanine] in the snails from Tromsø could indicate this population may have been in the transition between aerobic and anaerobic metabolism. This has also been demonstrated in *L. saxatilis* that increase the production of alanine (caused by degradation of phosphagens) indicates anaerobic metabolism (see Sokolova and Pörtner 2003, Lannig et al. 2010 also Zhang et al. 2011b).

Finally, it is interesting to note that the populations showing the most moderate decrease in growth under high CO_2 conditions are those able to increase their metabolic rates under these conditions. However, even if central populations possess a greater physiological ability for compensation, over longer time scales they may incur higher energy costs, which could lead to ecological and life-history trade-offs imposed by energy budget reallocations (Sibly and Calow 1986).

The variations in metabolic strategies that have been demonstrated among populations of *L. littorea* are likely to influence population dynamics and biogeography of *L. littorea*. Most specifically, given that the most vulnerable populations appear to be those at the southern- (Vigo) and northern-most (Tromsø) positions of the distributional range, we might expect this species to show range edge reductions or range shifts, as already observed in an array of terrestrial and marine taxa (Southward 1995, Parmesan 1996, Parmesan et al. 1999, 2000, Easterling et al. 2000, Perry et al. 2005), with future increasing pCO_2 levels (IPCC 2007). Other possible ways in which populations of *L. littorea* will be able to cope with environmental change could be through shifting their metabolic pathways, either *via* plastic responses (Lannig et al. 2010, see this chapter) or by adapting to the future conditions, although the time scales for species to adapt compared to the rapidity at which environmental changes are occurring may render this strategy unlikely (see discussion in Pistevos et al. 2011, Sunday et al. 2011, Chan et al. 2011). All-in-all, changes to the biogeography or dynamics of populations of *L. littorea* has the potential to greatly affect ecological functions within North Atlantic marine intertidal habitats (Christen Calosi Widdicombe *pers. comm.*).

Chapter 6

General Discussion

The general aim of this thesis was to increase our understanding of the potential effects of ocean acidification and temperature on the physiology and morphology of marine intertidal organisms. The edible periwinkle *Littorina littorea* was used as a model species as it is known to play an important ecological role in intertidal ecosystems, i.e. by controlling the abundance of sessile organisms such as algae and barnacles (Petraitis 1983), and has also been shown to be amenable to experimental laboratory studies, including those investigating the potential effects of ocean acidification (e.g. Bibby et al 2007). This thesis has addressed the specific questions: i) whether elevation of pCO_2, temperature and their interaction disrupt metabolic rates and energy metabolism of *L. littorea* (Chapter 2); ii) if this disruption impaired shell growth (Chapter 3); iii) whether morphological responses to ocean acidification varied among populations of *L. littorea* across its latitudinal range (Chapter 4); and iv) whether physiological and metabolic responses to ocean acidification also varied across these populations (Chapter 5).

The main objective of Chapter 2 was to assess whether *L. littorea* maintained its metabolic rate and energy status under medium-term exposure to ocean acidification and elevated temperature, and whether there was a synergistic effect from these two factors; i.e. did snails enter a hypometabolic state due to metabolic depression following through exposure to ocean acidification and warming as suggested by Pörtner et al. (1998). I investigated such responses using whole-organism metabolic rates, adenylate energy nucleotide (ATP, ADP, AMP) concentrations, total adenylate nucleotides (TAN), adenylate energy charge (AEC), and the concentrations of metabolic end products (succinate and lactate).

Main conclusion of this chapter was that ocean acidification supresses metabolic rates, reduces energy metabolism and increases production of metabolic end products; and

that, combined with elevated temperature, CO_2 has an even more deleterious effect on energy metabolism, but not on metabolic rates and production of metabolic end products. In fact, metabolic rates were higher, and metabolic end product overall lower, in high temperature treatments compared with the elevated CO_2 treatment, which could indicate on one hand that there is a higher energy demand for cellular processes or, on the other hand that under elevated temperature snails are more reliant on aerobic metabolism. Similar physiological responses were also found in the penaeid shrimp *Metapenaeus joyneri* (Dissanayake and Ishimatsu 2011), as well as in the brittle star *Ophiura ophiura* (Wood et al. 2010). My results were also consistent with those from the study of Lanning et al. (2010) on the interactive effect of high CO_2 and temperature in the oyster *Crassostrea gigas*, although methodological differences here should be taken into account. Lanning et al. (2010) found that that oyster showed a lower metabolic rate under high pCO_2 at low temperature (15 ˚C) but a higher rate when temperature was increased *via* ramping at 25 °C. In addition, ATP in the gill of *C. gigas* was lower under high CO_2 and temperature, concomitant with an increase in succinate, but there was no effect on the cellular energy demand for ion regulation via Na^+/K^+-ATPase under chronic hypercapnia or temperature. It was likely that *C. gigas* may have shifted its ionic exchange mechanism as in *L. littorea* from Na^+/K^+-ATPase to Na^+/H^+, which is known to be more energy efficient (Pörtner et al. 2000, Melatunan et al. 2011). The same mechanism may also have been utilized by *C. gigas* when exposed to high CO_2 and temperature.

The main aim of Chapter 3 was to assess the effects of ocean acidification and increased temperature, again in isolation and combined, on shell growth in *L. littorea*. In particular this chapter was aimed at at assessing how ocean acidification and thermal

conditions affect shell plasticity. This study was carried out to test the idea that future climate change and ocean acidification might impair the growth of gastropod shells, leading to changes in shell sizes and shapes, which could ultimately have implications in terms of water loss (Warthon 1996, Perrot et al. 2007) and vulnerability to predators (e.g. Trussell 1997, Trussell and Etter 2001, Rundle and Brönmark 2001, Cotton et al. 2004). My main findings were that ocean acidification, temperature and the combination of these factors negatively affected shell growth and led to changes in the shell shape of *L. littorea*. These findings are consistent with previous studies on marine gastropods (e.g. Nienhuis et al. 2010, Findlay et al. 2010a, b, Chan et al. 2011, Gaylord et al. 2011, Pistevos et al. 2011).

In addition, the data generated for shell growth and morphological plasticity in this chapter allowed me to make a comparison between the biometric parameters and those generated for physiological and biochemical responses in Chapter 2. In order to explore the potential relationship between percentage of shell morphological changes and physiological responses of *L. littorea* I used regression analysis to explore whether ATP levels were a good predictor of shell morphometric parameters and water loss. The main result of these analyses was that there were a significant positive relationship between metabolic energy levels ($\log_{10}$ [ATP]) and percentage change in shell weight (R^2 = 0.355, df = 63, $p < 0.0001$, Fig. 6.1A) and percentage change in thickness-2 (R^2 = 0.224, df = 63, $p < 0.0001$, Fig. 6.1B). This suggests that the observed disruptions in shell growth and shell thickness may be mediated by the alteration of physiological and biochemical processes restrictions in ATP production induced by ocean acidification and elevated temperature, rather than the decrease in Ω saturation status as previously thought (but see Pörtner 2008, Findlay et al. 2009, Findlay et al. 2011).

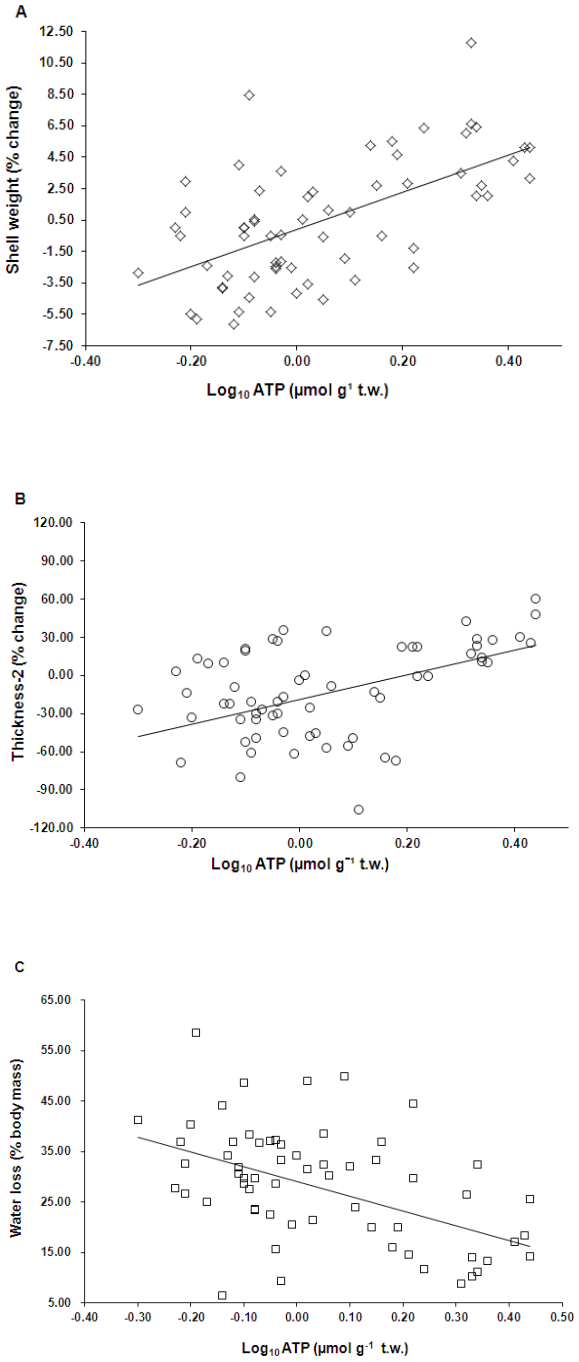

Figure 6.1. The relationship between $\log_{10}$ ATP content and A) percentage change in shell weight [Y = 11.854x + 0.1235, df = 63, R^2 = 0.3552, p < 0.0001], B) percentage change in shell thickness-2 [Y = 96.144x + 18.906, df = 63, R^2 = 0.2239, p < 0.0001], and C) water loss [Y = -29.151x + 29.043, df = 63, R^2 = 0.2295, p < 0.0001], in individual snails kept for 30 d under different temperature and pCO_2 conditions.

Further, 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 experiencing lower levels of ATP may have a reduced ability for exercise and this the ability to close their operculum tightly and/or for prolonged periods of time in order to prevent water loss. This possibility was confirmed by a significant negative relationship between water loss and $\log_{10}$ ATP levels (Fig. 6.1C) ($R^2 = 0.230$, df = 63, $p < 0.0001$). Hence, it appears that water loss in *L. littorea* could increase during emersion as a result of the disruption of physiological functions resulting from increased haemolymph acidosis and reduced energy production caused by an exposure to ocean acidification conditions (see Pörtner et al. 2004, Pörtner 2008) and elevated temperature (Sokolova and Pörtner 2001). This analysis suggests that metabolic energy may provide the basis for morphological plasticity (Findlay et al. 2009, 2011), as well as water loss (Sokolova and Pörtner 2001). It is relevant here to report that it has been suggested that alterations in metabolic energy due to exposure to ocean acidification rather than shell passive 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 may underpin the observed decrease in shell growth in molluscs (e.g. Michaelidis et al. 2005, Gaylord et al. 2011).

In Chapters 4 and 5 the focus was extended beyond a single population of *Littorina littorea* by investigating how different populations from across its latitudinal range responded in terms of their shell growth (Chapter 4), metabolic rates and metabolic profiles (Chapter 5) under high CO_2 conditions. As for my single population I also had the opportunity to use and synthesise the data reported in Chapter 2 and 3 in order to gain a holistic view of how the responses of different populations to ocean acidification may vary.

The main finding of Chapter 4 was that there is, indeed, population variation in the morphological response to ocean acidification. Snails from the southern- (Vigo) and northern-most (Tromsø) limits of distribution in the Eastern Atlantic show comparable reductions in shell wet weight, shell width and shell thickness compared with populations from the centre of the range. Greater reductions of these shell traits at the southern- and northern-most range edges could be caused by lower $CaCO_3$ saturation states. Some studies have shown that $CaCO_3$ polymorphic aragonite are lower than might be predicted at higher latitudes (see for example Orr et al. 2005, Bates et al. 2009, Fabry et al. 2009, Yamamoto et al. 2009 see also Riegl 2003), but this would only explain the reduction in shell growth at the northern edge. Another way of looking at this result is to focus on the relatively higher values in mid-range populations, which suggest they can compensate for the effects of ocean acidification. The question of whether biological reasons underlining these differences, are likely to be complex and may involve biotic and abiotic factors, for example, food availability, wave exposure, predators density and microclimatic conditions.

The main findings in Chapter 5 are that metabolic rates decreased linearly with increasing latitudes under current CO_2 conditions, but this pattern was changed when snails were exposed to high CO_2 conditions. The lower metabolic rates of northern populations under control conditions and, hence, the lack of latitudinal compensation is consistent for example with findings for gammarid amphipods at 79° N (see Marshal et al. 2011, Rastrick and Whiteley 2011, Whiteley et al. 2011 and see also Peck and Conway 2000).

In addition, it is not surprising that snails at southern ranges have higher metabolic rates than those in the centre and north, which may be linked to local temperature that relatively higher in southern than northern ranges. Hence, the metabolic costs of living in the south are likely to be higher (see Whiteley et al. 2011). There is no compensation of metabolic rate by northern snails, so-called metabolic cold-adaptation (Scholander 1953, Steffensen 2002, Whiteley et al. 2011) suggesting that, at low temperatures, energy requirements for basal metabolism and other energy demanding processes (e.g. for protein turn-over rates and ionic transport exchange) are low (Whiteley et al. 1997).

Surprisingly exposure to ocean acidification seems to impose a lowering of metabolic rates in the southern populations to levels comparable to those measured in the northern populations, and lower than those measured in central latitudes. This result is consistent with previous studies, which have shown that ocean acidification led to a decrease in metabolic rate in various taxa of marine organisms (e.g. Reipschläger and Pörtner 1996, Pörtner et al. 1998, Michaelidis et al. 2005, Metzger et al. 2007, Rosa and Seibel 2008, Small et al. 2010 but see for example Wood et al. 2008, Gutowska et al. 2010), and further I have shown that a great degree of variation is found also among populations.

In addition, ocean acidification has been shown, in some taxa, to lead to an increase in metabolic rates (e.g. Wood et al. 2008, 1010, Beniash et al 2010, Christensen et al. 2011). In Chapter 5 it shows that populations of *L. littorea* at central range latitudes increase their metabolic rates under high CO_2 conditions, and thus are able to carry out an active response to this environmental challenge. Furthermore, our results show that metabolic responses to ocean acidification greatly differ even among populations of the

same species (see for example Spicer et al. 2011, Stumpp et al. 2012, and discussion in Parker et al. 2010).

Metabolic depression is a common adaptive strategy for many populations living under high environmental variability particularly those are living in the intertidal zone (Storey and Storey 1990, Guppy and Withers 1999, Storey and Storey 2004), which is likely to have evolved as a physiological strategy to allow an organisms to match energy production and demand under hypoxia and hypercapnia (Pörtner et al. 2000, Fabry et al. 2008, Lamare et al. 2011, Melatunan et al. 2011). However, this mechanism is thought to be used only for short-term hypercapnia (Fabry et al. 2008), causing the slowdown of energy-expensive processes such as protein synthesis (Albright et al. 2011) and thus be too expensive to be used on longer-term exposure to environmental challenges (Michaelidis et al. 2005).

