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Assessing the impact of increasing seawater temperature and acidity on marine organisms using ophiuroid brittlestars as an experimental model

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Assessing the impact of increasing seawater temperature and acidity on marine organisms using ophiuroid brittlestars as an experimental model

Abstract

Hannah Louise Wood

Ocean acidification is a consequence of rapidly increasing atmospheric levels of carbon dioxide and, in tandem with increasing sea temperature, poses a significant threat to marine life. A series of mesocosm experiments have been conducted at the Plymouth Marine Laboratory (Plymouth, UK) and the Kings Bay Marine Laboratory (Ny-Alesund, Svalbard) where ophiuroid brittlestars were used as a model to investigate the physiological response of marine organisms to ocean acidification and ocean warming. A 'whole organism' approach was adopted to elucidate the primary physiological responses, trade offs and conflicts that occurred. Three ophiuroid species of differing lifestyle and habitats were chosen to give an insight into how such factors influenced a species' response to ocean acidification and warming; the infaunal *Amphiura filiformis,* the epibenthic *Ophiura ophiura,* both temperate, and the Arctic epibenthic *Ophiocten sericeum.* There was a similar physiological response of metabolic upregulation across all three species. All species survived the lowest pH exposures (6.8 for *A. filiformis*, 7.3 for *O. ophiura* and *O. sericeum*) and showed signs of synergy between increasing water temperature and ocean acidification with the effects of lowered pH amplified as temperature increased. Beyond this, whilst specific responses differed between species, some similarity was observed between the epibenthic species *O. ophiura* and *O. sericeum* which both reduced arm regeneration and motility at lowered pH. In contrast, *A. filiformis* increased arm regeneration and suffered arm muscle wastage that appeared unsustainable. Differences in response and long term vulnerability to ocean acidification related to lifestyle (infaunal

versus epibenthic) were consolidated by the Energy Limitation Model whereby the response of a species to ocean acidification is based on the increased cost of maintaining the acid-base balance of extracellular fluids. The ability to do this, and where the energy is taken from to do so, rationalises the variety of physiological responses seen between species. The results of the investigations described in this thesis indicate that even species with low regulatory capacity may survive ocean acidification. However, lifestyle may have a determining role in where energy trade offs are made to maintain acid-base balance and this may ultimately determine species survival.

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I feel incredibly privileged to be able to do a job that I love, and still find it quite crazy that I am effectively paid to indulge my curiosity. When I started out on my PhD I thought how hard can it be, it's just like a job right? The past three years have been challenging, fun, at times hard, and much more than a job. Steve told me then that if I didn't cry at least three times and want to throw it all in at least once then it wasn't a PhD. Well I have cried at times but I have never wanted to give it up, and that's testament to the people I'd like to thank here.

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Publications & Presentations:

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- Oral presentation at the 42nd European Marine Biology Symposium, Kiel, Germany. Aug. 2007
'*Quantifying the physiological effects of ocean acidification on* **Amphiura filiformis**'

- Oral presentation at the Marine Institute conference, Plymouth, UK. Dec. 2007
'*Quantifying the physiological effects of ocean acidification on* **Amphiura filiformis**'

- Oral presentation at the Acid Ocean Public Seminar, Plymouth, UK. Apr. 2008
'*Investigating the impact of ocean acidification & increasing temperature on the physiology of benthic organisms*'

- Poster presentation at the 2nd Oceans in a high CO_2 world conference, Monaco. Oct. 2008
'*A whole organism approach to the physiological impacts of ocean acidification*'

- Poster presentation at the IMBER workshop, Plymouth, UK. Jan. 2009
'*A whole organism approach to the physiological impacts of ocean acidification*'

- Oral presentation at the Ocean Acidification conference, Plymouth, UK. Apr. 2009
'*Understanding physiological responses to ocean acidification using a whole organism approach*'

- Oral presentation at the EPOCA meeting, Plymouth UK. June 2009
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CHAPTER ONE

General Introduction

OCEAN ACIDIFICATION

The oceans are decreasing in pH as a result of a rapid increase in the concentration carbon dioxide (CO_2) in the atmosphere and its subsequent dissolution into the oceans; this phenomenon has been termed Ocean Acidification (Caldeira & Wickett 2003; Sabine et al 2004; Orr et al 2005; Pörtner et al 2008; Feely et al 2008). Since the start of the industrial revolution, atmospheric levels of CO_2 have been rising at a rate far greater than previously experienced in the Earth's history primarily a result of burning fossil fuels. The oceans are a natural carbon sink and have so far absorbed approximately half of all anthropogenically produced CO_2 (Siegenthaler & Sarmiento 1993; Sabine et al 2004). When CO_2 enters the ocean it reacts with seawater and alters the balance of the carbonate system (Zeebe & Wolf-Gladrow 2001), producing hydrogen ions (thus increasing the acidity reflected in a lowering of the value of pH) and reducing carbonate availability (Orr et al 2005). It had been assumed that the natural buffering capacity of the carbonate system was able to buffer the hydrogen ions released as CO_2 entered the ocean, however the current rate of CO_2 uptake may be overwhelming this buffering process (Caldeira & Wickett 2003). Seawater pH currently ranges between 7.8 and 8.2 and is already on average 0.1 pH unit lower than it was prior to the industrial revolution (Caldeira & Wickett 2003; IPCC 2005). Predictions based on realistic scenarios for future CO_2 emissions suggest that ocean pH will decrease by a further 0.3 - 0.4 by 2100 (Caldeira & Wickett 2003; Steinacher et al 2009).

Ocean acidification is recognised as a major threat to marine life (Raven et al 2005; Fabry et al 2008; Widdicombe & Spicer 2008) through both increasing the acidity of seawater (reduced pH) and by causing seawater to become corrosive to calcium carbonate structures (*via* reduced carbonate saturation level). Ocean acidification will affect shallow coastal waters first as CO_2 dissolves across the sea surface (Caldeira & Wickett 2003; Sabine et al

2004). Recent evidence indicates that some coastal areas are already exposed to corrosive conditions (carbonate saturated states < 1) due to seasonal periods of upwelling (Feely et al 2008), and the cold polar waters which have a greater capacity to absorb carbon dioxide are expected to become seasonally undersaturated with respect to aragonite (and thus become corrosive) within the next decade (IPCC scenarios A2 & B1) and complete undersaturation of the water column in the Arctic is predicted before the end of this century (Steinacher et al 2009).

Ocean Acidification is just one of the consequences of anthropogenic induced changes to our atmospheric composition. As a result of global warming sea surface temperature is now on average 0.7 °C warmer than at the start of the industrial revolution (IPCC), and a further increase of 3 – 6 °C is forecast by the end of this century (Marine Climate Change Impacts Partner, MCCIP). As ocean acidification and global warming are both caused by increased levels of atmospheric CO_2 it is apparent that marine organisms and ecosystems will be exposed simultaneously to these two stressors. The relative importance of these two factors may differ with habitat and region; in the tropics where the warm waters result in a low CO_2 absorption capacity temperature may impact upon marine organisms before ocean acidification reaches a significant level while in the colder polar waters where ocean acidification will cause the largest global ocean pH change models suggest that increasing temperature will amplify the effects of ocean acidification (Steinacher et al 2009). Clearly temperature is an important climate change variable that should be considered when investigating ocean acidification.

Major changes in the marine environment in the past have taken place on a timescale of thousands of years, and have had significant impacts on marine fauna; 'rapidly' increasing CO_2 is cited as the cause of the mass extinction of marine species in the Permian/Triassic (Twitchett 2007; Mander et al 2008). The current change in atmospheric CO_2 levels is

occurring 100 times faster than any changes previously seen in the earth's history (Raven et al 2005), the resultant ocean acidification is changing the physico-chemical properties of seawater which have remained relatively stable for the past 24 million years (Pearson & Palmer 2000).

BIOLOGICAL IMPLICATIONS OF OCEAN ACIDIFICATION

Ocean acidification can impact upon marine organisms through the effects of reduced pH, and also through increased CO_2 (hypercapnia). In this thesis the response to ocean acidification covers the responses that arise from both of these pathways. Different species and groups of marine animals vary in their ability to cope with and compensate for hypercapnia and lowered pH (e.g. Pörtner et al 2004, 2005; Fabry et al 2008; Widdicombe & Spicer 2008) with implications for marine trophic interactions. The ability to predict how marine invertebrates will respond to ocean acidification, and more importantly, the understanding to forecast the potential for species to survive in future, warmer more acidic oceans is a high priority for the scientific community.

Results from ocean acidification studies to date have not yet resulted in any unified understanding, with a long and varied list of responses including altering extracellular acid – base balance (Pörtner et al 2005; Miles et al 2007; Widdicombe and Spicer 2008), reducing growth (Michaelidis et al 2005) and reducing larval fitness (Dupont et al 2008). The concomitant decreasing availability of carbonate ions with decreasing pH suggests that species with calcium carbonate shells or skeletons, such as molluscs, crustaceans and echinoderms, may be among the first to be affected (Orr et al 2005). Calcification entails binding carbonate ions and calcium from seawater within an organic matrix to create a hard shell or skeleton. Surface waters are currently supersaturated with carbonate ions

(Royal Society, 2005); it is therefore readily available for marine organisms to make calcium carbonate structures. As ocean pH decreases, so will carbonate availability which may result in reduced rates of calcification (e.g. Gattuso et al 1999), and carbonate undersaturation at low pH will increase pressure upon the calcification process by causing dissolution of pre existing calcium carbonate shells and skeletons (e.g. Feely et al 2004). More recently the ability of a species to maintain internal acid-base balance has been suggested as a defining factor in a species susceptibility to ocean acidification (Pőrtner et al 2008; Widdicombe and Spicer 2008).

Synergisms of ocean acidification with increasing seawater temperature

The biological implications of temperature change on marine organisms are far reaching and have long been recognised (e.g. Allee 1923; Genner et al 2004; Huntsman & Sparkes 1925). While much is known of short term responses to elevated temperatures (Brett 1970; Kinne 1970; Somero 1987) studies of more long term responses lag far behind. This said, there is a growing literature on temperature induced changes in species distributions (Spicer & Gaston 1999; Somero et al 1996; Stillman 2002; Gaston et al in press), the mismatch in food availability with reproduction/growth windows, and increasing occurrences of competitive 'invasive' species. However studies investigating both temperature increase and ocean acidification are far fewer in number despite their potential to provide a far more realistic assessment of organism and ecosystem response to future environmental change than studies limited to a single factor. The few such multi-factorial studies published to date highlight that the combination of ocean acidification and warming can have a much larger impact than would have been expected from summing the impacts of the individual factors determined in isolation; for example the crab *Cancer pagurus* was shown to be more sensitive to increased temperature under low pH conditions (Metzger et

al 2007). There is an urgent need for more studies that investigate how both seawater pH and temperature interact in their effect on biological systems.

PHYSIOLOGY; UNDERSTANDING MECHANISMS

Research into ocean acidification has tended to focus on one or two particular aspects of an organism's physiology, such as growth (e.g. Michaelidis et al 2005), calcification (Gazeau et al 2007), acid-base balance (Spicer et al 2007; Miles et al 2007) and metabolism (Langenbuch & Pőrtner 2004; Michaelidis et al 2005). As a result, how ocean acidification impacts upon an organism as a whole is not yet determined; while these single endpoint studies give a useful insight into what changes may occur in future oceans, it remains difficult to determine whether these observations are a direct response to ocean acidification or a secondary indirect consequence to another physiological change.

Physiological mechanisms within an organism are interdependent and a change to one process often impacts upon several others (Sibly & Calow 1986). This concept of conflicts and trade offs between physiological processes (Principle of Allocation, Sibly & Calow 1986) qualifies the supposition that some of the physiological responses of ocean acidification recorded to date are 'knock-on effects' rather than primary physiological responses to ocean acidification. To understand these primary physiological implications requires consideration of the integrated physiology of an organism as a whole. To understand what physiological mechanisms are going to be affected and how, it is necessary to investigate several parameters within an organism to identify all processes that are being affected, and separate out the primary and secondary responses.

There may be no common factors to the response to ocean acidification, there is not yet enough data to determine this; but the study of the whole organism physiology will be useful in identifying common primary physiological responses, and taking this 'whole organism approach' further to the study of several related species will allow those effects specific to habitat or lifestyle to be separated from more generic physiological responses.

ECHINODERMS AS A MODEL

Echinoderms are a large and diverse phylum with around 7000 species incorporating Sea Cucumbers (Holothuroidea), Seastars (Asteroidea), Sea Urchins (Echinoidea) as well as Brittlestars (Ophiuroidea). Their prevalence extends to all aspects of the marine realm; echinoderms are found from the intertidal to deep-sea, in the topics and polar waters, and infaunal to epibenthic. Their prevalence and presence in most marine environments makes them an ideal model for the study of ocean acidification for this wide spread distribution allows related species from different habitats and environments to be used to consider how these factors may influence a response to ocean acidification. The basic biology of several common echinoderms is relatively well understood, and the genome of the urchin *Strongylocentrotus purpuratus* is nearly fully sequenced, further facilitating molecular level studies of echinoderm. There are already some studies of echinoderms and ocean acidification (e.g. Miles et al 2007; Kurihara & Shirayama 2004; Shirayama & Thornton 2005; Dashfield et al 2008); while these are mainly limited to urchins, the knowledge and insight these studies provide, combined with the factors described above, make echinoderms a good model for the investigation of the physiological responses to ocean acidification.

Echinoderms are calcifying organisms; their skeletons are composed of magnesium calcite which is particularly susceptible to dissolution as ocean pH decreases (Shirayama & Thornton 2005). Another key characteristics of many echinoderm species is their unique ability to regenerate lost appendages (Bowmer & Keegan 1983; Bannister et al 2005); starfish and brittlestars can grow new arms following loss or damage, a scenario that frequently occurs through predation and damage by trawling (Ellis & Rogers 2000). Regeneration offers the unique opportunity to study mechanisms of tissue and nerve growth, manipulate an individual's energy budget and to look at the formation of calcified material under specific environmental conditions. Brittlestars are particularly suited to regeneration studies for their thin arms grow relatively quickly; the arms of *Amphiura filiformis* can grow up to 1cm a week, whereas in comparison the regenerating arm of an asteroid seastar takes several months to regrow a comparable amount due to the thicker arm and greater tissue complexity they possess.

Brittlestars (Ophiuroidea), one of the five classes of echinoderms are abundant in many marine habitats of the UK, are often 'keystone' species or ecosystem engineers (Jones et al 2004) and can be maintained within the laboratory. Some species, such as the infaunal *Amphiura filiformis* have been well studied, and there is ample data with respect to both the ecology (e.g Bowmer & Keegan 1983) and also the physiology of processes such as regeneration (e.g. Dupont & Thorndyke 2006). Some temperate brittlestar species are found in abundance on the south coast of the British Isles and can be collected relatively easily in large numbers by boat.

THESIS AIMS

The aim of this thesis is to understand the physiological impacts of ocean acidification taking a more holistic, 'whole organism' approach than has been attempted hitherto.

This aim will be addressed by pursuing the following objectives:

1. To investigate the physiological impacts of ocean acidification using a 'whole organism' approach. (Chapter 2)

2. To consider the importance of both intrinsic (species biology) and extrinsic (temperature) factors that may influence the response to ocean acidification. (Chapter 3)

3. To consider how impacts at the physiological level may impact upon a species functional role at an ecosystem level. (Chapter 4)

4. To explore the extent lifestyle determines the response to ocean acidification by investigating species from different lifestyles. (Chapter 5)

5. To investigate synergistic effect of increasing seawater temperature on the physiological impacts of ocean acidification, using a whole organism approach. (Chapters 5 & 6)

6. To explore the extent habitat determines the response to ocean acidification by investigating species from different habitats. (Chapter 6)

7. To consider whether there are common themes in the physiological response of species to ocean acidification. (Chapters 7 & 8)

These objectives were achieved through a series of mesocosm experiments carried out on three ophiuroid brittlestar species; the infaunal *Amphiura filiformis* and the epibenthic *Ophiura ophiura*, both temperate, and the Arctic epibenthic *Ophiocten sericeum*.

CHAPTER TWO

Ocean Acidification may increase calcification rates- but at a cost

Aspects of this Chapter are included in:

Wood H L, Spicer J I & Widdicombe S (2008) Ocean acidification may increase calcification- but at a cost. *Proc. R. Soc. Lond. B - Biol. Sci.* **275 (1644):** 1767-1773

ABSTRACT

Ocean acidification is the lowering of pH in the oceans as a result of increasing uptake of atmospheric carbon dioxide. Carbon dioxide is entering the oceans at a rate greater than ever before, reducing the oceans' natural buffering capacity and lowering pH. Previous work on the biological consequences of ocean acidification has suggested that calcification and metabolic processes are compromised in acidified sea water. In contrast, this chapter shows, using the ophiuroid brittlestar *Amphiura filiformis* as a model calcifying organism, that some organisms can increase the rates of many of their biological processes (in this case, metabolism and the ability to calcify to compensate for increased seawater acidity). However, this up-regulation of metabolism and calcification, potentially ameliorating some of the effects of increased acidity comes at a substantial cost (muscle wastage) and is therefore unlikely to be sustainable in the long term.

INTRODUCTION

The ophiuroid brittlestar *Amphiura filiformis* is an important bioturbator, and ecosystem engineer which lives in the sediment where it suspension feeds by extending two arms into the overlying water (Loo et al 1996). The feeding process results in the creation of a burrow environment, whereby arm undulation causes aerated water and food to move down one arm channel into a chamber where the central disk of the brittlestar is situated. Water is then forced up and out of the second arm channel. *Amphiura filiformis* occurs at high densities (often > 100 ind. m^2 but up to 3000 ind. m^2 have been recorded, Rosenberg 1995) and, where present, has a dominating effect on the surrounding sediment *via* bioturbation (O'Reilly et al 2006). *A. filiformis* is a key species in many seafloor communities and changes to this species' survival as a result of ocean acidification may have implications for these communities.

Work on the effect of acidification on echinoderms is currently restricted to investigations of survival, growth, and extracellular acid-base balance in a limited number of groups, mainly echinoids (e.g. Shirayama & Thornton 2005, Miles et al 2007). One of the key characteristics of many echinoderm species is their ability to regenerate, which involves alterations in calcification rates (Bowmer & Keegan 1983, Bannister et al 2005). However, we know nothing of the effect of CO_2 induced acidification on such regeneration or how different aspects of an organism's physiology change and interact as a response to the pressures of ocean acidification. Consequently, here the effect of CO_2-induced acidification on the ability of a calcifying organism (the ophiuroid brittlestar *Amphiura filiformis*) to regenerate calcium carbonate structures (arms) is investigated. In addition, the potential energetic costs associated with regeneration in terms of metabolism and reproduction have been examined. Using sediment filled cores supplied with filtered sea

water, brittlestars were exposed for 40 days to varying degrees of acidification; nominally a control of pH 8.0, the worst case scenario for the end of the century 7.7, a 2300 scenario of 7.3 and finally 6.8.

METHODS

Sediment Collection

On 16th November 2006, twenty four (16 for experimental use and 8 spare) undisturbed cores of sediment were collected from an area of moderately sorted sandy mud 100 m north of Plymouth Breakwater (50°20.090N, 4°08.520W); water depth was approximately 10 m. The cores were collected by sub-sampling from a 0.1 m^2 box corer. Plastic cores (10 cm diameter, 20 cm long) were pushed into the sediment to a depth of 15 cm. Each core was then gently removed from the box-core, sealed on the bottom with a plastic cap and returned to the Plymouth Marine Laboratory mesocosm within a few hours of collection. Once in the mesocosm the cores were placed randomly in a recirculating seawater system (S = 36 PSU, T = 12 °C) for 12 d. During this time any organisms that emerged from the cores were removed. Similarly cores that showed signs of marked burrow development were discarded to minimise the presence of macrofauna in the sediment. On the 16th November 2006 sixteen sediment cores were transferred from the recirculating seawater system to the experimental holding system where each core was supplied with filtered sea water (10 µm and 1 µm Hydrex filters), *via* silicone tubing, at a rate of 8 ml min^{-1}. The water was continually supplied and excess water flowed over the top of the core and was drained away.

Animal Collection

On 28th November 2006, specimens of *Amphiura filiformis* were collected from an area of muddy sand (water depth approximately 12 m) inside Plymouth Sound (50°20.598N, 4° 08.155W) using a 0.1 m^2 van Veen grab. Individuals with a disk diameter > 5 mm were

gently hand sorted from the sediment to prevent damage to the brittlestars' delicate arms. Only individuals with 5 intact arms were collected. The brittlestars were held in covered holding buckets (diam. = 30 cm, no more than 20 individuals per bucket), filled with sea water (S = 36 PSU, T = 12 °C) and were transported back to the mesocosm within 4 hours of collection. On arrival at the mesocosm 20 brittlestars were randomly allocated to each of the four acidified seawater treatments (pH = 8.0, 7.7, 7.3, 6.8). Each pH treatment contained four cores each core with five individuals of *A. filiformis*. Half of the individuals in each treatment had one arm removed; the remaining individuals had two arms removed. Arm excision was carried out by pinching the arm with fine forceps approx. 5 mm from the disk. Each individual was then weighed (A and R HF3000G balance accurate to 0.01 g) and placed in a sediment core. The experimental containers were covered with black cloth to minimize light penetration. All individuals had successfully burrowed after 24 hours.

Seawater acidification

The pH of the sea water supplied to the cores was decreased gradually over the next 7 d until the desired experimental pH was achieved. Acidified sea water was supplied from a header tank (vol. = 450 l) in which sea water supplied to the cores was replaced by an equal volume coming in from a 15,000 l recirculating supply tank topped up twice weekly with water collected approximately 10 nautical miles offshore (50°15'N, 04°13'W, water depth ~55 m). Seawater acidification followed closely the methods described by Widdicombe & Needham (2007) and was achieved by controlled bubbling of CO_2 into the header tank. After the 7 day acidification period the exposure experiment ran for a further 40 days.

Sampling

On 16th January 2007 the sediment of each core was gently extruded from the bottom upwards. Any brittlestars visible from the edge of the core were carefully removed by hand. The sediment was then gently washed over a 1 mm mesh sieve to retrieve all the brittlestars. Oxygen uptake rates of all individual brittlestars were obtained as a measure of metabolism (method described below). Following this the brittlestars were preserved for subsequent analyses. Half were placed in labelled plastic bags and immediately frozen at -80°C whilst the other half were fixed in Bakers formal calcium (+ 2.5 % NaCl).

Physico-chemical status of acidified waters

The water in each header tank, plus the water flowing out of the silicon supply tubes, was analysed three times a week for total carbon dioxide content (tCO_2), pH_{NIST}, (probe calibrated with National Institute of Standards and Technology, NIST, buffers) salinity and temperature. tCO_2 was measured on 100 µl subsamples using an automated carbon dioxide analyser (make: CIBA Corning 965 UK). pH_{NIST} was estimated using a pH electrode (make: Mettler Toledo LE413) calibrated with NIST standardised buffers. Salinity was measured with a conductivity salinometer (make: WTW LF197). Temperature was measured using a probe combined with the pH meter as detailed above (accurate to 0.1 °C). The data from the supply tubes are presented because the 6.8 treatment pH rose between the header tank and tube output; the header was adjusted so that the tube output gave the required pH. No additional food was supplied during the experiment; *A. filiformis* can switch to deposit feeding when there is insufficient food or flow of the water column (O'Reilly et al 2006).

Measurement of oxygen uptake

Rates of oxygen uptake by individual brittlestars were measured using an established closed-respirometry technique (Pomory & Lawrence 1999). Individuals were gently rinsed with filtered sea water to remove any adhering sediment before being placed into a respirometer chamber (vol. = 1 l) immersed in sea water of appropriate pH (T = 13 °C, S = 36, filtered through Hydrex 10 and 1 μm filters) and the respirometer was sealed for 4 h. After this time the respirometer was inverted to mix its contents, the lid was removed, and a water sample (vol. = 60 ml) quickly taken for analysis. Measurements of dissolved oxygen were made with an automated titration system with a photometric endpoint (Williams & Jenkinson 1982). Chemical reagents were based on Carritt & Carpenter (1966). Oxygen saturation was calculated from published equations for oxygen solubility in sea water (Benson & Krause 1984). The oxygen uptake was calculated as the difference in dissolved oxygen between the experimental bottle and the mean of controls for the same seawater pH, all data being corrected for barometric and water vapour pressures, the former measured on the day of the experiment. Respirometers without brittlestars were also run exactly as described above in order to estimate background respiration rates by bacteria.

Measurement of arm regrowth

Regrowth was discernable from the original arm by having a lighter coloured appearance. The length from the point of colour change to the arm tip was measured by straightening the arm along a straight edge of a ruler then using Vernier callipers (accurate to 0.1 mm) to measure the exact length.

Measurement of the calcium content of arms

From each frozen individual, a 5 - 10 mm section of established arm (fully formed, prior to exposure) and a similar length of regrown arm was removed, rinsed in distilled water before being dried for 4 h in an oven (T°C = 85). Each section was weighed using a microbalance (Ohaus Adventurer AR0640 accurate to 0.0001 g), dissolved in nitric acid (vol. = 5 ml, 15 % pro analysis). The sections in acid were heated on a hot plate to aide acid digestion. The dissolved samples were diluted with distilled water to achieve a concentration within the range (determined by prior investigation) of the atomic absorption spectrophotometer (Varian SpectrAA 50 accurate to 1- 2 % RSD) used to determine calcium concentration (as in Spicer & Eriksson 2003). Results are expressed as percentage calcium mass of arm mass. Arms which were not attached to brittlestars were also exposed for 7 days to acidified seawater at the same pH levels as were used in the main experiment. The calcium content of these "dead" arms was also determined. By comparing the values from "living" and "dead" arms it was possible to isolate the effect of chemical dissolution on arm calcium content from biological processes such as calcification or buffering by active calcium carbonate dissolution.

Measurement of egg size

Individuals were removed from Baker's solution, their arms excised close to the disk and the arms were replaced in the Baker's solution. Each disk was then placed into small glass vial (vol. = 20 ml) and dehydrated using a sequence of increasing ethanol concentrations (30 min in each of 50 %, 70 %, 95 % (repeated) and finally 95 % ethanol/ Monomer (1:1)). Disks were left in 2-hydroxyethyl methacrylate monomer overnight and then embedded in monomer with activator (Lewis & Bowen 1985) before being left to set for 24 h. Once removed from the monomer and air dried for 12 h, a glass knife was used to cut transverse

sections through the disk which were then stained with Lee's methylene blue/basic fuchsin. The plane of section that intersected the greatest number of eggs through the centre (thereby allowing accurate measurement of diameter) was chosen for each animal and the feret diameter of these eggs captured under high power magnification (x 40, Reichert Polyvar microscope) in a digital image provided by a microscope mounted camera (Optronics Magnafire model S99802). The image was analysed using image analysis software (Image-Pro Plus v4.5 Media Cybernetics) and the mean egg feret diameter, μm (greatest diameter measurement of the egg, based on assumption that eggs are not perfectly spherical) was calculated from diameter of all the eggs measured in each individual. The total number of eggs was not measured as one field of section may not show all eggs.

Assessment of arm structure

The previously excised arms were removed from Bakers solution, dehydrated, embedded, sectioned and stained using the methods described above. This time, however, longitudinal rather than transverse sections were cut. Each section was examined under low power magnification (x 10) to view shape and muscle coverage, and high power magnification (x 40) to view calcium carbonate structure of spines, outer edge and internal area of skeletal structure (Reichert Polyvar microscope).

Statistical Analysis

All statistical analyses were carried out using Minitab 14. Two way analysis of variance (ANOVA) was used to test for effects of pH treatment and number of arms regenerating on oxygen uptake, calcium content, arm regeneration rate, egg size and arm structure. A Kolmogorov- Smirnov test was used to test for normality. Non parametric data were either log or square root transformed to normalise.

RESULTS

Effect of ocean acidification on calcification in *Amphiura filiformis*

One of the most surprising results is that there was no decrease in the total amount of calcium carbonate in individuals exposed to acidified water. Indeed, individuals from lowered pH treatments had a greater percentage of calcium in their regenerated arms than individuals from control treatments, indicating a greater amount of calcium carbonate (2-way ANOVA using $\log_{10}$ transformed data, Table 2.1 c). Established arms had a significantly lower percentage of $CaCO_3$ content than regenerated arms (Figure 2.1 c); however number of arms removed had no effect. The interaction between pH and arm type (established or regenerated) was significant due to the different amount of calcium found in each of the arm types; the regenerated arms had significantly greater calcium levels than established ones (Figure 2.1 c). This was due to the more developed skeletal structure seen in the established arms. Throughout the exposure, and therefore period of regeneration, the brittlestars were maintained in sediment cores (also collected from Plymouth Sound) in order to simulate natural conditions for the species. The inorganic carbon levels (%) for this sediment are 2.207 (± 0.176) (S. Widdicombe unpubl. data). However, it is unlikely that the sediment is being used as a carbon source for the calcification process; a previous study found no change in the carbon (TIC) content of the same type of sediment (fine muddy) containing species including *Amphiura filiformis* after a 20 week exposure to pH of 7.3, 6.5 and 5.6 (Widdicombe et al 2009).

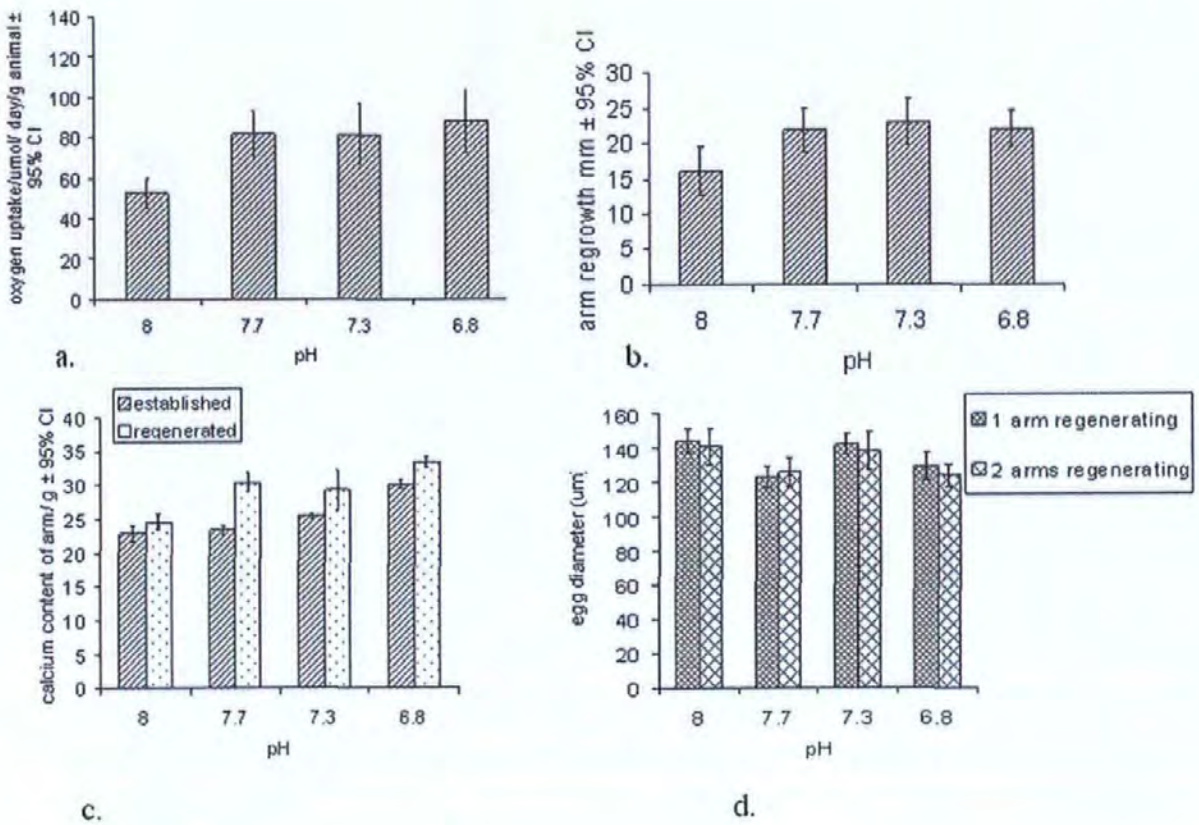


Figure 2.1. Impact of seawater pH on **a.)** oxygen uptake (μmol O_2/g/ day^{-1}), **b.)** length of arm regeneration (mm) **c.)** calcium content of established and regenerated arms (%), **d.)** egg feret diameter (μm) following a 40 day exposure. All values are means ± 95 % confidence intervals).

Table 2.1. 2-way ANOVA comparing the impact of seawater pH (8.0, 7.7, 7.3, 6.8) and number of arms regenerating, 'regen' (1 or 2) on **a)** oxygen uptake ($\mu mol/g/d^{-1}$). n = 80, data square root transformed. **b.)** length of arm regeneration (mm). n = 40 and normally distributed. **c).** mean egg feret (μm). n = 40, normally distributed **d.)** Calcium content of arm (%). Comparison of regenerating arms (1 or 2 regenerating) but also against established arms (collectively denoted as regen) n = 60, data log_{10} transformed. Significance level = 0.05.

a.