If metabolic depression can only be used as a short-term strategy by animals exposed to hypercapnia, increased metabolic rates could thus be advantageous for animals under hypercapnia to help compensate for the lowering of metabolic rates and aerobic scope in general (Metzger et al. 2007, Pörtner and Farrell 2008). But it is likely to influence performance and has fitness costs. Wood et al. (2008, 2010) provided clear evidence that the brittle stars *Amphiura filiformis* and *Omphiura ophiura* increased metabolic rate under high CO_2 conditions, however this led to arm degeneration. Lannig et al. (2010) also showed that, even though the oyster *C. gigas* increased metabolic rate under high CO_2 conditions, this was concomitant with decreased ATP and glycogen while alanine increased in the mantle cavity. This change in energy use may reduce energy turn-over

rates (Whiteley 1997), inhibiting growth and lowering functional activity (Leung and Furness 2001).

Furthermore, this chapter also shows that *L. littorea* populations from northern range not only maintain lower metabolic rates than other populations, but also maintain relatively constant metabolic rates when kept under high CO_2 conditions. Regardless, of whether or not the strategy to maintain lower metabolic rates under high CO_2 conditions is adaptive (e.g. Pörtner 1990, 1994 also Childress and Seibel 1998), it proves disadvantageous to the organisms in terms of the large decrease in growth observed. Lower metabolic rates reduce energy production and protein synthesis rates (Whiteley 1997, Whiteley et al. 2011) which may explain reduced growth (Fabry et al. 2008, Lannig et al. 2010). Indeed northern range populations (Trondheim and Tromsø) showed very different metabolomic fingerprints and metabolic responses in general, with a higher production of energy storage metabolites (e.g. thymine and uracil) and amino acids (e.g. valine and lysine), which are likely to be indicative of living under high environmental challenges. Lannig et al. (2010) found that increased energy storage increases ability for physiological maintenance, defence and repair when animals are in stressful conditions. They also found that ATP and succinate (the main products of cellular metabolism in marine gastropods – Sokolova and Pörtner 2001, 2003, Melatunan et al. 2011) were not significantly different in the muscle of the oyster *C. gigas* when exposed to hypercapnia compared with those exposed under normocapnia at 15 °C consistent with the present study. The lack of a significant difference in ATP production among populations in normal and high CO_2 conditions is probably due to high rates of ATP replenishment under high CO_2 conditions to maintain cellular homeostasis (see Hochachka 1985 see also Lannig et al. 2010). The high levels of

thymine and uracil suggests that these metabolities may have been used as endogenous fuels for ATP formation via pyrimidine nucleotide metabolism (e.g. convert thymine + deoxyribose-1-phosphate ↔ thymidine + P_i and thymidine + ATP ↔ dTMP + ADP – see Allen 1961, Cao et al. 2006).

Metabolite analysis on the effects of ocean acidification after short term exposure to the two higher temperatures (25 and 35 °C) show that snails from Trondheim are distinct compared to all other populations, with a lower production of betaine and taurine, and higher production of alanine and valine compared with the other populations. This result was consistent with those from Lannig et al. (2010) who showed that, when exposed to high CO_2, the oyster *C. gigas* produced higher levels of alanine. Interestingly, energy storage used as fuel for the production of energy ATP was not distinctive in the foot muscle of snails from Trondheim. Here snails may have been relying on the production of amino acid groups to maintain osmotic balance by increasing rates of osmo-ionic transport regulation (Burg and Ferraris 2008).

For future predicted levels of ocean acidification and warming in 2100, the current results suggest that populations of *Littorina littorea* at the southern- (42° N) and northern-most (70° N) limits of geographical distribution are more vulnerable than populations at central range (48 – 55° N). At the same time, populations living at the northern range edge (Tromsø) may have a greater vulnerability to ocean acidification than those at the southern range edge. In snails from Tromsø the production of alanine was reduced by 46% in high CO_2 conditions when compared to snails kept under normal CO_2 conditions, and this may reduce the production of glucose (*via* alanine

glucose pathway glycogenesis pathway, see King 1996) and could also impede ion and osmo-regulatory mechanisms (e.g. see Preston et al. 2002).

Finally, this thesis has described for the first time the existence of a latitudinal pattern of vulnerability to ocean acidification in a marine organism, and helps to advance this our understanding of the metabolic and morphological implications of this stressor. These findings will be useful in helping to interpret existing data on intertidal species' responses to ocean acidification and warming. This may help to demonstrate the value of taking a macrophysiological approach to the investigation on biological responses to complex climatic changes.

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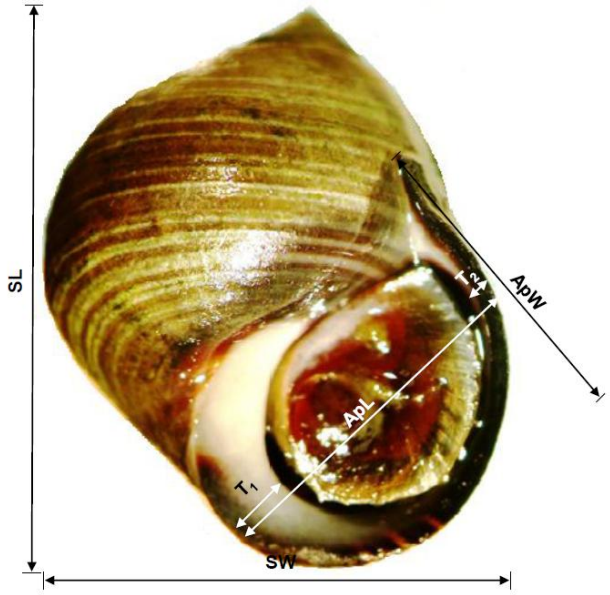
Appendix 2A

Mean physiological and biochemical parameters

Treatment Condition	**O_2 uptake** (µmol g^{-1} t.w. h^{-1})	**ATP** (µmol g^{-1} t.w.)	**ADP** (µmol g^{-1} t.w.)	**AMP** (µmol g^{-1} t.w.)	**AEC ratio**	**TAN** (µmol g^{-1} t.w.)	**succinate** (µmol g^{-1} t.w.)	**D-lactate** (µmol g^{-1} t.w.)
Current temp. & current pCO_2 [Control]	3.83 ± 0.21	2.15 ± 0.10	0.66 ± 0.08	0.37 ± 0.04	0.79± 0.01	3.18 ± 0.16	1.40 ± 0.07	0.90 ± 0.08
Elevated temp. & current pCO_2 [Elevated temp.]	3.23 ± 0.20	1.01 ± 0.08	0.47 ± 0.05	0.19 ± 0.03	0.75 /± 0.02	1.67 ± 0.12	1.62 ± 0.11	1.93 ± 0.12
Current temp. & elevated pCO_2 [Elevated pCO_2]	2.64 ± 0.18	1.01 ± 0.07	0.43 ± 0.06	0.24 ± 0.06	0.73 ± 0.02	1.68 ± 0.11	2.32 ± 0.14	2.54 ± 0.28
Elevated temp. & elevated pCO_2 [Combined]	3.18 ± 0.18	0.79 /± 0.04	0.25 ± 0.04	0.12 ± 0.03	0.80 ± 0.02	1.16 ± 0.07	1.26 ± 0.09	1.51 ± 0.11

NOTE. – Mean and standard error (SE) for O_2 uptake, adenylate nucleotide (ATP, ADP, AMP) concentrations, total adenylate nucleotide (TAN), adenylate energy ratio (AEC) and metabolic-end products (succinate and D-lactate) of the common periwinkle *L. Littorea* exposed to different combinations of elevated pCO_2 temperature.

Appendix 3A. Biometrics of the common periwinkle *Littorina littorea* shells were measured using images taken after and before 30 d exposure to different combinations of pCO_2 temperature. The measurements included shell length (SL), width (SW), shell aperture length (ApL), aperture width (ApW), thickness-1 (T_1) and thickness-2 (T_2).



Appendix 3B. Final mean values for shell biometric traits and resistance and water loss of the snail *L. littorea* exposed for 30 d to different combinations of pCO_2 and temperature. Log_{10} mean ATP content values used in correlation with other biometric traits and water loss are also reported. Data are given as mean values (± SE).

Treatment	**Shell weight** (% change)	**Shell length** (% change)	**Shell width** (% change)	**Aspect ratio** (% change)	**Shell thick-1** (% change)	**Shell thick-2** (% change)	**Aperture length** (% change)	**Aperture width** (% change)	**Aperture ratio** (% change)	**log_{10} ATP** ($\mu mol\ g^{-1}$)
Current temp. & current pCO_2 [Control]	6.39 ± 0.48	10.46 ± 0.77	7.49 ± 0.61	3.06 ± 0.86	23.87 ± 1.98	25.50 ± 2.31	7.01 ± 0.82	19.25 ± 1.36	-15.59±2.52	0.33±0.02
Elevated temp. & current pCO_2 [Elevated temp.]	1.35± 0.43	6.24 ± 0.50	8.20 ±/0.62	-2.36 ± 0.01	15.86 ± 1.51	9.73 ± 2.30	10.18 ± 0.59	22.04 ± 0.71	-15.80±1.28	-0.01±0.03
Current temp. & elevated pCO_2 [elevated pCO_2]	1.56± 0.57	7.58 ± 0.44	9.65 ± 0.45	-2.45 ± 0.01	5.40 ± 1.76	-43.22 ± 3.89	1.70 ± 0.58	12.49 ± 0.61	-12.64 ±0.99	-0.01±0.03
Elevated temp. & elevated pCO_2 [Combined]	-1.75 ± 0.34	4.97 ± 0.40	7.81 ± 0.66	-3.31 ± 0.01	15.80 ± 2.28	-23.18 ± 3.39	9.76 ± 0.73	20.30 ± 0.30	-15.09±2.06	-0.11±0.02

Appendix 3C. Percentage change for shell biometric traits and resistance and water loss of the snail *L. littorea* exposed for 30 d to different combination of pCO_2 temperature. Data are given as mean values (± SE).

Treatment	Shell weight (g)	Shell length (mm)	Shell width (mm)	Aspect ratio	Shell thick-1 (mm)	Shell thick-2 (mm)	Aperture length (mm)	Aperture width (mm)	Aperture ratio	Water Loss (% body mass)
Current temp. & current pCO_2 [Control]	1.91±0.04	15.84±0.11	13.57±0.10	1.17±0.01	1.88±0.03	0.69±0.11	8.99±0.1	9.35±0.1	0.97±0.01	19.54±2.39
Elevated temp. & current pCO_2 [Elevated temp.]	1.64±0.03	14.91±0.11	12.77±0.09	1.17±0.01	1.83±0.02	0.59±0.01	8.84±0.1	8.77±0.1	1.01±0.01	25.01 ±2.50
Current temp. & elevated pCO_2 [elevated pCO_2]	1.81±0.03	15.44±0.10	13.32/±0.09	1.16±0.004	1.68±0.03	0.50 ±0.01	8.79±0.1	7.50±0.1	1.18±0.02	31.38 /±1.90
Elevated temp. & elevated pCO_2 [Combined]	1.67±0.03	15.00±0.12	13.15±0.11	1.14±0.1	1.78±0.03	0.55 /±0.01	8.81±0.1	8.21±0.1	1.09±0.02	38.82 ±2.34

Appendix 4.1. A brief of sea surface temperatures (SSTs) and land surface temperature (LSTs) profile in each collection site.

Mean annual sea surface temperatures (SSTs) in Vigo ranged from 11.7 – 20.8 °C all over the year with mean maximum temperatures occurring in summer (16°C), although warmer temperature was also occur in the late autumn (15.4 °C) (Martinez-Urtaza et al. 2008). The mean land surface temperature (LSTs) in Vigo varied over the year, however the lowest level occurred during the winter (8.2 – 10.9 ºC) between December to early March whilst the warmest temperature was found in summer (17.4 – 19.7 ºC) with a maximum recorded temperature of 23 ºC (http://www.holidaycheck.com/). While mean SST in the sub-arctic region include Trømso (northern part) ranged between 6 – 8.5ºC (Kristensen et al. 2004) and can reach up to 15 ºC in summer (Whiteley et al. 2011). The LSTs varied during the year with the lowest temperature of -4 to -2 ºC between December to March and warmest temperature of 9 – 11 ºC between June to August. However during extreme climate condition the LSTs can drop down between -10 to -15 ºC in the autumn and winter and between 15 – 20 ºC in the summer (Sokolova and Pörtner 2001) or even more in extreme conditions in which the tide pool temperature can reach up between 35 – 45 °C (Sokolova and Pörtner 2003). Although both temperatures of SSTs and LSTs in these range-edges are highly different one to another, there is a tendency that within the nearest sites SSTs and/or SSTs are comparable. Annual SSTs of Vigo and Ile de Re are similar and likewise to Trondheim and Tromsø. While annual LSTs between Roscoff and Millport is slightly different but in term of annual LSTs they are almost similar (see Cooper 1958 and Hughes et al. 2011). In general, the SST in mid-ranges latitudes varied over the year with mean SSTs ranged between 7 – 15ºC. Period of cold occurred during winter between January to

March with mean SSTs of 6.8 – 7.4 ºC while warmest between July to September with the SSTs of 13.1 – 13.5 ºC (Hughes et al. 2007).

Appendix 4.2

Effect of 14 days exposure to normal and high pCO_2 at weight and morphological traits of *Littorina littorea*. Data are presented in percentage of proportion change at the final and beginning of the exposure period for each parameter. The bold type represents the high pCO_2 non-bold values under normal pCO_2conditions.

Population	Weight Changes (%)	Morphological changes (%)								
		Shell width	Shell length	Aspect ratio	Aperture width	Aperture length	Aperture shape	Aperture area	Thickness-1	Thickness-2
1	4.14 ± 0.39	1.64 ± 0.21	0.89 ± 0.14	-0.81 ± 0.29	0.86 ± 0.16	1.49 ± 0.67	0.55 ± 0.48	0.29 ± 0.16	4.06 ± 0.77	25.17 ± 2.32
	-2.90 ± 0.24	**-2.50 ± 0.13**	**-0.12 ± 0.12**	**0.65 ± 0.17**	**-0.27 ± 0.11**	**-1.05 ± 0.70**	**-1.78 ± 0.82**	**-0.96 ± 0.67**	**-2.99 ± 0.81**	**-29.46 ± 1.57**
2	4.24 ± 0.29	1.51 ± 0.22	1.36 ± 0.21	-0.17 ± 0.25	1.39 ± 0.25	4.97 ± 0.80	4.78 ± 0.86	2.36 ± 0.51	8.15 ± 0.88	30.95 ± 2.96
	-1.03 ± 0.22	**-1.28 ± 0.19**	**-0.55 ± 0.19**	**0.70 ± 0.20**	**-1.05 ± 0.25**	**-5.83 ± 0.69**	**-4.80 ± 0.74**	**-6.16 ± 0.80**	**-5.57 ± 0.85**	**-16.84 ± 0.91**
3	1.76 ± 0.24	1.49 ± 0.16	0.15 ± 0.18	-1.38 ± 0.23	0.39 ± 0.31	1.11 ± 0.64	0.57 ± 0.48	-0.14 ± 0.10	4.75 ± 0.82	22.91 ± 1.64
	-0.45 ± 0.25	**-1.50 ± 0.18**	**-0.47 ± 0.17**	**0.98 ± 0.24**	**-2.88 ± 0.31**	**1.34 ± 0.70**	**3.97 ± 0.83**	**-6.10 ± 0.67**	**-4.09 ± 0.84**	**-21.42 ± 1.15**
4	2.60 ± 0.29	2.11 ± 0.20	1.56 ± 0.23	-0.58 ± 0.20	0.08 ± 0.07	1.96 ± 0.32	-2.88 ± 0.70	0.38 ± 0.12	2.48 ± 0.68	32.04 ± 1.72
	-0.48 ± 0.29	**-1.22 ± 0.13**	**-1.71 ± 0.11**	**-3.29 ± 0.45**	**-3.80 ± 0.42**	**-10.19 ± 0.66**	**-6.34 ± 0.81**	**-6.06 ± 0.97**	**-23.90 ± 0.77**	**-12.95 ± 1.56**
5	3.11 ± 0.39	1.05 ± 0.22	1.23 ± 0.19	1.15 ± 0.21	0.79 ± 0.24	-0.14 ± 0.53	-3.57 ± 0.70	-0.13 ± 0.02	1.44 ± 0.71	9.61 ± 2.29
	-1.20 ± 0.38	**-0.25 ± 0.17**	**0.41 ± 0.14**	**0.65 ± 0.19**	**-0.06 ± 0.18**	**-2.19 ± 0.67**	**-3.63 ± 0.71**	**1.50 ± 0.66**	**-12.25 ± 0.68**	**-22.74 ± 1.01**
6	3.36 ± 0.31	1.25 ± 0.10	1.42 ± 0.15	0.16 ± 0.19	-0.03 ± 0.29	0.88 ± 0.37	0.72 ± 0.49	-3.40 ± 0.85	8.67 ± 0.71	20.75 ± 1.95
	-3.59 ± 0.27	**-1.86 ± 0.19**	**-0.29 ± 0.13**	**1.57 ± 0.24**	**-0.85 ± 0.32**	**0.71 ± 0.60**	**1.38 ± 0.80**	**-1.58 ± 0.55**	**-3.83 ± 0.87**	**-24.81 ± 2.25**

Appendix 4.3

Shell length width ratios calculated at the initial state and after 14 days exposure period in normal (385 µatm) and high pCO_2 (1000 µatm) for six populations of *Littorina littorea*. Values in bold indicate the ratio under high pCO_2 light indicate the ratio under normal pCO_2.

Ratio	Population					
	1	2	3	4	5	6
Initial L/W	1.174±0.007	1.184±0.005	1.208±0.005	1.191±0.005	1.180±0.004	1.153±0.004
	1.171±0.007	**1.174±0.004**	**1.191±0.005**	**1.202±0.006**	**1.175±0.004**	**1.167±0.005**
Final L/W	1.166±0.007	1.182±0.005	1.192±0.005	1.184±0.005	1.183±0.004	1.155±0.004
	1.199±0.007	**1.182±0.004**	**1.203±0.005**	**1.196±0.006**	**1.183±0.004**	**1.182±0.005**

Appendix 5.1.

NMR spectroscopy and spectral processing methods

All 360 dried polar extractions were suspended with sodium phosphate buffer in 90% H_2O and 10% D_2O (0.1 M, pH 7.0) containing 0.5 mM 2,2,3,3-D4-3-(Trimethylsilyl) propionic acid sodium salt (TMSP) (GOSS Scientific Instruments). 2D ^{1}H J-Resolved NMR spectra (JRES) were recorded at 500.11 MHz using an NMR spectrometer (Bruker Avance DRX-500, Bruker Optic GmbH, Ettlingen, Germany). JRES spectra were used in this study as the separation of chemical shift and coupling information into two axes can alleviate spectral crowding problems. 2-D JRES NMR spectra were acquired using 8 transients per increment collected into 16 k data points and a total of 32 increments. Datasets were zero-filled to 128 points in F1. The SEM window function was applied in the direct (chemical shift) dimension and sine-bell in the indirect dimension prior to Fourier transformation Viant et al. (2003) and Parsons et al. (2007). The chemical shift axis was calibrated to TMSP at 0.0 ppm and the skyline projections (pJRES) calculated (Parsons et al. 2007). All processing steps were done using TopSpin v3.0 (Bruker GmbH).

Initial inspection of the data matrix showed some poor quality spectra, which were removed. These were on rows 118, 239, 254. Three regions of the spectra were excluded to remove signal from TMSP, residual water and downfield noise. The chemical shift ranges for these regions were -5 to 0.6 ppm, 4.379 to 5.223 ppm; and 10 to 12 ppm respectively. Spectra were binned between 10 and 0.2 with a bin width of 0.005ppm. Spectra were normalized using Probabilistic Quotient Normalization (Byrne and Sommer 2011). Spectra were noise filtered, with the noise threshold set to 3 times the standard deviation of a region of known noise (9.5 to 10 ppm). For a variable to be retained, it was required that at least 10 samples must have signal above the threshold.

Spectra were glog transformed (BIOINFORMATICS Vol. 19 no. 8 2003, pages 966–972 DOI: 10.1093/bioinformatics/btg107), with the glog lambda parameter set to = 1.38e-9. This resulted in 1103 chemical shift points remaining after noise filtration.

To estimate the variability of the extraction process, the median RSD for six technical replicates was calculated. These technical replicates were six identical samples, homogenized and pooled, which were extracted in parallel. The median RSD was found to be 12.7%, which is comparable to the 12.5 % obtained by Parson et al. (in press) for pJRES spectra of six technical replicates of 3-spined stickleback European flounder liver (Parson et al. in press). The lambda parameter for the glog-transformation applied to the main study dataset was optimized using the six technical replicates using in-house developed MATLAB code (Parson et al. in press). The lambda parameter was calculated to be 1.38e-9.

The relative concentrations of metabolites in the samples was calculated using the web-based automated identification and quantification data mining tool FIMA (Field Independent Metabolite Analysis), developed at the University of Birmingham (http://www.bml-nmr.org/). This software package makes use of a library of metabolite spectra (Ludwig et al. 2010) and outputs a vector of relative metabolite concentrations for each sample.

Appendix 5.2.

A. Mean (± SE) percentage changes of shell weight and respiration rate of *L. littorea* after 14 d exposure periods under normal and high pCO_2. The O_2 uptake was measured after exposure to different air temperatures (15, 20, 25, 30, and 35 °C) for 3h.

Population	O_2 uptake (μmol O_2 g^{-1} t.w. h^{-1})	
	Normal pCO_2	High pCO_2
1	2.91 ± 0.15	2.20 ± 0.11
2	2.43 ± 0.10	2.18 ± 0.10
3	2.54 ± 0.13	2.90 ± 0.17
4	2.20 ± 0.10	2.81 ± 0.13
5	1.65 ± 0.10	1.59 ± 0.11
6	1.52 ± 0.11	1.68 ± 0.10

B. O_2 uptake rates of *L. littorea* measured after exposure to different pCO_2 for 14 d and air temperatures (given values) for 3h.

Population	pCO_2 level	Air temperature (°C)				
		15	20	25	30	35
1	Normal pCO_2	2.3500	3.3339	2.9305	3.4947	2.4321
	High pCO_2	2.3458	2.1348	2.6256	1.6663	2.2355
2	Normal pCO_2	3.3878	2.3725	2.1471	1.9254	2.3020
	High pCO_2	2.1931	2.3225	2.1471	2.1541	2.0264
3	Normal pCO_2	3.3100	2.4592	2.2252	2.3885	2.3140
	High pCO_2	3.4664	2.7785	2.2587	3.1230	2.8830
4	Normal pCO_2	2.1234	2.6358	2.0687	2.2111	1.9218
	High pCO_2	2.4889	3.6893	3.0965	2.1278	2.6602
5	Normal pCO_2	2.7500	1.3692	1.3645	1.3216	1.4203
	High pCO_2	1.9953	1.7993	1.1942	1.3943	1.6764
6	Normal pCO_2	2.7106	1.5657	1.0782	1.1148	1.1382
	High pCO_2	2.0902	1.1976	1.5989	1.2831	2.2271

APPENDIX 6.1

Publication 1. Exposure to elevated temperature and pCO_2 reduces respiration rate and energy status in the periwinkle *Littorina littorea*.

CHICAGO JOURNALS

Exposure to Elevated Temperature and Pco2 Reduces Respiration Rate and Energy Status in the Periwinkle Littorina littorea
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Exposure to Elevated Temperature and P_{CO_2} Reduces Respiration Rate and Energy Status in the Periwinkle *Littorina littorea*

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ABSTRACT

In the future, marine organisms will face the challenge of coping with multiple environmental changes associated with increased levels of atmospheric P_{CO_2}, such as ocean warming and acidification. To predict how organisms may or may not meet these challenges, an in-depth understanding of the physiological and biochemical mechanisms underpinning organismal responses to climate change is needed. Here, we investigate the effects of elevated P_{CO_2} and temperature on the whole-organism and cellular physiology of the periwinkle *Littorina littorea*. Metabolic rates (measured as respiration rates), adenylate energy nucleotide concentrations and indexes, and end-product metabolite concentrations were measured. Compared with values for control conditions, snails decreased their respiration rate by 31% in response to elevated P_{CO_2} and by 15% in response to a combination of increased P_{CO_2} and temperature. Decreased respiration rates were associated with metabolic reduction and an increase in end-product metabolites in acidified treatments, indicating an increased reliance on anaerobic metabolism. There was also an interactive effect of elevated P_{CO_2} and temperature on total adenylate nucleotides, which was apparently compensated for by the maintenance of adenylate energy charge via AMP deaminase activity. Our findings suggest that marine intertidal organisms are likely to exhibit complex physiological responses to future environmental drivers, with likely negative effects on growth, population dynamics, and, ultimately, ecosystem processes.

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Introduction

A significant increase in the burning of fossil fuels, industrial emissions, and levels of deforestation over the past 200 yr has resulted in an increase in atmospheric P_{CO_2} (Petit et al. 1999; Augustin et al. 2004; Siegenthaler et al. 2005). This increase is predicted to have profound implications for the global climate. In addition, around 30% of the CO_2 emitted in the past 250 yr has been taken up by the oceans, leading to substantial changes to the physicochemical conditions of seawater (Caldeira and Wickett 2003; Feely et al. 2004; Orr et al. 2005), such as significant alterations to pH, $[CO_3^{2-}]$ (Feely et al. 2004), and aragonite and calcite saturation states (Ω_{ara} and Ω_{cal}, respectively; Morse et al. 2006). Current models predict that this situation will worsen with an increase of atmospheric P_{CO_2} to 730–1,020 ppmv by the year 2100 (IPCC 2007), resulting in a further reduction in oceanic pH of 0.3–0.4 with a further consequent drop in $[CO_3^{2-}]$, Ω_{ara}, and Ω_{cal} (Caldeira and Wickett 2005; IPCC 2007; Gangstø et al. 2008). At the same time, global surface ocean temperatures are predicted to increase globally by 3°–5°C over the same period (IPCC 2007; Sokolov et al. 2009).

The predicted changes to seawater P_{CO_2} and temperature are expected to exert a negative impact on the developmental, ecological, and physiological functions of marine organisms (e.g., Bibby et al. 2007; Rosa and Seibel 2008; Dupont and Thorndyke 2009; Todgham and Hofmann 2009; Findlay et al. 2009*b*; O'Donnell et al. 2010; Small et al. 2010; Hale et al. 2011; Pistevos et al. 2011; Whiteley 2011). In particular, ocean acidification has been shown to alter metabolic rates (e.g., Rosa and Seibel 2008), net calcification (Findlay et al. 2009*a*; Ries et al. 2009), and hemolymph P_{O_2} levels (Metzger et al. 2007). It has been suggested that some marine animals may respond to exposure to elevated P_{CO_2} by reducing their metabolic rates (e.g., Pörtner et al. 1998; Langenbuch and Pörtner 2002; Seibel and Walsh 2003; Rosa and Seibel 2008; Small et al. 2010), although Wood et al. (2010) showed that the ophiuroid brittlestar *Ophiura ophiura* up-regulated its metabolic rate in response to low pH hypercapnia.

Temperature is also a primary driver for physiological, life-history, and ecological functions of ectotherms (Cossins and Bowler 1987), and therefore global warming is likely to affect physiological function, growth, and distribution of species (Southward et al. 1995; Stillman 2003; Pörtner and Knust 2007;

Koeller et al. 2009). Temperature has been shown to greatly affect respiration rates and energy metabolism in ectotherms (Prosser 1991; Angilletta 2009). For example, in the periwinkle *Littorina saxatilis* it has been shown that, during emersion, increased acclimation temperature leads to (i) an increase in oxygen consumption rates, (ii) alteration of the activation energy of aerobic metabolism (*Ea*), and (iii) the onset of anaerobiosis (as a response to the insufficient oxygen supply to tissues at elevated temperatures; Sokolova and Pörtner 2003). These effects accounted for a significant depletion of "high-energy" phosphates and accumulation of end products of fermentation (Sokolova and Pörtner 2001).

The combined exposure to elevated P_{CO_2} and temperature may exert different effects, by acting antagonistically, additively, or synergistically, on different functions. This makes it difficult to predict the direction and intensity of organisms' responses to environmental changes. Physiological studies of the jumbo squid *Dosidicus gigas* have shown a metabolic rate reduction of 30% on combined exposure to elevated P_{CO_2} and temperature (Rosa and Seibel 2008) and a possible energy deficit in the brittlestar *O. ophiura* (Wood et al. 2010). To date, however, there have been no in-depth investigations of the interactive actions that ocean acidification and elevated temperature will likely exert on the fundamental whole-organism and intracellular physiological responses of marine ectotherms. Here, we investigate such responses using whole-organism metabolic rates, adenylate energy nucleotide (ATP, ADP, AMP) concentrations, total adenylate nucleotides (TAN), adenylate energy charge (AEC), and the concentrations of metabolic end products (succinate, lactate) in the common periwinkle *Littorina littorea*, an abundant, widespread species within intertidal ecosystems of northwest Europe and the Atlantic coast of North America (Fretter and Graham 1962; Brawley et al. 2009).

Material and Methods

Animal Collection

Adult individuals of *Littorina littorea* (shell width = 13–15 mm) were collected during May 2009 from the rocky intertidal at Hannafore Point in Looe Bay (Cornwall, United Kingdom; −50°20′36.87″N, 4°27′16.83″W). Animals were transported to the laboratory within 2 h and were maintained for 10 d in aerated filtered seawater (pH 8.01, salinity = 35.0) at 15°C in two plastic aquaria (56 L, 130 individuals in each aquarium). Animals were fed every second day ad lib. on *Ulva lactuca* and *Fucus serratus* throughout this period.