Source	d.f.	SS	MS	F	P
pH	3	54.299	18.1	7.03	**< 0.001**
regen	1	3.954	3.9545	1.54	n/s
pH*regen	3	15.505	5.1683	2.01	n/s
Error	72	185.45	2.5757		
Total	73	259.21			

b.

Source	d.f.	SS	MS	F	P
pH	3	200.9	67	2.99	**0.05**
regen	1	10	10	0.45	n/s
pH*regen	3	139.4	46.5	2.07	n/s
Error	32	717.2	22.4		
Total	39	1067.5			

c.

Source	d.f.	SS	MS	F	P
pH	3	0.7695	0.2565	16.58	**< 0.001**
regen.	2	0.2719	0.1359	8.79	**0.001**
pH*regen	6	0.274	0.0457	2.95	**0.016**
Error	48	0.7427	0.0155		
Total	59	2.0582			

d.

Source	d.f.	SS	MS	F	P
pH	3	1489.8	496.593	1.4	n/s
regen	1	23	23.014	0.06	n/s
pH*regen	3	432.4	144.121	0.41	n/s
Error	32	11361.3	355.039		
Total	39	13306.4			

Sediment pH profiles from another acidification study using sediment cores (Dashfield et al 2008) show that the pH of the sediment is lower than that of the overlying water even

under normocapnic conditions; the pH is 7.64 at a depth of 5 cm, the depth at which *Amphiura filiformis* is typically found. However, after a 4 week exposure to mild hypercapnic conditions (overlying water pH 7.7) the sediment pH at 5 cm deep was still 7.64, whilst more severe hypercapnia (pH 7.3 and 6.5) only reduced sediment pH at 5 cm depth by 0.16 and 0.22 pH units respectively. In a study by Widdicombe et al (unpubl. data) cores of both muddy and sandy sediment were exposed to acidified seawater (pH 7.8, 7.4 and 6.8) for 60 days. After this time oxygen profiles were measured through the sediment. It was demonstrated that seawater acidification had no significant impact on the sediment oxygen profiles in either the sand or the mud, indicating no increase in sediment anoxia. pH imaging of *Nereis succinea* burrows showed that the porewater pH was dependant on the burrow profile, animal size and rate of irrigation, with high porewater pH associated with periods of irrigation (Zhu at al 2006). *A. filiformis* continually ventilate their burrows by arm undulation; therefore the pH of their burrow porewater is expected to be related to surface water pH rather than the surrounding sediment. As such the burrowing lifestyle of this study species is not counteracting or altering the experimental pH conditions created for the purposes of this study and the results shown are as a result of altering seawater pH.

To disentangle the direct chemical effect of pH on the calcium carbonate within *Amphiura filiformis* arms from the active biological processes used by the species to maintain calcium carbonate structures, a separate 7 day exposure at all four pH treatments was carried out on 'dead' arms. The arms were removed from the animal, frozen for a period in excess of 7 days to - 80 °C to kill, and then brought back to seawater temperature. In this experiment the dead arms were placed in small pots with no sediment and supplied continuously with seawater of appropriate pH. Under these conditions calcium levels decreased with pH (Figure 2.2). As these arms were detached from the individual and therefore could not replenish the calcium carbonate skeleton, the decrease in calcium

indicates that this structure is susceptible to dissolution at lowered pH. Therefore in live (attached) arms an increased rate of calcification is required merely to maintain calcium carbonate structures in their original condition. In regenerated arms, calcium levels were greater in those organisms exposed to acidified sea water than in those held in untreated seawater (Figure 2.1 c). This was true for all three levels of acidified sea water. The data from the detached (dead) arms (Figure 2.2) showed that lowered pH caused dissolution of arm calcium carbonate. Therefore, where these three lowered pH treatments appear to have had a similar response, there was actually an increasing rate of calcification with lowered pH. Calcium carbonate in established arms was also affected by lowered pH. At pH 6.8 calcium levels increased, and at pH 7.7 and pH 7.3 calcium levels were equal to the control, indicating *A. filiformis* actively replaced calcium carbonate lost by dissolution.

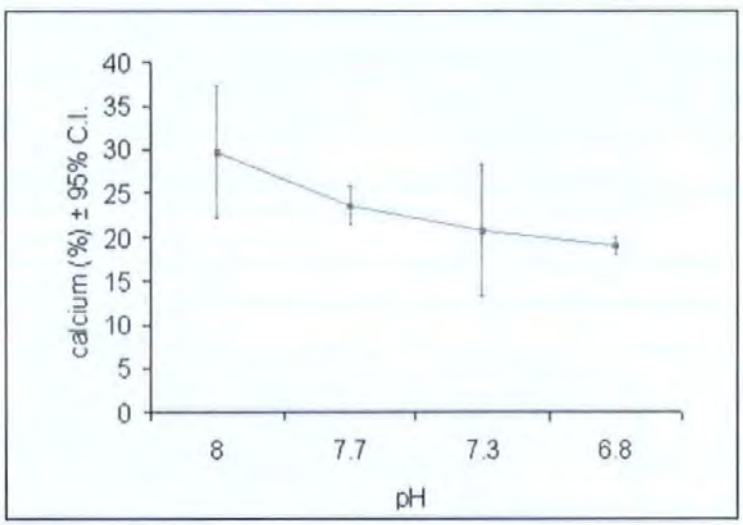


Figure 2.2. Calcium content (%) of arms which had been exposed to lowered pH after being removed from animal.

Effect of ocean acidification on *Amphiura filiformis* metabolism

Rates of oxygen (O_2) uptake (as a measure of metabolic rate), or mO_2, were significantly greater at reduced pH (7.7, 7.3 and 6.8) than in controls (pH 8) (Table 2.1 a). However, mO_2 was not significantly different between the three lowered pH treatments (Table 2.1 a). Increased rates of physiological processes that require energy are paralleled by an increase in metabolism; this relationship is seen with growth and metabolism here in the results.

Effect of ocean acidification on *Amphiura filiformis* growth and regrowth

Seawater acidification stimulated arm regeneration. After the 40 days exposure, the length of the regenerated arm was greater in acidified treatments than in the controls (2-way ANOVA, Table 2.1 b, Figure 2.1 b). This increased rate of growth coincided with increased metabolism. Regeneration was not affected by the number of arms removed, nor was there a significant difference in any of the physiological parameters measured as a result of having two arms regenerating instead of one. The ability to regenerate lost arms faster meant a reduction in the length of time animal function (e.g. burrow ventilation and feeding) was compromised by reduced arm length.

The biological cost of ocean acidification

The internal structure of *Amphiura filiformis* arms was affected by pH (Figure 2.3); muscle wastage occurred at lowered pH. Longitudinal sections of the arm showed distinct loss of muscle mass as pH decreased. In each arm segment there are four sections separated by the calcium carbonate skeleton. In the control individuals these sections were filled with muscle. As pH decreased large empty spaces were clearly visible (Figure 2.3). Candia Carnevali et al (2001) found muscle de-differentiation occurred in regenerating arms as a

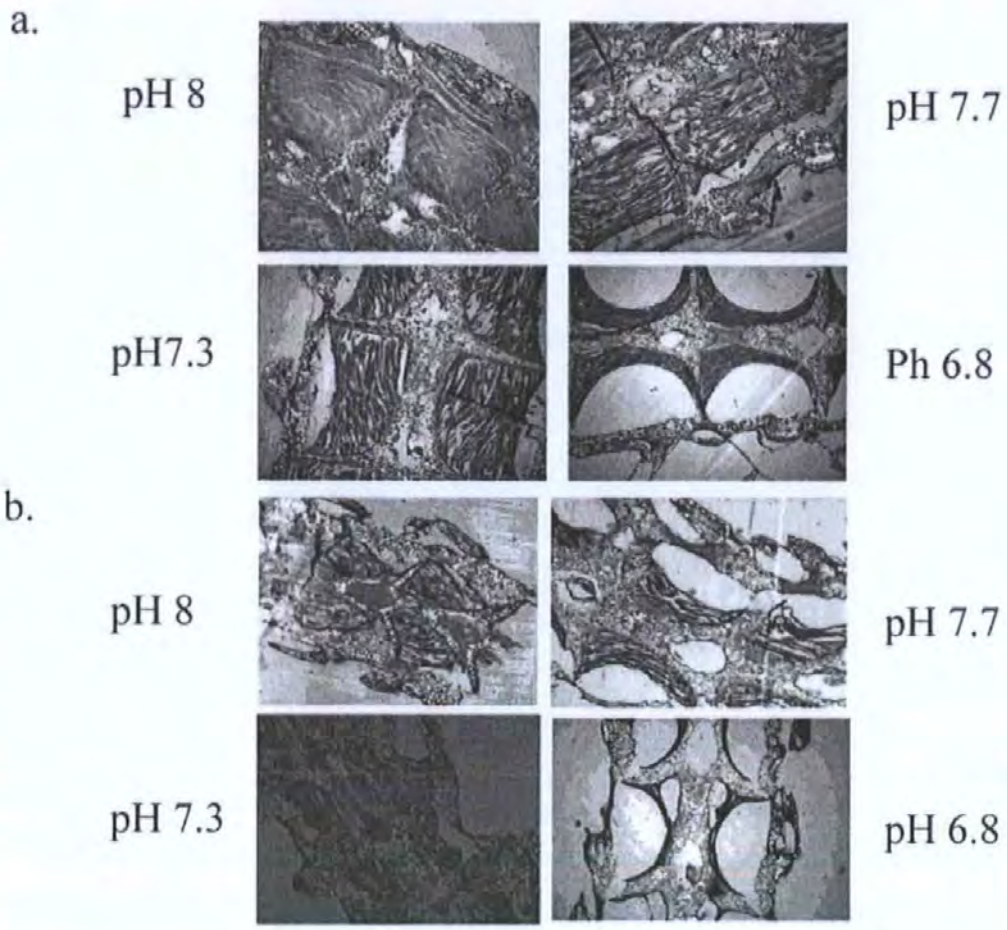


Figure 2.3. Longitudinal cross sections of **a.)** established arms **b.)** regenerated arms (x 10 mag.) mounted in methacrylate resin and stained with Lee's basic blue fuchsin.

result of PCBs (the pollutants Polychlorinated biphenyls), which appeared visually similar to muscle loss. However the de-differentiated muscle showed a change in structure not seen in our study; the arm muscle from lowered pH samples has the same visual structure as the controls, with just less present. In addition our results showed muscle loss in established arms as well as regenerating, whereas Candia Carnevali et al (2001) found de-differentiation in the regeneration process. The absence of muscle as a result of lowered

pH is not de-differentiation or an inability to synthesise muscle tissue under hypercapnia, but rather muscle loss.

In conclusion arms can be regenerated under hypercapnic conditions but they are unlikely to function as well as arms regenerated under normal conditions. *A. filiformis* uses its arms to collect food particles and irrigate its burrow. Muscle loss can be expected therefore to result in a loss of arm movement which in turn will affect both feeding and respiration and ultimately survival. This species is also predated on by the commercial flatfish dab, *Limanda limanda* (Bowmer & Keegan 1983), which crop the arms extended into the water column. If the muscle mass in these arms is significantly reduced, so too is the nutritional value, indicating the effects of ocean acidification could be transferred between trophic levels.

Effect of ocean acidification on *Amphiura filiformis* reproduction and mortality

Egg size (feret diameter) and structure were not affected by seawater acidification (2 way ANOVA, Table. 2.1 d, Figure 2.1 d). However, the timing of this study (Dec – Jan) falls in a latent period of egg growth; development of eggs laid down the previous autumn typically begins in March (Bowmer 1982). Therefore, while no degeneration of eggs was found in this study, egg development may still be affected by hypercapnia. A further experiment encompassing the egg growth phase is required to assess the impact of ocean acidification on egg development. A study by Lowe et al (unpubl. data) has found that the process of vitellogenesis in the surface dwelling ophiuroid *Ophiura ophiura* was disrupted by lowered pH, highlighting the potential for disruption in the growth phase. Spermatogonia were not investigated in the current study as all individuals sampled were female. While the sex ratio of *A. filiformis* is thought to be 1:1, patchiness in the

distribution of sexes has been documented (Bowmer 1982), which may explain the absence of males from the samples fixed for gonadal assessment.

While some ophiuroids reallocate energy from gonadal to somatic growth, and a decrease of egg size is seen when arm regeneration is undertaken, this was not seen in *Amphiura filiformis* (Figure 2.1 d). Ocean acidification has the potential to also affect reproductive success indirectly; as a broadcast spawner, *A. filiformis* must come to the sediment surface to spawn. This behaviour requires the arms to move the individual through the sediment and should arm muscle wastage reduce motility then individuals may release gametes within their burrows and far fewer gametes would enter the water column; significantly reducing reproductive success.

The duration of this experiment (40 days) was chosen to investigate long term physiological responses to hypercapnia. Shirayama and Thornton (2005) have elegantly demonstrated with echinoids that mortality as a result of a 0.05 pH decrease (560 ppm) may only occur after several months. Interestingly, even at high levels of hypercapnia (the 6.8 treatment crosses the threshold into acidic water, i.e. < pH 7.0) investigated here, no mortality was observed. In light of the results regarding the trade-off between calcification and muscle mass it is likely that mortality at low pH will occur as an indirect result of lowered pH; and this may take longer than the experimental duration. Any loss, or impairment, of an important ecosystem engineer (Jones et al 1994) will profoundly affect the biotic and abiotic environment where they occur; therefore the potential for loss of this species would alter ecosystems on a large geographical scale.

DISCUSSION

All previous ocean acidification studies on benthic marine invertebrates have reported reduced calcification rates (Gazeau et al 2007) and hypometabolism (Michaelidis et al 2007) as common outcomes. Here we have shown the opposite; that in some species at least, ocean acidification can increase both the rate of calcification and metabolism. These results change the face of predictions for future marine assemblages with respect to ocean acidification. Whereas it was previously assumed that all calcifiers would be unable to construct shells or skeletons, and inevitably succumb to dissolution as carbonate became undersaturated, we now know that this is not the case for every species. However, by investigating the functional consequences of hypercapnia and lowered pH at an organism level rather than focusing on a single process we have also detected a cost to these increased activities. Arm muscle mass decreased with pH, i.e. as calcification and metabolism increase. There was a trade-off between maintaining skeletal integrity and arm function. pH decreased arm muscle mass by causing the brittlestar to utilise the muscle as an energy source. As muscle loss was seen in established as well as regenerated arms it is clearly not just a failure to synthesise muscle tissue under hypercapnic conditions. For this particular ophiuroid species the loss of muscle mass experienced at low pH has implications for survival and ecosystem function; arm movement is necessary for feeding (Loo et al 1996), burrow aeration (Woodley 1975) and predator avoidance (O'Reilly et al 2006). In areas where this animal is present, burrow creation and irrigation by *Amphiura filiformis* is responsible for up to 80 % of all bioturbation (Vopel et al 2003), therefore the effects of ocean acidification will also alter the surrounding environment. Results of a previous study indicate this trade-off of increased calcification against reduced muscle mass is occurring in other species; Shirayama & Thornton (2005) found that the decrease in test thickness did not account for total mass loss of the echinoderms *Hemicentrotus*

pulcherrimus and *Echinometra mathaei* exposed to hypercapnic conditions. These species may also be decreasing muscle mass as a cost of increasing calcification and metabolism. Here we show that *A. filiformis,* and almost certainly other species, will attempt to cope with changes in seawater acid-base balance. Unfortunately it appears that the physiological responses to combat the effects of ocean acidification may themselves reduce survival and fitness as much as acidification itself.

The Intergovernmental Panel for Climate Change (IPCC) predicts that under their worst case scenario of carbon dioxide emissions, seawater pH will reach our experimental level of 7.7 by 2100. Here we show that some species at least can modulate their biological processes in response to ocean acidification and while calcified structures are affected by ocean acidification, so too is the rest of the animal. Such trade-offs are likely to be present in other species but to identify these future studies need work at the organism rather than the process level. To place the importance of calcification above other factors without empirical evidence leads to false assumptions and therein the capacity of some species to respond effectively may be overlooked.

CHAPTER THREE

Impact of Ocean Acidification on arm regeneration in the brittlestar *Amphiura filiformis* is growth rate & temperature dependent

Aspects of this Chapter are included in:

Wood H L, Spicer J I, Widdicombe S, Thorndyke M C & Dupont S Impact of Ocean Acidification on arm regeneration in the brittlestar *Amphiura filiformis* is growth rate & temperature dependent *J. Exp. Mar. Biol. Ecol. (In Review)*

ABSTRACT

Ocean acidification and increasing environmental temperatures are two consequences of anthropogenically-driven climate change that will clearly impact all marine taxa. However, it is crucial to recognise that there are also other extrinsic and intrinsic factors that impact life processes and may modulate responses to climate change related stressors. In this chapter, arm regeneration in the brittlestar *Amphiura filiformis* is used to investigate the potential energetic trade offs under conditions of reduced seawater pH in parallel with intrinsic (relative amount of tissue lost) and extrinsic (temperature) factors known to modulate regeneration and differentiation rates. I show that at low pH, both regeneration and differentiation rates are significantly increased. However, low pH only has a significant impact at high temperature, when arm regeneration rates are high (i.e. large amount of tissue removed) but relatively little effect when regeneration rates are low (small amount of tissue lost and/or low temperature). Consequently, to assess the real impact of ocean acidification on any organism it is essential to take account of intrinsic factors (seasonal cycles, age, etc.) as well as extrinsic factors such as natural variation of the environment (e.g. temperature).

INTRODUCTION

The ophiuroid brittlestar *Amphiura filiformis* is an ecologically important infaunal species that mixes and oxygenates benthic sediments generating an environment that is habitable to other species (Vopel et al 2003) whilst also promoting the exchange of nutrients between the sediment and overlying water (Chapter 4). As such this species can be considered as an "Ecosystem Engineer" (Jones et al 1994). It lives in mud burrows with the arms being the only part of the body extending into the water column for suspension feeding (Woodley 1975; Loo et al 1996; Solan & Kennedy 2002). Because of this behaviour it experiences frequent sublethal predation (Sköld & Rosenberg 1996) and its arms are an important food source for commercial fish species including juvenile dab *Limanda limanda* (Bowmer & Keegan 1983). *Amphiura filiformis* has developed (like several other echinoderms) the capacity of arm regeneration as an adaptive response to this disturbance (Dupont & Thorndyke 2006). It has developed structural, chemical and behavioural adaptations to manage predation including arms specialised for autotomy and regeneration (Wilkie 1978; Dupont & Thorndyke 2006), bioluminescence (Herring 1995), a chemically induced alarm response (Rosenberg & Selander 2000), and a photoperiodic pattern of suspension-feeding (Rosenberg & Lundberg, 2004). Nevertheless, predation does occur and its cost is important (Sköld & Rosenberg 1996). The costs of sub lethal predation can be *direct*: the demand for additional energy to regenerate the arm; and *indirect*: the loss of function, e.g. food acquisition (Lawrence & Vasquez 1996) or burrow oxygenation (Nilsson 1998; 1999). In predated *A. filiformis*, undamaged arms are used for feeding and the regenerating arms tend to be kept below the surface (in burrows) and not used for feeding (Bourgoin 1987; Grober 1988).

Regeneration in brittlestars has been studied extensively and appears to be dependent on environmental factors. For example, the regenerate grows faster at higher temperature (Thorndyke et al 2003). The main factor determining regeneration and differentiation rates is the length lost (Dupont & Thorndyke 2006); energy allocated during regeneration (growth in length vs. differentiation) is correlated with the quantity of tissue lost from a single arm. The observed trade-off between growth and differentiation during regeneration is a balance between cost and benefit that presumably has been selected by a long history of sub lethal predation in *Amphiura filiformis* (Dupont & Thorndyke 2006). Thus arms cut close to the disc ('proximal', a large length lost [LL]), regenerate faster in length than those cut close to the tip ('distal', small LL), but their differentiation is slower. In contrast, those with small LL grow slower in length but differentiate more rapidly. This appears adaptive. An arm cut at the tip (small LL) is still able to extend into the water column for feeding but lacks essential sensory organs located at the tip. Investing energy in rapid differentiation leads to a rapid recovery of this functionality and the autotomized arm is quickly functional for feeding. By contrast if an arm is lost close to the disc, rapid differentiation is useless since a short functional arm is not able to reach the water column for feeding so a rapid increase in length is of more adaptive value (Dupont & Thorndyke 2006; Clark et al 2007).

Amphiura filiformis has been shown to be able to not only survive but also regenerate lost arms during short-term exposure (40 days) to lowered pH conditions (Chapter 2). However, survival over the longer-term may not be possible given the concomitant important muscle loss also seen at the lowest pH. Here the impact of future ocean acidification and temperature change on regeneration in *A. filiformis* is investigated using temperature and length lost as extrinsic and intrinsic mechanisms respectively, to manipulate regeneration rate. Given the natural variability in regeneration rate, the impact

of ocean acidification is expected to vary according to factors responsible for these variations, including length lost and temperature.

METHODS

Animal and sediment collection

In January 2008, 90 individuals of *Amphiura filiformis* were collected from a depth of ~12 m in Plymouth Sound (50° 20.598 N, 4°08.155 W) using a 0.1 m^2 van Veen grab. Individuals were carefully hand sorted from the sediment to prevent damage to the arms; only those with 5 intact arms were collected. The brittlestars were held in covered holding buckets (vol. = 10 l, 30 individuals per bucket), filled with sea water also from the collection site and thus of the salinity and temperature experienced at the time of collection (S = 36, PSU, T = 12 °C). Ten litres of muddy sand sediment was collected on the same day from the same location also using a 0.1 m^2 van Veen grab. The sediment was sieved over a 2mm mesh to remove all large fauna. The brittlestars and sieved sediment were transported back to the Plymouth Marine Laboratory (PML) mesocosm facility within 4 h of collection. Once in the mesocosm a 2.5 cm layer of the sieved sediment was placed into each of three aquaria (vol. = 10 l) then filled with filtered (1 µl) seawater. After the sediment had settled (approx. 1 h) brittlestars were transferred to these aquaria (30 individuals per aquarium) which were then submerged in a large (vol. = 125 l) recirculating sea water (S PSU = 36, T °C = 12) holding tank. These individuals were used for the first lower temperature exposure experiment. In Feb 2008, an additional 90 *A. filiformis* individuals and sieved sediment (vol. = 10 l) were collected and prepared exactly as described above. This material was used for the second, higher temperature exposure experiment.

Seawater acidification

In both experiments, seawater pH was adjusted and maintained using a computerized control system which regulated the addition of gaseous CO_2. Seawater was acidified in header tanks (vol. = 450 l) where the acidified seawater supplied to the aquaria was replaced by an equal volume of untreated seawater supplied from a large supply tank (vol. = 15,000 l). Seawater acidification followed very closely the method described by Widdicombe & Needham (2007). Three different pH treatments were used: 8.1 (control), 7.7 and 7.3. The control pH was set as 8.1 as this was the pH of the overlying water at the time of collection. The experimentally lowered pH's were chosen as the worst case scenario for the end of the century (pH 7.7) and a 2300 scenario of pH 7.3.

Manipulation of Regeneration: Temperature and Arm Amputation

Regeneration rates were manipulated using two temperatures and six Length Lost (LL) classes. Two exposure experiments were conducted in the mesocosm facility of the Plymouth Marine Laboratory (PML). Both experiments investigated the impact of changing seawater pH on the growth and functional recovery of arms in the brittlestar *Amphiura filiformis*. The first experiment was run at a temperature of 11.5 °C whilst the second was run at a higher temperature of 15 °C. The two experiments were run concurrently rather than simultaneously since in order to maintain the accuracy of the water pH manipulations the temperature needed to be altered prior to the pH alteration and two temperatures could not be maintained within the facility at the same time. To this end specific attention was paid to collecting the individuals from the same site on both occasions, and keeping the protocols identical for both experiments. Both experiments were carried out while the individuals were dormant with respect to reproductive activity.

Only individuals with no visible sign of recent regeneration were selected for the experiment (N = 180). Regeneration rates were manipulated using LL (Dupont & Thorndyke, 2006). Fifteen individuals were randomly assigned to each of 6 Length Lost (LL) treatment levels (10, 20, 30, 40, 50 and 60 mm of arm removed). For each individual, disk diameter was measured and the point of amputation was determined using calculations supplied by Dupont & Thorndyke (2006) which provided the cutting point for a range of disc diameters (1 – 7 mm in 0.1 mm subdivisions). One arm was then amputated from each of the brittlestars by placing pressure with a scalpel blade between the arm segments at the appropriate distance from the disk. Before arm amputation brittlestars were anaesthetized by submersion in 3.5 % w/w $MgCl_2$ in artificial seawater for a minimum of 3 min. For each individual the arm amputated was the 1st arm clockwise to the madreporite. Each 'Length Lost' treatment class was divided into 3 groups (5 individuals per group) and the groups were randomly assigned to 1 of 3 pH treatment levels of 8.1 (control), 7.7 and 7.3.

Experimental set up

After amputation, 10 individuals from across the six LL classes were allocated to each of 9 aquaria (vol. = 5 l) containing sieved sediment (≈ 2.5 cm deep) and normal (pH ≈ 8) seawater. The animals were distributed so that each aquarium contained individuals from each of the 6 LL classes. Once the individuals had recovered from anesthetic and had burrowed into the sediment the aquaria were placed into the experimental system. Three replicate aquaria, each containing 10 individuals, were used for each pH treatment (8.1, 7.7 and 7.3). Each aquarium was supplied with seawater from a header tank of the designated pH using a peristaltic pump (40 $ml.min^{-1}$). Excess water flowed over the aquaria and ran to waste. The brittlestars were fed on green algae (*Pavlova* sp.) at 4.8 million $cells.ml^{-1}$.

Measurement of carbonate parameters

Seawater pH_{NIST} and temperature (°C) were constantly monitored in the header tanks and the mesocosm respectively. Seawater pH_{NIST}, tCO_2, salinity and temperature (°C) were recorded weekly (Table 1) from the exposure aquaria and header tanks using a Mettler-Toledo pH meter, a Ciba-Corning 965D Total CO_2 Analyser, Olympic Analytical Service and a WTW LF197 combination temperature and salinity probe respectively. The remaining carbonate parameters were calculated using CO2SYS (Pierot et al. 2006) with the constants supplied by Mehrbach et al (1973) refitted by Dickson & Millero (1987) and the KSO_4 dissociation constant from Dickson (1990).

Measuring arm regeneration and differentiation

After 7, 14, 21 and 28 days of exposure the overlying water in each aquarium was removed, and artificial seawater containing 3.5 % w/w $MgCl_2$ was added to anesthetise the brittlestars. After approx. 3 minutes individuals were removed and the following measurements obtained: disc diameter in mm, number of spontaneous autotomy events, total length of regenerated arm (RL) and length of the differentiated portion of regenerated arm (DL). Differentiated arm was identified by the the presence of segmentation and, or spines; these parameters and a full description of the identification of differentiated tissue are in Dupont & Thorndyke (2006). The latter two measurements were extracted from micrographs obtained under low power (x 12) using a Nikon Coolpix 995 camera mounted on a Wild Heerbrugg model 157940 microscope. Measurements were taken using Image-Pro Plus v4.5 Media Cybernetics software. Regeneration rate (RR in $mm.week^{-1}$), differentiation rates (DR1 in $mm.week^{-1}$ and DR2 in $\%.week^{-1}$) were calculated using methods described in Dupont & Thorndyke (2006). Once measured each individual was carefully returned to a bucket and placed on top of the sediment which was submerged in

seawater of the appropriate pH freshly drawn from the header tank. The brittlestars typically took around 20 min to recover from anaesthetisation and rebury into the sediment.

Statistical analysis

Each individual was identified from measurements of disc diameter, length of longest arm and position of amputation, thus allowing individual growth profiles to be constructed for each individual throughout the course of the experiment. The regeneration rate and differentiation rate was calculated for each individual thus the serial time points from each brittlestar were turned into one data point to represent the regeneration/differentiation of that individual; therefore although repeated measures were taken from the same individual this provided only one data point for the statistical analysis; this methodology was developed and is discussed in detail in Dupont & Thorndyke (2006).

Neither RR nor DR data were normally distributed (Kolmogorov-Smirnov test). Consequently RR data were Log_{10} transformed to achieve homogeneity of variance (Kolmogorov-Smirnov test). and were then analysed using two-way analysis of variance (ANOVA) run on Minitab V.15 statistical software.

Neither measures of differentiation (DR1 and DR2) were normally distributed so were analysed using the permutational MANOVA (PERMANOVA) procedures introduced by McArdle & Anderson (2001) and Anderson (2001). PERMANOVA+ routines (beta version, Anderson et al 2008), are an 'add-in' to the PRIMER 6 software; these procedures make more restrictive assumptions than a fully non-parametric approach, but crucially the multivariate PERMANOVA method operates on a similarity matrix and avoids unrealistic normality (or other distributional) assumptions. It does this by exploiting permutation to

generate null hypothesis distributions for its pseudo-F statistics; the latter constructed by exact analogy with the standard F statistics for corresponding univariate ANOVA designs.

RESULTS

Experimental conditions

The water carbonate parameters within the aquaria and header tanks were stable throughout the experiment as were temperature and salinity. The water conditions of the aquaria are shown in Table 3.1 as these are the most relevant representation of the experimental conditions. While pH is known to alter with depth in the sediment, the continued burrow ventilation of *Amphiura filiformis* is thought to ensure burrow pH reflects that of the overlying water rather than the surrounding sediment (Zhu et al 2006). Both temperature treatments at pH 7.7 were undersaturated with respect to aragonite (Ω_{arag}) (indicated in bold, Table 3.1).

Table 3.1. Summary of water parameters (mean) calculated from samples taken from all aquaria across the experimental period, ± 95% confidence interval. tT indicates temperature treatment; High = 15 °C, Low = 11.5 °C. tpH indicates target pH, Sal = Salinity (psu), T = temperature (°C), TA = Total Alkalinity ($mEq.L^{-1}$), Ω_{ca} = Omega Calcite, Ω_{ar} = Omega aragonite.

tT	tpH	Sal	T	pH	TA	Ω_{Ca}	Ω_{Ar}
High	**8**	33.79 ± 0.23	11.57 ± 0.29	8.08 ± 0.01	2044.51 ± 104	2.53 ± 0.12	1.61 ± 0.08
High	**7.7**	33.95 ± 0.20	11.54 ± 0.29	7.70 ± 0.00	1913 ± 102	1.07 ± 0.06	0.68 ± 0.04
High	**7.3**	33.93 ± 0.22	11.57 ± 0.33	7.31 ± 0.01	1924 ± 89	0.46 ± 0.02	0.29 ± 0.013
Low	**8**	34.12 ± 0.31	15.04 ± 0.08	8.05 ± 0.03	1929 ± 194	2.11 ± 0.33	1.35 ± 0.21
Low	**7.7**	34.08 ± 0.31	15.02 ± 0.09	7.72 ± 0.02	1989 ± 204	1.20 ± 0.15	0.77 ± 0.10
Low	**7.3**	34.13 ± 0.34	14.85 ± 0.06	7.37 ± 0.03	1906 ± 169	0.69 ± 0.08	0.44 ± 0.05

Impact of pH on regeneration rate

Regeneration rate (RR) increased with increasing amount of arm length lost (LL) (Figure 3.1). RR was significantly greater at 15 °C than 11.5 °C across all pH treatments (Figure 3.1, Table 3.2). At the higher temperature RR was consistent with the rates presented in an earlier investigation of regeneration in *Amphiura filiformis* (Dupont & Thorndyke 2006). At the low temperature the RR/LL curve did not differ significantly between pH treatments (Figure 3.1, Table 3.3).

There was also a significant effect of pH and temperature on regeneration rate (RR), and a significant interaction between these factors (Table 3.3, Figures 3.1 & 3.4). Unlike the low temperature pH results, at the higher temperature there was a clear effect of pH on the RR/LL relationship (Figure 3.1), with RR considerably greater in the pH 7.7 treatment. At pH 7.3 the RR remained greater than the control when a large amount of arm was lost (LL < 3 mm) but then converged to similar RR at greater LL (Figure 3.1).

Impact of pH on differentiation rate

There was a significant difference in the differentiation rate 2 (DR2) between the two temperature treatments, and also a significant interaction between pH and arm length lost (LL) (Table 3.4 a, Figure 3.2). At the lower temperature, there was a significant effect of LL on DR2 (Table 3.4 b) but not pH (Figure 3.2). Actual differentiation rate (DR1) shows greatly increased differentiation rates (Figure 3.3), however these are a function of overall increased regeneration, as illustrated by the percentage of regenerate showing functional recovery (DR2, Figure 3.3).

Impact of pH on spontaneous autotomy

There was a significant interaction between temperature and pH treatment (Table 3.4 c). The number of autotomies was lower in the low temperature treatment compared to the high temperature (Figure 3.4), and whereas in the low temperature treatment there was no effect of pH, at the high temperature the number of autotomies increased with lowered pH (Figure 3.4).

Table 3.2. One way ANOVA results indicating the impact of seawater temperature on regeneration rates (RR) at length lost of a.) 10 mm, b.) 20 mm and c) 30 mm. Temperatures compared are 11.5 °C and 15 °C.

a.