Mecososm Setup

A factorial design incorporating seawater with two levels of both P_{CO_2} and temperature representing current conditions (385 ppmv/pH 8.0 and 15°C) and predicted conditions for the year 2100 (1,000 ppmv/pH 7.6 and 20°C; Caldeira and Wickett 2005; IPCC 2007; Sokolov et al. 2009) were used. Individual snails were haphazardly allocated to one of these four P_{CO_2} × temperature treatment levels (64 individuals per treatment) and exposed for 30 d. A CO_2/air-equilibration microcosm was set up for each treatment in a temperature-controlled room maintained at 15°C (12L : 12D), as a modified version of the equilibration flow-through systems used by Widdicombe and Needham (2007), Dashfield et al. (2008), and Widdicombe et al. (2009). Briefly, each CO_2 equilibration system consisted of a header tank (80 L) in which the seawater was either aerated by bubbling normal air or acidified by bubbling pure CO_2 gas. From each of the four header tanks seawater was gravity fed (600 mL min^{-1}) continuously to one of four exposure tanks (23 cm × 15 cm × 14 cm, 5 L), which were held in larger holding tanks (60 cm × 35 cm × 15 cm, 32 L). Each exposure tank contained 16 plastic pots (45 mL) with 25 holes (diameter = 3 mm), each containing a single individual snail (mean shell width ± SD = 12.9 ± 5.3 mm). The excess water from the exposure tanks flowed into the holding tanks and was transferred into a large plastic container (45 cm × 36 cm × 35 cm, 56 L), aerated, and recirculated via a submersible pump (EP68; Hengtong Aquarium, Hengtong, Taiwan) to the header tanks. Fifty percent of the seawater in each header tank and sump was replaced weekly, debris was removed every 2 d, and deionized water was added as needed to maintain stable salinity levels and guarantee good water quality (i.e., to minimize ammonia accumulation). CO_2 gas was released into the header tank using a multistage CO_2 regulator (EN ISO 7291; GCE, Worksop, United Kingdom) connected to a flip-flop control solenoid valve (ORIFICE 3/16 Closed System; Peter Paul Electronics, New Britain, CT) controlled by a calibrated pH controller (pH-201 Digital; Dream Reef, Humberston, United Kingdom).

Seawater temperature in two of the holding tanks was increased to 20°C using aquarium heaters (Rio 1700; Aqua Vital, Bristol, United Kingdom); the other two holding tanks were maintained at 15°C by the ambient conditions in the temperature-controlled room where the experiment was conducted. Seawater temperature, salinity, pH, total dissolved inorganic carbon, and dissolved oxygen in exposure tanks were measured daily. Temperature and salinity were measured using a handheld multimeter (YSI 85; YSI, Yellow Springs, OH), and pH was measured using an Inlab 413SG pH electrode and Sevengo pH meter (Mettler-Toledo Analytical; Sonnenbergstrase, Switzerland), employing the National Bureau of Standards (NBS) pH standards. Total dissolved inorganic carbon was measured using a CO_2 analyser (965D; Corning, Cambridge). Carbonate system parameters that were not directly measured were calculated using CO_2SYS (Pierrot et al. 2006), employing constants from Mehrbach et al. (1973) refitted to the NBS pH scale by Dickson and Millero (1987) and the KSO_4 dissociation constant from Dickson (1990).

Metabolic Rates

Metabolic rates of individual snails were measured using oxygen uptake as a proxy following the method developed by Spicer and Erickson (2003). Six individual snails were selected haphazardly from each aquarium (96 individuals in total), placed individually in a blacked-out glass jar (70 mL) to rest (following

preliminary tests) for 30 min. O_2 levels were measured right before closing the jars and after 85 min using an O_2 meter (model 781; Strathkelvin Instruments, Glasgow, United Kingdom) equipped with an O_2 electrode (1302 electrode; Strathkelvin Instruments). Oxygen uptake was calculated as the delta of the O_2 levels at the beginning and at the end of the incubation period (expressed as μmol O_2 g^{-1} h^{-1} wet weight). No snail was exposed to hypoxic conditions (i.e., below 80% O_2 saturation).

Intracellular Metabolites and Metabolic End Products

For the preparation of tissue, four snails were pooled haphazardly from each aquarium and inspected for possible infections. Individual foot muscles (approximately 0.2 g) were quickly dissected, cleaned in fresh seawater, frozen immediately in liquid nitrogen, and stored at −80°C. For extraction, frozen tissue was powdered using a precooled pestle and mortar. To remove protein, the powdered tissue was extracted in perchloric acid with the ratio of one part of tissue in two parts of perchloric acid (0.9 M). The extracted tissues were centrifuged using a microcentrifuge (Sorvall Legend Micro 17; Heraeus Biofuge Pico, Crown Way, United Kingdom) for 10 min at 3,000 *g* at 4°C. The supernatant was transferred to another microcentrifuge tube, and the precipitate was extracted again in perchloric acid (0.2 M) using one part of precipitate to three parts of perchloric acid. The supernatants from both extractions were then pooled, and the pH of the solution was adjusted to 6.0–6.5 using 2.0 M potassium hydroxide, after which they were left resting for 1 h in an ice bath. After rapid centrifugation for 3 min at 10,000 *g*, the extracts were stored at −80°C.

All reagents were purchased from Sigma Aldrich (Poole, United Kingdom) except for enzymes, which were purchased from Roche Diagnostics (Mannheim, Germany). Concentrations of energy metabolites ATP, ADP, and AMP and end-product accumulation (succinate, D-lactate, and L-lactate) were determined spectrophotometrically in the perchloric acid extracts (Bergmeyer 1985). The assay procedures for measurement of adenylate nucleotides were described in protocols provided by Calzyme Laboratories (Surrey, United Kingdom) that were modified on the basis of the reaction described by Strominger et al. (1959), whereas the end products were assayed using commercial kits (Megazyme International Ireland, Wicklow, Ireland; succinate, K-SUCC II/5; D-lactate, K-DATE 03/06; L-lactate, K-LATE 03/06). However, L-lactate was not detected using this method.

The AEC index, a biomarker used to assess the disruption of energy status due to environment challenges (Luca-Abbott et al. 2000), was calculated using the formula

$$\mathrm{AEC} = \frac{[\mathrm{ATP}] + \frac{1}{2}[\mathrm{ADP}]}{[\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}]}. \quad (1)$$

TAN has been used as a proxy to gauge the physiological condition of an organism following environmental anaerobiosis associated with cyclical variations in temperature, dissolved oxygen, and pH (Dehn 1992; Luca-Abbott et al. 2000; Suska and Scotnicka 2010) and is here employed to assess the effect of the interaction between elevated P_{CO_2} and temperature on the pool of nucleotides available for ATP synthesis. It is calculated as

$$\mathrm{TAN} = [\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}]. \quad (2)$$

Statistical Analyses

The effect of P_{CO_2}, temperature, and their interaction on O_2 uptake, energy metabolism, and metabolic end products were analyzed using a two-way ANCOVA, with tank as a random factor nested within P_{CO_2} × temperature and weight of total body tissue as a covariate. All data met assumptions for normality as untransformed data or after $\log_{10}$ transformation (maximum $Z_{64} = 1.181$, $P = 0.123$). Variances were homogeneous for O_2 uptake and other metabolic parameters (maximum $F_{3,60} = 1.721$, $P = 0.172$) but were not homogeneous for [ADP], [succinate], and [D-lactate] (minimum $F_{3,60} = 3.223$, $P = 0.029$). In light of the fact that our experimental design included four treatments with a minimum of 16 replicates per treatment per measurement, we assumed that the ANCOVA design employed should be tolerant of deviation from the assumption of normality and heteroscedasticity (Sokal and Rohlf 1995; Underwood 1997). Tank had a significant effect on respiration rate, [ATP], and TAN measured in this study (minimum $F_{12,48} = 1.966$, $P < 0.039$) but did not have a significant effect on [ADP], [AMP], AEC, [succinate], and [D-lactate] (maximum $F_{12,48} = 1.506$, $P = 0.156$). However, in the cases where the term tank was found significant, removing this factor (tank effect) from the calculation caused no change in the significance of the main factors, and thus tank effect is considered marginal. All analyses were conducted using SPSS 17.

Results

Microcosm Parameters

Seawater physiochemical parameters were stable over the experimental period (see Table 1). Mean P_{CO_2} values were 428 ± 17 ppmv in the control treatments and 998 ± 30 ppmv in the acidified treatments, resulting in mean pH's of 8.04 ± 0.005 and 7.66 ± 0.003 in control and acidified treatments, respectively. Mean temperatures under control and elevated conditions were 15.1° ± 0.1°C and 20.3° ± 0.1°C, respectively.

Metabolic Rates

Metabolic rates of individual *Littorina littorea*, here measured as O_2 uptake, were significantly lower by an average of 31% under elevated P_{CO_2} conditions compared with those in control snails after the 30-d exposure period (Table A1; Table B1 in the online edition of *Physiological and Biochemical Zoology*; Fig. 1). In addition, elevated temperature caused no significant changes in metabolic rates in comparison to control P_{CO_2} conditions. However, under elevated P_{CO_2} conditions temperature

Table 1: Physicochemical parameters of the seawater in the mesocosm unit

Parameter	Current Temperature	Elevated Temperature	Elevated Pco_2	Elevated Pco_2 and Temperature
$[O_2]$ (mg L^{-1})	6.75 ± .01^{A}	7.09 ± .15^{A}	6.68 ± .17^{A}	6.85 ± .17^{A}
Salinity (ppt)	34.77 ± .03^{A}	35.12 ± .05^{A}	35.06 ± .04^{A}	35.12 ± .07^{B}
Temperature (°C)	14.43 ± .06^{A}	20.91 ± .09^{B}	14.89 ± .05^{A}	20.68 ± .08^{B}
pH	8.03 ± .01^{A}	8.04 ± .01^{A}	7.67 ± .01^{B}	7.65 ± .00^{B}
DIC	1,596 ± 49.77^{A}	1,573 ± 39.81^{A}	1,596 ± 40.25^{A}	1,723 ± 46.41^{A}
TA (μequiv kg^{-1})[a]	1,726 ± 52.15^{A}	1,743 ± 44.06^{A}	1,627 ± 40.43^{A}	1,767 ± 47.71^{A}
Pco_2 (μatm)[a]	428 ± 17.22^{A}	428 ± 13.21^{A}	998 ± 29.91^{B}	1,185 ± 33.28^{C}
$[HCO_3^-]$ (μmol kg^{-1})[a]	1,489 ± 46.51^{A,B}	1,444 ± 36.02^{B}	1,518 ± 38.31^{A,B}	1,633 ± 43.97^{A,C}
$[CO_3^{2-}]$ (μmol kg^{-1})[a]	89.79 ± 3.53^{A}	114.31 ± 5.01^{B}	40.58 ± 1.30^{C}	51.50 ± 1.79^{C}
Ω_{cal}[a]	2.14 ± .08^{A}	2.73 ± .12^{B}	.97 ± .03^{C}	1.23 ± .04^{C}
Ω_{ara}[a]	1.37 ± .05^{A}	1.78 ± .08^{B}	.62 ± .02^{C}	.80 ± .03^{C}

Note. Data are means ± SE for seawater physicochemical parameters measured or calculated during the duration of the experiment: oxygen concentration (O_2), salinity, temperature (°C), pH (National Bureau of Standards scale; Mettler-Toledo pH meter, Luton, United Kingdom), dissolved inorganic carbon (DIC; Total CO_2 Analyser, CIBA Corning 965D, Essex, United Kingdom), total alkalinity (TA; Alkalinity Titrator, AS-ALK2, Apollo SciTech, Bogart, GA) using the method developed by Dickson et al. (2007), carbon dioxide partial pressure (Pco_2), bicarbonate and carbonate ion concentration ($[HCO_3^-]$ and $[CO_3^{2-}]$), and calcite and aragonite saturation states (Ω_{cal} and Ω_{ara}). Superscript capital letters indicate a significant difference among treatments by the post hoc Bonferroni test of one-way ANOVA ($P < 0.05$), according to a 95% confidence interval test for estimated marginal means.

[a]Parameters were calculated using the CO_2SYS program (Pierrot et al. 2006), employing the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

appeared to outweigh CO_2, leading to a significant increase in metabolic rate (+18%; Fig. 1) as indicated by the presence of a significant interaction between temperature and Pco_2 (Table B1). Finally, there was also a significant negative relationship between tissue weight and O_2 uptake (Table B1).

Energy Metabolites

The mean concentration of [ATP] ranged from 2.15 μmol g^{-1} t.w. under control conditions to 1.01 μmol g^{-1} t.w. under the high Pco_2 and temperature conditions, with levels under control Pco_2 conditions at 15°C comparable to those reported previously for *Mytilus edulis* (Beis and Newholmes 1975) and *Littorina saxatilis* (Sokolova and Pörtner 2003; Table A1).

Mean [ATP] was significantly lower after 30 d of exposure to elevated CO_2 and temperature in isolation (−53% in both cases) and even more so under the combined condition (−63%), compared with that under control conditions (Tables A1, B1; Fig. 2*a*). A similar pattern was found for [ADP], although the elevated Pco_2 treatments did not show any significant difference (Table B1; Fig. 2*b*). Mean [AMP] was highest and significantly different from any of the other treatments under control conditions, lowest under combined elevated Pco_2 and temperature conditions, and intermediate under elevated Pco_2 and temperature conditions in isolation, with these two treatments showing no significant difference between each other (Tables A1, B1; Fig. 2*c*). In general, elevated Pco_2 led to a comparable degree of decrease in mean [AMP] at both temperatures tested here, as no significant interaction was found (Table B1).

Mean TAN showed the same basic pattern of changes as [ATP] across the different treatments, with a significant decrease in mean TAN of 48% under both elevated Pco_2 and elevated temperature conditions and a further reduction by 64% under combined conditions (Tables A1, B1; Fig. 2*e*). Mean AEC also showed a pattern comparable to that observed for [ATP] with the exception that the mean level measured under combined conditions was similar to that found for the control; thus, elevated temperature exerts a different effect on mean AEC under control and elevated Pco_2 conditions, as indicated by the existence of a significant interaction (Tables A1, B1; Fig. 2*d*).

Metabolic End Products

Levels of succinate found in the tissues of *L. littorea* were similar after 30 d of exposure to control, elevated temperature, and combined conditions, but under high Pco_2 conditions significantly higher levels were found (Fig. 3*a*). Hence, the effect of hypercapnia is dependent on temperature, as indicated by a statistically significant interaction (Table B1). Compared with that in control snails, mean [D-lactate] was higher in snails exposed to either elevated Pco_2 or elevated temperature, and these groups were comparable with each other (Fig. 3*b*). However, D-lactate in animals exposed to the combined conditions was lower and not significantly different from levels in control animals, giving rise to a significant interaction between Pco_2 and temperature (Table B1; Fig. 3*b*). A significant negative correlation was also found between the weight of the individual (i.e., without the shell) and [D-lactate], indicating that smaller individuals accumulated more lactate than large ones. No relationship between individual weight and [succinate] was found.

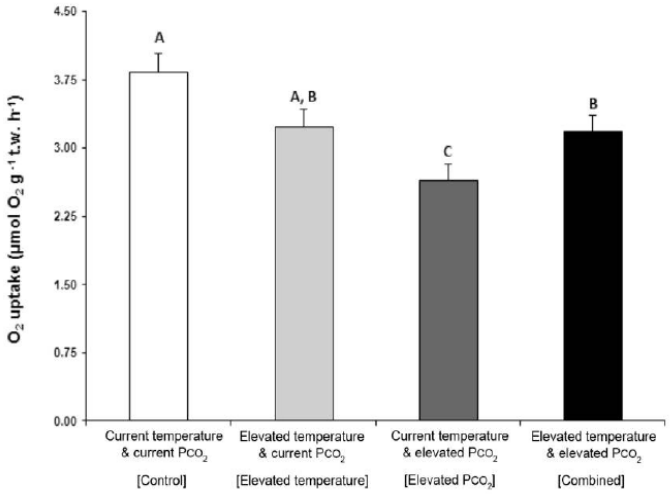

Figure 1. O_2 uptake in the snail *Littorina littorea* exposed to different combinations of P_{CO_2} (428 or 998 ppmv) and temperature (15° or 20°C): control (*white*), elevated temperature (*light gray*), elevated P_{CO_2} (*dark gray*), and elevated P_{CO_2} and temperature (*black*). Values are means ± SE. Significantly different treatments ($P \leq 0.05$) are indicated by different letters (according to the 95% confidence interval test).