Source	d.f.	Seq SS	Adj SS	Adj MS	F	P
Temp	1	1097083	1097083	1097083	4.94	0.036
Error	25	5552718	5552718	222109		
Total	26	6649801				

b.

Source	d.f.	Seq SS	Adj SS	Adj MS	F	P
Temp	1	0.35286	0.35286	0.35286	8.5	0.007
Error	28	1.16218	1.16218	0.04151		
Total	29	1.51505				

c.

Source	d.f.	Seq SS	Adj SS	Adj MS	F	P
Temp	1	0.51983	0.51983	0.51983	25.84	<0.001
Error	27	0.54318	0.54318	0.02012		
Total	28	1.06301				

Table 3.3. ANOVA results indicating the impact of seawater pH and temperature on regeneration rates. LL denotes Amount of arm removed (length lost). Temp = environmental temperature. Significant P values (to 0.05) shown in bold

Source	d.f.	Seq SS	Adj SS	Adj MS	F	P
pH	2	1.11317	1.05109	0.52555	31.53	**< 0.001**
Temp	1	3.79371	3.78388	3.78388	227.02	**< 0.001**
LL	5	2.12715	1.9149	0.38298	22.98	**< 0.001**
pH*Temp	2	0.90159	0.86314	0.43157	25.89	**< 0.001**
pH*LL	10	0.32697	0.2975	0.02975	1.78	0.069
Temp*LL	5	0.36589	0.35387	0.07077	4.25	**0.001**
pH*Temp*LL	10	0.32814	0.32814	0.03281	1.97	**0.042**
Error	130	2.16683	2.16683	0.01667		
Total	165	11.12346				

Table 3.4. a.) PERMANOVA results indicating the impact of seawater pH and temperature on differentiation rates. **b.)** PERMANOVA results indicating the impact of seawater pH and length lost on rate of functional recovery (DR2) within the low temperature treatment of 11.5 °C. **c.)** 2-Way ANOVA indicating the impact of seawater pH and temperature on differentiation rates. LL = amount of arm removed (length lost), pH = pH treatments and temp = temperature treatment (11.5 °C & 15 °C). Significant results are shown in bold.

a.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
pH	3	0.417	0.139	4.266	**0.0072**	9951
temp	1	1.8344	1.8344	56.298	**0.0001**	9839
LL	5	0.8435	0.1687	5.1775	**0.0005**	9951
pH*temp	1	8.53E-02	8.53E-02	2.6185	0.1089	9815
pH*LL	15	1.9124	0.12749	3.9128	**0.0001**	9928
temp*LL	5	0.14623	2.92E-02	0.89756	0.4798	9947
pH*temp*LL	5	6.48E-02	1.30E-02	0.3979	0.8439	9966
Res	124	4.0403	3.26E-02			
Total	159	9.738				

b.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
pH	2	0.80294	0.40147	2.4341	0.0953	9957
LL	5	2.1942	0.43883	2.6607	**0.0287**	9953
pH*LL	10	2.8849	0.28849	1.7492	0.0839	9918
Res	71	11.71	0.16493			
Total	88	17.597				

c.

Source	d.f.	Seq SS	Adj SS	Adj MS	F	P
Temp	1	8.0278	8.0278	8.0278	11.2	**0.002**
pH	2	21.0556	21.0556	10.5278	14.69	**< 0.001**
Temp*pH	2	17.0556	17.0556	8.5278	11.9	**< 0.001**
Error	30	21.5	21.5	0.7167		
Total	35	67.6389				

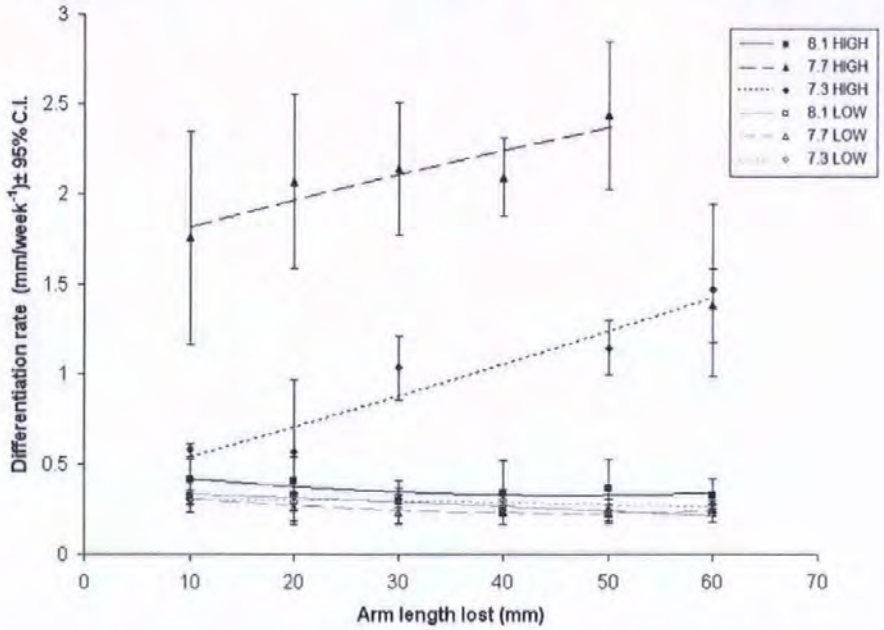


Figure 3.1. Regeneration rate of *Amphiura filiformis* at the three pH and two temperature treatments across a range of length lost values. Mean values ± 95 % confidence intervals

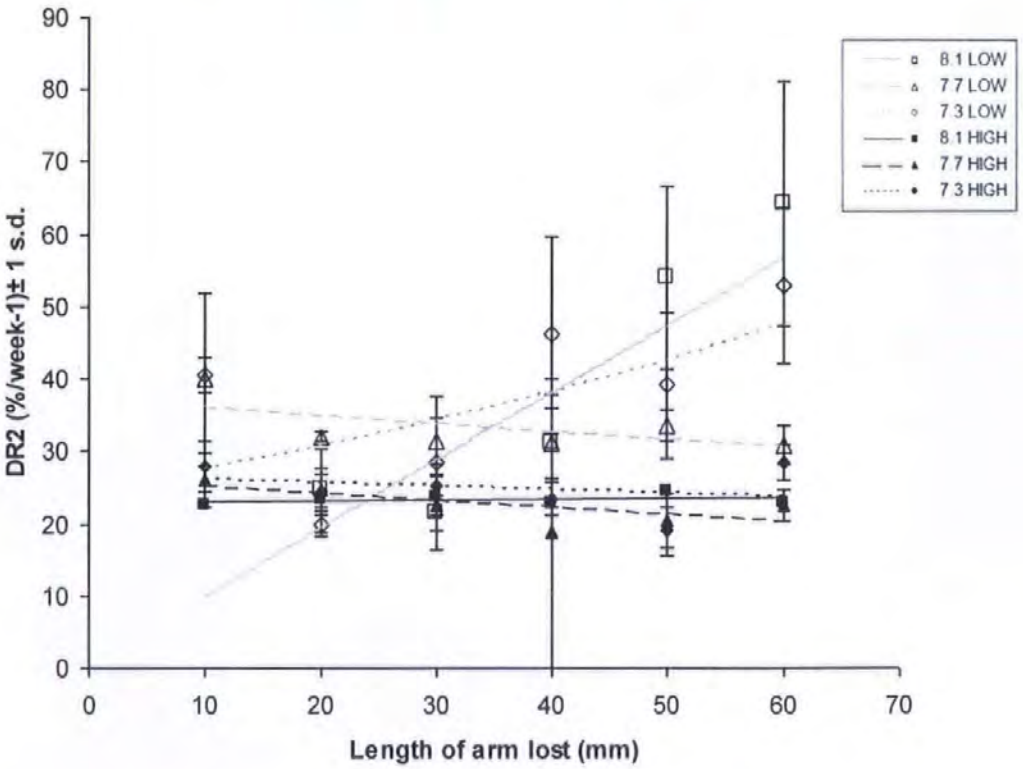


Figure 3.2. Differentiation rate of *Amphiura filiformis* at the three pH and two temperature treatments across a range of length lost values. Values shown are means ± 95 % confidence intervals.

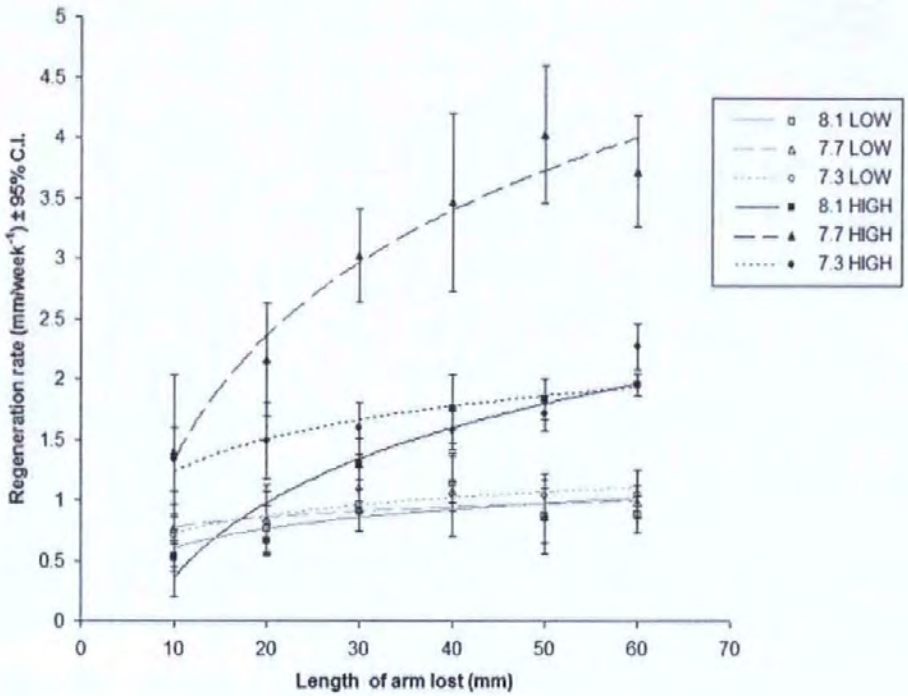


Figure 3.3. Functional recovery (DR2) of *Amphiura filiformis* expressed as percentage recovery per week. Results presented for 11.5 °C (LT) and 15 °C (HT) temperature treatments and pH treatments of pH 8.1 (control), 7.7 and 7.3. Legend denotes each length lost class (mm). Values shown are means ± 95% confidence interval.

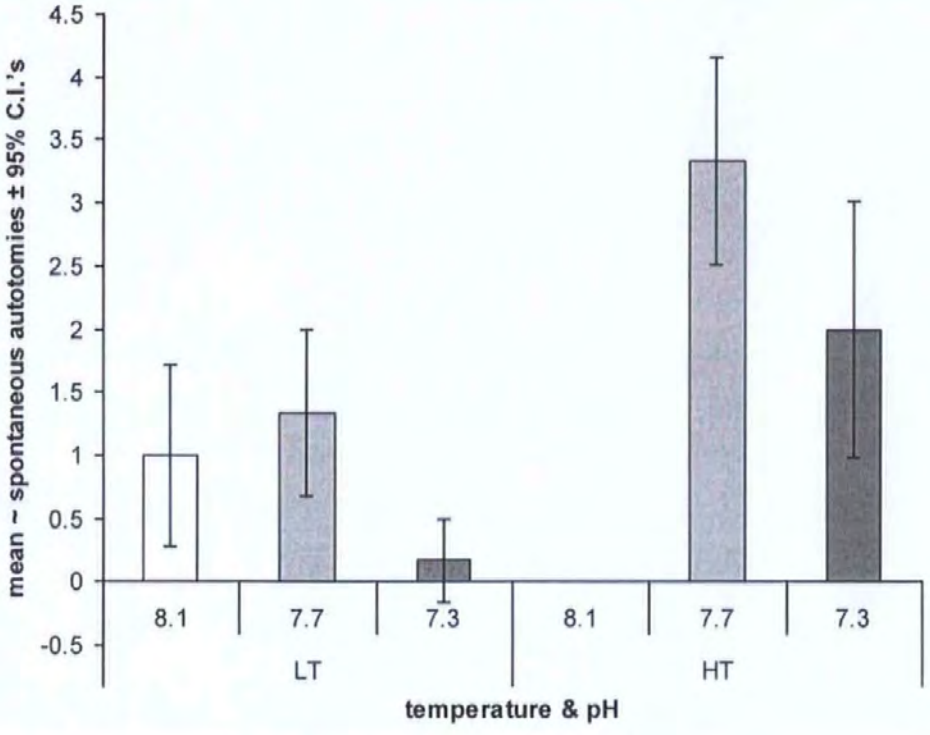


Figure 3.4. Mean number of spontaneous autotomy. Results presented for 11.5 °C (LT) and 15 °C (HT) temperature treatments and pH treatments of pH 8.1 (control), 7.7 and 7.3. Values shown are means ± 95 % confidence intervals.

DISCUSSION

In this study a range of LL and temperatures were used to manipulate arm regeneration and differentiation rates in the brittlestar *Amphiura filiformis* against various backgrounds of pH chosen to reflect predicted future OA levels. The LL range reflects the variety of sub lethal predation events likely to be experienced by natural populations of *A. filiformis*. The temperatures used in this study are similar to the normal range experienced by the sample population while the experimental OA levels fall within those predicted for the end of the century.

Interaction between regeneration rates and pH

This study found that the effect of pH on arm regeneration in *A. filiformis* is dependent on regeneration rate. At the lower regeneration rate (low temperature and/or small LL) there was no effect of pH on RR or DR, but when regeneration rate was increased (high temperature and high LL) there was a marked difference in response between the pH treatments. This comprised an up-regulation in both regeneration and differentiation rates at lowered pH. Up-regulation of metabolism, growth and calcification at lowered pH has previously been shown in *A. filiformis* (Chapter 2). Those findings were based only on experiments at 15°C, the same as the highest temperature treatment used in the current study and highlights the importance of running multifactorial experiments in order to obtain a clearer picture of responses to a combination of factors.

Arm regeneration in *A. filiformis* is similar to that observed in the crinoid model *Antedon mediterranea*. It is an epimorphic blastemal process, in which new tissues arise from active proliferation of migratory undifferentiated cells (amoebocytes and coelomocytes), which

accumulate at the end of the nerve cord. Arms appear to extend from the tip with segmental maturity occurring in a temporally regulated fashion and proximal-distal direction (Candia Carnevali et al 1995, 1999; Bonasoro et al 1998; Thorndyke et al 2001). In terms of histogenesis the regenerative events involve the development of new structures from migratory stem cells, which proliferate actively, and in both cases there is a significant contribution from dedifferentiated cells, in particular dedifferentiating muscle cells (myocytes) (Biressi et al 2009). The degree of de-differentation can vary, and during exceptionally high stress conditions, such as in explants, a more extensive use of myocytes is often observed in order to sustain a higher demand for new cells (Candia-Carnevali et al 1998). It then follows that the phenomenon of regeneration involves a number of 'energetic trade-offs' according to the level, type and timing of the stressor(s). Moreover when there are multiple stressors, as in our experiments, then the trade-offs are likely to be even more acutely regulated.

There is an apparent compounding of stressor affects with the most important observation being that effects are most prevalent when regeneration rates are high and thus cell proliferation, energy consumption and general metabolic demands are also high. Thus, when there is a large LL and a high temperature, the impact of OA is most dramatic. This emphasizes the fact that to assess the real impact of OA other extrinsic factors such as temperature should be included, but also any other intrinsic factor that may affect metabolism and growth and then modulate the response to low pH (e.g. seasonal hormonal changes, life history, age.)

As noted earlier, regeneration is in effect a modified stress response that involves increased growth, proliferation, de-differentiation and re-differentiation, which in echinoderms commonly involves muscle cells as the source of the "stem cells" that underpin the development of new tissues in the regenerate. This gives the appearance of muscle loss and

degeneration typically observed in many stress responses since the muscle cells are in effect "recycled" as new tissues (Candia Carnevalli et al 1998). This can become limiting at very low pH as illustrated by the major muscle loss observed under such conditions in an earlier study (Chapter 2). Previously it was not clear whether the raised metabolic rate was a response to the increased regeneration rate induced by lowered pH, or if the lowered pH caused metabolism to increase directly and this in turn stimulated faster regeneration rates. However, the current observation that the OA response is modulated by an intrinsic biological factor (LL) provides ample evidence that an increased regeneration rate can be regulated directly and is not necessarily simply a response to an increased metabolic rate.

Trade off between growth and differentiation

The relationship between regeneration and differentiation rates at control pH (8.1) shows that when amputation is proximal (LL is 30 mm or greater), energy is invested in growth rather than differentiation, as in a previous study (Dupont & Thorndyke 2006); and this effect is clearly amplified as temperature increases. The effect of pH is seen most dramatically at this high temperature where regeneration rates between LL differ greatest; the RR/DR relationship alters with pH only in the higher growth rate treatments where the proxy measure of functional recovery, DR2 (Dupont & Thorndyke 2006) is greater at the higher temperature, and a pH effect is seen here to increase functional recovery with the amount of arm lost. Here, in addition to increased regeneration rate; there is a prioritisation of functional recovery over regaining maximum arm length particularly at large LL. Considered in the context of the biology of *Amphiura filiformis,* if these individuals are prioritising shorter but functional arms this may suggest a change in behaviour to facilitate the use of these arms; perhaps accepting a shorter amount of arm exposed within the water column or moving to a shallower position within the sediment. A 'not perfect, but better than nothing' approach to arm recovery may be adopted in a situation where both food and

oxygen are under increased demand (Chapter 2), and functional arms are a necessity to supply these.

Ecological consequences

To establish if the effect of OA related on regeneration rate is adaptive, and what this would mean in terms of the likely response of this species (and potentially other sensitive taxa) to predicted future OA it is necessary to analyse the profit and loss account for increased regeneration under the various stress parameters tested. Echinoderms, as any other biological system, are dependent upon energy. Energy is limiting, either extrinsically or intrinsically, and thus external biotic and abiotic factors which affect the acquisition and retention of energy are important selective pressures (Lawrence 1990; 1991; Lawrence & Vasquez 1996). *Amphiura filiformis* possesses exceptional regenerative abilities (Dupont & Thorndyke 2006) but regeneration requires energy beyond that needed for the normal processes of maintenance, growth, and reproduction. The allocation of an energy budget to regeneration is of importance and must be balanced against the individual's other needs to be sure that the benefits of regeneration exceed the fitness cost. The regeneration of a functional arm, rapidly and at low energy cost is essential to increase the overall level of energy available, *via* more efficient feeding.

By the end of this century, when average seawater pH is predicted to be pH 7.7, the results of this study indicate *Amphiura filiformis* will probably put more energy into the overall regeneration process (higher regeneration/differentiation rates) and proportionally less energy into growth in length, instead regaining arm function earlier. However, the biology of the species may limit the negative impact of OA on the regeneration process and then on the organism as a whole. (i) In nature, most autotomy occurs more distally (Bowmer & Keegan 1983) and so in most cases RR will be low and thus the effect of OA can be

predicted to be low. (ii) Regeneration should not be considered as a permanent stress. Sub lethal predation and subsequent regeneration is a frequent but seasonal phenomenon. Predation pressure is higher in spring and summer and regeneration rate is dependent on environmental factors such as temperature and food availability (Sköld et al 1994) and our data from spontaneous autotomies, which is a good measure of stress (Dupont & Thorndyke 2006) show that this warmer seasonal time is a time that the animals are also going to be more stressed at future lower pH conditions. Most individuals collected present evidence of regeneration events (Sköld & Rosenberg 1996) but this occurs over a long period of time in a long lived species and lasts only months depending on extrinsic and intrinsic parameters (Dupont & Thorndyke 2006) (iii) Temperature also varies seasonally and *A. filiformis* only experiences high temperatures (e.g. more than 11 °C) for up to 6 months every year. In consequence, the potentially detrimental effects of OA will be limited in time and most of the regeneration process could occur under more favourable conditions (low temperature and small LL). Nevertheless, in some situations (environments) multiple (extrinsic) stressors might be present and thus, when present, likely to modulate the impact of predicted future OA. It is known that when exposed to multiple stressors, the 'stress-like' regeneration process seems to be "over-expressed" with increased growth and differentiation rates, increasing use of stem cells (e.g. increased dedifferentiation of myocytes), etc. This typical "stress-response" of regeneration has been well documented for stressors such as pollutants, temperature as well as ocean acidification (D'andrea et al 1996; Candia Carnevali et al 2001a, b; 2003; Gunnarsson et al 1999; Selck et al 2004; Granberg 2004; Bannister et al 2004; Barbaglio et al 2004; Wood et al 2008). However, the observed increase in regeneration rates appears to have some limitations. The RR decreases at pH 7.3 compared to pH 7.7 at high temperatures and LL and DR already decreases when LL is higher than 50 mm at high temperature. In consequence, a combination of additional stressors will narrow individual tolerance to pH and thus

increase the response to OA in the future ocean. This may be a major concern with respect to the global warming that will likely accompany OA.

Even though there may be apparently good recovery the likely longer term fitness impacts that might result from the described elevation in regeneration rate and process are still unknown. For example, is fecundity reduced if the regenerative event occurs during the reproductive season? Earlier studies suggest that quantitatively, energy allocation to regeneration is not dependent on the number of arms lost. The same quantity of energy is allocated (from both the remaining proximal part of the arm and the disc) irrespective of whether one or more arms are lost (Nilsson 1998; 1999). However, the potential effects of several compounded stressors might have on this is unknown. Thus it is going to be important to adopt a 'whole organism' approach to the investigation of the potential impact of OA, temperature and extent of regeneration as combined stressors.

Summary

In predicting the potential impacts of Ocean Acidification (OA) on marine taxa the acknowledgment that ocean pH will not change in isolation to other events is critical. The responses of marine organisms to OA will take place against an existing background of both extrinsic and intrinsic parameters. These interactions are key to the understanding of how species and ecosystems will respond to, and cope with, future environmental conditions. Using the natural process of arm regeneration in the burrowing ophiuroid *Amphiura filiformis* as a proxy, it is shown that the response to lowered pH differed significantly depending on the rate of regeneration, a phenomenon that is also modulated by temperature and length lost. This highlights the danger of characterising responses to ocean acidification in isolation from other factors. Arm regeneration rates in *A. filiformis* vary according to the relative amounts of tissue lost at autotomy following the sub-lethal

predation that is common in this species. Here it is clear that when regeneration rates are high (large tissue loss and high temperatures) then lowered pH has a significant additive Effect on rate. This seems to be linked to the natural stress response that underpins regeneration, a response that is also regulated by temperature and can include substantial tissue re-modelling using dedifferentiated muscle as the source for new tissue components (Biressi et al 2009), and may become an increasing issue as temperature increases.

The message then is clear. In order to be able to predict likely impacts of OA in our future oceans the broad spectrum of both extrinsic and intrinsic factors need to take into account. These factors already provide the established background for the regulation of fitness, seasonality and life history events, and cannot be ignored in the research of ocean acidification

CHAPTER FOUR

The influence of hypercapnia and *Amphiura filiformis* on sediment nutrient flux - will ocean acidification affect nutrient exchange?

Aspects of this Chapter are included in:

Wood H L, Spicer J I, Widdicombe S (2009) The influence of hypercapnia and macrofauna on sediment nutrient flux - will ocean acidification affect nutrient exchange? *Biogeosci.* **6:** 2015 -2024

ABSTRACT

Rising levels of atmospheric carbon dioxide and the concomitant increased uptake of this by the oceans is resulting in hypercapnia-related reduction of ocean pH. Research focussed on the direct effects of these physicochemical changes on marine invertebrates has begun to improve our understanding of impacts at the level of individual physiologies. However, CO_2-related impairment of organisms' contribution to ecological or ecosystem processes has barely been addressed. The burrowing ophiuroid *Amphiura filiformis*, which has a physiology that makes it susceptible to reduced pH, plays a key role in sediment nutrient cycling by mixing and irrigating the sediment, a process known as bioturbation. Here I investigate the role of *A. filiformis* in modifying nutrient flux rates across the sediment-water boundary and the impact of CO_2- related acidification on this process. A 40 day exposure study was conducted under predicted pH scenarios from the years 2100 (pH 7.7) and 2300 (pH 7.3), plus an additional treatment of pH 6.8. This study demonstrated strong relationships between *A. filiformis* density and cycling of some nutrients; *A. filiformis* activity increases the sediment uptake of phosphate and the release of nitrite and nitrate. No relationship between *A. filiformis* density and the flux of ammonium or silicate were observed. Results also indicated that, within the timescale of this experiment, effects at the individual bioturbator level appear not to translate into reduced ecosystem influence. However, long term survival of key bioturbating species is far from assured and changes in both bioturbation and microbial processes could alter key biogeochemical processes in future, more acidic oceans.

INTRODUCTION

Soft sediments are an important coastal benthic habitat and host many of the biogeochemical processes that underpin ecosystem function in shallow shelf seas. In particular, nutrient cycling (the 'recycling' of nutrients both within and between the benthic and pelagic systems), is strongly driven by the biological and chemical processes that occur within the sediment; e.g. bacteria mineralise dissolved and particulate organic nutrients from the debris that sinks to the sea floor (Dale & Prego 2002). The transformation of nutrients (e.g. denitrification, nitrification and anammox) is primarily performed by bacteria and, therefore, nutrient cycling is strongly affected by the presence and activity of key microbial groups. In turn, the type and distribution of these microbes within the sediment is ultimately determined by their surrounding geochemical environment (e.g. Satoh et al 2007). In addition to determining the microbial communities present, geochemistry can also affect nutrient flux directly. For example, the flux of silicon is dependent upon both the substrate compound availability on the sediment surface and the oxygen distribution within the sediment (Hartikainen et al 1996). Therefore, biological processes that set or modify the geochemical nature of the sediment, such as the presence and activity of large infaunal animals, are critically important for nutrient cycling.

The impacts of burrowing macrofauna on nutrient cycling are numerous (Bird et al. 1999, Christensen et al 2000). As well as increasing the surface area of sediment available for nutrient exchange (Fenchel, 1996), it has also been suggested that the burrow itself creates a favourable environment (i.e. more than the sediment surface) for some of the bacteria involved in nutrient cycling (Henriksen et al 1983; Kristensen et al 1985). Burrow irrigation transfers both oxygen and nutrients from the pelagic system into the deep sediment. In addition, bioturbation, resulting from the presence and activity of

macroinvertebrates, mixes the top layers of sediment and water (Eckman et al 1981), further increasing the reaches of the sediment-water nutrient exchange, refreshing compound availability on the sediment surface and oxygenating the top layer of sediment. These cumulative effects of infaunal organisms on the sediment environment can enhance nutrient cycling directly through changing sediment geochemistry and indirectly by determining the nature and function of the resident flora and fauna (Mayer et al 1995; Satoh et al 2007). This habitat modification is termed ecosystem engineering (Lawton 1994).

Amphiura filiformis is an important bioturbator and ecosystem engineer; where present it has a dominating effect on the surrounding sediment *via* bioturbation (O'Reilly et al 2006). The current study uses a controlled laboratory experiment to expose *A. filiformis* in sediment cores to CO_2 lowered pH water conditions. pH treatments used are control, pH 7.7 (expected by the end of this century), 7.3 (predicted for 2300) and a finally at pH 6.8. Several densities of *A. filiformis* were tested to determine the extent to which nutrient flux is mediated by the presence of this bioturbator and assess the degree to which this biological mediation of nutrient cycling may be altered under future scenarios of high CO_2.

METHODS

Experimental Set-up

Ninety undisturbed muddy-sand sediment cores were collected (Jan 2007) from a subtidal site (~ 10 m depth) within Plymouth Sound, UK, (50° 20.598N, 4° 08.155W). The cores were collected by sub-sampling from a 0.1m^2 box corer. Plastic cores (10 cm diameter, 20 cm long) were pushed into the sediment to a depth of 15 cm. Each core was then gently removed from the box-core, sealed on the bottom with a plastic cap. The cores were returned to the laboratory at Plymouth Marine Laboratory (PML) and maintained in a recirculating seawater system (S = 36 PSU, T = 12 °C) until required in the experiment described below. Cores that displayed evidence of burrowing activity during this time were not used. Two weeks after the collection of the sediment cores, 500 individuals of *Amphiura filiformis* (disc diameter 3 – 6 mm, intact and with no signs of recent regeneration), were collected from the same location using a 0.1 m^2 van Veen grab. Individuals with a disk diameter > 5 mm were gently hand sorted from the sediment to prevent damage to the brittlestars' delicate arms. Only individuals with 5 intact arms were collected. The brittlestars were held in covered holding buckets (diam. = 30 cm, no more than 20 individuals per bucket), filled with sea water (S = 36 PSU, T = 12 °C) and were transported back to PML within 4 hours of collection. The brittlestars were kept overnight in a recirculating seawater system (S = 36 PSU, T = 12 °C), before visibly healthy individuals were selected for use in experiments.

Eighty sediment cores that showed no signs of animal presence (e.g. burrows) were haphazardly allocated to 1 of 4 different CO_2-acidified treatment levels (nominal pH = 8.0, 7.7, 7.3, 6.8) and within each pH treatment haphazardly allocated to 1 of 5 brittlestar

density levels (0, 4, 8, 12 or 16 indiv.core^{-1}). The chosen density levels are within the natural range of densities; 4 to16 indiv./core = 512 to 2051 indiv./m^2. Each pH treatment (8.0, 7.7, 7.3 and 6.8) consisted of four replicate cores of each brittlestar density (20 cores per pH treatment) in addition to the cores with no brittlestars which were to provide baseline information of the sediment nutrient fluxes at each pH level. Sediment cores were transferred to large holding containers within the PML seawater acidification facility (hereafter referred to as 'mesocosm') where each core was continually supplied with filtered seawater of the allocated pH at a rate 8ml min^{-1} using a peristaltic pump. The excess water overflowed from the core and drained out of the larger holding container. These containers were draped with black cloth to reduce direct light penetration from the fluorescent lighting directly above the containers that were on for 10 hours a day. The remainder of the time the mesocosm was in darkness.

Alteration of water pH was achieved by sparging CO_2 into header tanks (vol. = 500 l) using a negative feedback system whereby a pH probe (Walchem S650CD) in the header tank continually feeds the pH to a computer control which turns on a fine bubbling of CO_2 (control, achieved *via* a solenoid attached to the CO_2 regulator) when the pH rises above a given set point. Once the desired pH is reached the CO_2 supply is stopped. By continual mixing and virtue of the large volume of the header tank this system can maintain water to a set pH level with an accuracy of 0.002 pH units. Further details of the PML acidification facility are presented in Widdicombe & Needham (2007). The exposure experiment was run for a period of 40 days during which time the brittlestars were not fed; while primarily a suspension feeder, *A. filiformis* are known to switch to deposit feeding when there is little food available in the water column (Buchanan, 1964).

Physico-chemical status of acidified waters

The water in each header tank, plus the water flowing out of the silicon supply tubes, was analysed three times a week for total carbon dioxide content (tCO_2), pH_{NIST}, salinity and temperature. tCO_2 was measured from 100 µl subsamples of seawater using an automated carbon dioxide analyser (CIBA Corning 965 UK). pH_{NIST} was measured using a pH electrode (Mettler Toledo LE413) calibrated with NIST standardised buffers. Salinity was measured with a conductivity salinometer (WTW LF197). Temperature was measured using a probe combined with the pH meter as detailed above (accurate to 0.1 °C).

Sampling

At the end of the exposure period, a 50 ml sample of the water overlying the sediment was taken from each core. The water sample was filtered through a GFF filter and stored in an acid washed Nalgene® bottle. In addition a 50 ml sample of the water from each inflow tube was collected in the same way. All water samples were immediately frozen and stored frozen (T = -20 °C) to await analysis. This sampling was repeated on 3 consecutive days. Samples were analysed, after thawing, using a nutrient autoanalyser (Branne & Luebbe, AAIII) for ammonium, nitrate, nitrite, silicate and phosphate concentrations using standard methods (Brewer & Riley 1965; Grasshoff 1976; Mantoura & Woodward 1983; Kirkwood 1989; Zhang & Chi 2002). The cores were then emptied and the live *Amphiura filiformis* counted to provide survival data.

Statistical analysis

Nutrient fluxes were calculated using Equation 1 from Austen (2006) and Widdicombe & Needham (2007).

$$F_x = \frac{(C_i - C_o) \times Q}{A}$$

(Equation 1)

where F_x is the flux of nutrient x ($\mu mol.m^{-2}.h^{-1}$), C_i is the mean concentration of nutrient x in the inflow water (μM), Co is the mean concentration of nutrient x in the core water (μM), Q is the rate of water flow through the core ($l.h^{-1}$) and A is the core area (m^2). A positive F_X value indicates the nutrient is being taken up by the sediment, and a negative value indicates nutrient being released from the sediment into the water.

Nutrient flux data were not normally distributed so were analysed using the permutational MANOVA procedures introduced by McArdle & Anderson (2001) and Anderson (2001). These procedures make more restrictive assumptions than a fully non-parametric approach, but crucially the multivariate PERMANOVA method operates on a similarity matrix and avoids unrealistic normality (or other distributional) assumptions. It does this by exploiting permutation to generate null hypothesis distributions for its pseudo-F statistics; the latter constructed by exact analogy with the standard F statistics for corresponding univariate ANOVA designs. Here, we have used the PERMANOVA+ routines (beta version, Anderson et al. 2008), which are an 'add-in' to the PRIMER 6 software.

RESULTS

Experimental conditions and *Amphiura filiformis* survival

The analysis of nutrient fluxes for cores containing no *A. filiformis* indicated that the sediment used in the current study was a sink for nitrate (104.95 ± 23.04), and a source of nitrite (-0.14 ± 0.77), ammonium (-35.01 ± 11.48), silicate (-70.58 ± 41.11) and a slight source of phosphate (-1.41 ± 0.97). Values given are means (μmol m^{-2} h^{-1}) ± 95 % confidence intervals.

The water parameters within the cores and header tanks were monitored throughout the experiment to ensure stability of the acid base-status of the water (Table 4.1). In all treatments pH, alkalinity, salinity and temperature remained constant throughout the experiment. Water became undersaturated with respect to calcite at pH 7.3 and with respect to aragonite at pH 7.7. The sediment in all cores appeared healthy based on the oxygenated colour of the surface and the lack of animals appearing on the surface, which is often an indication of anoxia or otherwise contaminated sediment. Three cores (one each from pH = 7.7, 7.3 and 6.8 treatments) were removed from the experiment after the base caps failed during the experiment causing the water to drain out of the core resulting in air exposure and the *Amphiura filiformis* present to die. In the remaining cores *A. filiformis* survival was always 100 % with feeding arms visible above the surface.

Table 4.1. Summary of water conditions throughout experiment. tpH = target pH, TA = Total Alkalinity (mEq.l^{-1}), Sal = Salinity (psu) T = temperature (°C), Ω_{Ca} = Omega calcite, Ω_{Ar} = Omega aragonite. Values are means ± 95 % confidence intervals.

tpH	pH	TA	Sal	T	ΩCa	Ωar
8	7.98 ± 0.003	1726 ± 105	36.04 ± 0.03	11.93 ± 0.07	1.81 ± 0.11	1.16 ± 0.07
7.7	7.69 ± 0.01	2483 ± 59	36	12.04 ± 0.09	1.45 ± 0.03	0.93 ± 0.02
7.3	7.39 ± 0.01	2214 ± 76	36	11.97 ± 0.07	0.53 ± 0.02	0.34 ± 0.01
6.8	6.76 ± 0.04	2470 ± 93	36.06± 0.04	12.09 ± 0.16	0.17 ± 0.02	0.11 ± 0.01

Impact of *Amphiura filiformis* on nutrient cycling

Nitrate, nitrite and ammonium. *Amphiura filiformis* presence and density did not alter ammonium flux under normocapnic conditions (Figure 4.1), while the flux of nitrite out of the sediment was significantly increased (Table 4.2) as *A. filiformis* density increased. The flux of nitrate from the sediment into the water column increased with density of *A. filiformis* individuals present (Figure 4.2).

Silicate and phosphate. The sediment uptake of phosphate significantly increased with increasing *Amphiura filiformis* density (Table 4.2), while the sediment release of silicate remained stable and unchanged by the density of *A. filiformis* (Table 4.2, Figure 4.3).

Effect of pH on nutrient flux

A significant direct effect of pH on flux rate was only seen for nitrate (Figure 4.2, Table 4.2) where a decreasing pH caused a reduction in the uptake of nitrate to such an extent that the sediment changed from being a sink to become a source of nitrate between pH 7.3

and pH 6.8. None of the other nutrients measured (ammonia, nitrite, silicate & phosphate) responded directly to changes in pH (Table 4.2).

Impact of pH on the *A. filiformis*-nutrient flux relationships

There was a significant interaction between pH and *Amphiura filiformis* density exhibited for nitrate flux (Table 4.2). At pH 8.0 *A. filiformis* density had no effect on the flux. However, at reduced pH where sediment uptake of nitrate was suppressed, the presence of *A. filiformis* increased the positive flux, to some extent mitigating the suppression by lowered pH. At pH = 6.8 the sediment switched to become a source of nitrate to the water column; however, this nutrient loss was reduced with an increase in *A. filiformis* density (Figure 4.2).

Despite no direct effect of pH or *A. filiformis* density (under normocapnic conditions) on ammonium flux rate, a significant interaction effect was identified between these two main factors (Table 4.2); whilst no relationship between *A. filiformis* density and ammonium flux was seen in control or pH 7.7 treatments, increasing *A. filiformis* density caused increased ammonium release from the sediment at pH 7.3 and 6.8. Phosphate flux (Figure 4.3 a) showed no response to *Amphiura filiformis* density at control pH, whereas at pH 7.7 the sediment uptake increased with increased *A. filiformis* density, this relationship remained but was far weaker at pH 7.3, and the trend reversed at pH 6.8 where phosphate release from the sediment was enhanced at increased *A. filiformis* density. Silicate flux out of the sediment exhibited no discernable effect of *A. filiformis* density in the control, pH 7.7 or pH 7.3 treatments (Figure 4.3 b). In the pH 6.8 treatment there is a density effect, where flux of silicate out of the sediment increased with *A. filiformis* density.

Table 4.2. Effect of pH and *Amphiura filiformis* density on sediment nutrient flux determined using PERMANOVA analyses of two crossed, fixed factors, pH: treatment pH levels (control, 7.7, 7.3, 6.8), De = A. filiformis density (0, 4, 8, 12, 16 individuals per core). Significant pseudo P values (to 0.05 significance) shown in bold.

Nitrate						
Source	d.f.	SS	MS	Pseudo-F	P(perm)	perms
pH	3	908580	302860	57.987	**0.001**	999
De	4	68824	17206	3.2944	**0.011**	998
pH*De	12	154680	12890	2.4681	**0.008**	997
Res	151	788650	5222.9			
Total	170	1880800				
Nitrite						
Source	d.f.	SS	MS	Pseudo-F	P(perm)	perms
pH	3	81.014	27.005	0.30205	0.834	998
De	4	5335.8	1333.9	14.92	**0.001**	995
pH*De	12	400.29	33.357	0.37311	0.966	999
Res	151	13500	89.404			
Total	170	19461				
Ammonium						
Source	d.f.	SS	MS	Pseudo-F	P(perm)	perms
pH	3	25547	8515.6	1.455	0.217	999
De	4	26744	6685.9	1.1424	0.351	999
pH*De	12	140870	11740	2.0058	**0.022**	999
Res	151	883750	5852.7			
Total	170	1076800				
Silicate						
Source	d.f.	SS	MS	Pseudo-F	P(perm)	perms
pH	3	503330	167780	2.2124	0.069	999
De	4	500620	125160	1.6504	0.143	999
pH*De	12	1552900	129410	1.7065	**0.046**	999
Res	151	11451000	75835			
Total	170	13785000				
Phosphate						
Source	d.f.	SS	MS	Pseudo-F	P(perm)	perms
pH	3	843.61	281.2	1.6292	0.182	999
De	4	5786.9	1446.7	8.3821	**0.001**	999
pH*De	12	5154.6	429.55	2.4887	**0.009**	998
Res	151	26062	172.6			
Total	170	38131				

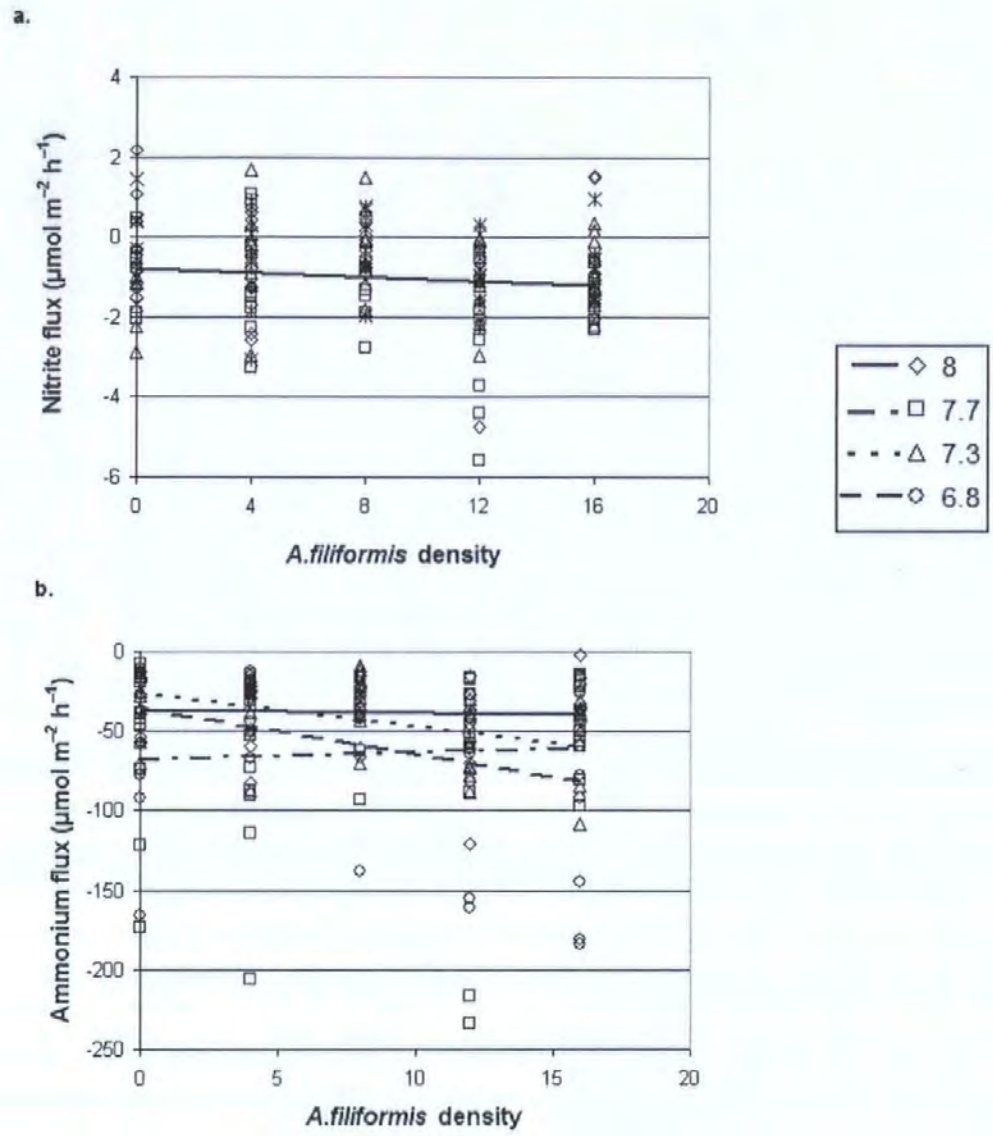


Figure 4.1. Relationship between *Amphiura filiformis* density (no. ind. / core) and **a)** nitrite (line indicates best linear fit for the entire dataset) and **b)** ammonium flux rate (best linear fit lines fitted for each pH).

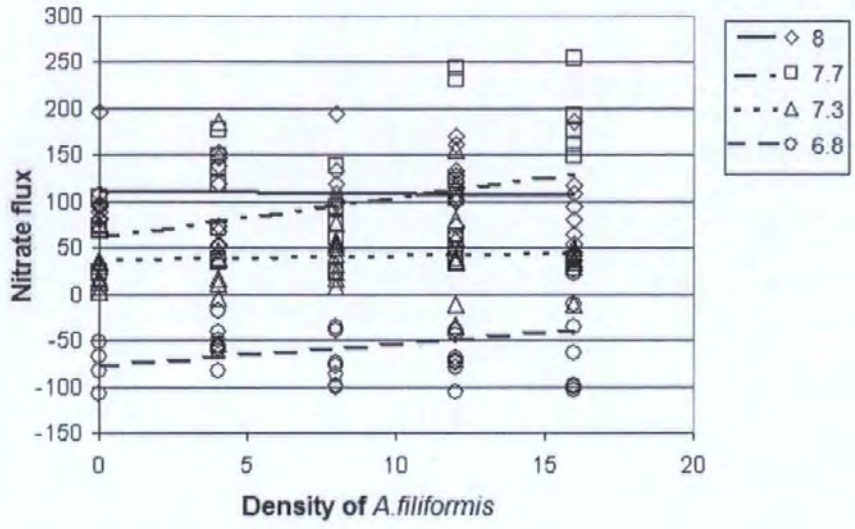
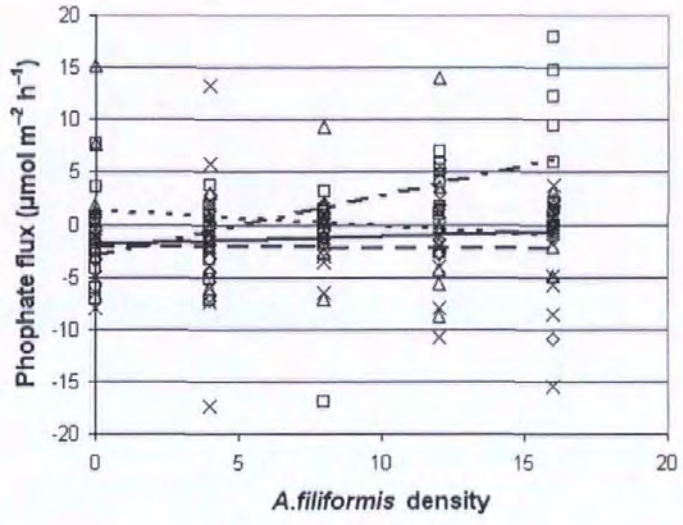


Figure 4.2. Relationship between *Amphiura filiformis* density (no. ind. / core) and nitrate flux, (best linear fit lines fitted for each pH).

a.



b.

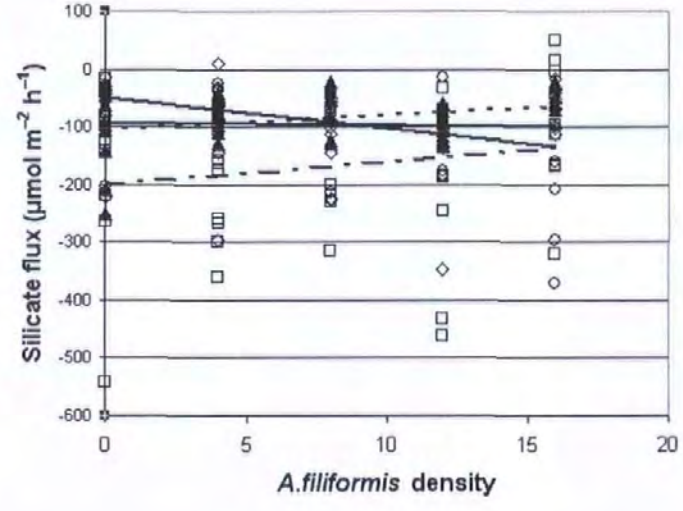


Figure 4.3. Relationship between *Amphiura filiformis* density ((no. ind. / core) and **a)** phosphate and **b)** silicate flux rate (best linear fit lines fitted for each pH).

DISCUSSION

This study has shown that *Amphiura filiformis* can be considered as an ecosystem engineer in modifying sediment nutrient cycling. The study has also demonstrated that changing seawater pH can have a significant effect on this organism's ecosystem engineering activities by altering the relationships between *A. filiformis* density and sediment nutrient fluxes.

Impact of *Amphiura filiformis* on nutrient cycling

Nitrate, nitrite and ammonium. In normocapnic conditions (control pH) there was no significant effect of *Amphiura filiformis* density on nitrate flux. This could imply, therefore, that neither nitrification nor denitrification were affected, or, more likely nitrate production was enhanced through burrow creation but this response was masked by a concomitant increase in nitrate sediment uptake; because the flux results obtained by the methods utilised in this study represent only net changes, an increase in both processes will therefore not be reflected in the results. The greater surface area provided by increased animal density also elucidates the significant increase in sediment nitrite release with increasing animal density; previous studies have shown burrows contain equal, and at certain depths greater, numbers of nitrite oxidising bacteria than the sediment surface (e.g. Satoh et al 2007) thus the greater the number of *A. filiformis* and therefore burrows present in a core, the more nitrification and denitrification will occur. While neither animal density nor pH significantly altered ammonium release from the sediment, the interaction between these factors was significant. This result suggests that the level of statistical discrimination available within each independent factor was insufficient to demonstrate a significant relationship between ammonium flux and either animal density or pH. However, the extra statistical power generated when comparing across both factors (12 d.f. compare with 3 d.f.

for pH and 4 d.f. for density) is sufficient to demonstrate that the biological control of ammonium flux was dependant on seawater pH. This indicates the importance of *A. filiformis* bioturbation on ammonium fluxes is greater under high CO_2 conditions.

Silicate and phosphate. Increasing levels of CO_2 can intensify and / or alter the relationship between *A. filiformis* density and the sediment flux of phosphate and silicate.

Phosphate flux significantly changed with density of *Amphiura filiformis* switching the sediment from a source to a sink. The transport of phosphate from water to sediment can be influenced by sorption. Our results suggest that the density dependant increase in phosphate uptake results from sediment oxygenation as a result of increased bioirrigation and therefore increased oxic adsorption of phosphate ions; the oxidised burrow walls of many burrows form an insoluble iron-manganese compound upon which sorption readily occurs (Aller 1980). Indeed, for *Nereis virens,* increased worm density resulted in increased sediment P uptake (Clavero et al 1994). It is also probable that microphytobenthos (MPB) utilises some phosphate; with more surface area and, with the presence of more burrows, the activity of MPB will also increase with animal density (Tang & Kristensen 2007). Thus, sediment P uptake was influenced by burrow, and in this case *A. filiformis*, presence (but not pH), as long as these brittlestars are able to maintain their burrows. Regardless of potential changes to irrigation rate, these phosphate data provide indirect evidence that *A. filiformis* irrigation function is not completely halted by lowered pH, for if this were the case a pH change to phosphate sorption should have been observed.

In the current study, silicate exhibited a consistent release from the sediment; a balance between oxic precipitation and silicaceous waste production by infauna. This is consistent

with other publications indicating a steady release of silicate from the sediment. (e.g. van der Loeff et al 1984; Tengberg et al 2004). Silicate flux initially appeared independent of both pH and *Amphiura filiformis* density treatments, suggesting *A. filiformis*, and its associated bioturbation and burrow creation played little role in silicate cycling. If the impact of *A. filiformis* on phosphate flux was due to oxygenation of the sediment and thus enhanced deposition then a change in silicate flux in response to changing animal density would have been expected. However, *A. filiformis* is likely to spend a proportion of its time deposit feeding on silicate rich sediment and therefore excreting silicate rich waste. This type of biological impact on silicate efflux has previously been suggested for other deposit feeders in ocean acidification studies; *Nereis virens* (Widdicombe & Needham 2007) and *Echinocardium cordatum* (Widdicombe et al 2009). Therefore, the net flux of silicate will be a balance between the oxic precipitation of silicate (uptake) and the excretion of silicate rich waste (release), both of which could increase with *A. filiformis* density. It is possible therefore that *A. filiformis* had a significant effect on both uptake and release processes but the overall net effect resulted in no significant change.

Effect of pH on nutrient flux

The significant effect of pH on nitrate flux was clearly visible as a reduction in sediment uptake demonstrated by the differing flux values when brittlestars were absent across pH treatments. The net sediment uptake or release of nitrate is mediated by the balance between a host of bacterially mediated biogeochemical processes; coupled nitrification - denitrification, dissimilatory nitrate reduction to ammonium and anammox. Consequently, the changes in nitrate flux observed in the current study could results from pH induced impacts on any one, or indeed all, of these processes. Currently, data which describe the response of these key processes to seawater acidification are limited. Huesmann et al (2002) reported decreased nitrification at lowered pH with a 50 % reduction at pH 7.0 and

90 % at pH 6.5; however, while denitrification bacteria are known to be influenced by pH (e.g. Knowles 1982; Lin & Shieh 2006) there are few data on pH ranges of marine sediment denitrifiers- most knowledge of *in situ* optima are derived from terrestrial soil systems (e.g. Bothe et al 2000). Based on previous results from Widdicombe & Needham (2007) and Widdicombe et al (2009), sediment uptake of nitrate would be expected to increase. In addition to fuelling bacterial pathways, nitrate in sediments can also be utilised by the microphytobenthos (MPB), thus altering N levels within the sediment (Lorenzen et al 1998). So any apparent change in nitrate uptake by the sediment in response to elevated levels of CO_2 could have resulted from a combination of bacterial and microalgal processes. The current study has shown that changes in seawater pH can have a significant effect on the net flux of nitrate across the sediment water interface. It is now imperative that further investigations identify the individual biogeochemical responses that underlay these net effects.

Net nitrite production in the sediment used for the current study was not shown to be affected by lowered pH over the timescale used here. In addition, net ammonium flux did not change as a result of pH, indicating that the bacterial processes which breakdown organic material and produce ammonium were not influenced by CO_2-related acidification hypercapnia. Alternatively, and as discussed for nitrate flux, the opposing processes responsible for ammonium generation and degradation could have been affected proportionally, resulting in no net change in flux. Again, this highlights the need to examine more closely the individual processes within the N cycling before predictions of net flux are possible.

Neither phosphate nor silicate fluxes were altered by CO_2-induced acidification alone. However it is likely that the effect of pH is hidden by the significant interaction between pH and *A. filiformis* density which is discussed further in the following section.

Impact of pH on the *A. filiformis*-nutrient flux relationships

Nitrate flux exhibited a significant interaction between pH and *Amphiura filiformis* density. In this case the presence of *A. filiformis* mitigated the suppression of nitrate uptake at lowered pH to some extent; at pH 7.7, returning the sediment nitrate consumption to control levels in the highest density cores. Increased *A. filiformis* density increased the positive flux of nitrate at pH 7.7 and pH 7.3. In the lowest pH treatment (pH 6.8) the flux became negative i.e. a source of nitrate to the water column. However, this release of nutrients from the sediment was reduced with increasing *A. filiformis* density. The significant positive influence on flux into the sediment (or in the case of the pH 6.8 treatment a reduction in the loss of nitrate from the sediment) could be a result of the burrow environment created by the *A. filiformis* presence; burrow irrigation and sediment bioturbation generally increases the supply of nitrate into the sediment environment where diffusive gradients cause absorption into the sediment where denitrification then occurs (Banta et al 1999). As such, the greater the density, the more irrigation and bioturbation and therefore sediment nitrate uptake. When the slope of the density vs. flux plots are examined it is seen that the influence of the *A. filiformis* density is greater in the pH 7.7 treatment than pH 7.3 and pH 6.8; this may be due to an optimal scenario whereby increased oxygen consumption at lowered pH reduces sediment oxidisation, yet muscle wastage is minimal thus irrigation continues to supply nitrate to the sediment. Both increased oxygen consumption and arm muscle wastage are physiological responses recorded in *A. filiformis* as a result of exposure to CO_2-related acidification (Wood et al 2008, Chapter 2). However, the effect of muscle wastage on irrigation capability and rate requires further investigation. The significant interaction between both pH and density was attributable to the differing influence of density which had no effect on the flux at control pH, but a significant effect at lowered pH as described above, This same pattern is also

seen in the significant interaction between pH and density for ammonium flux; where increasing *A. filiformis* density increases ammonium release from the sediment in the lowest two pH treatments but not at control pH or pH 7.7. While there is no significant interaction between pH and *A. filiformis* density seen in nitrite flux, it is likely, as previously mentioned, that a down regulation of both nitrite production and consumption has prevented such a change being seen when only net flux is recorded. Overall the interaction of pH and density effects on nitrate and ammonium fluxes indicate that the biological control (through bioirrigation) of the nitrogen cycle nutrients across the sediment-water boundary may become even more important in a high CO_2 future.

Phosphate and silicate, both of which rely on similar sediment-water exchange mechanisms, displayed a significant interaction between pH and density whereby density had no visible effect on fluxes at control pH. At pH 7.7 phosphate flux increased with increased *Amphiura filiformis* density whilst silicate flux, showed little effect of density at this level of pH. As pH decreased further no density effect is observed at pH 7.3 for either silicate or phosphate. By pH 6.8 both nutrients show a density effect, with increased *A. filiformis* density increasing the flux of both silicate and phosphate out of the sediment. The biological explanation for this response to both pH and *A. filiformis* density of phosphate flux could be attributed to changes in *A. filiformis* bioirrigatory function; as previously stated the increased sediment surface area in the higher density treatments, as a consequence of burrow presence, results in both a larger surface area for sorption of phosphate into the sediment. Both factors therefore explain the density dependant increase in sediment phosphate uptake seen slightly at control and more so at pH 7.7. The stronger response at pH 7.7 is probably a reflection of the previously documented (Wood et al 2008, Chapter 2) increased oxygen demand of *A. filiformis* at lowered pH which is expected to result in an increased rate of burrow irrigation and therefore supply of phosphate to the burrows where sorption occurs. The decreasing strength of this density response at pH 7.3

could reflect an inhibition of MPB production and therefore reduced demand for phosphate. By pH, 6.8 where sediment release of phosphate increases with *A.filiformis* density, MPB uptake of phosphate appears greatly diminished and given the documented muscle wastage within *A. filiformis* at low pH (Wood et al 2008) (Chapter 2) it is probable that burrow irrigation, while continuing, had slowed in rate.

The flux of silicate out of the sediment showed little impact of *Amphiura* presence at control pH, 7.7 or 7.3, however at pH 6.8 the flux of this nutrient out of the sediment increased. Given that silicate release from the sediment is a balance between oxic precipitation and silicaceous waste these data suggest that until pH 6.8, any increase in precipitation into the sediment as a result of the burrow surface area and irrigation due to *A. filiformis* is tempered by increased silicaceous waste produced by the presence of the same *A. filiformis.* This compensation in response breaks down at 6.8 whereby an increase in silicate release from the sediment is seen with increased *A. filiformis* density. As with phosphate, this suggests the rate of burrow irrigation has decreased as a result of arm muscle wastage so that while the brittlestars are still producing silicaceous waste they are not facilitating oxic precipitation.

Seasonal variation and importance of bioturbating species in regulating nutrient flux

Interestingly there was no evidence of nitrification inhibition at lowered pH as observed by Huesmann et al (2002) however; their data were derived from the response of nitrification in open water. Such work does not take into account the many contributory factors of nutrient supply and use which occur at the exchange between the sediment and overlying water. A perhaps more relevant benthic nutrient flux study into the effects of pH and macrofaunal presence by Widdicombe & Needham (2007) demonstrated a mixed response of flux to *Nereis virens* and pH, with some nutrients affected more than others. The basic

sink/source properties were the same as the sediment used in this study. The impact of *N. virens* on nitrite and nitrate flux were the same as found here for *Amphiura filiformis* strengthening the concept of that ecological function i.e. burrow building was more important than the identity of the burrower. In addition, the responses of silicate and ammonium fluxes to changing pH and *A. filiformis* density were similar to the density affect of *N. virens*. But *N. virens* had no influence on phosphate uptake but both density and the interaction of pH and density affected phosphate uptake; phosphate fluxes in this earlier study were approximately 70 times greater than the current study. The most contradictory results between these similar studies however, was that of nitrate. Whereas this present study found nitrate uptake was significantly reduced by pH, Widdicombe & Needham (2007) recorded a significant increase while the burrowing animal influence alone on nitrate flux was comparable between the two experiments. The difference cannot be attributed to species identity as the distinctive patterns were still present with no animals present, so additional explanations must be sought. One possibility could be that while the sediment in each study was from the same location, the sediment used in the current study was collected in early January whereas Widdicombe & Needham (2007) collected their sediment in June; when temperature and nutrient supply are dramatically different. Such seasonal differences have been documented in previous cases to result in a change in microphytobenthos (MPB) community structure (Hillebrand & Sommer 1999) and increases in benthic microalgae can significantly reduce coupled nitrification-denitrification (Risgaard-Petersen 2003). Consequently, the different observations presented by the two studies could be due to seasonal changes in the influence of MPB on nutrient cycling. It should also be considered that the nitrifying bacterial communities in the two studies were likely to be comprised of different species due to seasonal succession. The disparity in response, in particular with respect to nitrate, highlights the need to consider sediment nutrient flux and indeed all ecosystems in a holistic manner that

incorporates, or at a minimum considers results in the context of, temporal variability and any micro-organism level changes this may incur.

Conclusions

The results presented here confirm that *Amphiura filiformis* are important bioturbators that affect nutrient flux. The role of *A. filiformis* in nutrient cycling is not necessarily unique; rather a function of their bioturbation and burrow irrigation activities. It is probable that high densities (often > 100 ind. m^2 but up to 3000 ind. m^2 have been recorded, Rosenberg 1995) and in particular constant burrow irrigation, results in their dominance as a bioturbator and ecosystem engineer in soft sediments.

Physiological changes to *Amphiura filiformis* as a result of ocean acidification can impact on nutrient fluxes indirectly by changing the bioirrigatory activity of *A. filiformis* either directly through an increased demand for oxygen or food, or indirectly by muscle wastage that reduces the capacity to bioirrigate. In the case of nitrate at the intermediate altered pH of 7.7 the presence of *A. filiformis* even mitigates the effect of pH creating a situation whereby the biological control of nutrient flux is enhanced. It is still not clear whether arm movement is impaired in *A. filiformis* as a result of CO_2-related, muscle wastage; however the changes to both silicate and phosphate flux at pH 6.8, where the greatest muscle wastage is seen, support this. If muscle atrophy is ongoing, it would only be a matter of time before the brittlestar loses its feeding /irrigating ability and dies; in which case the question is can another macrofaunal burrower fill the same nutrient cycling niche? Most common bioturbators irrigate sporadically rather than continuously like *A. filiformis* and what difference this has will require further investigation.

Net nutrient exchange with the overlying water is an important sediment function. Through characterising the impact of *Amphiura filiformis* on this net nutrient exchange, and documenting how ocean acidification changes this relationship, this study has also highlighted the extent to which elevated levels of CO_2 can affect the important relationships which exist between the key components driving ecosystem function. It is also necessary to appreciate the manner by which these relationships vary naturally through space and time.

CHAPTER FIVE

Synergism of ocean acidification and temperature; the high cost of survival in the brittlestar *Ophiura ophiura*

Aspects of this Chapter are included in:

Wood H L, Spicer J I & Widdicombe S Synergism of ocean acidification and temperature; the high cost of survival in the brittlestar *Ophiura ophiura. J. Exp. Mar. Biol. Ecol. (In Review)*

ABSTRACT

This chapter investigates the synergistic effect of increased ocean acidity and temperature (40 days exposure) on a number of key physiological parameters in the epibenthic ophiuroid brittlestar, *Ophiura ophiura*. Metabolic upregulation is seen in response to lowered pH at low temperatures; however this is far outweighed by the response to elevated temperature (+ 3.5°C). This increased temperature is accompanied by an energetic trade off at low pH to maintain net calcification in undersaturated waters where dissolution of calcium carbonate occurs. This energy deficit results in a ~30% reduction in the rate of arm regeneration and decreased rate of movement. This understanding of how *O. ophiura* responds to ocean acidification, taking into account a synergistic effect of temperature, suggests that fitness and survival will indirectly be reduced through increased vulnerability to predation and slower recovery from arm damage.

INTRODUCTION

An energetic trade off resulting in muscle wastage has been found in the ophiuroid brittlestar *Amphiura filiformis* (Chapter 2) and a significant interaction in response to increasing seawater temperature and acidity (Chapter 3). However, *A. filiformis* is unusual within the ophiuroid group in that it is an infaunal suspension feeder whilst the majority of ophiuroid species are epibenthic and generalist feeders. Is the energy deficit seen in *A. filiformis* an artefact of is specialist and costly burrowing life strategy, or representative of the survival issues ophiuroids, and even echinoderms as a group will face in future more acidic oceans? To test this, we carried out an acidification exposure on the epibenthic brittlestar species *Ophiura ophiura* similar to the aforementioned experiments on *A. filiformis* (Chapters 2 & 3).

Despite being one of the most common ophiuroid species found on subtidal temperate sediments, and its documented ecological importance in the North Sea (Dahm 1993), knowledge of the general biology and ecophysiology of *Ophiura ophiura* is scarce. Mortensen (1927) made the first study of growth rate, later readdressed by Dahm (1993), and in 1966 Fell described the species' predilection for soft sediment habitats. While some aspects of the nervous system have been investigated in relative detail (e.g. Ghyoot et al 1994), ecophysiological information is limited to an evaluation of the behavioural response to chemical cues (Moore & Cobb 1985; Valentincic 1991).

In this present study the same physiological parameters were measured as in the *A. filiformis* exposure where muscle wastage was documented; metabolism, calcification, arm regeneration. In addition, the analysis of arm structure was changed from the qualitative measure used in Chapter 2, to quantitative methodology so that any changes to muscle

structure and coverage could be statistically tested. In light of subsequent publications, and the progression of the ocean acidification community to recognise the increasing need and value of multi-factorial experiments, temperature was added as a second variable to investigate synergies in the physiological response to both temperature and acidification.