Discussion

Our study shows that a marine intertidal invertebrate, *Littorina littorea*, undergoes a significant reduction in metabolic rate, as indicated by a decrease in respiration rate, when exposed for a prolonged period to either elevated P_{CO_2} in isolation or, to a lesser extent, to combined elevated P_{CO_2} and temperature. Most importantly, we show—for the first time, to our knowledge—that the metabolic reduction caused by prolonged exposure to elevated P_{CO_2} is combined with a significant disruption of aerobic metabolism.

Metabolic Rates

It has been widely reported that exposure to environmental hypercapnia, which leads to changes in extracellular acid-base balance, affects metabolic mode and respiration rate in a variety of marine invertebrates, including the sipunculid worm *Sipunculus nudus* (Reipschläger and Pörtner 1996; Pörtner et al. 1998), the bivalve mollusc *Mytilus galloprovincialis* (Michaelidis et al. 2005), the Humboldt squid *Dosidicus gigas* (Rosa and Seibel 2008), the edible crab *Cancer pagurus* (Metzger et al. 2007), and the velvet swimming crab *Necora puber* (Small et al. 2010). In all these cases, a decrease in O_2 uptake was reported at low pH induced by hypercapnia. In contrast, in the brittle star *Ophiura ophiura* an increase in O_2 uptake at low pH was observed (Wood et al. 2010), as was no significant change in standard metabolic rates in the cuttlefish *Sepia officinalis* (Gutowska et al. 2008). Our data demonstrate that littorinid snails experience a reduction in metabolic activity (−31%) comparable to that reported for most marine invertebrates exposed to elevated environmental CO_2. The capacity for metabolic reduction in molluscs as a response to environmental stress (e.g., hypoxia) is high (Guppy and Withers 1999). Substantial decreases in metabolism are used as a strategy to ensure survival by matching ATP supply and demand (Bishop and Brand 2000; Seibel and Walsh 2003). However, the reduction seen here was relatively modest compared with fully depressed, hypometabolic states in molluscs (≥80%; Guppy and Withers 1999), and there was no behavioral evidence that the animals had entered a fully hypometabolic state. Thus, we proposed that the term "depression" be used more cautiously within the context of metabolic rate reduction reported in most marine invertebrates as a response to exposure to ocean acidification.

The observed hypercapnia-induced metabolic reduction, which was similar to that found in *S. nudus* by Reipschläger and Pörtner (1996) and Pörtner et al. (2000), could be explained by a decrease in ATP demand, a decrease in ATP production, or both. Lowering of pH leads to an initial acid-base imbalance, requiring a variety of compensatory mechanisms (including ionic exchange) to maintain cellular pH homeostasis, with a concomitant requirement for ATP (Seibel and Walsh 2003). However, it has been suggested that changes in the method of cellular pH homeostasis—for example, a switch to the use of Na^+/H^+ and Na^+-dependent Cl^-/HCO_3^- exchange from the use of H^+-ATPase—leads to a reduction in the rate of hydrolysis of ATP required for this process (Pörtner et al. 2000). An alternative possibility could be hypercapnia-induced disruption to the supply of oxygen—for example, via effects on oxygen-transport proteins, such as hemocyanin—and hence disruption to the supply of ATP generated via aerobic metabolism (Seibel and Walsh 2003).

When elevated CO_2 and temperature were combined, their

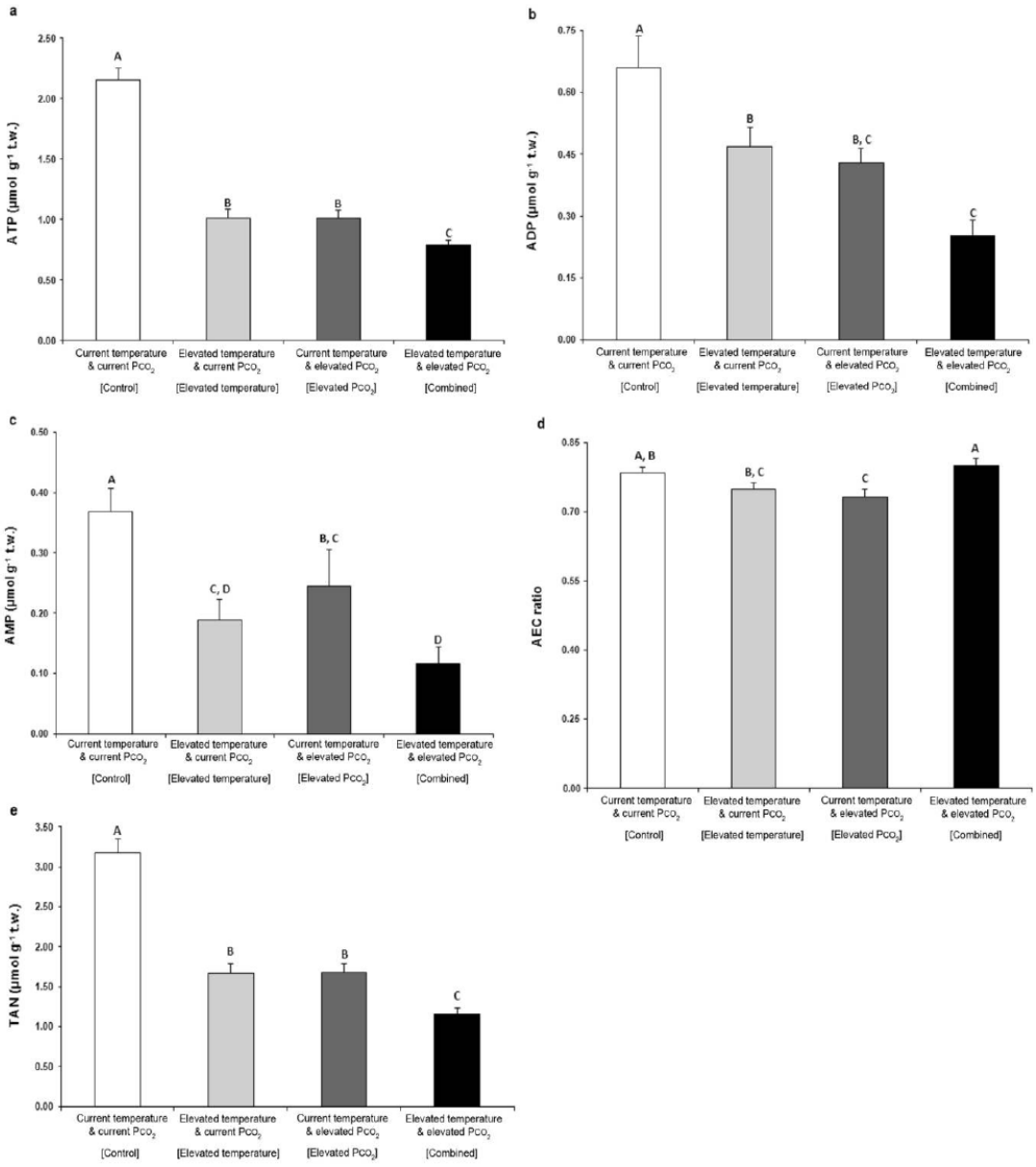

Figure 2. Cellular nucleotide energy of the snail *Littorina littorea* exposed to different combinations of Pco_2 (428 or 998 ppmv) and temperature (15° or 20°C). *a*, [ATP]; *b*, [ADP]; *c*, [AMP]; *d*, total adenylate nucleotides (TAN); *e*, adenylate energy charge (AEC) ratio. Histograms represent means ± SE determined for the four treatments used. Significantly different means ($P \leq 0.05$) are indicated by different letters (according to the 95% confidence interval test).

negative effect on O_2 uptake was much less pronounced, although there was still a significant reduction in metabolic rates compared with the control. Under this combined condition there was also a significant decline in TAN compared with the control, as well as compared with either elevated temperature or elevated CO_2 in isolation. As discussed in more detail below, this may indicate an increased demand for ATP and hence may explain why an additional increase in temperature led to an increase in the O_2 uptake rate by snails. A similar interactive effect between elevated Pco_2 (1 kPa) and temperature (20°C)

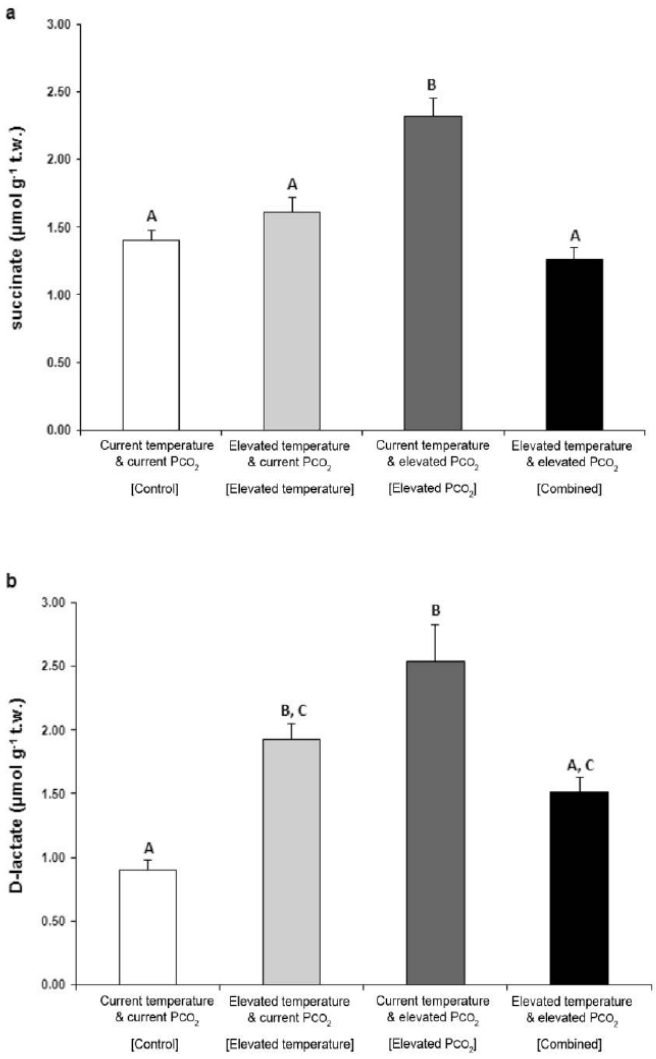

Figure 3. Cellular nucleotide energy of the snail *Littorina littorea* exposed to different combinations of P_{CO_2} (428 or 998 ppmv) and temperature (15° or 20°C). *a*, [succinate]; *b*, [D-lactate]. Histograms represent means ± SE determined for the four treatments used. Significantly different means ($P \leq 0.05$) are indicated by different letters (according to the 95% confidence interval test).

has also been shown in the penaeid crustacean *Metapenaeus joyneri* (Dissanayake and Ishimatsu 2011).

Energy Metabolism

Both elevated temperature and P_{CO_2} in isolation affected AEC and TAN in the foot tissue of the marine periwinkle *L. littorea*. The decreases in AEC were mainly due to decreases in [ATP], but without corresponding increases in [ADP] and [AMP]. Instead, levels of ADP and AMP were significantly lower compared with the control for either elevated temperature or elevated P_{CO_2} in isolation, leading overall to significant reductions in TAN. Although elevated temperature and P_{CO_2} had adverse effects, the AEC in both cases was still maintained above the levels (0.5–0.7) considered to represent a threshold of severe decline in energy status (Luca-Abbott et al. 2000). It therefore seems likely that the animals had successfully used substantial decreases in TAN to protect AEC, probably via activation of AMP deaminase (Gibbs and Bishop 1977), and hence depletion of AMP in response to an initial decrease in AEC (Chapman and Atkinson 1973). Nevertheless, although AEC was largely protected, the approximate halving of TAN might have the

consequence of reducing the snails' capacity for work, perhaps making them more vulnerable to additional environmental challenges due to a further increase in energy utilization (Giesy et al. 1981). As noted above, under elevated P_{CO_2} conditions *L. littorea* showed substantial metabolic reduction, which may be indicative of this energy limitation. To maintain homeostasis under energy limitation such as that shown to be caused by hypoxia, many aquatic organisms reduce ATP demand to match ATP production (Bishop and Brand 2000; Seibel and Walsh 2003), thereby entering a hypometabolic state where O_2 uptake rates are reduced to 5%–40% of normal levels (Storey and Storey 2004). Although respiration rate fell in our study, it was not to an extent where the animals would have been expected to enter a hypometabolic state (Shick et al. 1986; De Zwaan et al. 1991; Pörtner and Grieshaber 1993), and neither was there any indication that they had (i.e., they remained active and responsive).

It is of interest that combined elevated P_{CO_2} and temperature did not have a synergistic effect on either AEC or O_2 uptake rate. Nevertheless, the combined conditions did lead to a significant drop in TAN compared with elevated temperature or P_{CO_2} in isolation. This indicates, despite the normal AEC value, that combined elevated temperature and P_{CO_2} put further strain on the ability of snails to match ATP supply and demand, which may explain the relatively high O_2 uptake (comparable with the control). A likely consequence of this strain on energy metabolism is that the animals have lower capacity for growth, consistent with data on effects of elevated temperature and P_{CO_2} on growth of *L. littorea* (S. Melatunan, P. Calosi, S. D. Rundle, S. Widdicombe, and J. A. Moody, personal observation).

Metabolic End Products

Exposure of *L. littorea* to elevated temperature or P_{CO_2} in isolation led to a significant increase in D-lactate levels and, in the case of P_{CO_2}, in succinate levels. Both of these end products of anaerobic metabolism are required, for example, during both functional and environmental hypoxia (Pörtner et al. 1984; Grieshaber et al. 1994). D-lactate accumulation is more associated with short-term functional hypoxia brought about by animal behavior in which there is rapid reoxidation of NADH by pyruvate, which is catalyzed by D-lactate dehydrogenase and allows maintenance of ATP regeneration via glycolysis. In contrast, succinate accumulation is more associated with environmental hypoxia—that is, longer-term hypoxia brought about by environmental change—in which there is a slower reoxidation of NADH, catalyzed by Krebs cycle enzymes (Livingstone 1983). The ATP yield in the accumulation of succinate depends on the energy store used (either aspartate or glycogen), but it is greater than that obtained in the accumulation of D-lactate (Larade and Storey 2002).