The control pH treatments from this acidification study have been used to characterise the physiology of this species, and by choosing a high temperature treatment within the range currently experienced by this brittlestar, this study not only give an insight into the synergies of increasing temperature and acidity, but also provide a comparison of the physiology between summer and winter under present day normocapnic conditions. The brittlestars were exposed for a period of 40 days to one of three pH treatments: a control of pH 8.3, and lowered pH of 7.7 and 7.3. The experiment was repeated at two temperatures: firstly at winter ambient of 10.5 °C (low) and then at summer temperature of 15 °C (high).

METHODS

Location

The experiments in this study were carried out in the mesocosm facility at Plymouth Marine Laboratory, Plymouth UK.

Animal and sediment collection

In May 2008, 96 individuals of *Ophiura ophiura* were collected from a depth of ~6 m in Cawsand Bay, Plymouth Sound (50° 09.770N, 4° 11.500W) using a naturalist dredge. Intact brittlestars (those with 5 intact arms) were carefully removed from the dredge net by hand and placed in buckets (vol. = 5 l, 10 indiv. /bucket) with freshly collected seawater (S = 34.5 PSU, T = 10.5 °C). Muddy sand sediment was collected on the same day from the same location using a 0.1 m^2 van Veen grab. The sediment was sieved over a 2 mm mesh to remove all large fauna. The brittlestars and sieved sediment were transported back to the PML mesocosm within 4 h of collection. Once in the mesocosm a 7 cm layer of the sieved sediment was placed into each of six large (76 x 76 x 15 cm) experimental trays then filled with filtered (10 µm) seawater. After the sediment had settled (approx. 1 h) brittlestars were transferred to these trays (18 indiv. / tray). Each tray was supplied with natural seawater (S = 34.5 PSU, T = 10.5 °C) at a rate of 100 l / min. These individuals were used for the first lower temperature exposure experiment. In July 2008, an additional 96 *Ophiura ophiura* individuals and sieved sediment were collected and prepared exactly as described above, where the seawater temperature was 15 °C. This material was used for the second, higher temperature exposure experiment. The two temperature experiments

were conducted sequentially rather than concurrently in order to investigate the different physiological characteristics and responses of winter and summer condition individuals.

Arm Amputation

Only individuals with no visible sign of recent regeneration were selected for each experiment (N = 96). The brittlestars were assigned to one of the 12 Length Lost (LL) x pH treatments (pH 8.0, 7.7 or 7.3 and LL = 10/20/30/40 mm or Complete, i.e. no amputation) (N = 6/pH treatment). For each individual within the LL groups, the point of amputation from the arm tip was determined using a simple calculation based on the length of the complete arm:

Position of cut from tip = length of arm (mm) – A*

* where A = the length lost (mm) to be removed.

Prior to amputation the brittlestars were anaesthetized by submersion in 3.5 % w/w $MgCl_2$ in artificial sea water for a minimum of 3 min. Amputation was carried out by on the first arm clockwise to the madreporite by placing pressure with a scalpel blade between the arm segments at the appropriate distance from the disk. 'Complete' brittlestars were anesthetised but no amputation made.

Experimental Setup

Following amputation, disk diameter, longest arm length and length of remaining arm were recorded and the brittlestar left to recover from anaesthesia in one of the two replicate experimental trays of that treatment (18 indiv./tray). Recovery from the anaesthetic was typically 15 – 40 min. All treatment trays were supplied with seawater of ambient

temperature and pH (T = 10.5 / 15 °C, pH = 8.0) at this initial stage. Twelve hours after amputation the seawater supply to the experimental trays was switched to the appropriate pH header tank resulting in the trays reaching target pH after 12 hours. This was considered Day 0 for the exposure experiment. Brittlestars were fed every two days on a commercial algal feed (Shellfish Diet 1800, Instant Algae) supplied at 37 000 cells.min^{-1}.

Seawater acidification

In both experiments, seawater pH was adjusted and maintained using a computerized control system which regulated the addition of gaseous CO_2. Seawater was acidified in header tanks (vol. = 450 l) where the acidified seawater supplied to the aquaria was replaced by an equal volume of untreated seawater supplied from a large supply tank (vol. = 15000 l). Seawater acidification followed very closely the method described by Widdicombe & Needham (2007) whereby a pH controller (Webmaster) regulated carbon dioxide (CO_2) supply (delivered using metal spargers to achieve fine bubbles) to the header tanks *via* a solenoid. Three different pH treatments were used: 8.0 (control), 7.7 and 7.3. The control pH was set as 8.0 as this was the pH of the overlying water at the time of collection. The experimentally lowered pHs were chosen as the worst case scenario for the end of the century (pH 7.7) and a 2300 scenario of pH 7.3. All header tanks were set up and balanced to the desired pH level prior to animal collection; tubing to supply the experimental trays were connected at the experimental flow rate (10 ml/ min) and allowed to flow to waste from the experimental trays.

Measurement of carbonate parameters

Water pH_{NIST} and total carbon dioxide content (tCO_2), salinity and temperature (°C) of all experimental trays were monitored three times a week (summary: Table 5.1). tCO_2 was

measured from 100 µl subsamples of seawater using an automated carbon dioxide analyser (CIBA Corning 965 UK). pH_{NIST} was measured using a pH electrode (Mettler Toledo LE413) calibrated with NIST standardised buffers. Salinity and temperature were measured with a combined temperature- conductivity salinometer (WTW LF197) accurate to 0.1 °C (temp.) and 0.01 ppm (salinity). The remaining carbonate parameters were calculated using CO2SYS (Pierot et al 2006) with the constants supplied by Mehrbach et al (1973) refitted by Dickson & Millero (1987) and the KSO_4 dissociation constant from Dickson (1990).

Oxygen uptake

On Day 40 the six complete brittlestars (no amputation) from each treatment were placed in Perspex respiration chambers (vol. = 150 ml) with seawater from their exposure pH header tank. The brittlestars were allowed 30 min to acclimatise to being placed in the chambers. After this period the chambers were then sealed and left for two hours under dark conditions. The chambers were held within a larger tank supplied with water of the experimental treatment temperature to keep temperature constant. The oxygen content of the water was measured before and after the incubation period using an oxygen microelectrode (Strathkelvin Instruments SI130) linked to a calibrated meter (Oxygen Meter 781, Strathkelvin Instruments, Glasgow, UK). Oxygen saturation did not fall below 90 % in the chambers at any time. Control respirometers without brittlestars were also run exactly as described above in order to estimate background respiration rates by bacteria. In addition to correction for bacterial respiration, all data were corrected for barometric and water vapour pressures, the former measured on the day of the experiment. Oxygen consumption was calculated as µmol O_2 per mg of brittlestar wet mass per hour (µmol O_2 mg^{-1} h^{-1}).

Assessment of arm structure

Assessment of potential changes to the structure of arms intact prior to the experimental exposure (hereafter referred to as established arms) and arms regrown during the course of the experiment (hereafter referred to as regenerated arms) entailed examining muscle tissue and coverage, the calcium content of the arm as a proxy for calcification and the thickness of the epithelium, which is the organic matrix that covers the high-magnesium calcite mesoskeleton in echinoderms. After 40 days one established arm and the regenerated arm were removed from each brittlestar from the 40 mm LL class and preserved in Baker's formal calcium fixative. Once fixed, the arms were removed from Baker's fixative, placed in a glass beaker on an orbital shaker (slow) and decalcified using 40 ml Gooding and Stewart's decalcification fluid (5 % formic acid) for 18 hours. Following removal from the decalcification fluid the arms from each treatment were placed together into small glass vial (vol. = 20 ml) and dehydrated using a sequence of increasing ethanol concentrations (30 min in each of 50 %, 70 %, 95 % (repeated) and finally 95 % ethanol/ Monomer (1:1)). Arms were left in 2-hydroxyethyl methacrylate monomer overnight and then embedded in monomer with activator (Lewis & Bowen 1985) before being left to set for 24 h. Once removed from the monomer and air dried for 12 h, a glass knife was used to cut longitudinal sections through the arms which were then stained with Lee's methylene blue/basic fuchin. All arms were positioned flat within the methacrylate block thus ensuring the same plane of section was examined for each arm. This was verified under low power magnification (x 10). A central section was chosen for each arm, muscle shape and coverage was examined under low power magnification (x 10) then under high power magnification (x 60) the muscle nuclei within the microscope's field of vision (at x 60 magnification the muscle covered the whole field of view) were counted. This was repeated on five muscle blocks for each individual (arm) thus allowing a mean for each individual to be taken. Under medium power magnification (x 40 Reichert Polyvar

microscope) arm outer epithelium thickness was measured in a digital image provided by a microscope mounted camera (Optronics Magnafire model S99802). Using image analysis software (Image-Pro Plus v4.5 Media Cybernetics). Mean epithelium thickness for each individual (and established and regenerate arms) was calculated from three replicate measurements.

Motility

To complement the physiological measures of the arms described above in Assessment of Arm Structure, a physical measure of motility was taken. For each complete brittlestar in each treatment (N = 6/pH*temperature treatment) the righting time was recorded. Righting time is the time taken for the brittlestar to turn the right way up when placed upside down. This experiment was carried out within the experimental trays at the end of the experimental exposure (day 40). The overlying water depth was greater than the arm tip to tip diameter of the brittlestars so that lack of water did not influence the turnover time. Each brittlestar was turned over three times and an average righting time recorded to 0.01 of a second using a digital stopwatch which was started when the brittlestar was released upside down and stopped when all arms were flat on the surface the right way up.

Measurement of arm calcium & magnesium content

Calcification is defined as the deposition of calcium (and carbonate) ions into an organic matrix. Calcium carbonate, the mesoskeletal material of echinoderms, requires calcium and carbonate in equal amounts; therefore comparison of the amount of calcium between treatment arm samples can be used as a proxy to infer comparison of the total calcium carbonate between treatment arm samples. Any changes seen between treatments result from differences in calcification and/or dissolution rates over the course of the

experimental exposure, presuming that the individuals were randomly allocated to the treatments and thus the 'control' are representative of all individuals aside from changes caused by the experimental factors. Because echinoderm mesoskeletons are made of high-magnesium calcite the percentage of magnesium within the arm composition was also measured to look for changes. An established arm from the complete group (no surgical amputations) was removed from each brittlestar, rinsed in distilled water, frozen (-20 °C) then dried in a drying oven (60 °C). The methodology for determining calcium content closely followed the methodology used by Spicer & Eriksson (2003). The arm samples were weighed using a microbalance (Ohaus Adventurer AR0640 accurate to 0.0001 g), dissolved in nitric acid (vol. = 5 ml, 15 % pro analysis) over a gentle heat to aid digestion. The dissolved samples were then diluted with distilled water to achieve a concentration within the range of the optical emission spectrometer (Varian 725-65 ICP accurate to 1- 2 % RSD) used to determine calcium and magnesium concentrations. Results were expressed as percentage calcium (or magnesium) mass of arm mass.

Arm regeneration

These analyses were carried out on the 30 brittlestars (per pH treatment at each temperature) that made up the six LL regeneration classes. After 40 days the regenerated arm was photographed using a digital Nikon Coolpix 995 camera mounted on a Wild Heerbrugg model 157940 optical microscope. Each individual was identified using a combination of disk diameter, longest arm length and length of amputated arm, and the individual's I.D. recorded with the corresponding photograph number. The arm regenerate was clearly discernable from the existing arm as it was considerably smaller in diameter and lighter in coloration. The total length of regenerate and differentiated regenerate (which represents functional regenerate, Dupont & Thorndyke 2006) were measured using image analysis software (Image-Pro Plus v4.5 Media Cybernetics) calibrated with scale

photographs taken on the same microscope as the arm regenerate pictures. The average regeneration rate over the experimental period was calculated by dividing the total regenerate length by the exposure period in days. The amount of arm regrown and with functional capacity (functional regenerate) was calculated by dividing the differentiated length by the exposure period in days. This method does not allow for the slower growth rate initially following amputation, however, it gives a good approximation of rate, and importantly allows for a comparison between treatments

Statistical analysis

Parametric data analyses were run using Minitab 14. Non-parametric data were analysed using the PERMANOVA+ routines (beta version, Anderson et al 2008), which are an 'add-in' to the PRIMER 6 software. Homogeneity of variance was tested with the Kolmogorov-Smirnov Test.

Two-way analysis of variance (ANOVA) was used to test for effects of pH and temperature on epithelium thickness and calcium and magnesium content of arms. Rate of oxygen uptake, motility (righting time), muscle structure (# nuclei) and arm regeneration data (% recovered and % functional) were not normally distributed so were analysed using the permutational MANOVA (PERMANOVA) procedures introduced by McArdle & Anderson (2001) and Anderson (2001). These procedures make more restrictive assumptions than a fully non-parametric approach, but crucially the multivariate PERMANOVA method operates on a similarity matrix and avoids unrealistic normality (or other distributional) assumptions. It does this by exploiting permutation to generate null hypothesis distributions for its pseudo-F statistics; the latter constructed by exact analogy with the standard F statistics for corresponding univariate ANOVA designs.

RESULTS

Experimental conditions

The water carbonate parameters remained stable throughout the experiment both within the header tanks and the experimental trays (Table 5.1). The seawater was undersaturated with respect to aragonite at pH 7.7 in both temperature treatments of 10.5 °C (low) and 15 °C (high). The sediment within the experimental trays appeared healthy based on the oxygenated colour of the surface.

Table 5.1. Water parameters (mean ± 95 % confidence intervals) calculated from samples taken from all experimental trays across the experimental period. T treatment indicates the temperature treatment; High (HT) refers to 15 °C and Low (LT) to 11.5 °C. pH treatments refers to the target pH, Sal = Salinity (psu), T = temperature (°C), TA = Total Alkalinity ($mEq.L^{-1}$), Ω_{ca} = Omega calcite, Ω_{ar} = Omega aragonite.

pH	Temp.	pH	TA	Sal	T	Ω_{Ca}	Ω_{Ar}
8	LT	7.99 ± 0.03	2105 ± 54	34.46 ± 0.07	10.35 ± 0.3	2.14 ± 0.2	1.36 ± 0.1
	HT	7.95 ± 0.03	1955 ± 241	34.1 ± 0.3	15.18 ± 0.2	2.11 ± 0.3	1.35 ± 0.2
7.7	LT	7.62 ± 0.02	2120 ± 132	34.5 ± 0.4	10.75 ± 0.3	0.99 ± 0.1	0.63 ± 0.06
	HT	7.66 ± 0.02	2052 ± 238	34.1 ± 0.3	15.02 ± .01	1.12 ± 0.2	0.77 ± 0.1
7.3	LT	7.38 ± 0.02	2193 ± 132	34.5 ± 0.07	10.28 ± 0.2	0.60 ± 0.1	0.38 ± 0.03
	HT	7.42 ± 0.03	2024 ± 198	34.05 ± 0.4	14.76 ± 0.1	0.69 ± 0.1	0.44 ± 0.06

Mortality & Fecundity

Survival was 100 % at both temperatures and across all pH treatments. Gametes were not visible through the underside of the disk, or through the mouth opening in either of the two experiments or over the course of the experiment. In addition disk size did not alter

between start and end of the experimental exposure, which is a documented phenomenon related to reproductive state in some ophiuroids (O'Connor et al 1983).

Metabolism

Rate of oxygen uptake, used as a proxy for metabolic rate, was found to increase at lowered pH in the 10.5 °C (low) temperature treatment. However, in the high (15 °C) treatment there was no significant difference in oxygen uptake across the pH treatment (Figure 5.1). There was a significant interaction (Table 5.2) between temperature and pH, due to a pH effect seen at low temperature but not in the high (15 °C) treatment where oxygen uptake was significantly greater than at the low temperature (Table 5.2, Figure 5.1).

Arm structure

In established arms, no voids were visible around the muscle sections, and the area of muscle appeared visually unchanged across both temperature and pH. Quantitative analysis of muscle density showed the same, with no significant difference in muscle density between temperature and pH treatments (Table 5.3). Regenerated arms had significantly lower density muscle than established arms (Table 5.3). A significant interaction between temperature and type of arm ('regen') can be attributed to the switch in higher muscle density from low temperature to high temperature between established and regenerated arms (Figure 5.2). There was no significant effect of pH on the muscle density in either regenerated or established arms Table 5.3). Epithelium thickness did not differ across either temperature or pH factors, nor between established and regenerated arms (Table 5.4).

Motility

Within the low temperature treatment righting time decreased at pH 7.3, however, in the high temperature treatment the righting time increased at pH 7.3 (Figure 5.3) resulting in a significant interaction between temperature and pH (Table 5.5). Righting time was faster at the higher temperature at pH 8 and 7.7, and faster at the low temperature at pH 7.3 (Figure 5.3).

Calcification

The mesoskeleton contains an average of 86 % calcium carbonate and 4 % magnesium carbonate, leaving the remaining 10 % to be comprised of organic material. The amount of calcium carbonate and magnesium was altered by neither temperature nor pH (Table 5.6), thus the brittlestars across all treatments had the same net calcification throughout the experiment.

Arm regeneration

Regeneration and functional recovery (differentiation) were significantly affected by temperature, pH and LL (Table 5.7). The rate of regeneration (RR) within the low temperature treatment was unaffected by the length of arm lost (Figure 5.4), and the rate was similar between all pH treatments. At the high temperature regeneration rate was significantly faster than at the low temperature. The significant effect of length lost is due to the LL dependent increase of RR. However this is an artefact of the way in which RR is calculated; for all high temperature pH treatments the full LL was recovered within the 40 day exposure for the 10 and 20 mm LL classes. Thus the regenerate length from which rate was calculated stopped at 10 or 20 mm resulting in a lower RR being calculated. Neither

30 nor 40 mm in length was reached by any pH treatment therefore these give a better representation of RR. Within the high temperature treatment pH 7.3 RR was lower than the control and pH 7.7, which were not significantly different from each other (Figure 5.4). There was little difference between the rate of regeneration (RR) and functional recovery (differentiation rate) and the same pattern was seen as for RR (Figure 5.5).

Table 5.2. PERMANOVA results for two-way analysis of oxygen uptake. The two factors were temperature (temp) and pH. Significant results indicated in bold.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	1.8792	1.8792	135.67	**0.0001**	9810
pH	2	0.44168	0.22084	15.944	**0.0002**	9953
temp*pH	2	0.35562	0.17781	12.837	**0.0005**	9961
Res	26	0.36013	1.39E-02			
Total	31	3.1223				

Table 5.3. Three-way PERMANOVA indicating the effect of temperature (temp), pH and type of arm (regen) on arm muscle density. Density measured as number of nuclei in a standardized area of central section muscle tissue, examined in cross section. Significant results indicated in bold.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	1.33E-03	1.33E-03	8.66E-02	0.77	9833
pH	2	5.35E-02	2.67E-02	1.7356	0.1849	9951
Re	1	4.3823	4.3823	284.56	**0.0001**	9847
temp*pH	2	9.63E-02	4.81E-02	3.1252	0.0528	9931
temp*regen	1	9.37E-02	9.37E-02	6.083	**0.0173**	9850
pH*regen	2	2.65E-02	1.33E-02	0.86186	0.4304	9955
temp*pH*regen	2	1.50E-03	7.50E-04	4.87E-02	0.9565	9951
Res	47	0.72383	1.54E-02			
Total	58	5.3019				

Table 5.4. Three-way ANOVA indicating the effect of temperature (temp), pH and type of arm (regen) on arm epithelium thickness.

Source	d.f.	Seq. SS	Adj. SS	Adj. MS	F	P
Regen	1	1.127	2.154	2.154	0.84	0.365
Temp.	1	0.551	1.055	1.055	0.41	0.526
pH	2	3.046	2.942	1.471	0.57	0.569
Regen*Temp.	1	1.59	1.019	1.019	0.4	0.533
Regen*pH	2	9.01	9.731	4.866	1.89	0.163
Temp.*pH	2	1.254	1.06	0.53	0.21	0.815
Regen*Temp.*pH	2	0.643	0.643	0.321	0.12	0.883
Error	48	123.826	123.826	2.58		
Total	59	141.047				

Table 5.5. Two-way PERMANOVA indicating the effect of temperature (temp. and pH on mobility, measured as the time (sec) taken to right when turned upside down. Significant results indicated in bold.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	3.03E-03	3.03E-03	8.26E-02	0.7765	9850
pH	2	9.75E-02	4.87E-02	1.3281	0.2878	9962
temp*pH	2	0.48013	0.24007	6.5432	**0.0035**	9967
Res	28	1.0273	3.67E-02			
Total	33	1.6451				

Table 5.6. Two-way ANOVA indicating the effect of temperature (temp), pH and arm type (regen) on the mineralogical structure of the arm mesoskeleton. **a.)** Calcium content, which can be used as a proxy for calcium carbonate, and **b.)** Magnesium content.

a.

Source	d.f.	Seq. SS	Adj. SS	Adj. MS	F	P
temp	1	0.1514	0.13	0.13	0.23	0.634
pH	2	0.3236	0.3084	0.1542	0.27	0.764
regen	1	0.0311	0.0423	0.0423	0.07	0.786
temp*pH	2	0.0951	0.0768	0.0384	0.07	0.935
temp*regen	1	0.0901	0.0973	0.0973	0.17	0.681
pH*regen	2	0.8686	0.8971	0.4486	0.79	0.459
temp*pH*regen	2	0.4474	0.4474	0.2237	0.39	0.677
Error	70	39.8689	39.8689	0.5696		
Total	81	41.8761				

b.

Source	d.f.	Seq. SS	Adj. SS	Adj. MS	F	P
temp	1	0.2596	0.2304	0.2304	0.4	0.527
pH	2	0.3511	0.3331	0.1665	0.29	0.748
regen	1	0.0271	0.0378	0.0378	0.07	0.798
temp*pH	2	0.1061	0.0862	0.0431	0.08	0.927
temp*regen	1	0.1246	0.1332	0.1332	0.23	0.63
pH*regen	2	0.9614	0.9909	0.4954	0.87	0.424
temp*pH*regen	2	0.4475	0.4475	0.2237	0.39	0.677
Error	70	39.9204	39.9204	0.5703		
Total	81	42.1977				

Table 5.7. Two-way PERMANOVA indicating the effect of temperature (temp), pH and length lost (LL) on a.) the length of arm regenerated over 40 days, and b.) the length of regrown arm functional (differentiated) after 40 days. Significant results shown in bold.

a.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	43.463	43.463	32.062	**0.0001**	9822
pH	2	8.9954	4.4977	3.3179	**0.0392**	9940
LL	3	16.125	5.375	3.9651	**0.0107**	9951
temp*pH	2	4.5901	2.295	1.693	0.1902	9948
temp*LL	3	26.684	8.8948	6.5616	**0.0006**	9957
pH*LL	6	25.69	4.2816	3.1585	**0.0085**	9952
temp*pH*LL	6	17.952	2.992	2.2071	**0.0467**	9950
Res	92	124.71	1.3556			
Total	115	268.01				

b.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	47.111	47.111	33.924	**0.0001**	9848
pH	2	9.8809	4.9405	3.5576	**0.0326**	9947
LL	3	15.906	5.3019	3.8179	**0.0108**	9958
temp*pH	2	5.2067	2.6033	1.8746	0.1659	9957
temp*LL	3	29.097	9.699	6.9841	**0.0004**	9946
pH*LL	6	26.854	4.4757	3.2229	**0.0073**	9953
temp*pH*LL	6	18.052	3.0086	2.1665	0.0517	9954
Res	92	127.76	1.3887			
Total	115	280.4				

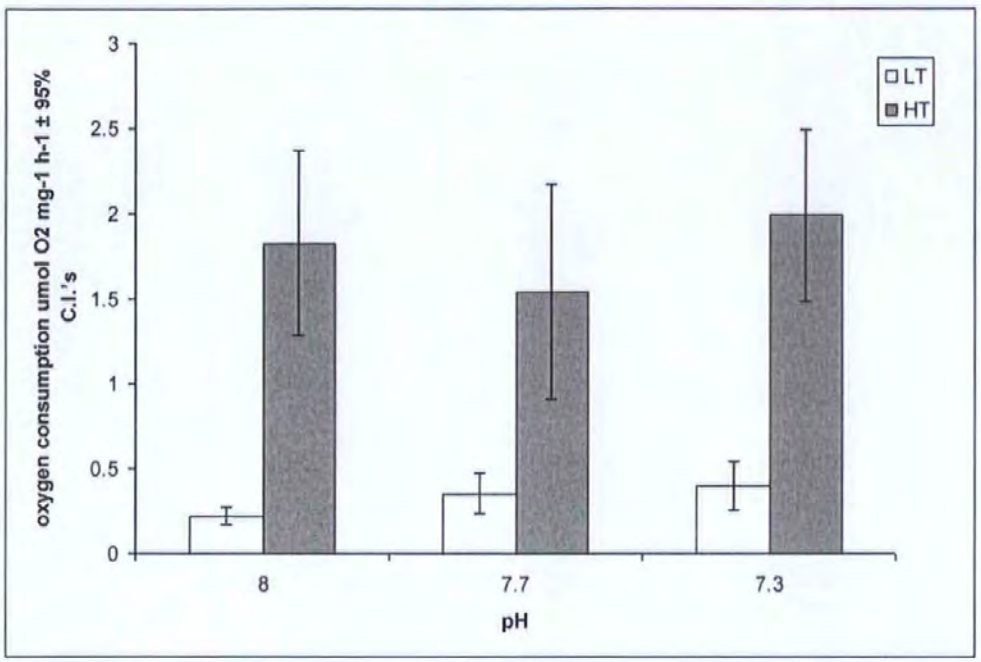


Figure 5.1. Mean oxygen uptake rate (μmol O_2 mg^{-1} h^{-1}) as a proxy of metabolic rate. LT = low temperature (10.5 °C), and HT = high temperature (15 °C).

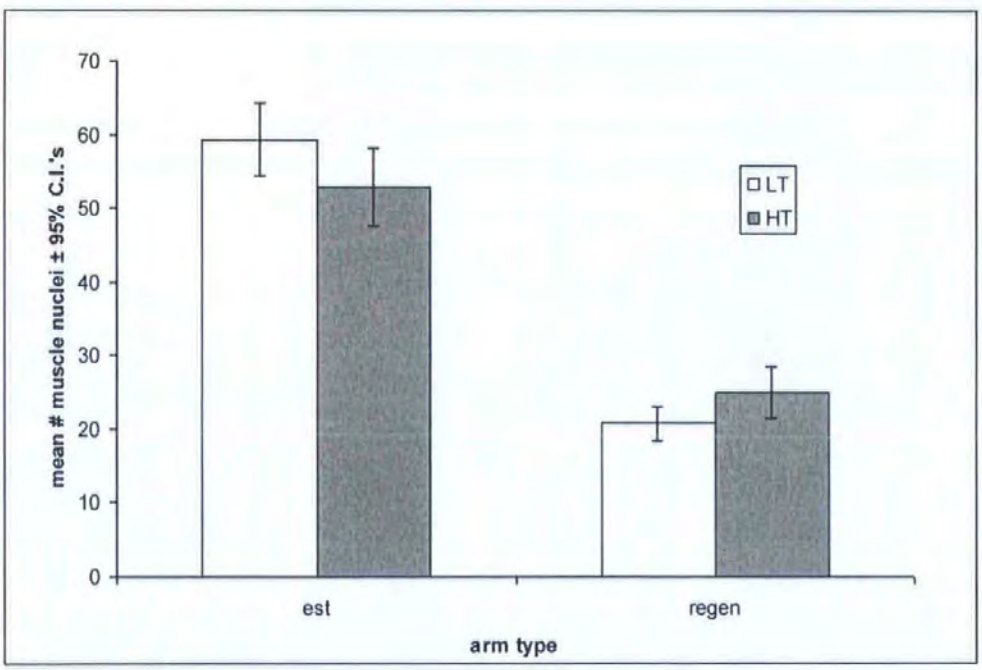


Figure 5.2. Mean muscle density (# nuclei). LT = low temperature (10.5 °C), HT = high temperature (15 °C) exposure. Arm type on x axis refers to established (est) or regenerated (regen) arms. Data from all pH treatments pooled as not significantly different.

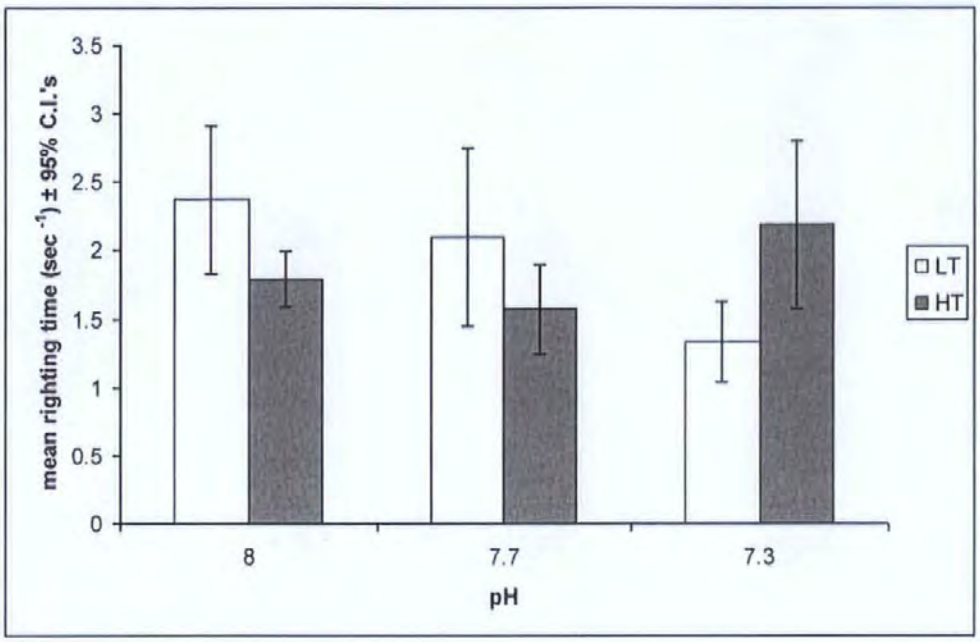


Figure 5.3. Effect of temperature and pH on mobility, expressed here as righting time (in sec). LT = low temperature (10.5 °C), HT = high temperature (15 °C) exposure.

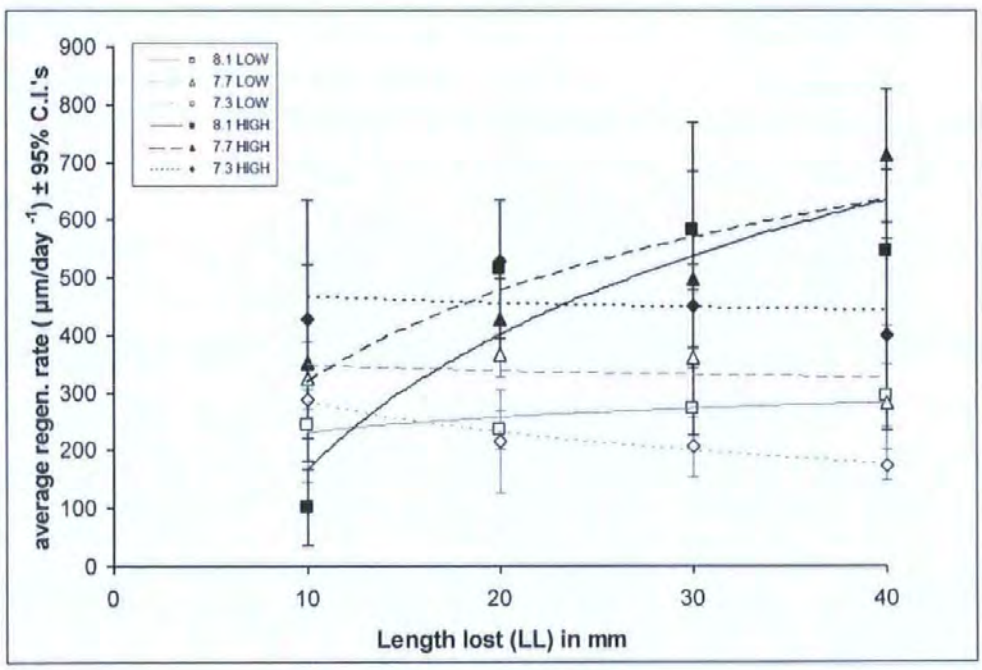


Figure 5.4. Mean arm regeneration rate in *Ophiura ophiura* at the three pH and two temperature treatments (LT = 10.5 °C HT = 15 °C) across a range of length lost values.