Because of the length of exposure used in our study (30 d), we assumed that the levels of D-lactate and succinate were steady state levels—that is, that the rate of accumulation in each case matches the rate of loss (e.g., in the case of succinate as a consequence of further metabolism to propionate and acetate; De Zwaan et al. 1976; Zebe 1977; Schöttler 1980). Hence, differences in lactate and succinate levels probably arose from differences in accumulation rate, with the caveat that differences in steady state levels could also arise from differences in the rate of loss. Hence, it seems that elevated temperature and P_{CO_2} in isolation lead to an increased reliance on anaerobic metabolism by the snails involving both cytosolic and mitochondrial compartments (Sokolova and Pörtner 2003). Levels of succinate were similar to those seen by Sokolova and Pörtner (2003) when *Littorina saxitilis* was exposed to elevated temperature in water for short periods (18 h). However, in the same study no accumulation of lactate was found, in contrast to the substantial increases in D-lactate seen here, suggesting that littorinid species may exhibit complex forms of metabolic plasticity (Greenway and Storey 2001). We also showed that prolonged exposure to elevated P_{CO_2} and temperature in combination had clear effects on energy metabolism, probably adversely affecting the supply of ATP and requiring compensatory changes in ATP demand. Although the snails were able to maintain AEC at relatively normal levels, it is questionable whether they would have been able to continue to do so if faced with fluctuations in O_2 supply, such as are seen in an intertidal ecosystem. Hence, it would be interesting in the future to look at the ability of *L. littorea* to cope with acute stress by, for example, following changes in anaerobic metabolic end products, the adenylate nucleotide pool, and arginine phosphate levels during air exposure at elevated temperature (Sokolova and Pörtner 2001) after prolonged exposure of the animals to elevated P_{CO_2} and temperature in combination.

Here we have provided evidence that the marine intertidal gastropod *L. littorea* undergoes physiological disruption under high P_{CO_2} and temperature conditions in isolation as well as combined. Metabolic reduction was characterized by decreased rates of O_2 uptake and decreased energy status (AEC and TAN). Increased levels of metabolic end products (D-lactate and succinate) also indicated an increased reliance in anaerobic metabolism under elevated temperature and/or P_{CO_2} conditions. Although snails were able to survive under future global change conditions (no mortality was observed during the study period), they experienced a (chronic) mismatch between energy supply and demand, which could make them more vulnerable to further environmental alternations that often occur in the intertidal environment (such as hypoxia; see Larade and Storey 2009). In the context of global climate change, this keystone intertidal gastropod might face difficulties in maintaining physiological homeostasis, and this could ultimately influence growth, reproduction, and population dynamics. Moreover, while our results may well represent a worst-case scenario, it is also possible that changes in environmental conditions may go beyond the scenario predicted for surface oceans (Thomsen et al. 2010), given that the intertidal ecosystem is characterized by a complex pattern of seawater P_{CO_2} levels (Agnew and Taylor 1986), notwithstanding the species' ability to rapidly adapt to future climatic change scenarios (Collins and Bell 2004, 2006; Findlay et al. 2011; Pistevos et al. 2011; Sunday et al. 2011).

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APPENDIX A

Table A1: Mean physiological and biochemical parameters

Treatment Condition	O_2 Uptake (μmol g^{-1} t.w. h^{-1})	ATP (μmol g^{-1} t.w.)	ADP (μmol g^{-1} t.w.)	AMP (μmol g^{-1} t.w.)	AEC Ratio	TAN (μmol g^{-1} t.w.)	Succinate (μmol g^{-1} t.w.)	D-lactate (μmol g^{-1} t.w.)
Control	3.83 ± .21	2.15 ± .10	.66 ± .08	.37 ± .04	.79 ± .01	3.18 ± .16	1.40 ± .07	.90 ± .08
Elevated temperature	3.23 ± .20	1.01 ± .08	.47 ± .05	.19 ± .03	.75 ± .02	1.67 ± .12	1.62 ± .11	1.93 ± .12
Elevated P_{CO_2}	2.64 ± .18	1.01 ± .07	.43 ± .06	.24 ± .06	.73 ± .02	1.68 ± .11	2.32 ± .14	2.54 ± .28
Combined	3.18 ± .18	.79 ± .04	.25 ± .04	.12 ± .03	.80 ± .02	1.16 ± .07	1.26 ± .09	1.51 ± .11

Note. Data are means ± SE for O_2 uptake, adenylate nucleotide (ATP, ADP, AMP) concentrations, total adenylate nucleotides (TAN), adenylate energy charge (AEC) ratio, and metabolic end products (succinate, D-lactate) of the common periwinkle *Littorina littorea* exposed to different combinations of elevated P_{CO_2} and temperature.

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APPENDIX 6.2

Manuscript to MEPS – under review

Publication 2. Marine gastropod plastic responses to the combined effects of ocean acidification and elevated temperature.

Marine gastropod shell plastic responses to the combined effects of ocean acidification and elevated temperature

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Abstract. Phenotypic plasticity is a fundamental mechanism by which organisms can alter their morphology, life history or behaviour in response to environmental changes. Here we investigate plastic responses in shell morphology in the intertidal gastropod *Littorina littorea* in response to predicted levels of pCO_2 and temperature, focusing on shell traits known to relate to protection from predators (size and thickness) and resistance to desiccation (shape). Both low pH and elevated temperature disrupted the overall investment in shell material, with snails in acidified seawater and elevated temperature in isolation or in combination showing lower shell growth rates than control individuals. Percentage increase in shell length was also lower for individuals kept under combined acidified seawater and elevated temperature, and percentage 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 rate of snails, measured as percentage water loss, was also lower at low pH and high temperature. Counter to predictions, however, water loss was only weakly related with shell biometric measures but was negatively correlated with ATP concentrations. As ATP concentration was positively correlated with shell thickening and weight, it seems that the combined effects of low pH and elevated temperature may occur 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 and Scheiner 2004). Given the drastic 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 explicitly examine the extent to which species can exhibit plastic responses to predicted levels of environmental change.

Marine intertidal habitats are highly heterogeneous and contain numerous species that exhibit plastic responses to natural environmental variation in, for example, wave action (e.g. Gaylord 2000) and predation pressure (e.g. Boulding and Alstyne 1993). At the same time, marine environments are exposed to anthropogenically-induced environmental variation, and current predictions suggest that environmental conditions in marine systems are likely to undergo a shift towards lowered pH (due to increased in aqueous [CO_2]) and increased temperatures, as a result of increased atmospheric carbon dioxide (CO_2) levels (Caldeira and Wickett 2003, Sokolov et al. 2009). These conditions are expected to cause severe alterations to marine organisms' physiology, development and behaviour (e.g. Bibby et al. 2007, Todgham and Hofmann 2009, Munday et al. 2009) culminating in changes to community structure and ecosystem function (e.g. Wootton et al. 2008, Feng et al. 2009, Hale et al. 2011).

Calcification plays an important role in the plastic responses of many marine taxa that alter their exoskeleton size and shape in response to environmental variation (e.g. Cotton et al 2004, Irie and Fischer 2009), and this key physiological process is also

affected by both reduced seawater pH, carbonate ion concentration (ocean acidification), and temperature (e.g. Rodolfo-Metalpa et al. 2009). Early evidence suggested that calcification rates decrease with decreasing pH (e.g. Gazeau et al. 2007), but more recent experimental studies have shown that calcification can also increase in some taxa at low pH (Findlay et al. 2011, Ries et al. 2009). Similarly, temperature increases may lead to the disruption of calcification in marine ectotherms (e.g. Irie 2006). Given that elevated temperature and reduced pH are both induced by elevated atmospheric CO_2 levels, marine ecosystems will be exposed simultaneously to these two key environmental stresses. Consequently, the true impacts of elevated pCO_2 on the function of marine calcifiers are likely to be greater than previously thought (e.g. Feng et al. 2009).

Marine gastropods depend heavily on a calcareous shell for protection against predation, wave exposure and desiccation. The shell morphology within this group has been shown to vary widely in response to spatial and temporal environmental variability (Avery and Etter 2006), e.g. in temperature (Irie 2006) and predation threat (e.g. Trussell and Nicklin 2002, Cotton et al. 2004). 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 or 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, and thickness) and desiccation (shell aperture size and shape).

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. The two pH_{NBS} levels selected were based on current (8.0) and predicted values for the year 2100 (7.7) corresponding to the global ocean pCO_2 of 380 and 1000 ppm, respectively (Caldeira and Wickett 2003). Two water temperature levels were used, 15±0.1°C, which correspond to the monthly sea surface temperature (SST) at the collection site (Joyce 2006) and 20±0.1°C, which assumes an increase of +5°C in line with future prediction for global warming trend for sea surface temperatures (Sokolov et al. 2009). At the time of collection, we also measured local seawater temperature, which was 14 °C (determined using a YSI 85 handheld multimeter, YSI Inc., Yellow Springs, USA).

Animal collection and preparation

Littorina littorea individuals (shell width 13-15 mm) were collected in May 2009 from the rocky intertidal shore at Hannafore Point in Looe bay, Cornwall (50° 20' 36.87'' N and 4° 27' 16.83'' W). Individuals were returned to the laboratory within two hours and. before being introduced into the experimental set-up. were maintained in two large plastic aquaria (capacity 56 l, 130 individuals in each aquarium) for ten days in aerated seawater at 15°C salinity 33 ppt. Individuals were fed on *Ulva lactuca* and *Fucus serratus ad libitum* every second day throughout this period.

116 *Mesocosm setup*

117 Four CO_2/air-equilibration mesocosm (one per treatment) were set up in a control-
118 temperature room (CT-room) maintained at 15°C (12 h light and 12 h dark), as modified
119 versions of the equilibration flow-through systems used by Widdicombe and Needham
120 (2007). Briefly, gas of CO_2-air mixed was passed through the water in header tank and
121 pump *via* gravity to the experimental unit. Seawater pH was monitored using pH
122 controller (Aqua Digital PH-201, Reef dreams Inc., Hampshire, UK). Excess seawater
123 from the experimental unit was left flowing *via* gravity into a common sump (50 cm
124 length, 45 cm width and 35 cm height), where seawater was degasified *via* vigorous air
125 bubbling with a common air pump. From here with a submersible pump (EP68,
126 Hengtong Aquarium Co. Ltd., Hengtong, Taiwan) the header tanks were resupplied
127 with seawater at controlled pH, which triggered the injection of new CO_2 *via* the CO_2
128 controller (CO_2 solenoid, Peter Paul Electronic Inc. New Britain, USA) until the set pH
129 was reached again. A submergible pump in the header tanks allowed for a rapid
130 homogenisation of the physic-chemical parameters of the seawater. In addition, 50% of
131 the seawater in the experimental system was changed weekly and distilled water added
132 as needed to avoid salinity fluctuations and ammonia build-up.

133 Four aquaria (23 cm length, 15 cm width and 15 cm height) with forty-eight holes (ø 10
134 mm, 9 and 15 holes on each side of the sight) were placed on each experimental unit (65
135 cm length, 38 cm width and 15 cm height, for a total of 16 aquaria). Sixteen plastic pot
136 (20 ml, ø 316 mm and 5 cm height) containing each individual animal were fit on each
137 aquarium for 30 d exposure period. The holes on each aquaria and pot were made to
138 ensure seawater pass through on each system. Hence, a total of 256 individuals were
139 used in the experiment. For details on the physico-chemical parameters within the
140 mesocosm and carbonate system calculations see Appendix A.

141

142 *Biometric measurements*

143 Shell morphological parameters known as the expression of phenotypic plasticity in
144 response to predation susceptibility (Brönmark and Miner 1992, Hoverman 2010), were
145 calculated as the proportional difference between measurements at the start before the
146 experiment and after 30 d exposure. These parameters were shell length and width,
147 aperture length and width, shell thickness of the inner lip of the shell (which lies
148 alongside the columellar axis in the posterior aperture of the shell, referred to hereafter
149 as *thickness-1*), and thickness of the outer lip (the growing tip lying along the anterior
150 portion of the shell, hereafter *thickness-2*), as well as total weight of the intact
151 individual (Cotton et al. 2004) (Appendix B). These parameters were also used to
152 calculate measures related to shell shape, including aspect ratio (shell length:shell
153 width) and aperture ratio (shell aperture length:shell aperture width).

154

155 All measurements were carried out on images collected with a digital camera (Coolpix
156 4500, Nikon UK Ltd., Kingston upon Thames, UK) mounted on a light microscope
157 (SDZ-IR-P, Kyoma Optical, Co. Ltd, Tokyo, Japan). Each image was measured using
158 image processing UTHSCSA Image tool program for Windows 2003 calibrated using a
159 micro meter (1.000 ± 0.001 mm) that has been widely used and provide more accuracy
160 than using conventional method of digital calipers (Rundle et al. 2004, Al-Mazrouai
161 2008, Gaylord et al. 2010). Differences between initial and final parameter measures
162 were expressed as percentage change. Finally, shell and tissues were separated and
163 weighed.

164

165

166 *Water loss*

167 Water loss was measured in a sub-set of six snails from each replicate aquarium (total n
168 = 96 individuals), using equation (1) (Sokolova and Pörtner 2001):

169

170 $$WL = \frac{W_{in} - W_{exp}}{W_{in} - W_{dry}} \text{x } 100\%$$

171

172 Where WL is water loss (%) while W_{in}, W_{exp} and W_{dry} are initial weight, weight after a
173 given exposure period and final dry weight of snail (mg) respectively. Briefly, W_{in} and
174 W_{exp} were determined with a digital scale (PF-203, Fisher Scientific, Leicestershire,
175 UK) before and after the exposure in air to 30°C for 6 h on an aluminium tray in a
176 programmable oven (Sokolova and Pörtner 2001). After exposure, snails were returned
177 to their individual pots in the original experimental tanks to acclimate for 2 h and then
178 were dried at constant temperature of 100°C for 24 h to determine dry weight (W_{dry}).

179

180 *Statistical analyses*

181 The effects of elevated pCO_2, temperature and their interaction on total wet weight,
182 biometric shell characteristics (shell length and aspect ratio, aperture length, width and
183 aperture ratio, shell thicknesses-1 and -2) and water loss were analysed using a two-way
184 ANCOVA, with exposure aquaria as a random factor nested into pCO_2*temperature.
185 Aquaria had a significant effect on most parameters measured in this study (minimum
186 $F_{1,255} = 1.937$, $p = 0.031$) with the exception of percentage change in shell aspect,
187 aperture length and water loss (maxim $F_{1,96} = 0.921$, $p = 0.535$). However, in those cases
188 where the term aquaria was found significant, removing the factor 'Aquaria' causing
189 such effect did not change the patterns of significance of the main factors, and thus
190 aquaria effect is considered marginal. Where found not significant the term 'Aquaria'

were 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 aspect where no transformation was beneficial (minimum $Z_{256} = 1.476$, $p = 0.026$). Variances were homogeneous for percentage change aspect ratio and water loss (maximum $F_{15,240} = 1.420$, $p = 0.158$), but not for the other variables (minimum $F_{15,240} = 1.758$, $p = 0.041$). In light of the fact that our experimental design included four 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 assumption of normality and heteroscedasticity (Sokal and Rohlf 1995). Pairwise comparisons were conducted using the 95% Confidence Interval test calculated for Estimated Marginal Means. Finally, a correlation analysis was conducted to verify the existence of a possible relationship between water loss and shell aperture aspect. All analyses were conducted using SPSS 17.