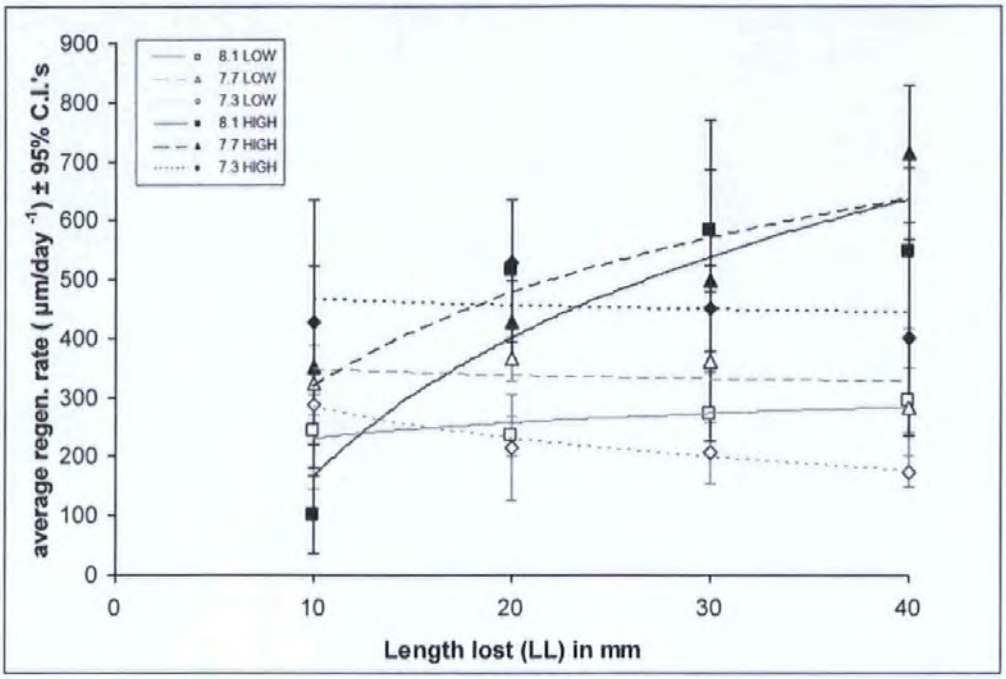


Figure 5.5. Rate of functional arm recovery in *Ophiura ophiura* at the three pH and two temperature treatments across a range of length lost values. Values shown are means ± 95 % confidence intervals.

DISCUSSION

This study has shown that *Ophiura ophiura* upregulated metabolism at high temperature with associated increases in speed of movement and arm regeneration. Lowered pH induces metabolic upregulation in winter conditions but not summer where this low pH is instead accompanied by reduced motility and regeneration.

Physiology of *Ophiura ophiura* under present day, normocapnic conditions

The metabolic rate of *Ophiura ophiura* was approximately seven times lower in the low temperature experiment compared to the high temperature treatment. This effect of temperature on rates of oxygen uptake has been seen in the brittlestar *Hemipholis elongata* (Christensen et al 2000). The temperature treatments over which these experiments were carried out represent average summer (15 °C) and winter (10.5 °C) temperatures where these *Ophiura ophiura* were collected thus providing an insight into the seasonal physiological characteristics of this species. The results of the metabolism investigation at control pH are consistent with *O. ophiura* being less active during the winter; and so supporting the hypothesis that *O. ophiura* are reproductively dormant throughout the winter months. *O. ophiura* is known to carry oocytes overwinter (Tyler 1977). However claims that year round spawning occurs based on the occurrence of ophioplutei from March through to October are unlikely to be true. It is more likely that, in common with other ophiuroid species such as *Amphiura filiformis* (Bowmer 1982) oocytes are laid down at the end of the spawning and then lay dormant over winter before development the following year. Due to the absence of gamete development between or during the experimental periods of this study, it is suggested that *O. ophiura* had either spawned prior to the experiment in May, or had yet to spawn (around late summer) and so had not yet begun gamete development.

Interestingly neither regeneration nor functional recovery rates are dependent on the amount of arm that has been lost as they are in the infaunal brittlestar species *Amphiura filiformis* (Dupont & Thorndyke 2006). In this latter species regrowth and functional recovery of arms is vital for burrow ventilation and feeding. There is little lag between arm regrowth and functional recovery in *Ophiura* which indicates that movement and/or sensory capability of arms is of greater importance than recovery of full length. This brittlestar species moves by using two side arms to 'row' along the sediment while one arms extends to the front as a sensory arm, and the remaining two arms at the rear are used to maintain stability (Fell 1966). Casual observations during the experiment showed that the partially amputated arm was always used as the frontal 'sensory' arm and in this case functional recovery may be advantageous. *O. ophiura* have been documented to be highly receptive to chemical cues (Moore & Cobb 1985) and chemosensory initiation of foraging behaviour occurs at low chemical concentrations (Valentincic 1991). However the location of these receptor sites is not clear from available literature. If these receptor sites are on the disc (internal or external) then chemo-sensing bears no relation to the importance of functional arm recovery; however the orienting response to such stimuli suggests there may be receptors on the arms. Where the number of arms damaged does not impede movement (i.e. two near complete arms for rowing are present) then prioritisation of arm functional recovery over length recovery may be an adaptive response. Muscle density analysis showed lower muscle density in newly regenerated arm, further indicating that movement may not be the key driver for functional recovery.

Ophiura ophiura is a comparatively heavily calcified brittlestar, with 90 % of the skeletal mass made up of high-magnesium calcite, in comparison to 60 % in the Arctic epibenthic species *Ophiocten sericeum* (Chapter 6), and the temperate infaunal species *Amphiura filiformis* (Chapter 2). This highly calcified mesoskeleton is likely to be an adaptation to the high predatory vulnerability their epibenthic lifestyle entails. Motility was directly

linked to metabolic rate under normocapnic conditions with a faster righting time seen in warmer water suggesting such movement is limited by energy availability.

Physiology of *Ophiura ophiura* under future, more acidic conditions

Ophiura ophiura metabolism increases in low pH seawater at low winter temperatures, but no increase is seen in warmer summer temperatures. The large seven-fold increase in metabolic rate due to temperature dwarfs the two-fold increase seen between pH 8 and pH 7.3, and the intermediate increase seen at pH 7.7 which can be expected by the end of this century (IPCC). Potentially then the already elevated metabolic rate at higher temperature generates enough energy to address the additional requirements elicited by low pH seawater. This certainly appears to be the case initially; unlike the infaunal ophiuroid *Amphiura filiformis* (Chapter 2) there is no indication of an energy deficit at low pH in terms of muscle density. But while both arm regeneration and righting time are improved by increasing temperature (regeneration rate increased and righting time decreased), the performance of the brittlestars at pH 7.3 did not match the control and pH 7.7 treatments. Regeneration rate and righting times here were equal to the control pH low temperature responses, despite the far greater metabolic rate of the former. Despite this the metabolic rate at this high temperature is not increased at this pH; the key question is whether this is because the limitations (to movement & regeneration) experienced are acceptable, or because the brittlestars are physiologically unable to further upregulate the metabolism and therefore compromised. As a common and one of the largest temperate epibenthic brittlestar species, *O. ophiura* is an important prey species for fish, especially haddock, plaice and dab (Fell 1966). The absence of elongated or sharp spines as present in other ophiuroids, means that *O. ophiura* relies on physical protection of its mesoskeleton and speed of movement; the significantly slowed mobility in the lowest pH treatment is likely to have significant implications for this latter method of predator avoidance, perhaps

further exasperated by slowed regrowth of arms already lost to predators which could also impede movement. The limitations to regeneration and movement appear ecologically significant; if the additional energy garnered from upregulated metabolism is not available for these important functions then it is being diverted to an additional process upregulated at low pH, and evidence here suggests that is calcification.

There is increasing empirical evidence that calcification is not only possible (e.g. *Ophiocten sericeum,* Chapter 6) but can also increase in low pH water in brittlestars (*Amphiura filiformis*, Chapter 2), gastropod molluscs (Bibby et al 2008) and barnacles (McDonald 2009) to name a few. *Ophiura ophiura* is a comparatively heavily calcified brittlestar, presumably due to the predatory vulnerability their epibenthic lifestyle entails. This high-magnesium calcium carbonate skeleton will experience dissolution in the undersaturated waters of pH 7.7 and 7.3. Despite this the calcium carbonate proportion of the skeleton remained unchanged in brittlestars exposed to these low pH conditions. Exposure of isolated *A. filiformis* arms to water of these pH confirm that calcium (as a measure of calcium carbonate) levels decrease through dissolution (Chapter 7), therefore the calcium carbonate lost by the same process of dissolution here in *O. ophiura* is replaced through active calcification. In this experiment the lower the pH, the lower the saturation of calcite was, and therefore the greater the rate of dissolution and demand for upregulation of calcification. This holds true at both temperatures and wherever calcite is undersaturated, which here was at both lowered pH treatments. The critical point is that a cost to the required upregulation of the rate of calcification (to maintain the same *net* calcification) is seen where metabolism cannot seemingly be increased further to compensate, and the pressure from dissolution is greatest. In the infaunal species *Amphiura filiformis* the cost of this energy deficit manifested as muscle wastage, a physical loss of tissue as the energy within the brittlestar was utilised to maintain burrow ventilation. For this current study, species movement may be conducive to survival but it is not critical to

the physical supply of oxygen and food, so muscle tissue was not utilised to fuel this at all costs. Instead, savings to the energy budget to meet the cost of life in low pH seawater were manifested as reduced energy expenditure on regeneration and slower movement, which in turn may have an indirect effect on species survival though increased vulnerability to predation.

Implications of climate change for the future of *Ophiura ophiura*

Ocean pH is decreasing, gradually increasing the energetic requirement of marine invertebrates to maintain their calcium carbonate structures. At the same time temperature is increasing, which for some species including *Ophiura ophiura,* requires greater metabolic up regulation than that induced by lowered pH. An upper limit in metabolic rate appears to limit a further response to lowered pH at even moderately high temperatures, and a deficit in the energy budget emerges. In *O. ophiura* suboptimal regeneration rates and motility are likely to have sub lethal impacts on survival and fitness. *O. ophiura* is both lethally and sub-lethally predated on by fish (Fell 1966), the latter results in regeneration of lost arms and both require fast movement to minimise successful attacks. In addition, this species is one of the two most common species caught as by-catch by commercial trawlers in the Irish and North Seas (Bergmann et al 1998; Ellis & Rogers 2000); the mechanical disturbance of the trawl itself, the crush trauma of the haul, and the physiological stress of air exposure and hypoxia all result in high levels of arm damage requiring regeneration to maintain fitness (Bergman & van Santbrink 2000). In future warmer, more acidic oceans mortality from both predation and by-catch is likely to increase as a result of reduced mobility and regeneration rates. If survival is significantly affected at a population level then the distribution of this ecologically important (Dahm 1993) species may change drastically.

CHAPTER SIX

Ocean warming and acidification; implications for the Arctic brittlestar *Ophiocten sericeum*

Aspects of this chapter are included in:

Wood H L, Spicer J I & Widdicombe S. Ocean warming and acidification; implications for the Arctic brittlestar *Ophiocten sericeum. Polar Biol.* (In Review)

ABSTRACT

The Arctic Ocean currently has the highest global average pH. However, due to increasing atmospheric CO_2 levels, it will become a region with one of the lowest global pH levels. In addition Arctic waters will also increase in temperature as a result of global warming. These environmental changes can form a significant threat for marine species, and in particular true Arctic species that are adapted to the historically cold and relatively stable abiotic conditions of the region. Consequently, in this chapter I investigated some key physiological responses of brittlestar *Ophiocten sericeum*, a polar endemic which can dominate benthic infauna, to a temperature increase of 3.5°C (to 8.5°C) above ambient (4°C) and CO_2 induced reduction in pH of 0.6 units (pH 7.7) and 0.9 units (pH 7.3) below ambient (pH 8.3). Metabolism was upregulated at low pH. Faster arm regeneration stimulated by increased temperature was counteracted by low pH; at pH 7.3 in the high temperature treatment the maintenance of calcium carbonate structures in undersaturated conditions accelerated the use of energy reserves thus resulting in a limitation to the rate of arm regeneration. An energy deficit is apparent in these circumstances of increased energetic costs associated with the combined factors of high temperature and low pH.

INTRODUCTION

Sea surface temperature is now 0.6 °C warmer than at the start of the industrial revolution (IPCC 2001), and a further increase of 3– 6 °C is forecast by the end of this century (MCCIP 2008). Marine Arctic ecosystems are already experiencing some measurable changes as a result of this warming (Hu & Pan 2009). On the 'warmer' west side of Svalbard many glaciers are retreating and decreasing in mass balance changes linked to increases in temperature (Wright et al 2006) and winter fjord ice cover is no longer guaranteed; Kongsfjord, did not freeze over between 2005 and 2008 where this was once a regular occurrence.

In addition to changing temperature in the Arctic, ocean acidification is affecting polar waters first with changes to aragonite saturation state (Ω_{arag}); 10 % of Arctic waters are projected to become undersaturated with respect to Ω_{arag} for at least one month a year when atmospheric CO_2 reaches 409 ppm which will be within the next decade (IPCC scenarios A2 & B1) and complete undersaturation of the water column is predicted before the end of this century (Steinacher et al 2009). While polar waters currently have the highest global average pH, the largest pH changes in ocean pH will occur in the Arctic Ocean (Steinacher et al 2009) resulting in one of the lowest global pH averages. Furthermore, in these regions increasing temperature and ocean acidification are predicted to have a synergistic effect; models have forecast ocean warming will further magnify ocean acidification changes in this region (McNeil & Matear 2007).

Such changes present marine fauna with the dual physiological challenges of increasing environmental temperature and decreasing environmental pH. These challenges may turn out to be even more pronounced for Arctic species; one of the few studies on the response

of a species found in the Arctic to temperature and pH found a narrowing of the thermal range in the boreal spider crab *Hyas araneus* (Walther et al 2009). Overall there are very few studies of the synergies between temperature and pH and even fewer investigating the susceptibility of Arctic species despite the large changes expected in the region.

It is not known whether the energy deficit found in *Amphiura filiformis* (Chapter 2) is unique to this species, or a more general response of ophiuroids to lowered pH. In the latter case, this energetic deficit would be expected to be greater in an Arctic ophiuroid where low abiotic variability may lead to reduced capability to adapt to environmental change. Within soft sediment benthic habitats around Svalbard, ophiuroid brittlestars are dominant in terms of both constancy and relative abundance (Piepenburg et al 1996). *Ophiocten sericeum* is one of the most abundant of these ophiuroids; it feeds primarily on detritus and plays an important role in nutrient cycling, as well as providing an important food source for fish including Atlantic cod (H Wood pers. Obs.). The dominance of brittlestars within Arctic benthic ecosystems inherently means that detrimental effects to this group as a result of increasing ocean temperature and acidity would have a disproportionally large impact on these benthic ecosystems.

To test whether the energy deficit seen in the burrowing temperate brittlestar *Amphiura filiformis* at low pH (Chapter 2) is more pronounced in Arctic species, we carried out an acidification exposure on the epibenthic brittlestar *Ophiocten sericeum* which is endemic to the Arctic (Paterson et al 1982; but c.f. Piepenburg & Schmid 1996) and abundant within the Barents Sea (Piepenburg et al 1997). In order to compare the response of this arctic species to *A. filiformis* the same parameters of metabolism, calcification, arm regeneration and arm muscle structure were examined. In addition to investigating a potential energy deficit as a result of pH, this present study also introduces a second variable of temperature to investigate potential synergies between these two changing

environmental variables. The brittlestars were exposed to one of three pH treatments: a control of pH 8.3, and lowered pH of 7.7 and 7.3, at one of two temperatures: summer ambient of 5 °C (control) and a higher treatment of 8.5 °C. The experiment was conducted for a period of 20 days in order to investigate long term rather than initial short term shock responses to altered pH and temperature.

METHODS

Location

All experiments were carried out during Aug 2008 at the Kingsbay AS Marine Laboratory, Ny Alesund, Svalbard (79° N).

Animal and sediment collection

Ophiocten sericeum (N = 108) were collected from a depth of ~10 m in Kongsfjord, Svalbard (78° 55′ N, 11° 56′ E) from the passage between Svalbard and the small island of Blomstrand. The glacier adjacent to the collection site deposits riverine sediments to the benthos resulting in clay dominated soft sediment. Brittlestars were collected using a small (30 cm wide) naturalist dredge deployed from a rigid hulled inflatable boat. Intact brittlestars (those with 5 intact arms) were carefully retrieved from the dredge net by hand and placed in buckets (vol. = 5 l, 10 indiv./bucket) filled with freshly-collected seawater (S = 34 PSU, T = 5 °C). Twenty litres of muddy-clay sediment were collected on the same day from the same location using a 0.1m^2 van Veen grab. The sediment was sieved (2 mm mesh) to remove all large fauna. Sediment and brittlestars were returned to the Marine Laboratory within 4 h of collection. Sieved Sediment (3 cm layer) was placed into each of eighteen trays (vol. = 5 l) which were then filled with filtered (10 µl) seawater (vol. ~ 2.5 l) and fitted with a supply tube which delivered seawater from a header tank at the rate of 10 $ml.min^{-1}$. Trays were left to settle (approx. 1 h) before brittlestars were added following the experimental manipulations (amputations) described below.

Arm Amputation

Only individuals without any visible sign of recent regeneration were selected for the experiment (N = 108). Individuals were haphazardly assigned to one of three groups: 25 % amputation, 70 % amputation or untreated, i.e. no amputation. For each individual within the first two groups, disk diameter was measured (Vernier calipers accurate to 0.1 mm) and the point of amputation from the arm tip determined using a simple calculation based on the length of the complete arm:

Position of cut from tip = length of arm (mm) x A*

* where A = the proportion equal to the % of arm to be amputated, either 0.70 (70 % amputated) or 0.25 (25 % amputated).

Prior to amputation the brittlestars were anaesthetized by submersion in 3.5 % w/w $MgCl_2$ dissolved in artificial seawater for a minimum of 3 min. Amputation was carried out by on the first arm clockwise to the madreporite by placing pressure with a scalpel blade between the arm segments at the appropriate distance from the disk. Untreated, 'Complete' brittlestars were anaesthetized but no amputation carried out.

Experimental Setup

Following amputation, disk diameter, longest arm length and (where appropriate) length of remaining arm were recorded (Vernier calipers accurate to 0.1 mm). Brittlestars were then assigned to one of the six pH x temperature treatments (pH 8.3, 7.7 or 7.3 and T = 5 °C or 8.5 °C) and left to recover from anesthesia in one of the three replicate experimental trays of that treatment (6 brittlestars/tray). Each of the six total treatments were carried out

using three replicate experimental trays to hold the randomly allocated brittlestars in order to avoid possible 'tank effects'. Recovery from the anesthetic typically lasted 15 – 40 min. All treatment trays were supplied with seawater of ambient temperature and pH (T = 5 °C, pH = 8.3) at this initial stage. Twelve hours after amputation the seawater supply to the experimental trays was switched to the appropriate pH and temperature header tank resulting in the trays reaching target pH and temperature after 12 h. This was designated Day 0 for the exposure experiment. Brittlestars were fed every two days on a commercial algal feed (Shellfish Diet 1800, Instant Algae) supplied at 37, 000 cells.min^{-1}.

Seawater acidification

Six header tanks (vol. = 200 l) were supplied with filtered (10 µm) seawater; 3 header tanks were supplied with ambient temperature seawater (5 °C) and the other 3 with higher temperature seawater (8.5 °C). pH manipulation was achieved in these header tanks using individual pH controllers (AquaDigital pH201) that regulated carbon dioxide (CO_2) supply (delivered using metal spargers to achieve fine bubbles) to the header tank, *via* a solenoid valve. The pH probes connected to the pH controllers were calibrated with NIST buffers and cross referenced with a bench-top pH meter and glass electrode (Mettler Toledo, also calibrated using NIST buffers). Each header tank (3 per temperature) was haphazardly assigned to a pH treatment of 8.3 (control), 7.7 or 7.3 and the pH controller set to the according value. Seawater drawn from the header tanks to supply to the experimental trays was replaced by an equal volume of untreated (with regards to CO_2 manipulation) filtered seawater supplied from one of two large supply tanks. Both storage tanks were identical except one storage tank contained a heater to produce the higher temperature treatment of 8.5 °C. All header tanks were set up and balanced to the desired pH level prior to brittlestar collection, tubing to supply the experimental trays were connected at the experimental flow rate (10 ml. min^{-1}) and allowed to flow to waste from the experimental trays.

Measurement of carbonate parameters

Water pH_{NIST} and total carbon dioxide content (tCO_2), salinity and temperature (°C) of all experimental trays were recorded three times a week (Table 1). tCO_2 was measured from 100 µl subsamples of seawater using an automated carbon dioxide analyser (CIBA Corning 965 UK). pH_{NIST} was measured using a pH electrode (Mettler Toledo LE413) calibrated with NIST standardised buffers. Salinity and temperature were measured with a combined temperature- conductivity salinometer (WTW LF197) accurate to 0.1 °C (temp.) and 0.01 ppm (salinity). The remaining carbonate parameters were calculated using CO2SYS (Pierot et al 2006) with the constants supplied by Mehrbach et al (1973) refitted by Dickson & Millero (1987) and the KSO_4 dissociation constant from Dickson (1990).

Oxygen uptake

On day 20 the six complete brittlestars (no amputation) from each treatment were placed in Perspex respiration chambers (vol. = 150 ml) with seawater from their exposure pH and temperature header tank. The brittlestars were allowed 30 min to acclimatise to the chambers. After this period chambers were sealed and left in darkness and at constant temperature for two hours. The oxygen content of the water was measured before and after the incubation period using an oxygen microelectrode (SI 130, Strathkelvin Instruments, Glasgow, UK) coupled to a calibrated meter (Oxygen Meter 781, Strathkelvin Instruments, Glasgow, UK). Respirometers were run without brittlestars, exactly as described above, in order to estimate background respiration rates by bacteria. Oxygen uptake was calculated as µl O_2 per mg of brittlestar wet mass per hour (µl $O_2.mg^{-1}.h^{-1}$). In addition to correction for bacterial respiration, all data were corrected for barometric and water vapour pressures, the former measured on the day of the experiment.

Assessment of arm structure

Assessment of potential changes to the structure of arms intact prior to the experimental exposure (hereafter referred to as established arms) entailed, (a) quantifying the density of muscle cells within muscle tissue and looking for changes in the size of the muscles, (b) quantifying the calcium content of the arm as a proxy for calcification, and (c) measuring the thickness of the epithelium, which is the organic matrix that covers the high-magnesium calcite mesoskeleton in echinoderms.

After 20 days one established arm was removed from each complete brittlestar and preserved in Baker's formal calcium solution and transported back to the UK. Here the arms were removed from Baker's solution, placed in a glass beaker on an orbital shaker (slow) and decalcified using 40 ml Gooding and Stewart's decalcification fluid (5 % formic acid) for 18 h. Following removal from the decalcification fluid the arms from each treatment were placed together into small glass vial (vol. = 20 ml) and dehydrated using a sequence of increasing ethanol concentrations (30 min in each of 50 %, 70 %, 95 % (repeated) and finally 95 % ethanol/ monomer (1:1)). Arms were left in 2-hydroxyethyl methacrylate monomer overnight and then embedded in monomer with activator (Lewis & Bowen 1985) before being left to set for 24 h. Once removed from the monomer and air dried for 12 h, a glass knife was used to cut longitudinal sections through the arms which were then stained with Lee's methylene blue/basic fuchsin. All arms were positioned flat within the methacrylate block thus ensuring the same plane of section was examined for each arm. This was verified under low power magnification (x 10, Reichert Polyvar microscope). A central section was chosen for each arm, muscle shape and coverage was examined under low power magnification (x 10) then under high power magnification (x 60) the muscle nuclei within the microscope's field of vision (at x 60 magnification the muscle covered the whole field of view) were counted. This was repeated on five muscle

blocks for each individual (arm) thus allowing a mean for each individual to be taken. Under medium power magnification (x 40, Reichert Polyvar microscope) arm outer epithelium thickness was measured from a digital image provided by a microscope mounted camera (Optronics Magnafire model S99802) using image analysis software (Image-Pro Plus v4.5 Media Cybernetics). Mean epithelium thickness for each individual was calculated from three replicate measurements.

Measurement of arm calcium & magnesium content

Calcification is defined as the deposition of calcium (and carbonate) ions into an organic matrix. Calcium carbonate, the mesoskeletal material of echinoderms, requires calcium and carbonate in equal amounts; therefore comparison of the amount of calcium between treatment arm samples can be used as a proxy to infer comparison of the total calcium carbonate between treatment arm samples. Any changes seen between treatments result from differences in calcification and/or dissolution rates over the course of the experimental exposure, presuming that the individuals were randomly allocated to the treatments and thus the 'control' are representative of all individuals aside from changes caused by the experimental factors. Because echinoderm mesoskeletons are made of high-magnesium calcite the percentage of magnesium within the arm composition was also measured to look for changes. In addition to the arm removed for assessment of structure, a second arm from the complete group (no surgical amputations) was removed from each brittlestar, rinsed in distilled water, frozen (-20 °C) then dried in a drying oven (60 °C). The dried arm samples were labeled and placed in airtight containers for transportation back to the UK. The methodology for determining calcium content closely followed the methodology used by Spicer & Eriksson (2003). The arm samples were weighed using a microbalance (Ohaus Adventurer AR0640 accurate to 0.0001 g), dissolved in nitric acid (vol. = 5 ml, 15 % pro analysis) over a gentle heat to aid digestion. The dissolved samples

were then diluted with distilled water to achieve a concentration within the range of the optical emission spectrometer (Varian 725-65 ICP accurate to 1- 2 % RSD) used to determine calcium and magnesium concentrations. Results were expressed as percentage calcium (or magnesium) mass of arm mass.

Arm regeneration

These analyses were carried out on the 70 % and 25 % amputated groups. After 20 d the regenerated arm was placed under an optical microscope (x 12) and photographed using a digital eyepiece camera (Digital Microscope Eyepiece, Ulead) connected directly to a laptop, *via* a USB cable. Each individual was identified using a combination of disk diameter, longest arm length and length of amputated arm, and the individual's I.D. recorded with the corresponding photograph number. The regenerated arm was clearly distinguishable from the existing arm as it was considerably smaller in diameter and lighter in coloration. The total length of regenerate and differentiated regenerate were measured using image analysis software (Image-Pro Plus v4.5 Media Cybernetics) calibrated with scale photographs taken on the same microscope as the arm regenerate pictures.

Spontaneous autotomy

The number of spontaneous autotomies was recorded on day 20. A spontaneous autotomy was defined as partial or complete loss of an arm other than surgical amputation for the experiment.

Statistical analysis

Parametric data analyses were run using Minitab 14. Non-parametric data were analysed using the PERMANOVA+ routines (beta version, Anderson et al 2008), which are an 'add-in' to the PRIMER 6 software. Homogeneity of variance was tested with the Kolmogrov-Smirnov Test.

Two-way analysis of variance (ANOVA) was used to test for effects of pH and temperature on rate of oxygen uptake, muscle structure (# nuclei), epithelium thickness. Three-way analysis of variance (ANOVA) was used to test for effects of pH, temperature and position of amputation on the number of spontaneous autotomies. Arm regeneration data (total regenerate and differentiated regenerate) and arm calcium/magnesium content data were not normally distributed so were analysed using the permutational MANOVA (PERMANOVA) procedures introduced by McArdle & Anderson (2001) and Anderson (2001). These procedures make more restrictive assumptions than a fully non-parametric approach, but crucially the multivariate PERMANOVA method operates on a similarity matrix and avoids unrealistic normality (or other distributional) assumptions. It does this by exploiting permutation to generate null hypothesis distributions for its pseudo-F statistics; the latter constructed by exact analogy with the standard F statistics for corresponding univariate ANOVA designs.

RESULTS

Experimental conditions

In all treatments pH, alkalinity, salinity and temperature remained stable throughout the experiment (Table 6.1). The seawater was undersaturated with respect to aragonite at pH 7.7 in both temperature treatments of 8.5 °C (high) and 5 °C (ambient). The sediment within the experimental trays appeared healthy based on the oxygenated colour of the surface. Only one brittlestar died during the experiment on day 7. All remaining individuals survived with no visible differences in behaviour of appearance between treatments.

Table 6.1. Summary of water parameters (mean) calculated from samples taken from all experimental trays across the experimental period, ± 95% confidence interval. Treatment refers to first temperature (LT= 5°C, HT = 8.5°C) and then to the pH treatment. Temp = temperature in °C, TA = titratable alkalinity ($mEq.L^{-1}$), Ω_{cal} = calcite saturation, Ω_{arg} = aragonite saturation.

Treatment	pH	Temp.	TA	Ω_{cal}	Ω_{arg}	Sal
HT 8.3	8.31 ± 0.02	8.46 ± 0.02	1889 ± 139	4.00 ± 0.37	2.53 ± 0.24	33.61 ± 0.05
HT 7.7	7.73 ± 0.03	8.46 ± 0.04	1801 ± 97	1.22 ± 0.12	0.78 ± 0.07	33.66 ± 0.03
HT 7.3	7.32 ± 0.03	8.54 ± 0.05	1797 ± 139	0.49 ± 0.05	0.31 ± 0.03	33.79 ± 0.26
LT 8.3	8.30 ± 0.03	4.97 ± 0.12	1846 ± 109	3.49 ± 0.35	2.20 ± 0.22	33.68 ± 0.16
LT 7.7	7.69 ± 0.02	4.96 ± 0.14	1872 ± 135	1.02 ± 0.10	0.64 ± 0.06	33.69 ± 0.08
LT 7.3	7.34 ± 0.02	4.93 ± 0.14	1857 ± 157	0.47 ± 0.05	0.29 ± 0.03	33.71 ± 0.09

Metabolism

Rates of oxygen uptake were recorded as a proxy measure of metabolic rate. Seawater pH had a significant (P= 0.002, Table 6.2 a) effect on the rate of oxygen uptake of *Ophiocten sericeum*, although there was no significant effect of temperature (Table 6.2 a). As pH decreased, metabolism increased (Figure 6.1).

Arm structure

The overall muscle mass of the arms appeared unchanged with respect to both temperature and pH; there were no noticeable voids between the muscle blocks and surrounding skeletal structure. However, quantitative analysis of density of the muscle tissue found that the high temperature treatment had significantly (P= 0.04, Table 6.2 b) lower muscle density than the ambient temperature treatment (Figure 6.2). There was no significant effect of pH on the muscle density (Table 6.2 b). No changes to epithelium thickness across either temperature or pH factors (Table 6.2 c).

Calcification

Under normocapnic (pH 8.3) conditions at control temperature of 5 °C, *Ophiocten sericeum* arms are comprised of 60 % calcium carbonate (average of 30 % calcium in control pH, ambient temperature, Figure 6.3) with 1.25 % magnesium, the remainder of the structure being organic. The percentage of arm mass that was calcium was significantly (P= 0.038, Table 6.3 a) lower in the higher temperature (8.5 °C) treatment, with no significant difference in calcium content between the pH treatments at either temperature (Figure 6.3). Because echinoderm mesoskeletons are made of high-magnesium calcite the percentage of magnesium within the arm composition was also analysed; there were no

significant differences in the magnesium content of arms across both temperature and pH treatments (Table 6.3 b) so temperature reduced calcification but pH did not.

Arm regeneration

The total length of arm regenerated and length of differentiated regenerate, which represents the regenerated arm with functional recovery (Dupont & Thorndyke, 2006), were not significantly different between those individuals with 25 % arm length amputated and 70 % amputated (Table 6.4 a); indicating neither regrowth nor differentiation rate is dependent on the amount of arm lost. Measures of regenerate were converted from total & differentiated length to percentage of lost arm regrown, and percentage of lost arm functionally present which have greater ecological relevance. The following results relate to these calculated measures (Tables 6.4 c & d) however PERMANOVA results for original length values (Tables 6.4 a & b) present the same factors as significant.

The percentage of lost arm regenerated after 20 days was significantly affected by temperature ($P = 0.042$) and pH ($P = 0.02$) treatments (Table 6.4 c), with pH showing the greatest effect at high temperature where regeneration rates were increased in the control and pH 7.7 treatments (Figure 6.4).

The amount of amputated arm that had not only regrown but also regained functionality was calculated using differentiation data. This 'functionally recovered' measurement was significantly different between both temperature and pH treatments (Figure 6.4) with a significant interaction between the factors ($P = 0.02$, Table 6.4 d). At the ambient temperature there was no difference in the percentage of functionally recovered arm regrown between the three pH treatments, however at the high temperature functionally

recovered regenerate is greater at 8.3 and 7.7 than other the high temperature treatment of pH 7.3, and also greater than all ambient temperature treatments (Figure 6.4, dark bars).

Spontaneous autotomy

Analysis of the number of spontaneous autotomies showed no difference across the pH treatments, but a significant interaction ($P = 0.007$) between the amount of arm removed ('regen') and temperature as a result of higher autotomy within the 70 % of arm removed group in the ambient temperature treatment compared to 25 % of arm removed at ambient temperature and both amputation classes at high temperature (Figure 6.5).

Table 6.2. ANOVA results indicating the impact of pH and temperature (temp) on a.) oxygen uptake (data square root transformed), b.) muscle density, measured as the number of muscle nuclei visible under x 60 magnification, c.) arm epithelium thickness (μm). Significant results (P) shown in bold.

a.

Source	d.f.	Seq SS	Adj SS	Adj MS	F	P
temp	1	0.0595	0.0595	0.0595	1.14	0.296
pH	2	0.82924	0.82924	0.41462	7.94	**0.002**
temp*pH	2	0.12443	0.12443	0.06221	1.19	0.321
Error	24	1.25262	1.25262	0.05219		
Total	29	2.26579				

b.

Source	d.f.	Seq SS	Adj SS	Adj MS	F	P
temp	1	516.31	459.22	459.22	4.65	**0.04**
pH	2	157.85	175.67	87.83	0.89	0.422
temp*pH	2	72.25	72.25	36.13	0.37	0.697
Error	28	2765.86	2765.86	98.78		
Total	33	3512.27				

c.

Source	d.f.	Seq SS	Adj SS	Adj MS	F	P
temp	1	0.4779	0.4966	0.4966	1.1	0.304
pH	2	0.0122	0.0119	0.0059	**0.01**	0.987
temp*pH	2	0.2507	0.2507	0.1253	0.28	0.76
Error	29	13.1378	13.1378	0.453		
Total	34	13.8786				

Table 6.3. PERMANOVA indicating the impact of pH and temperature (temp) on a.) arm calcium content, and b.) arm magnesium content Significant results (P) shown in bold.

a.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	155.66	155.66	4.2623	**0.0382**	9850
pH	2	105.65	52.827	1.4465	0.263	9945
temp*pH	2	87.506	43.753	1.1981	0.3225	9957
Res	35	1278.2	36.52			
Total	40	1604				

b.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	0.26637	0.26637	3.7527	0.0559	90943
pH	2	0.16027	8.01E-02	1.129	0.3561	95691
temp*pH	2	0.1495	7.48E-02	1.0531	0.3761	95584
Res	35	2.4843	7.10E-02			
Total	40	3.0203				

Table 6.4. PERMANOVA results indicating the impact of pH and temperature (temp) on a.) Total arm regeneration (mm), b.) total arm differentiation, c.) percentage of lost arm regrown and d.) % of lost arm regrown and regain functionality.

a.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	16.309	16.309	12.442	**0.0007**	9839
pH	2	21.796	10.898	8.3136	**0.0002**	9956
regen	1	4.6741	4.6741	3.5657	0.0717	9832
temp*pH	2	6.7132	3.3566	2.5606	0.0914	9954
temp*regen	1	1.2011	1.2011	0.91625	0.3508	9834
pH*regen	2	1.8547	0.92736	0.70745	0.5182	9958
temp*pH*regen	2	3.8956	1.9478	1.4859	0.239	9942
Res	48	62.921	1.3108			
Total	59	122.24				

b.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	64.114	64.114	15.785	**0.0001**	9828
pH	2	89.059	44.529	10.963	**0.0002**	9937
regen	1	4.28E-04	4.28E-04	1.05E-04	0.9908	9813
temp*pH	2	40.044	20.022	4.9293	**0.0129**	9953
temp*regen	1	5.87E-02	5.87E-02	1.45E-02	0.903	9827
pH*regen	2	0.14934	7.47E-02	1.84E-02	0.9818	9952
temp*pH*regen	2	6.64E-02	3.32E-02	8.17E-03	0.9934	9948
Res	48	194.97	4.0618			
Total	59	405.67				

c.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	0.81815	0.81815	4.3223	**0.0415**	9826
pH	2	1.6018	0.8009	4.2312	**0.0194**	9948
regen	1	0.51054	0.51054	2.6972	0.1048	9832
temp*pH	2	0.86549	0.43274	2.2862	0.1012	9954
temp*regen	1	0.33029	0.33029	1.7449	0.2038	9839
pH*regen	2	0.23349	0.11675	0.61677	0.5551	9945
temp*pH*regen	2	0.44182	0.22091	1.1671	0.3285	9950
Res	48	9.0857	0.18929			
Total	59	14.042				

d.

Source	d.f.	SS	MS	Pseudo-F	P(perm)	Unique perms
temp	1	4.9934	4.9934	10.827	**0.0021**	9812
pH	2	7.6735	3.8367	8.3191	**0.0008**	9958
regen	1	0.58502	0.58502	1.2685	0.2596	9858
temp*pH	2	3.7919	1.896	4.111	**0.0214**	9954
temp*regen	1	2.71E-02	2.71E-02	5.87E-02	0.81	9848
pH*regen	2	0.16193	8.10E-02	0.17555	0.8373	9956
temp*pH*regen	2	2.65E-02	1.33E-02	2.88E-02	0.9737	9948
Res	48	22.137	0.46119			
Total	59	40.454				

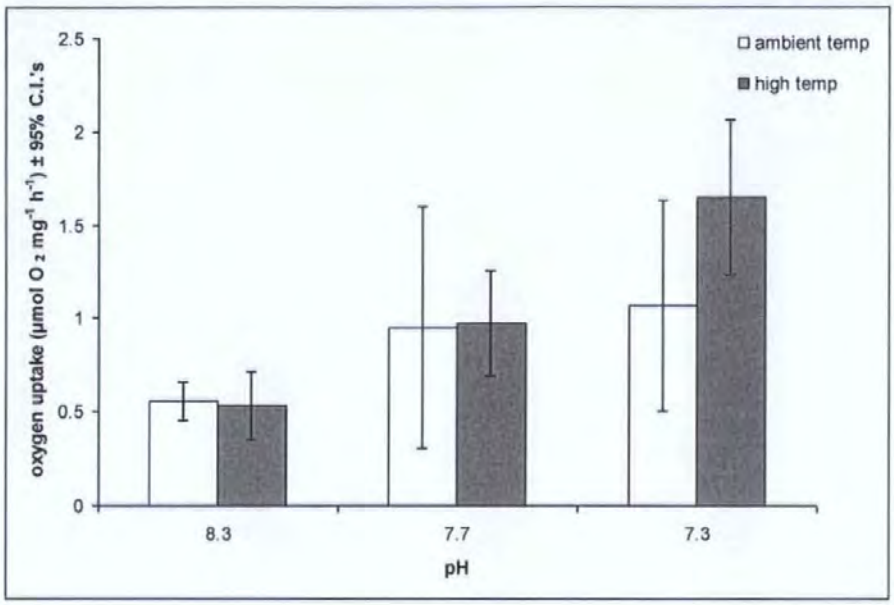


Figure 6.1. Mean oxygen uptake of *Ophiocten sericeum* at the three pH and two temperature treatments ± 95 % confidence intervals.

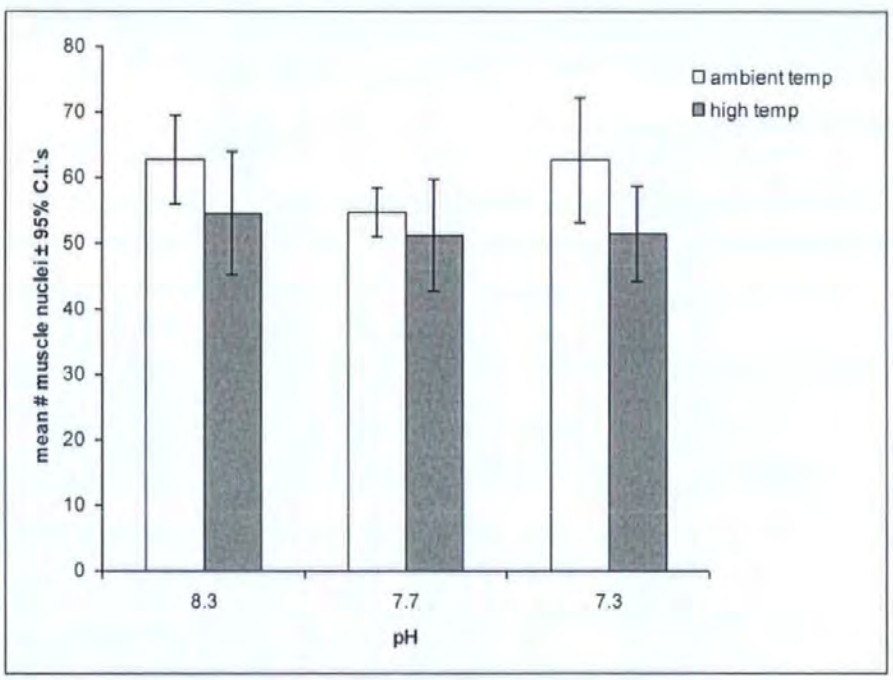


Figure 6.2. Mean number of nuclei in a standardised area of *Ophiocten sericeum* arm muscle compared across three pH and two temperature treatments ± 95 % confidence intervals.

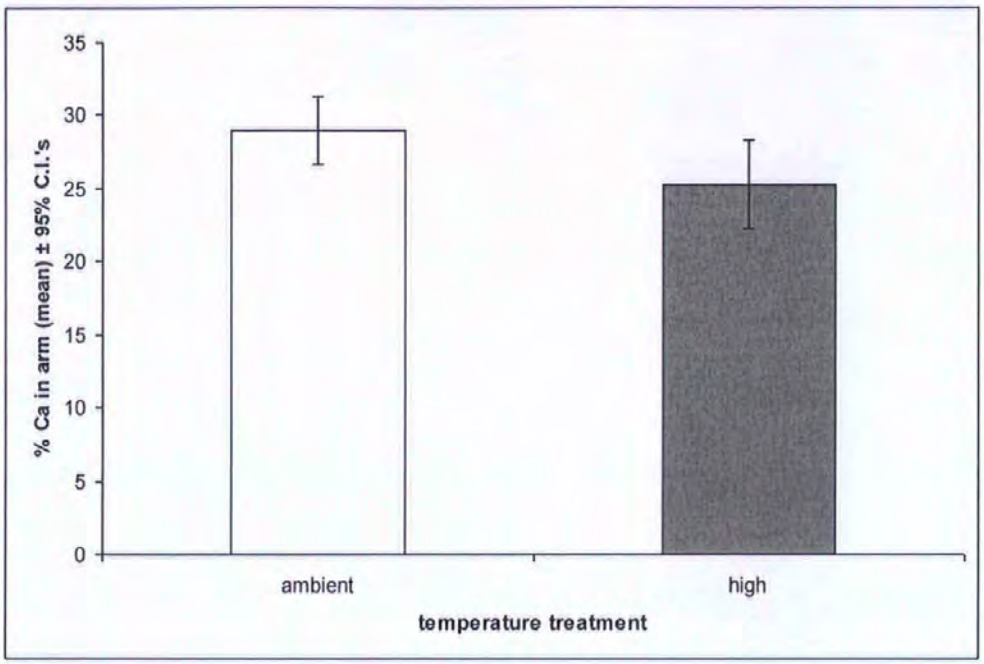


Figure 6.3. Mean percentage (± 95 % confidence intervals) of calcium in an established *Ophiocten sericeum* arm at the two temperature treatments of ambient (5 °C) and high (8.5 °C)

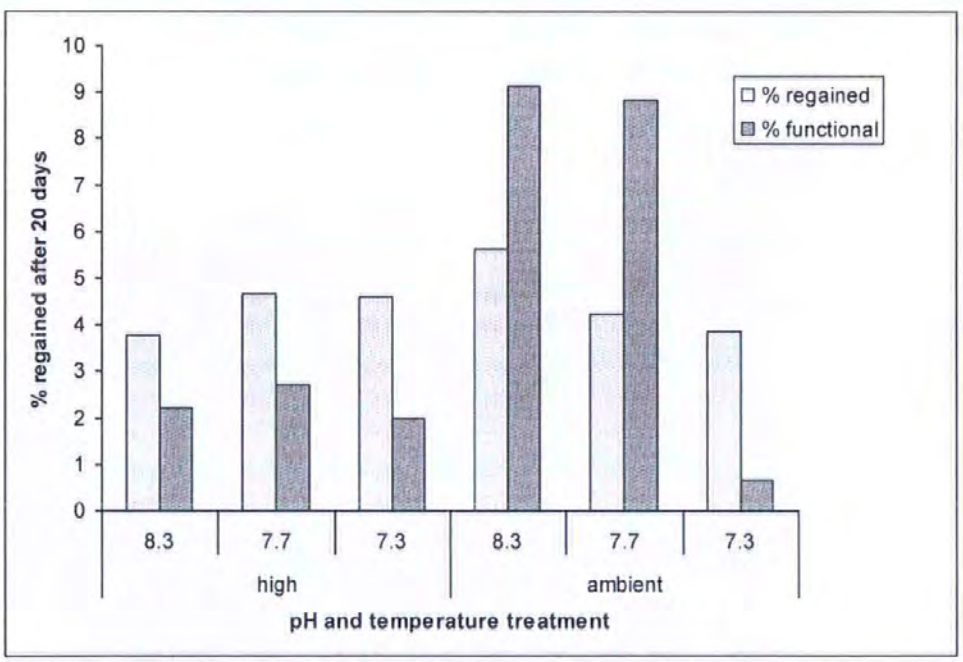


Figure 6.4. Mean (± 95 % confidence intervals) percentage of *Ophiocten sericeum* lost arm that has been regenerated (% regained) and arm functionally recovered (%) after 20 days across three pH treatments and the two temperature treatments of ambient (5 °C) and high (8.5 °C)

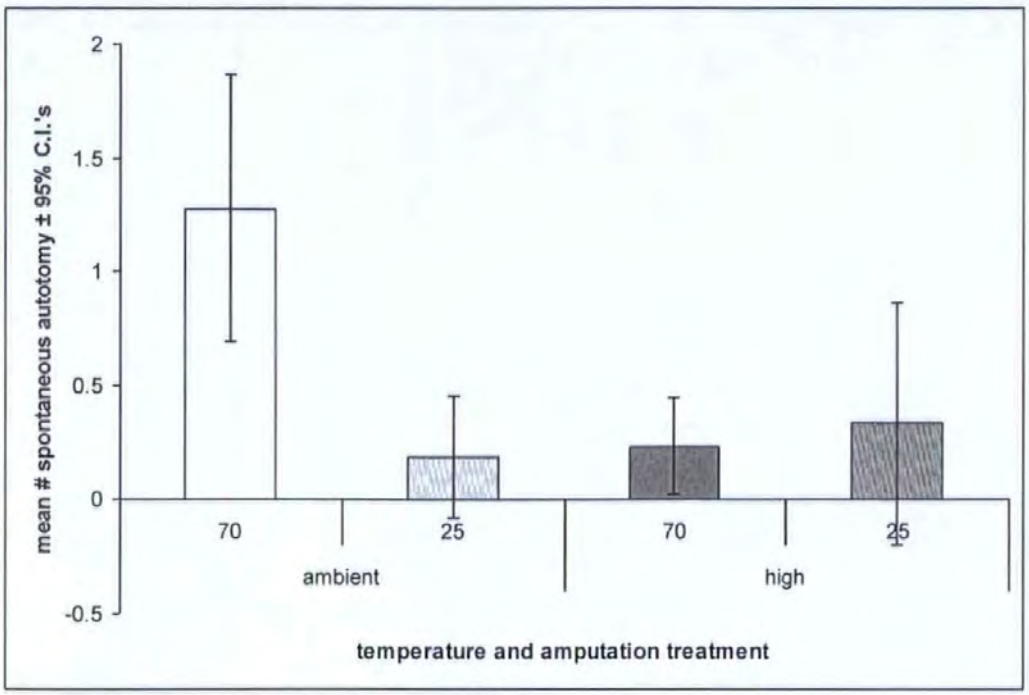


Figure 6.5. Mean (± 95 % confidence intervals) number of spontaneous arm autotomies in Ophiocten sericeum across the two temperature treatments of ambient (5 °C) and high (8.5 °C) and within each temperature the two levels of amputation: 70 % removed and 25 % removed.

DISCUSSION

Physiology of *Ophiocten sericeum* under present day conditions

Under ambient pH (8.3) and summer temperature (5 °C) conditions the Arctic ophiuroid brittlestar, *Ophiocten sericeum*, has a low metabolic rate compared to temperate species; mass adjusted oxygen uptake is approximately one third of that of *Ophiura ophiura* (Chapter 5), a temperate ophiuroid of similar life history, despite the later being larger and therefore expected to have a lower weight specific metabolic rate. This lower metabolism, even under arctic summer conditions, is likely an adaptation to the low energetic cost of living in this region with long periods of limited food availability due to sea ice (Smith et al 2000) and cold temperatures, and may further explain the viability of the high densities of epibenthic brittlestars found in the region despite this limited food availability (Piepenburg & Schmid 1996). Similarly arm regeneration is slow, with an average weekly regrowth rate of 0.63 mm compared to up to 4 mm a week seen in some temperate brittlestar species (Dupont & Thorndyke 2006). Neither regeneration nor differentiation rates are dependant on the amount of arm that has been lost as they are for another brittlestar species *Amphiura filiformis* (Dupont & Thorndyke 2006). Rather than being an arctic specific response, this difference is likely due to differences in lifestyle between the two species. *A. filiformis* is infaunal and regrowth and functional recovery of arms is vital for burrow ventilation and feeding. *O. sericeum* is epibenthic therefore upregulation of regeneration rate when a large portion of arm is lost is not advantageous because one shorter arm does not reduce mobility on the sediment surface (H Wood, pers. obs) nor impede feeding potential from the remaining arms.

Physiology of *Ophiocten sericeum* under future warmer, more acidic conditions

Ophiocten sericeum exhibited raised metabolism at lower pH which in turn requires increased energetic intake. On the basis of the skeleton mineralogy data presented here we suggest that at least part of the increased energetic demand seen in this present study is related to increasing the rate of calcification at low pH. This is not the first study to find metabolic increase as the result of low pH; similar results have been seen in other species including a brittlestar (Chapter 2), a decapod crustacean (Walther et al 2009) and an amphipod (Hauton et al 2009). In addition, a recent study on the early life stages of the sea urchin *Stronglyocentrosis drobachiensis* found that the efficiency of food assimilation was reduced in low pH conditions (M Thorndyke pers comm.). There have not yet been any similar investigations of a similar mechanism in adult individuals, however an element of the metabolic upregulation seen in this current study may also be as a result of less energy uptake from food at low pH. Interestingly there was no effect of temperature on metabolic rate as would be expected in a typical Q_{10} response. Given that this brittlestar experiences very little temperature variation (-1.8 °C to 4 °C from winter to summer in 2008, H Wood unpubl. data) the absence of a temperature response seems counterintuitive, particularly as the pH results demonstrate that an increase in metabolic rate is possible. One potential explanation for this is the cold temperatures of the experiment. Cold water holds more oxygen than warmer water; if this oxygen availability is above a critical threshold then the brittlestars are already taking in more oxygen than required simply as a function of its saturation in the seawater. Therefore provided the water increase to 8.5 °C does not surpass this critical oxygen threshold the brittlestars will have enough oxygen to fuel increased metabolic rate to a certain point before an increase in uptake is required. This principle is illustrated by a level rate of oxygen uptake between 12 °C and 16 °C (the cold end of the thermal range) in the eelpout *Zoarces viviparous* (Pörtner & Knust 2007) and may explain the seemingly counterintuitive results of this present study.

Regeneration and differentiation (functional recovery) was faster with increased temperature as has been observed in another ophiuroid species, *Amphiura filiformis* (Thorndyke et al 2003). Yet this increase was not observed at pH 7.3 where the rates were analogous to the ambient temperature rates. When the metabolic and regrowth responses are considered together it appears that the elevated metabolic demand at pH 7.3 could leave the individual insufficient energy to upregulate regeneration and functional recovery in the high temperature treatment.

The number of muscle cells in the arm muscle blocks (muscle density) was reduced in the high temperature treatment; this decrease is due to loss of muscle tissue, and if the experiment were to be carried out over a longer time period this muscle loss will result in visible reduction in the size of the arm muscles. The muscle is losing cells and therefore volume, quantifiable here on the small, cellular level, but the longer it continues and the greater the number of muscle cells lost, the higher up it will be seen, resulting in a visible loss of overall muscle area as there will simply not be enough tissue to fill the original muscle volume. These results lead us to suggest that *Ophiocten sericeum* will experience energetic deficits as a result of ocean acidification in conjunction with increased temperature. This decrease in muscle density does not correlate with metabolic upregulation, which occurred at low pH not high temperature. On the bases of these data we suggest firstly that the muscle wastage results from increased arm regeneration, and that regeneration rate is controlled independent of metabolism.

The pH treatments did not alter net calcification in the brittlestars despite both the 7.7 and 7.3 treatments being undersaturated with respect to aragonite (and calcite) and thus corrosive to their high magnesium calcite mesoskeletons. Presuming that dissolution of the mesoskeleton occurred in the low pH treatments, as has been demonstrated for another

ophiuroid (Chapter 2) then actual calcification in the low pH treatments must be greatly increased to compensate for this dissolution, a response that will further add to the increased energetic burden discussed above. The organic epithelium which separates the mesoskeleton from the environment did not change in thickness, nor was the proportion of magnesium, which increases the solubility of the structure (Andersson et al 2008), reduced in response to reduced pH. Thus in these respects there appears to be no adaptations to reduce the dissolution of the mesoskeleton. This adds weight to the idea that there was increased *actual* calcification in order to produce the same *net* calcification over the experimental period. Decreased net calcification in the high temperature treatment may be due to less energy available to allocate to the process due to increased regeneration. Alternatively it could be that, whether undersaturated or not, the saturation states were consistently lower in the high temperature treatments thus making the assimilation of carbonate ions a slower process. Upregulation of calcification under low pH conditions has also been recorded in other invertebrates (e.g. barnacles, McDonald et al 2009), and even the otoliths of fish (Checkley et al 2009), providing increasing empirical evidence that calcification in undersaturated (Ω_{cal} & Ω_{ar}) seawater is possible.

Even from the results of the relatively short experimental period (20 days) employed here there is evidence that ocean acidification may result in an energy deficit for *Ophiocten sericeum*. Intuitively such an outcome, which is manifest once energetic reserves are exhausted, will be more pronounced at higher temperature. This holds true here where increased regeneration rates accelerated the use of energy reserves. Longer term exposure is likely to have elicited the same response in the ambient temperature treatment where slower regeneration rates (growth being the primary energy output when this species is reproductively inactive) take longer to exhaust energy reserves and reveal this deficit through the loss of muscle tissue. Further to this, metabolic rate was increased in both the pH 7.7 and 7.3 treatments. Regarding the short term survival (over the next century) it is

important to determine whether over a longer time period the more subtle metabolic rate increase shown at pH 7.7 results in an energy deficit or whether it is sustainable; this outcome will certainly be key to this species' survival over the next century where pH 7.7 is fast becoming a depressingly realistic pH forecast (Orr 2009). It is clear that in the high temperature treatment used here there was the same type of response as found in Chapter 2 where a 40 day exposure was carried out on the temperate burrowing brittlestar *Amphiura filiformis*.

Implications for the future of Arctic species

Arctic subtidal habitats, while dynamic in some regard (e.g. high sedimentation rates near glaciers) are very stable in terms of water conditions; there is much lower variability in temperature and pH than experienced in analogous temperate habitats (Wlodarska-Kowalczuk et al 2004). As such, true Arctic species such as *Ophiocten sericeum* have evolved to maximize their fitness in this extreme environment at the expense of plasticity in physiological response in the same way as deep sea species which similarly inhabit in an abiotically stable environment (Gage & Tyler 1993). For example, deep sea species generally have low metabolic rates (Childress 1997) but in doing so lose the capacity to cope with environmental variability; the acid-base regulation under hypercapnic conditions of the deep sea crab *Chionoecetes tanneri* is far poorer than the closely related shallow water species *Cancer magister* (Pane & Barry 2007). The reduced abiotic range of true Arctic species combined with the greatest pH and temperature changes of all oceans over the 21st century (Steinacher et al 2009) combine to make Arctic ecosystems, theoretically at least, especially vulnerable.

Northwards geographical range shifts as a result of ocean warming (Mieszkowska et al 2005) are likely to result in the Arctic waters around Svalbard becoming hospitable for an

increasing number of boreal species. For the incoming boreal species the warmed Arctic waters will be at the cold end of their thermal range, while the true Arctic species will be at the upper end of their thermal range. The results of this study indicate a greater prevalence of pH effects at higher temperatures (also seen in other brittlestars, Chapters 3 & 5). Under such circumstances true Arctic species may face competition from invasive boreal species in addition to pH induced physiological stress not experienced by their boreal competitors. In future oceans true Arctic species may be outcompeted by such invasive species. In addition to impacting benthic invertebrates changing ocean temperature and pH will also affect phytoplankton (e.g. Langer et al 2006), bacteria, zooplankton (e.g. Comeau et al 2008). To fully understand and predict the winners and losers of this impending change requires much more research into how these factors will affect Arctic species, and an interdisciplinary approach to understand changing species interactions in addition to individual species/niche responses.

For the brittlestar dominated epibenthic fauna, the results of this study indicate in the short term at least, physiological adaptation to increased temperature and hypercapnia are possible. However from metabolism increase and early signs of muscle wastage the indication is that long term survival in warmer more acidic Arctic waters may not be sustainable.

CHAPTER SEVEN

Comparing the impact of high CO_2 on calcium carbonate structures in different marine organisms

Aspects of this chapter are included in:

Findlay H S*, **Wood H L***, Kendall M A, Spicer J I, Twitchett R J & Widdicombe S. Comparing the impact of high CO_2 on calcium carbonate structures in different marine organisms. *J. Exp. Mar. Biol Ecol. (In Review)*

** These authors contributed equally to this work.*

ABSTRACT

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

INTRODUCTION

Calcifying marine organisms (molluscs & foraminifera, crustacean, echinoderms and corals, coccolithophores reviewed in Fabry et al 2008) are predicted to be most vulnerable to decreasing oceanic pH (ocean acidification) because calcification rates may decrease as a result of reduced carbonate ion availability. However, the possibility for increased or maintained calcification under high carbon dioxide (CO_2) conditions originates from evidence that calcifying organisms are not reliant on carbonate ions to calcify. Investigations principally of molluscs (Wilbur 1964; Erez 2003) but also of corals (Al-Horani et al 2003; Erez 2003), barnacles (Bubel 1975) and echinoderms (Decker & Lennarz 1988) show that bicarbonate (HCO_3^-) or $CO_{2(aq)}$ and not carbonate (CO_3^{2-}) is the origin of the carbon used in calcification. When either HCO_3^- or CO_2 is the substrate for biogenic $CaCO_3$, the formation of $CaCO_3$ structures (calcification) should not be inhibited directly by decreasing CO_3^{2-} concentrations (*via* ocean acidification). Although not new, this information often seems to be overlooked when explaining decreases in net calcification. Furthermore, many of these organisms produce calcium carbonate ($CaCO_3$) at a crystallisation site isolated from the surrounding seawater (Wilbur 1964; Hart & Podolsky 2004; de Nooijer et al 2008).

Molluscan shell calcification takes place away from the surrounding ambient seawater, at a crystallisation site in the extrapallial space (Wilbur & Yonge 1964). Detailed investigations of shell-forming cells indicated that calcium transport and secretion may in part be dependant on metabolic energy derived from the generation of ATP. This has also been shown to be true in corals (review by Cohan & McConnaughey 2003). Additionally an increasing amount of glycogen has been found to be present in these shell-forming cells and this may provide a source of CO_2, which can be converted to CO_3^{2-} by the enzyme

carbonic anhydrase (Wilbur & Jodrey 1955) and used to form $CaCO_3$. In barnacles, calcification takes place in the mantle cavity and again, examination of the structure of shell-secreting cells reveals a large presence of glycogen and mitochondria (Bubel 1975). Ophiuroids possess a mesodermal skeleton, yet the epithelium is very thin and the internal barrier separating coelomic fluid from the test is not well developed. This structure can therefore be exposed to changing seawater chemistry. The skeletal structure of echinoderms is made of magnesium calcite and is therefore highly susceptible to dissolution at lowered pH. Current understanding of the calcification process in echinoderms is mainly based on echinoid studies, with little known of the process in ophiuroids (Hart & Podolsky 2005).

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

relevant for organism survival. In calcifying organisms calcification is integral in the control of other processes such as growth, metabolism and regulation of internal body pH (Pörtner 2008).

Five different calcifying organisms were used to assess the impacts of ocean acidification on aspects of whole animal physiology and calcification in this study: three mollusc species, a gastropod limpet (*Patella vulgata*), a gastropod snail (*Littorina littorea*), and a bivalve mussel (*Mytilus edulis*); one crustacean, a cirripede (*Semibalanus balanoides*); and one echinoderm, a brittlestar (*Amphiura filiformis*). Either the calcium (Ca^{2+}) concentration in the calcified structures or shell morphological parameters were measured as a proxy for a net change in calcium carbonate in live individuals exposed to lowered pH. In order to gain a basic understanding of the rates at which some of these organisms' calcium carbonate structures dissolve, the Ca^{2+} concentration was also measured in isolated shells and arms exposed to lowered pH. This measurement allowed quantification of the change in calcium carbonate when biogenic calcification was absent, which enabled the determination of a species' ability to calcify compared to dissolution across decreasing levels of pH and thus also across calcite and aragonite saturation states.

METHODS

The *Amphiura filiformis*, *Mytilus edulis*, *Littorina littorea* and *Semibalanus balanoides* experiments were initially carried out during studies with different aims to this investigation, focusing on other physiological, histological, and ecological impacts of ocean acidification, and hence the experiments were not all conducted at the same pH levels. The calcium and metabolism data for *A. filiformis* were previously presented in Chapter 2, some morphometric measurements and metabolic rate data for *L. littorea* have been published in Bibby et al (2007) and preliminary data from *S. balanoides* are presented in Findlay et al (in press). However the data presented on *M. edulis* and *P. vulgata* are novel to this study and the data on *A. filiformis* previously shown in Chapter 2, in addition to the data from Bibby et al (2007) and Findlay et al (in press) have been reanalysed. Information on other physiological impacts is also brought together using both examples from the studies mentioned above and other literature, as well as paleoecological examples to gain a greater understanding of the processes impacting the whole organisms.

Experimental set ups

The *Amphiura filiformis*, *Patella vulgata*, *Mytilus edulis* and *Littorina littorea* experiments were carried out using acidified seawater by means of pH adjustment through bubbling of CO_2 into header tanks, and drawing water from these header tanks into the experimental containers as described in Widdicombe & Needham (2007). For details of the *A. filiformis* experiment see Chapter 2; the *P. vulgata* experiment was run alongside the *A. filiformis* experiment. 10 *P. vulgata* individuals were placed in replicate 5 l containers at each pH condition; briefly the pH levels for these two experiments were 8.0, 7.7, 7.3 and 6.8. The *M. edulis* experiment is detailed in Beesley et al (2008) with pH levels set at 8.0, 7.8, 7.6

and 6.8. The *L. littorea* experiment is detailed in Bibby et al (2007), where only two pH conditions were examined: pH 8.0 and 6.45. The *Semibalanus balanoides* experiment was carried out in tidal microcosm systems (Findlay et al 2008) containing high CO_2 - air detailed in Findlay et al. (in press), with two pH conditions: pH 8.0 and 7.7. Table 7.1 presents overall information on exposure conditions and state of the organisms, while Table 7.2 presents information on the seawater conditions and carbonate system. In all experiments pH (NBS scale, Mettler-Toledo pH meter), dissolved inorganic carbon (DIC) (Ciba-Corning 965D Total CO_2 Analyser, Olympic Analytical Service), temperature and salinity (WTW LF197 combination temperature and salinity probe) were recorded throughout the experimental periods. Total alkalinity, bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and the saturation states for aragonite and calcite were all calculated from pH and DIC using CO2sys (Pierrot et al 2006) with dissociation constants from Mehrbach et al (1973) refit by Dickson & Millero (1987) and KSO_4 using Dickson (1990).

Measurement of calcium content

The calcium carbonate composition of the shells (*P. vulgata*, *M. edulis* and *S. balanoides*) or arms (*A. filiformis*) was estimated by analysing the calcium ion (Ca^{2+}) concentrations as a proxy for any changes in calcification or dissolution. Live individuals produce calcium carbonate (i.e. calcify) during shell growth, however there may also be some dissolution of the shell or indeed some abiotic influence of shell formation. The dissolution factors, as discussed in the introduction, may be enhanced in high CO_2 conditions. Ca^{2+} ions are abundant in seawater and hence are not limiting. Formation of $CaCO_3$ involves combining inorganic carbon with Ca^{2+} ions. Therefore any observed changes in Ca^{2+} ions should indicate how the shell structure changes over time through calcification and dissolution processes. This principle is the same whether measuring radio-active labelled Ca^{45} incorporation into shells (Comeau et al 2009) or Ca^{2+} content via spectrophotometric

techniques. Comparing the concentration of Ca^{2+} ions in shells/arms of non-living calcified structures (shells and arms) with the concentrations in the structures from live animals provides an estimate of a organism's ability to calcify relative to any dissolution or abiotic effects because biogenic calcification will not be taking place in dead individuals.

All shells and arms were taken at the end of each experiment and frozen at -20 °C for further analysis. Concentration of Ca^{2+} ions was measured using methods described in Spicer & Eriksson (2003); briefly this involved dissolving the shells and arms in 10 % nitric acid after rinsing in distilled water, drying and weighing and the total Ca^{2+} concentration determined using atomic absorption spectrophotometer (Varian SpectrAA 50). The proportion of calcium (Ca^{2+} %) in the shell or arm (mg Ca^{2+} mg $shell^{-1}$) was calculated from the known total mass of the shell or arm (mg) and the volume of acid used in the digest (l).

RESULTS

All five species showed a response to acidified conditions with perhaps the most surprising result being that four of these five had increased levels of calcium in low pH conditions (Figure 7.1).