RESULTS

Shell weight, size and shape

Mean percentage change in shell wet weight, shell length and shell shape was significantly lower under low pH and elevated temperature conditions in isolation (Table 1 and Fig. 1a and b). The percentage change in shell weight was three and five times higher, respectively, under current temperature conditions (6.4%) compared with acidified seawater (1.6%) and acidified and elevated temperature in combination (-1.8%) (Fig. 1a, Appendix C). Hereinafter, the percentage change in shell length was respectively lower under elevated temperature (5.83%), acidified seawater (8.39%), and interaction between elevated pCO_2 and temperature (4.35%) compared to current

temperature conditions (10.31%) (Appendix C). Shell length in snails kept under elevated pCO_2 was also lower than the current temperature condition however there is no significant mean different found (Fig. 1b). In addition, the percentage change of shell shape was lower under elevated temperature (-2.37%), elevated pCO_2 (-2.45%), and interaction (-3.31%) compare to current temperature condition (3.06%). As a result, shells were significantly heavier and longer under current temperature conditions compared with those under combined elevated pCO_2, elevated temperature and interaction conditions; weights, lengths and shell shape for the other two treatments did not significantly differ between each other (Fig. 1a and b). Percentage increase in shell width was not affected by elevated pCO_2, elevated temperature or their interaction (Table 1).

Shell Thickness

Both measures of shell thickness decreased at the higher temperature under normal pH conditions but increased with temperature under acidified conditions (Fig. 1c and d) giving a significant interaction between $pCO2$ and temperature (Table 1). Mean percentage change in shell thickness-1 (inner lip) was significantly higher under current temperature conditions (23.9%), lower under acidified conditions (5.4%), and intermediate for the other two treatments (both c16%) (Fig. 1c). Mean percentage change in shell thickness-2 (outer lip) was also significantly higher (25.5%) under current temperature conditions, and was actually lower due to negative growth under acidified conditions (-43.2%), and intermediate for the other two treatments, which did significantly differ from each other (Fig. 1d).

241 *Shell aperture size and shape*

242 Percentage aperture length increase was significantly affected by elevated temperature
243 (10.2% increase) and by pCO_2 and elevated temperature in combination (9.8%) (Table
244 1) (Fig. 1e). The lowest value for percentage change in aperture length was recorded
245 under acidified conditions (1.7%), whilst control conditions were found to be
246 intermediate (7.0%) (and significantly different) to the elevated temperature treatments
247 and the acidified treatment (Fig. 1e). Mean percentage increase in aperture width and
248 mean percentage changes in aperture shape (i.e. aperture ratio) were not significantly
249 affected by pCO_2 or temperature (see Table 1).

250

251 *Water loss*

252 Mean percentage water loss increased under both low pH (31.4%) and at elevated
253 temperature (38.8%) conditions (Appendix D), these two factors showing a negative
254 additive effect as both had a significant effect on water loss in isolation; no significant
255 effect of their interaction was detected (see Table 1 and Fig. 2a). Water loss increased
256 with mean percentage change in aperture ratio, but this trend was marginally significant
257 ($r^2 = 0.030$, df = 95, p = 0.09, Fig. 2b).

258

259 ## DISCUSSION

260 A complex pattern of responses to the exposure to ocean acidification and global
261 warming appears to be emerging across phylogenetically diverse taxa (e.g. Martin et al.
262 2008, Rodolfo-Metalpa et al. 2009, Ries et al .2009, Hale et al. 2011). Here we show
263 that plastic responses to environmental parameters predicted to change through
264 alterations to the climate can also be complex within a species. Specifically, we show
265 that shell morphometric traits in the marine gastropod *Littorina littorea*, respond

266 differently to elevated pCO_2 and elevated temperature, with a mixture of single,
267 additive, synergistic and no effects. As the traits investigated potentially underpin the
268 ability of this intertidal gastropods to protect itself from predators and avoid desiccation,
269 our results suggest that exposure to future global change scenarios (Caldeira and
270 Wickett 2003, Sokolov et al. 2009) may alter the tolerance of this species and,
271 ultimately, its fitness and survival.
272
273 *Shell growth, thickness and shape*
274 Under control (i.e. current) temperature conditions, the shells of *L. littorea* were heavier
275 and longer than those in animals grown under low pH and elevated temperature in
276 isolation. For example, increased seawater acidity caused a reduction in shell growth of
277 the mussels *Mytilus galloprovincialis* (Michaelidis et al. 2005) and exposure to low pH
278 seawater in larvae of the Mediterranean pteropod *Cavolinia inflexa* (Comeau et al.
279 2010) and the mussels *Mytilus edulis* (Gazeau et al. 2010) and *Mytilus californianus*
280 (Gaylord et al. 2011) also reduced shell thickness. Moreover, elevated temperatures
281 have also been reported to induce smaller metamorphic size in the gold-ringed cowry
282 *Monetaria annulus* (Irie and Fisher 2009), and to cause disruptions of the metabolism,
283 growth and fitness in the periwinkle *Littorina saxatilis* (Sokolova and Pörtner 2001).
284 Not surprisingly, in *L. littorea* the combined exposure to both low pH and elevated
285 temperature had a greater negative effect on shell growth than either of these factors in
286 isolation, a result in line with those for other marine calcifying organisms exposed
287 simultaneously to elevated pCO_2 and temperature (e.g. Rodolfo-Metalpa et al. 2009).
288 *Littorina Littorea* also shows a lower shell thickness under acidified conditions. This
289 reduction in shell thickness occurred mainly at the growing tip (thickness-2) rather than
290 more centrally on the body whorl. Dissolution at the growing tip of the shell under

291 acidified seawater conditions has previously been reported in the planktonic pteropod
292 *Clio pyramidata* (Orr et al. 2005). Such reductions in shell thickness in marine calcifiers
293 have been considered to be the result of either dissolution of calcium carbonate
294 structures exposed to acidified seawaters (e.g. Orr et al. 2005, Michaelidis et al. 2005,
295 Nienhuis et al. 2010) or insufficient deposition of calcium carbonate material (see
296 Findlay et al. 2011). A separate study conducted with the same experimental unit
297 describes that total shell dissolution over 30 d exposures to elevated pCO_2 (1293±16.85
298 ppm, mean ± SE) in isolated shells of *L. littorea* is higher by 1.5 - 4.0 folded times
299 (ranged between 3 – 15%) compared to that observed in occupied shells (1.7 – 4.1%)
300 (Melatunan S., Calosi P., Rundle D.S., Widdicombe S., and Moody A.J., *pers. observ.*).
301 This indicates that shell dissolution still occurs in such shells but has a relatively small
302 impact on total shell weight. Despite the fact that a different population of *L. littorea* to
303 that one tested here are known to continue to calcify under elevated pCO_2 conditions
304 (Findlay et al. 2011), our results appear to point out that under acidic conditions a
305 distinctive reduction in carbonate calcium deposition may occur at the growing tip of
306 the shell.
307 Here, we have also shown that elevated pCO_2 exerts a different effect on shell thickness
308 at different temperatures. In fact in the acidified treatments, shell thickness was higher
309 under elevated temperature. This may be due to the increase in calcite and aragonite
310 saturation states with increasing temperature and thus a decrease in the rate of passive
311 dissolution, with Ω calcite being just below and just above values of 1 under acidified
312 and combined acidified and elevated temperature conditions, respectively. Whilst we
313 did not explicitly test for shell strength, a reduction in shell thickness may likely lead to
314 a reduction in shell compression load (e.g. Gaylord et al. 2011). As thicker apertural lips
315 are likely to provide better defence against shell crushing predators (Vermeij et al. 1987,

316 Bourdeau 2010) the morphological changes reported here may increase susceptibility to
317 predation (Boulding and Alstyne 1993, Trussell and Etter 2001).
318 Shell shape in aquatic gastropods is also thought to play an important role in predator
319 defence. A globular shell shape in the freshwater snail *Physa spp*. has more resistance to
320 crushing predators, such as crayfish, than an elongated shape (DeWitt et al. 2000), and
321 species of marine intertidal gastropods possessing shells with a larger aspect ratio (i.e.
322 with a more elongated shape) were found to be more vulnerable to crab predation,
323 possibly due to a reduced handling efficiency in shells with a flatter more discoid shape
324 (Cotton et al. 2004).
325 Here we show that proportional change in shell shape in *L. littorea* is affected
326 significantly by elevated temperature, elevated pCO_2 and the combined effect of these
327 factors. Shells kept under current temperature conditions were more elongated but had a
328 more globular shape under other treatment conditions. This finding suggests that in
329 more acidic, warmer conditions snails produce a shell shape that may be less susceptible
330 to crab predation. As shell thickness is reduced under low pH and elevated temperature,
331 acquiring a more globose shape may enable snails to compensate for a possible
332 reduction in shell strength.
333
334 *Shell aperture plasticity and water loss*
335 The marine gastropod *Thais lapillus* increases operculum size (Gibson 1970), the land
336 snail *Cepaea spp*. reduce aperture sizes (Goodfriend 1986) and the common limpet
337 *Patella spp*. decreases base-aperture size (Cabral 2007) in order to maintain constant
338 body temperatures and reduce desiccation. Overall, shells of *L. littorea* exposed to
339 elevated temperature conditions were more elongate compared to the more rotund shape
340 found under current temperature conditions. This altered shell shape may have negative

341 consequences for desiccation rates. Most shelled gastropods and barnacles reduce
342 desiccation by completely closing the open aperture area with the operculum (Shick et
343 al. 1988). However, this strategy may reduce the rate of oxygen uptake, thus impairing
344 organismal production of energy metabolites (Sokolova and Pörtner 2001).
345 Consequently, it can be concluded that under a future global change scenario *L. littorea*
346 may be exposed to a significantly increased desiccation risk, unless thermoregulatory
347 behavioural plastic responses can mediate this situation (as suggested for terrestrial
348 ectotherms by Huey and Tewksbury (2009).
349
350 *A metabolic basis for plasticity and water loss*
351 In general, increased ocean acidity can cause internal acidosis, interfere with
352 homeostatic functions, affect metabolism and ultimately disrupt growth and fitness
353 (Pörtner 2008). It has been suggested that alterations in metabolic energy due to
354 exposure to ocean acidification rather than shell passive dissolution will be more likely
355 to impact calcification (Findlay et al. 2011). In fact, as calcium transport and secretion
356 in shell-forming cells of molluscan and corals is partly ATP-dependent (see Findlay et
357 al. 2011 for a review), disruption of energy metabolism may underpin the observed
358 decrease in shell growth in various molluscs (e.g. Michaelidis et al. 2005, Gaylord et al.
359 2011, this manuscript). In a parallel investigation of the physiological responses of *L.*
360 *littorea*, we have shown that a 30 d exposure to low pH, elevated temperature and their
361 interaction caused a significant drop in both oxygen consumption rates and ATP levels
362 (Melatunan et al. *in press*). Combining these findings for ATP with those on shell
363 plasticity we find a positive significant correlation between metabolic energy levels
364 ($\log_{10}$ [ATP]), percentage change in shell weight ($R^2 = 0.355$, df = 63, $P < 0.0001$, Fig.
365 3a) and percentage change in thickness-2 ($R^2 = 0.224$, df = 63, $P < 0.0001$, Fig. 3b).

366 This suggest that the observed disruptions in shell growth and shell thickness plastic
367 responses may be mediated by the alteration of physiological processes induced by
368 ocean acidification and elevated temperature, rather than the decrease in Ω saturation
369 status as previously thought (Pörtner 2008, Findlay et al. 2009, Findlay et al. 2011).
370 Further, as energy metabolites play a key role in energy transduction in the intracellular
371 space, underpinning whole-organism exercise capacity, it is possible that individuals of
372 *L. littorea* experiencing lower levels of ATP may have a reduced ability to close their
373 operculum tightly in order to prevent water loos. Thus not surprisingly, we also found a
374 significant negative relationship between water loss and $\log_{10}$ ATP levels (Fig. 3c) (R^2 =
375 0.230, df = 63, $P < 0.0001$). Hence, it appears that water loss in *L. littorea* will increase
376 during emersion as a result of the disruption of physiological functions resulting from
377 increased haemolymph acidosis and reduced energy transduction exerted by ocean
378 acidification (for review see Pörtner et al. 2004) and elevated temperature (Sokolova
379 and Pörtner 2001).
380
381 In general, phenotypic plasticity enables organisms to respond to environmental
382 variability (West-Eberhard 2003), and can be defined as a measure of 'organismal
383 malleability' (Huey and Berrigan 1996). Here we report an in-depth investigation of
384 plastic responses for a marine invertebrate to the combined exposure of elevated pCO_2
385 and temperature that will likely occur in the near future; according to projected pCO_2-
386 pH and SST levels (Solomon et al. 2007, Sokolov et al. 2009). Under future global
387 climate scenarios, individuals of the periwinkle snail *L. littorea* might be predicted to be
388 smaller in size, with thinner, more rotund shells; further, observed changes in shell
389 aperture will likely cause an increase in individuals' water loss during emersion. The
390 pattern of responses observed here is rather complex but clearly suggest that global

391 climate change will likely have far reaching consequences for the ecology of marine
392 organisms. In addition, we propose a mechanistic (ATP-based) explanation for changes
393 in marine organisms' plastic responses and water loss, thus providing a direct link
394 between organismal energy metabolism, phenotypic responses, and functional
395 vulnerability to global climate change.
396
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406

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561

562 TABLE 1. The effect of elevated pCO_2 and temperature on different shell traits and water

563 loss in the common periwinkle *Littorina littorea*. Degrees of freedom (df), mean of

564 square (MS), F-ratio (F), probability level (p) are reported.

Trait	Source	df	MS	F	p
Shell Wet Weight (% change)	pCO_2	1	1006.2	26.5	< **0.0001**
	temperature	1	1113.6	29.4	< **0.0001**
	interaction	1	47.9	1.262	0.283
	aquaria	12	37.9	2.95	**0.001**
Shell Length (% change)	pCO_2	1	274.9	5	**0.046**
	temperature	1	746.7	13.5	**0.003**
	interaction	1	41	0.7	0.406
	aquaria	12	55.3	3.2	< **0.0001**
Shell Width (% change)	pCO_2	1	49.6	0.7	0.429
	temperature	1	20.7	0.3	0.607
	interaction	1	103.9	1.4	0.259
	aquaria	12	74.2	3.8	< **0.0001**
Shell Aspect (% change)	pCO_2	1	667.3	18.7	< **0.0001**
	temperature	1	631.7	17.7	< **0.0001**
	interaction	1	332.8	9.3	**0.003**
Shell Thickness-1 (% change)	pCO_2	1	5489.2	12.8	**0.004**
	temperature	1	91.1	0.2	0.653
	interaction	1	5423.3	12.7	**0.004**
	aquaria	12	429.8	1.9	**0.031**
ShellThickness-2 (% change)	pCO_2	1	143970.9	52.8	< **0.0001**
	temperature	1	12.3	0.01	0.948
	interaction	1	24581.5	9.01	**0.011**
	aquaria	12	2727.7	4.9	< **0.0001**
Aperture length (% change)	pCO_2	1	523.5	17.2	< **0.0001**
	temperature	1	2015.4	66.2	< **0.0001**
	interaction	1	382.5	12.6	< **0.0001**
Aperture width (% change)	pCO_2	1	1155.2	2.3	0.156
	temperature	1	1795.9	3.6	0.84
	interaction	1	403.8	0.8	0.389
	aquaria	12	504.9	10.3	< **0.0001**
Shell Aperture Aspect (% change)	pCO_2	1	511.5	0.4	0.526
	temperature	1	6.9	0.01	0.941
	interaction	1	287.8	0.2	0.633
	aquaria	12	1196.1	7.4	< **0.0001**
Water Loss (%)	pCO_2	1	4116.7	30.2	< **0.0001**
	temperature	1	1085.4	8	**0.006**
	interaction	1	1.1	0.01	0.929

565

566 **Figure legends**

567 FIG. 1. Effect of 30 d exposure to different combinations of $pCO_2$and temperature on the
568 percentage change on: (a) shell wet weight, (b) shell length, (c) shell thickness-1, (d) shell
569 thickness-2, (e) aperture length, of the snail *L. Littorea* exposed at different temperatures
570 under: a) elevated pCO_2; and b) current pCO_2 levels. Histograms represent mean values ± SE
571 determined for the four employed treatments: current temperature (white), elevated
572 temperature (light grey), elevated pCO_2 (dark grey), elevated pCO_2*temperature (black).
573 Significantly different means ($p \leq 0.05$) are indicated by different letter in capital letter (A, B,
574 C, and D), according to the 95% Confidence Interval test for Estimate Marginal Means
575 (EMM).

576

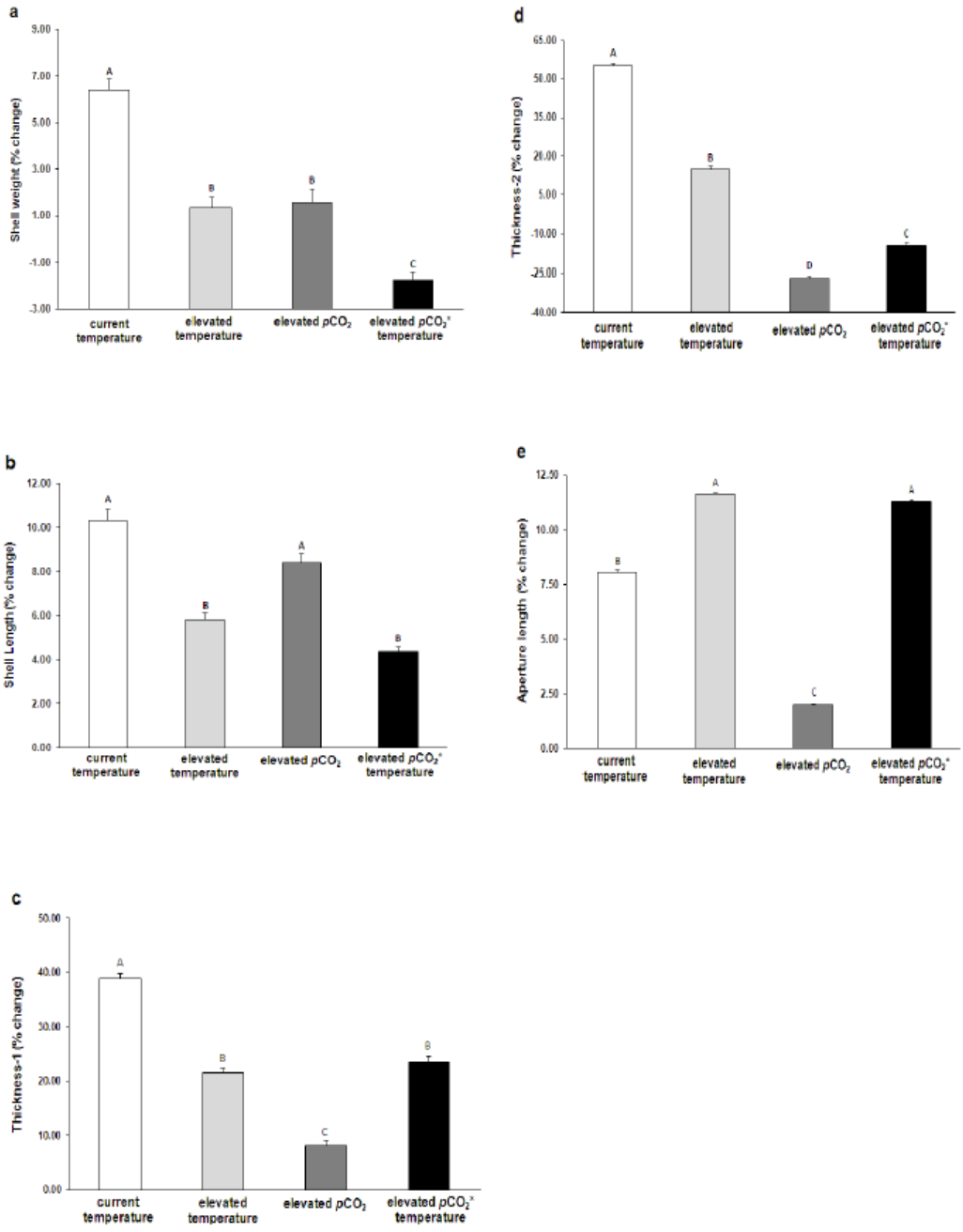
577 FIG. 2. Effect of 30 d exposure to different combinations of $pCO_2$and temperature on: (a)
578 percentage of water loss and (b) relationship between shell percentage change in aperture
579 ratio and water loss of the snail *L. Littorea* exposed at different temperatures under: a)
580 elevated pCO_2; and b) current pCO_2 levels. Histograms represent mean values ± SE
581 determined for the four employed treatments: current temperature (white), elevated
582 temperature (light grey), elevated pCO_2 (dark grey), elevated pCO_2*temperature (black).
583 Significantly different means ($p \leq 0.05$) are indicated by different letter in capital letter (A, B,
584 C, and D), according to the 95% Confidence Interval test for Estimate Marginal Means
585 (EMM). Full line in the scatter plot data trend: equation, r^2, degree of freedom (df), a
586 probability value (p) are provided. [$Y = 0.1766x + 30.312$, $df = 95$, $r^2 = 0.0301$, $p = 0.091$].

587

588 FIG. 3. Relationship between $\log_{10}$ ATP content and a) percentage change of shell weight [Y
589 = 11.854x + 0.1235, df = 63, $r^2 = 0.3552$, $p < 0.0001$], b) percentage change of thickness-2
590 [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 individual snails kept for 30 d under different temperature and pCO_2 conditions. Full line in the scatter plot data trend: equation, r^2, degree of freedom (df), a probability value (p) are provided.

595 **Figure 1**



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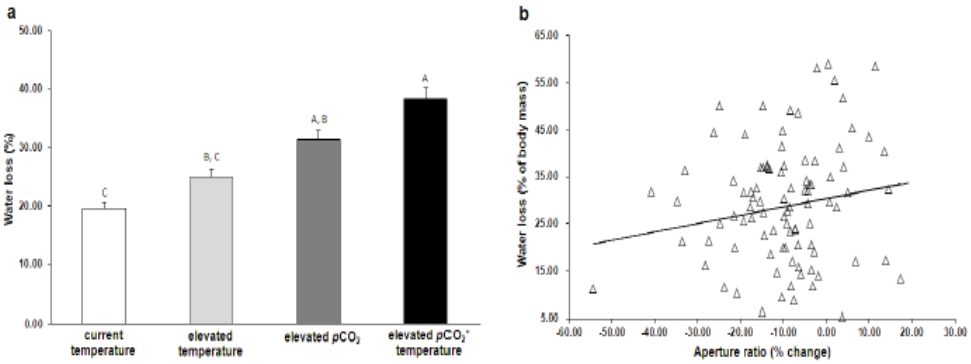
599 **Figure 2.**

600



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607 **APPENDIXES**

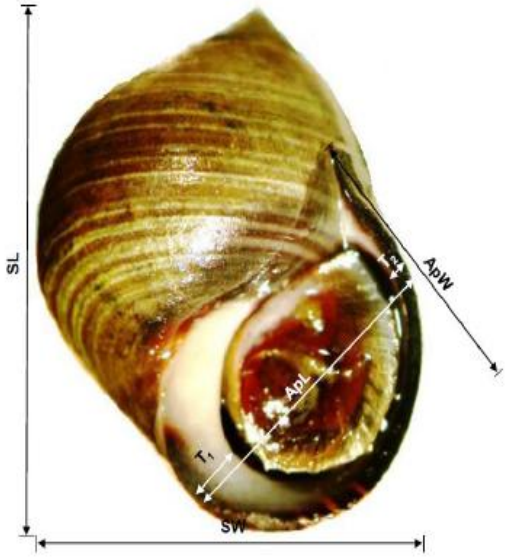
608 **Appendix A.** Mean and SE for seawater physico-chemical parameters measured or calculated
609 during the duration of the experiment: oxygen concentration (O_2), salinity, temperature (°C),
610 pH (NBS scale, Mettler-Toledo pH meter, Luton, UK), dissolved inorganic carbon (DIC,
611 Total CO_2 Analyser, CIBA Corning 965D, Essex, UK), total alkalinity (TA, Alkalinity
612 Titrator, AS-ALK2, Apollo SciTech, Bogart, USA) using method developed by Dickson et al
613 (2007), carbon dioxide partial pressure (pCO_2), bicarbonate and carbonate ion concentration
614 ($[HCO_3^-]$ and $[CO_3^{2-}]$), calcite and aragonite saturation state (Ω_{cal} and Ω_{ara}). * indicates
615 parameters that were calculated using the CO_2SYSs program (Pierrot *et al*. 2006), using the
616 dissociation constants of Mehrbach *et al*. (1973) as refitted by Dickson and Millero (1987).
617 Letter on the bracket $\{^{(a),\ (b),\ (a,\ b),\ \text{or}\ (a,\ c)}\}$ indicate significant different among treatment using
618 Post Hoc Bonferroni test of one way ANOVA ($p < 0.05$), according to 95% Confidence
619 Interval test for Estimate Marginal Means (EMM).

620

Parameter	Current temperature.	Elevated temperature	Elevated pCO_2	Elevated pCO_2 * temperature
[O2] (mg l^{-1})	6.75 / $_{0.01}$ $^{(a)}$	7.09 / $_{0.15}$ $^{(a)}$	6.68 / $_{0.17}$ $^{(a)}$	6.85 / $_{0.17}$ $^{(a)}$
Salinity (ppt)	34.77 / $_{0.03}$ $^{(a)}$	35.12 / $_{0.05}$ $^{(a)}$	35.06 / $_{0.04}$ $^{(a)}$	35.12 / $_{0.07}$ $^{(b)}$
Temperature (°C)	14.43 / $_{0.06}$ $^{(a)}$	20.91 / $_{0.09}$ $^{(b)}$	14.89 / $_{0.05}$ $^{(a)}$	20.68 / $_{0.08}$ $^{(b)}$
pH	8.03 / $_{0.01}$ $^{(a)}$	8.04 / $_{0.01}$ $^{(a)}$	7.67 / $_{0.01}$ $^{(b)}$	7.65 / $_{0.00}$ $^{(b)}$
DIC	1596 / $_{49.77}$ $^{(a)}$	1573. / $_{39.81}$ $^{(a)}$	1596 / $_{40.25}$ $^{(a)}$	1723 / $_{46.41}$ $^{(a)}$
TA (µequiv kg-1)*	1726 / $_{52.15}$ $^{(a)}$	1743 / $_{44.06}$ $^{(a)}$	1627 / $_{40.43}$ $^{(a)}$	1767 / $_{47.71}$ $^{(a)}$
pCO2 (µatm) *	428 / $_{17.22}$ $^{(a)}$	428 / $_{13.21}$ $^{(a)}$	998 / $_{29.91}$ $^{(b)}$	1185 / $_{33.28}$ $^{(c)}$
[HCO3-] (µmol kg^{-1}) *	1489 / $_{46.51}$ $^{(a, b)}$	1444 / $_{36.02}$ $^{(b)}$	1518 / $_{38.31}$ $^{(a, b)}$	1633 / $_{43.97}$ $^{(a, c)}$
[CO32-] (µmol kg^{-1}) *	89.79 / $_{3.53}$ $^{(a)}$	114.31 / $_{5.01}$ $^{(b)}$	40.58 / $_{1.30}$ $^{(c)}$	51.50 / $_{1.79}$ $^{(c)}$
Ωcal *	2.14 / $_{0.08}$ $^{(a)}$	2.73 / $_{0.12}$ $^{(b)}$	0.97 / $_{0.03}$ $^{(c)}$	1.23 / $_{0.04}$ $^{(c)}$
Ωara *	1.37 / $_{0.05}$ $^{(a)}$	1.78 / $_{0.08}$ $^{(b)}$	0.62 / $_{0.02}$ $^{(c)}$	0.80 / $_{0.03}$ $^{(c)}$

621

622 **Appendix B.** Biometrics of the common periwinkle *Littorina littorea* shells were measured
623 using images taken after and before 30 d exposure to different combinations of pCO_2 and
624 temperature. The measurements included shell length (SL), width (SW), shell aperture length
625 (ApL), aperture width (ApW), thickness-1 (T_1) and thickness-2 (T_2).
626



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Appendix C. Percentage change for shell biometric traits and resistance and water loss of the snail *L. littorea* exposed for 30 d to different combination of pCO_2 and temperature. Also log_{10} mean ATP content values used in correlation with other biometric traits and water loss are reported. Data is given as mean values ± standard error.

Treatment	Shell weight (% change)	Shell length (% change)	Shell width (% change)	Aspect ratio (% change)	Shell thick-1 (% change)	Shell thick-2 (% change)	Aperture length (% change)	Aperture width (% change)	Aperture ratio (% change)	log_{10} ATP ($\mu mol\ g^{-1}$)
Current temperature	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	0.33/ 0.02
Elevated temperature	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	-0.01/0.03
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	-0.01/0.03
Elevated pCO_2* temperature	-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	-0.11/0.02

640 **Appendix D**. Final mean values for shell biometric traits and resistance and water loss of the snail *L. littorea* exposed for 30 d to different

641 combination of pCO_2 and temperature. Data is given as mean values ± standard error.

642

Treatment	Growth (g)	Shell length (mm)	Shell width (mm)	Aspect ratio	Shell thick-1 (mm)	Shell thick-2 (mm)	Aperture length (mm)	Aperture width (mm)	Aperture ratio	Water Loss (% body mass)
Current temperature	1.91/ $_{0.04}$	15.84/ $_{0.11}$	13.57/ $_{0.10}$	1.17/ $_{0.007}$	1.88/ $_{0.03}$	0.69/ $_{0.11}$	8.99/ $_{0.08}$	9.35/ $_{0.10}$	0.97/ $_{0.01}$	19.54 / $_{2.39}$
Elevated temperature	1.64 / $_{0.03}$	14.91/ $_{0.11}$	12.77/ $_{0.09}$	1.17/ $_{0.005}$	1.83/ $_{0.02}$	0.59/ $_{0.01}$	8.84/ $_{0.06}$	8.77/ $_{0.09}$	1.01/ $_{0.01}$	25.01 / $_{2.50}$
Elevated pCO_2	1.81 / $_{0.03}$	15.44/ $_{0.10}$	13.32/ $_{0.09}$	1.16/ $_{0.004}$	1.68/ $_{0.03}$	0.50 / $_{0.01}$	8.79/ $_{0.08}$	7.50/ $_{0.09}$	1.18/ $_{0.02}$	31.38 / $_{1.87}$
Elevated pCO_2 * temperature	1.67 / $_{0.03}$	15.00/ $_{0.12}$	13.15/ $_{0.11}$	1.14/ $_{0.05}$	1.78/ $_{0.03}$	0.55 / $_{0,01}$	8.81/ $_{0.07}$	8.21/ $_{0.13}$	1.09/ $_{0.02}$	38.82 / $_{2.34}$

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