Over the respective experimental exposures, the Ca^{2+} % of shells of live *Patella vulgata* and *Semibalanus balanoides* and the arms of live *Amphiura filiformis* either remained constant or increased significantly (ANOVA, $F_{2,59} = 16.58$, $p < 0.001$) compared to the control as the pH treatments decreased (Figure 7.1 a). The Ca % in the shells of live *Mytilus edulis* (Figure 7.1 a) did not differ significantly compared to the controls as pH decreased. These changes occurred despite the seawater in the low pH treatments having lower calcite and aragonite saturation states (Table 7.2) due to a reduction in carbonate ions. In some cases, treatments were completely undersaturated for $CaCO_3$, with calcite becoming undersaturated at ~ pH 7.3 and aragonite becoming undersaturated at ~ pH 7.6.

The Ca^{2+} % in isolated shells of *P. vulgata*, *M. edulis* and *S. balanoides*, and arms of *A. filiformis*, decreased over the exposure period (7 d) compared to the controls (Figure 7.1 b). The percent change in Ca^{2+} % d^{-1} (overall increase or decrease) relative to the control showed that Ca^{2+} % d^{-1} in isolated *M. edulis* shells decreased by up to 1.5 % d^{-1} while live shells did not differ from the control (Figure 7.2). A similar pattern was exhibited by *P. vulgata*, *S. balanoides* and *A. filiformis* (Figure 7.2). The decrease in % Ca^{2+} observed in the isolated shells and arms of all four species correlates strongly with a decrease in carbonate ion concentration (Figure 7.2 b), yet this decline is not observed in the live individuals in any of the species.

All the morphological shell parameters in *L. littorea* (width, height, thickness, area, perimeter, aperture area, and aperture perimeter) increased in low pH treatments compared to the control (Figure 7.1 c & d): there was ~67 % more growth in shell height, ~30 % more growth in shell width and ~40 % more growth in shell thickness under low pH conditions compared to the control. This increased growth implies that acidification was not preventing the animals from producing their shells and hence formation of $CaCO_3$ was possible at lowered pH. There were no measures taken of the mineral structure of the shell and therefore it cannot be ascertained whether there was any impact on shell structure however both calcite and aragonite were undersaturated in the low pH treatment, indicating that dissolution is likely to have been occurring in the low pH treatment.

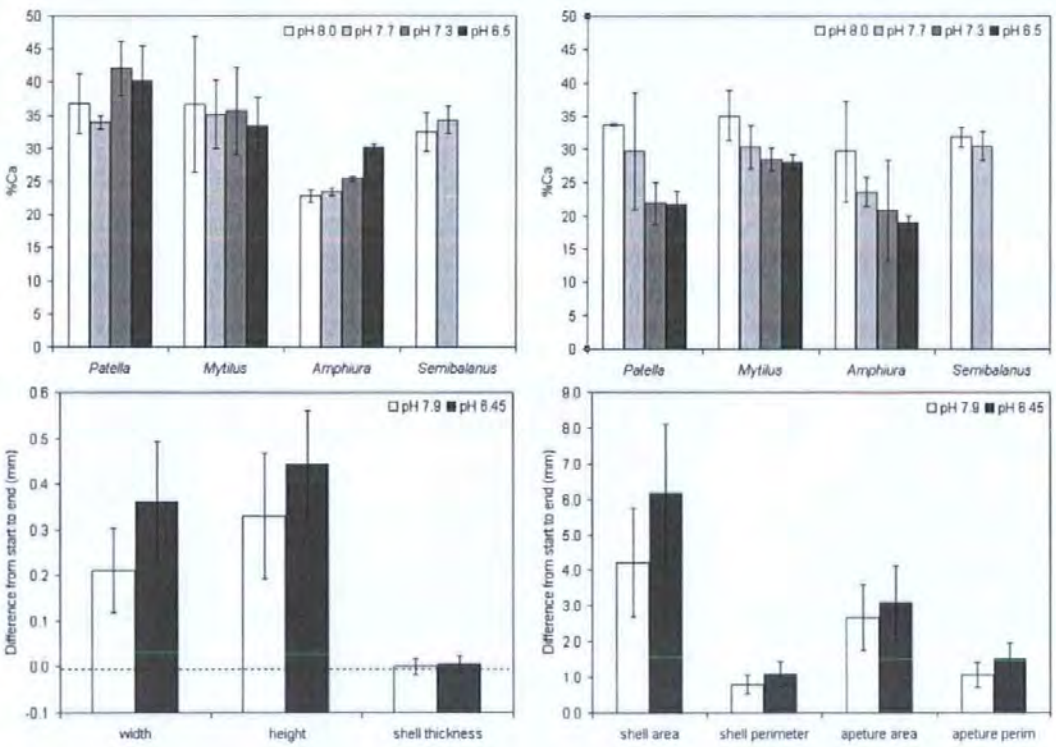


Figure 7.1. Calcium ion concentration (% of total structure) in the shells of **a.)** live and **b.)** dead *Patella vulgata, Mytilus edulis, Semibalanus balanoides* and arms of *Amphiura filiformis* (from Chapter 2) **c.)** & **d.)** Mean difference (start - end) in shell parameters of *Littorina littorea* where values above zero represent an increase (mm). Error bars represent 95% confidence intervals.

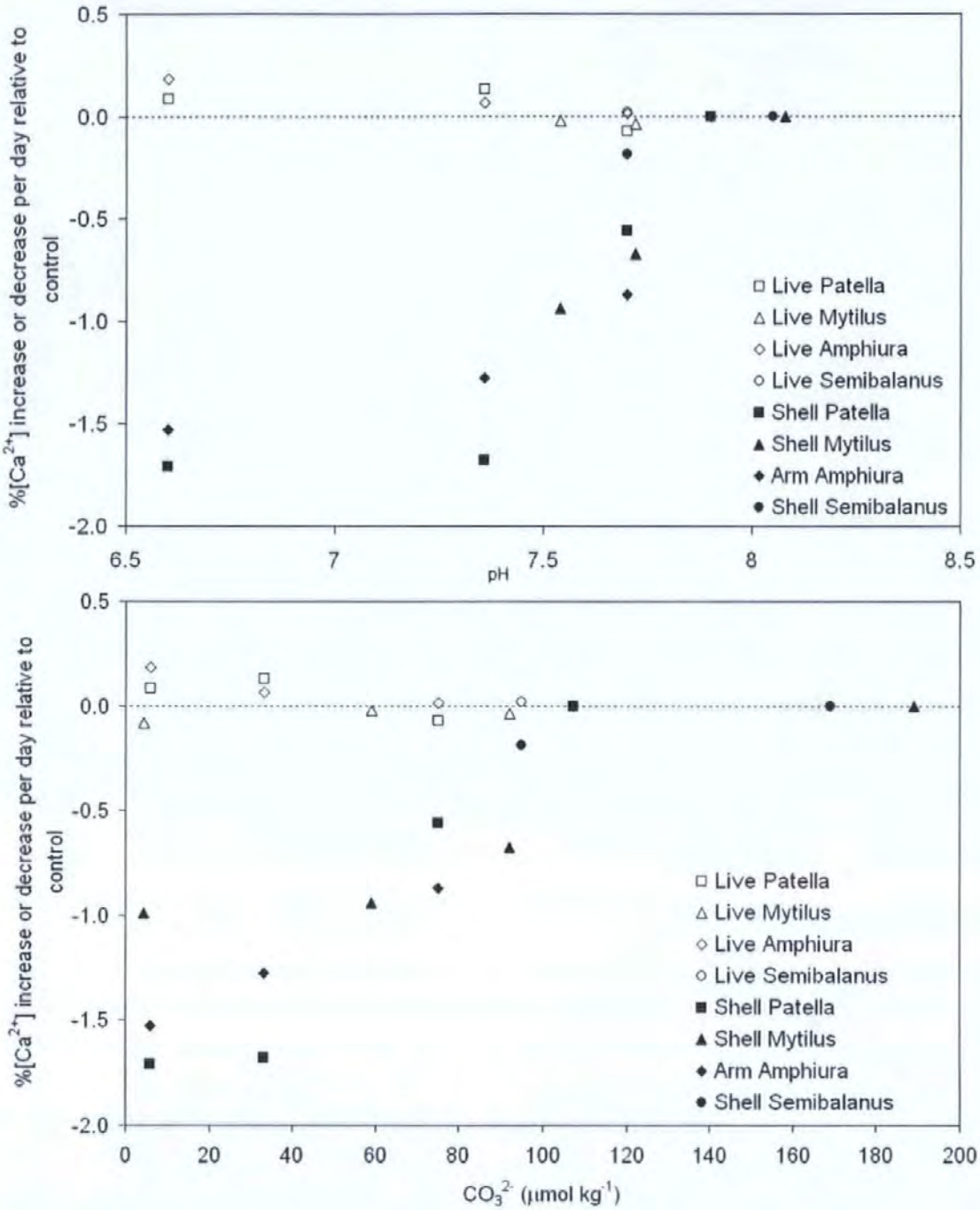


Figure 7.2. The increase or decrease in calcium ion concentration (percentage total structure) in each treatment a.) actual pH measured in the exposure tanks b.) calculated carbonate ion concentration in the exposure tanks, of live and dead *Patella vulgata* shells, live and dead *Amphiura filiformis* arms, live and dead *Mytilus edulis* shells and live and dead *Semibalanus balanoides*. The means are standardised to an increase or decrease per day, assuming that there was a linear change over the experimental time period.

Table 7.1. Experimental information for each species, detailing when the experiments were carried out, where the animals were collected from, what reproductive state the animals were in, how long the exposures lasted for and the mean pH of each exposure treatment.

Species	Date of experiment	Collections location	Adult reproductive state	Exposure period	Treatments (pH)	Feeding
Mytilus edulis	Sep. – Nov.	Trebarwith Sand, Cornwall, UK	Spawned *prior* to day 30	60 days	8.08, 7.72, 7.54, 6.41	Mixed algal diet (Shellfish Diet 1800®, Reed Mariculture)
Patella vulgata	Dec. – Jan.	Wembury Bay, Devon, UK	Dormant	40 days	7.88, 7.70, 7.36, 6.60	Preconditioned biofilm slides
Littorina littorea	Nov. – Dec.	Wembury Bay, Devon, UK	Dormant	15 days	7.97, 6.63	3 algal sp. *Ulva lactusa, Ascophyllum nodosum, Fucus serratus*
Amphiura filiformis	Dec. – Jan.	Plymouth Sound, Devon, UK	Dormant	40 days	7.87, 7.69, 7.36, 6.80	Deposit feeding within the cores
Semibalanus balanoides	Nov. – Feb.	Looe Bay, Cornwall, UK	Dormant	104 days	8.07, 7.70	Mixed algal diet (Shellfish Diet 1800®, Reed Mariculture)

Table 7.2. System data (mean ± 95% confidence interval) for the pH used in each of the five experiments. Salinity, temperature, pH & DIC data were measured, all other data (A_T = total alkalinity; CO_3^{2-} = carbonate ion concentration; $\Omega_{calcite}$ = calcite saturation state; $\Omega_{aragonite}$ = aragonite saturation state) were calculated from pH and DIC using CO2sys with the solubility constant of Mehrbach et al, (1973) refit by Dickson & Millero (1989).

		Mytilus edulis	*Patella vulgata*	*Littorina littorea*	*Amphiura filiformis*	*Semibalanus balanoides*
	Temp	17.74 ± 0.24	14.83 ± 0.39	15	14.83 ± 0.39	11.88 ± 0.06
	Sal	35.13 ± 0.07	36	35	36	35.60 ± 0.11
Control	pH	8.08 ± 0.09	7.88 ± 0.04	7.96 ± 0.04	7.89 ± 0.05	8.07 ± 0.03
pH-1		7.72 ± 0.12	7.70 ± 0.03		7.69 ± 0.03	7.70 ± 0.03
pH-2		7.54 ± 0.09	7.36 ± 0.07		7.37 ± 0.10	
pH-3		6.41 ± 0.22	6.60 ± 0.06	6.64 ± 0.06	6.60 ± 0.06	
Control	DIC (mmol kg^{-1})	1.88 ± 0.65	1.92 ± 0.11	1.24 ± 0.22	1.94 ± 0.11	1.88 ± 0.89
pH-1		1.95 ± 0.62	2.05 ± 0.14		2.04 ± 0.14	2.05 ± 0.83
pH-2		1.97 ± 0.70	2.04 ± 0.15		2.08 ± 0.15	
pH-3		2.39 ± 0.24	1.83 ± 0.23	2.54 ± 0.24	2.39 ± 0.24	
Control	$\Omega_{calcite}$	4.37 ± 1.34	2.56 ± 0.38	1.76 ± 0.12	2.79 ± 0.38	3.22 ± 0.34
pH-1		2.12 ± 0.63	1.81 ± 0.22		1.86 ± 0.22	1.59 ± 0.13
pH-2		1.36 ± 0.45	0.81 ± 0.32		0.95 ± 0.32	
pH-3		0.10 ± 0.08	0.14 ± 0.04	0.15 ± 0.02	0.16 ± 0.04	
Control	$\Omega_{aragonite}$	2.83 ± 0.87	1.66 ± 0.25	1.13 ± 0.07	1.80 ± 0.25	2.07 ± 0.22
pH-1		1.37 ± 0.41	1.17 ± 0.14		1.19 ± 0.14	1.02 ± 0.09
pH-2		0.88 ± 0.29	0.52 ± 0.21		0.61 ± 0.21	
pH-3		0.06 ± 0.05	0.09 ± 0.02	0.10 ± 0.01	0.10 ± 0.02	
Control	Alkalinty (µEq kg^{-1})	2160 ± 697	2094 ± 120	1363 ± 213	2099 ± 120	2086 ± 101
pH-1		2069 ± 635	2175 ± 153		2125 ± 153	2115 ± 95
pH-2		2019 ± 707	2134 ± 156		2067 ± 156	
pH-3		2057 ± 620	1943 ± 232	2116 ± 155	1981 ± 232	
Control	CO_3^{2-} (µmol kg^{-1})	189 ± 57.98	107.4 ± 16.07	76 ± 5.1	117.1 ± 16	144.3 ± 20
pH-1		91.84 ± 27.40	75.8 ± 9.3		77.9 ± 9.3	71.6 ± 12.9
pH-2		58.88 ± 19.54	33.7 ± 13.77		39.9 ± 13.8	
pH-3		4.20 ± 3.47	5.8 ± 1.65	6.7 ± 0.90	6.4 ±1.65	

DISCUSSION

These results indicated that there was a large amount of dissolution taking place on isolated shells and arms while the presence of a live animal within its calcium carbonate structure offset this dissolution; although the dissolution rate observed here may have been greater than might be expected to occur *in vivo*. *In vivo* dissolution remains an expected response to lowered pH primarily due to both the continued external exposure of $CaCO_3$ structures to the lowered pH water in the shell bearing species (*Mytilus edulis, Littorina littorea, Patella vulgata,* and *Semibalanus balanoides*) and the poor internal regulatory capacity of both *Amphiura filiformis* and the aforementioned species. Continued exposure and poor internal regulatory capacity results in the internal fluids having similar chemical composition to the surrounding seawater, therefore the mesoskeleton and inner shell surface respectively are also bathed in lowered pH fluid. Our results showing continued presence and in some cases growth of calcified structures demonstrates that the animals are still able to produce $CaCO_3$, i.e. calcify, thus replacing the $CaCO_3$ lost through dissolution. This supports the hypothesis that calcification in molluscs, crustaceans and echinoderms relies on either HCO_3^- or CO_2 and is not dependent on the CO_3^{2-} concentration or calcite/aragonite saturation states but may be related to metabolism (Lewis & Cerrato 1997). Perhaps more importantly it demonstrates that there is a great degree of biological control on calcification with complex links to other physiological processes (e.g. Pörtner 2008). In some instances organisms were are able to completely overcome dissolution to increase their levels of calcium carbonate, while in other organisms levels were simply maintained (*Mytilus*).

Understanding how biological processes such as calcification influence the oceans' natural feedback mechanisms is fundamental when attempting to predict how the oceans'

carbonate system will change in the future. Models indicate that under ocean acidification $CaCO_3$ saturation states will become undersaturated (Caldeira & Wickett 2003) leading to increased $CaCO_3$ dissolution. It has been shown here, however, that biogenic calcium carbonate formation may increase or remain constant despite falling carbonate saturation levels and associated increasing dissolution (Andersson et al 2006). Future net calcium carbonate production will represent a trade off between the antagonistic processes of calcification and dissolution. Dissolution may exert a cost, physically or energetically on organisms and additional impacts of hypercapnia and acidosis on metabolism and physiology may also interfere with an animal's homeostatic function (Pörtner 2008).

Recent experiments focusing on a single physiological process, such as growth of calcifying organisms under hypercapnia, potentially overlook the possibility that increased calcification may have counteracted some, or all, shell dissolution that was occurring at the same time as the animals were growing (e.g. Michaelidis et al 2005; Gazeau et al 2007; Cooper et al 2008). Shell growth or net calcification may appear to be slower or reduced under hypercapnic conditions compared to the control, yet this may be a result of increased dissolution rates or impairment to other physiological processes, not necessarily a reduction in the animals' ability to calcify. Increasing evidence is appearing in the literature which agrees with the results of this study: McDonald et al (2009) show another barnacle species (*Amphibalanus amphitrite*) to continue, and possible even increase, calcification in conditions with pH 7.4; Arnold et al (2009) demonstrate larval lobsters (*Homarus gammarus*) are able to lay down calcium carbonate structure in pH conditions 0.3 units below the control levels; Checkley et al (2009) show young fish have enhanced aragonite otolith growth when grown under elevated CO_2; Maier et al (2009) showed that although there was a decrease in calcification in cold-water corals, overall they showed a positive net calcification at aragonite saturation states below 1, and longer-term experiment

suggest that they may actually maintain or even increase calcification over longer timescales at low pH (U. Riebesell, pers comm.).

While the five species presented in this study are all benthic calcifiers, they vary greatly in lifestyle, and therefore it needs to be considered whether the abiotic environments differ in their natural pH conditions. The most notable exception is the brittlestar *Amphiura filiformis* which lives within the sediment which is naturally lower in pH (Dashfield et al 2008). However it has been shown (Zhu et al 2006) that burrow irrigation results in porewater pH reflecting the overlying water rather than that of the sediment; it can be assumed this is the case for *A. filiformis* which continually ventilates its burrow. The remaining species investigated in this study were all intertidal, and studied under immersed conditions (except *S. balanoides*, which was studied under tidal conditions), thus the altered seawater pH reflects the conditions these species experienced. Under natural conditions these species typically, with the exception of *Littorina littorea*, shut down during emersion. Therefore their internal pH may decrease for short term periods due to the build up of respiratory CO_2, however this does not mirror these experiments due to the short term nature of these episodes, and because these current experiments result in the total immersion, both internally and externally, of the animal in lowered pH seawater.

The findings here also have implications for the understanding of past episodes of CO_2 rise, ocean acidification and biodiversity crisis, and find support in recent paleoecological studies. The fossil record is an archive of global-level experimental data on the response of the biosphere to climatic and environmental change, and understanding past changes allows us to place the present-day crisis in its historical and scientific context. The geochemical and paleontological proxies that are used to estimate past levels of atmospheric CO_2, such as the stomatal index of fossil leaves (McElwain et al 1999) and the

carbon isotope signature of ancient soil carbonates (Cerling 1991), demonstrate that CO_2 has fluctuated over the Phanerozoic and at times in the past has greatly exceeded present-day levels and the maximum predictions for the coming century (Royer et al 2004), albeit on very different timescales to the present-day crisis. All of the major mass extinction events of the past 500 million years show evidence of associated climate change, including CO_2 rise and global warming (Twitchett 2006). The Late Triassic mass extinction event, for example, occurred during a relatively fast 400% rise in atmospheric CO_2 levels from ca. 600 to 2400 ppm (e.g. McElwain et al 1999; Beerling & Berner 2002) and increased dissolution may have had a leading role to play in the extinctions of marine invertebrates (Hautmann 2004). Measurements of bivalve size and shell thickness through this event demonstrated a temporary reduction in size but increase in shell thickness (Mander et al 2008), which would be a predicted response to increasing acidification based on our laboratory studies. The timescale of present day climate change is faster than the events recorded in the fossil record, where changes are more likely to result from evolutionary adaptation. However such evidence does support the survival and continued calcification potential of benthic invertebrates in a high CO_2 world. In addition, the metabolic change seen in paleoecological data (Hautmann 2006) is consistent with the results of some recent ocean acidification studies highlighted here (e.g. Chapters 2, 5 & 6; Bibby et al 2007) which also found increased calcification and metabolism in species today under ocean acidification conditions.

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

response have shown that several are impacted as a consequence of the up regulation of calcification and metabolism; for example, there was increased muscle degradation in *Amphiura filiformis* (Chapter 2), a lowered predation avoidance response in *Littorina littorea* (Bibby et al 2007), and reduced health in *Mytilus edulis* (Beesley et al 2008). Other investigations, with similarly small changes in pH, show that acid-base balance cannot be maintained in other mollusc and echinoderm species under acidified conditions (Michaeladis et al 2005; Miles et al 2007). Indeed Table 7.1 illustrates that all species that showed increased % Ca^{2+} were in a dormant reproductive state, while *M. edulis*, which spawned during the exposure, only had maintained and slightly lower % Ca^{2+}. This again hints at differences in energy allocation and metabolic demand resulting in different effects. A longer term (6 month) sea urchin acidification experiment (Shirayama & Thornton 2005) appears to provide evidence that some species are not able to maintain a high rate of calcification in order to overcome an increased rate of dissolution. The decrease seen in test thickness (Shirayama & Thornton 2005) did not account for total mass loss of *Hemicentrotus pulcherrimus* and *Echinometra mathaei* indicating a loss of soft tissue, as seen in *A. filiformis* (Chapter 2). Ocean acidification therefore may not directly result in a reduced ability to calcify, but it does appear to cause negative impacts on all tested organisms. This highlights the importance of bringing together the current literature to gain a holistic insight when evaluating parameters such as calcification but also the need to investigate other processes in both calcifying and non-calcifying species.

Paleoecological studies of past episodes of CO_2 rise provide some data concerning longer term changes. One characteristic of extinction episodes, especially those associated with CO_2 rise such as the Late Permian and Late Triassic events, is a dramatic decline in the size of marine organisms (the Lilliput effect) (Hautmann 2006; Twitchett 2007; Mander et al 2008). The costs associated with the need for increased calcification may have a role to

play in this phenomenon. Changes in shell mineralogy, from aragonite to calcite, have also been observed in Triassic-Jurassic bivalves and interpreted as reflecting a need to conserve energy as metabolic rates increased (Hautmann 2006). This change in mineral structure, which may also be an adaptation to ocean acidification by benthic calcifiers today, reduces metabolic costs of calcification indirectly because calcite is less prone to dissolution and hence the rate at which the structure needs to be replenished in low pH conditions is reduced. There are apparent differences in the deep-sea coral ecosystems between the North Atlantic and the North Pacific, the latter of which has much a shallower aragonite saturation horizon (ASH). In the North Pacific six of the seven stylasterid species of coral (Cairns & Macintyre 1992), used calcite to form their spicules and skeletons, yet only 10% of all known stylasterid species produce calcite instead of the more soluble aragonite. Cold-water corals have also been found living close to the ASH, suggesting they have mechanisms to cope with high rates of dissolution, yet they do not flourish or form large structure, as in the North Atlantic (Guinotte et al 2006). At marine volcanic CO_2 vent sites, although the abundance of calcifying animals' decreases with increasing pH, these organisms are nonetheless found under acidified seawater conditions (Hall-Spencer et al 2008).

Conclusion

Results from both the laboratory and from paleoecological records suggest that animals are capable of altering their biology to be able to cope with a decrease in pH. As research now homes in on realistic scenarios, we may find that within the predicted pH ranges, at least in terms of producing calcium carbonate, animals are able to compensate. Other physiological processes are more likely to be impacted as a cost to increased energy expenditure of producing calcified material in a more acid ocean, therefore organisms may grow less (i.e.

become smaller on average, as is evident from experimental and paleoecological data) and/or over longer timescales they may change their mineralogical structures. While work to date (See Fabry et al 2008 for review) has made some steps in determining physiological responses to high levels of CO_2, research should focus on whole animal physiology in both non-calcifying and calcifying organisms as well as investigate the possibility of mineral and size changes over longer time-scales.

CHAPTER EIGHT

GENERAL DISCUSSION

The physiological response of calcifying benthic invertebrates to ocean acidification; a common theme of energy limitation?

INTRODUCTION

Ocean acidification alters the physico-chemical properties of the water and as such acts as an environmental stressor. Marine organisms are literally bathed in seawater and the impact of ocean acidification on seawater chemistry can change how a marine organism interacts with this abiotic environment thus affecting internal processes; this makes ocean acidification inherently a physiological concern. As such all investigations of the response of a species to ocean acidification are effectively addressing how ocean acidification impacts upon the species' physiology; whether that investigation focuses directly on the physiological processes (e.g. growth, Michaelidis et al 2005; acid-base balance, Spicer et al 2007; whole organism physiology, Chapter 2, Chapter 4) or instead looks at changes expressed at a higher organisational level, e.g. population or ecosystem level effects, which are the indirect consequences of changes to the physiology of the individuals therein (e.g. Widdicombe et al 2009).

All such experimental approaches are essential in formulating possible implications of ocean acidification, prerequisite to predicting the long term implications on marine ecosystems, biodiversity, goods and services. To date there has been little standardisation between studies (but c.f. the recently compiled 'Best Practice Guide' from the EPOCA consortium): they have differed in timescale (from hours to months), pH treatments (extreme changes of 1+ pH units, to more subtle changes ~0.1 pH unit) and methodology of pH manipulation (e.g. CO_2 *versus* acid addition). Therefore it is perhaps not surprising that the picture that has emerged is one of conflicting results, leading to the view that there is, as yet, no unified physiological response to ocean acidification across all, or even across specific groups (functional/ taxonomic), of species. One exception to this was Pörtner et al (2008), who postulated that acid-base regulation capacity may be a key determinant of the

survival prospects of marine invertebrates to ocean acidification; this present discussion compliments some of these principles. To my knowledge this is the only published attempt to present a unified physiological response.

In this chapter I present a new model. Based on the insight gained from both the chapters in this thesis, and also published data, I present a model developed to characterise the physiological response to ocean acidification which, at present, appears to be common to all adult calcifying benthic invertebrates. To present this model I will first outline the basic physiological cost to ocean acidification, introduce the model itself, then expand upon this by discussing the influence of lifestyle and level of activity on an organism's response to ocean acidification (and therefore the model). Finally I will consider areas where greater understanding is required before the model can be applied. Throughout this chapter I will use case studies to demonstrate how the expectations of the model are reflected in empirical data. These case studies are initially dominated by echinoderms, as the premise for the model is based around the work in this thesis; however, case studies based on the findings of other researchers are also used where appropriate and demonstrate the wider applicability of this model.

ACID-BASE BALANCE REGULATION

The acidity of a liquid is determined by the amount of hydrogen ions (H^+) it contains; as the uptake of CO_2 by seawater continues and ocean pH decreases, the internal extracellular fluids of marine invertebrates also undergo a concomitant rise in H^+ *via* a diffusion gradient between the seawater and internal fluids. Internal processes and structures, from cell membranes to enzymatic reactions, have an operational pH outside of which they cease to function effectively (Reipschläger & Pörtner 1996). The mechanisms by which a

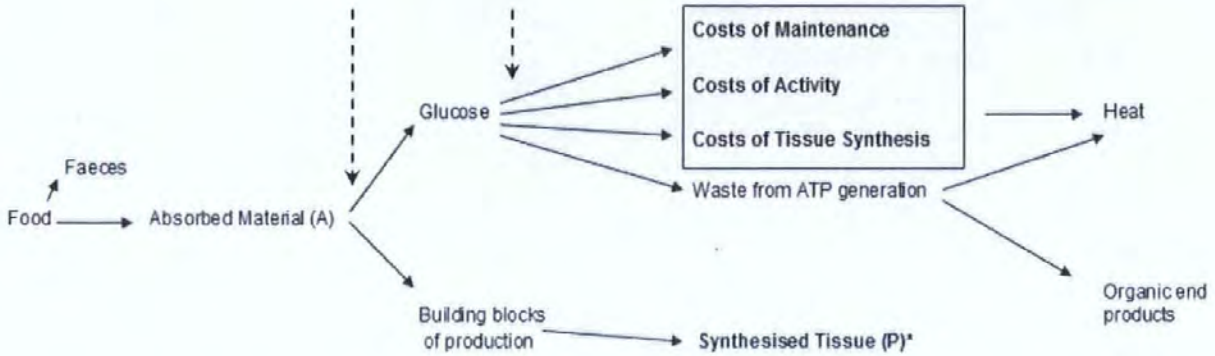
species maintains this extracellular pH when the external environment changes is relatively well known (Truchot 1975, 1979, 1983; Toews et al 1983; Heisler 1984, 1993; Lindinger et al 1984; Cameron 1986, 1989; Cameron & Iwama 1987, 1989; Taylor & Spicer 1991; Clairborne & Evans 1992; Clairborne et al 2002; Pörtner et al 1998; Truchot & Forgue 1998; Langenbuch & Pörtner 2002; Hayashi et al 2004) and may define the most fundamental susceptibility to ocean acidification at the cellular level (Widdicombe & Spicer 2008; Pörtner et al 2008). Higher organisms such as fish use ion transport and exchange to actively buffer extracellular pH changes (Hayashi et al 2004), and appear to be unaffected by lowered pH in the surrounding seawater (Melzner et al 2009), at least where the water remains alkaline (Hayashi et al 2004). Calcifying benthic invertebrates, with the exception of crustaceans (e.g. Truchot 1983), do not have the same ion regulatory capability as fish and rely on passive buffering to regulate extracellular pH. Bicarbonate is accumulated within the extracellular fluid to compensate for the increased H^+ (Henry et al 1981; Taylor & Spicer 1991; Walsh & Milligan 1989; Reipschläger & Pörtner 1996). Many benthic invertebrates appear to source this bicarbonate from the internal dissolution of their calcium carbonate skeleton (Michaelidis et al 2005; Shirayama & Thornton 2005; Spicer et al 2006; Miles et al 2007). The effectiveness of this passive buffering mechanism seems to vary between species. The echinoderm *Psammechinus miliaris* could not fully compensate its extracellular pH when exposed to seawater of pH 7.4 (Miles et al 2007) despite small but significant increases in bicarbonate, while indirect evidence from long term survival suggests other echinoderms are better at compensation e.g. *Amphiura filiformis* (Chapter 2) and *Ophiura ophiura* (Chapter 5) survived 40 day exposures to pH 7.3, *Pisaster ochraceus* (Gooding et al 2009) 780 ppm (approx pH 7.8) for 70 days and *Ophiocten sericeum* pH 7.3 for 20 days (Chapter 6). Even within the crustaceans, which possess relatively well developed ion exchange mechanisms, alongside recourse passive buffering via shell dissolution (e.g. de Fur and McMahon 1984), there is still considerable

variation between species in the ability to compensate extracellular acidosis at low environmental pH. The decapod crustacean *Necora puber* showed full acid-base compensation at pH 7.3 (Spicer et al 2007) during a 7 day exposure, while the shrimp *Palaemon pacificus* experienced greater mortality attributed to incomplete acid-base compensation at 1000 ppm (approx pH 7.7) after 30 weeks (Kurihara et al 2008).

Regulation of extracellular acid-base is essential for an organism's survival but energetically expensive (Pörtner et al 2000). The energy requirement for acid-base balance compensation is increased by ocean acidification, and it is the recognition of this increased cost that is the key to understanding the physiological response of marine organisms to ocean acidification as set out in the new model presented here; The Energy Limitation Model.

THE ENERGY LIMITATION MODEL

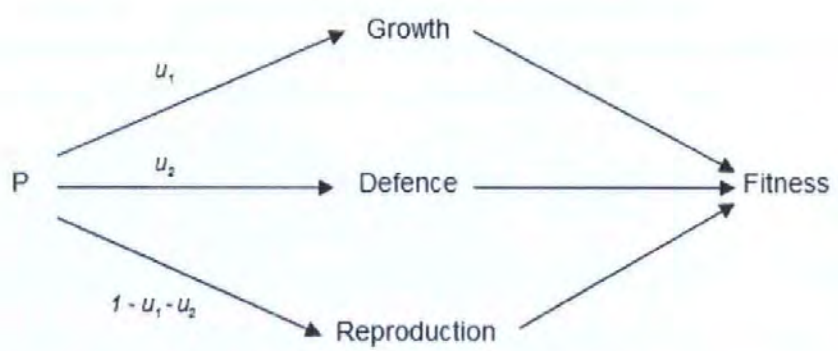
The energy input required to maintain all maintenance activities, including acid-base balance (also known as extracellular pH, and hereafter referred to as pH_e, Langenbuch & Pörtner 2002) in hypercapnic seawater has implications for an organism's energy budget (Sibly & Calow 1986). When the cost of maintenance is increased there are two options, to increase energy intake, or to reallocate energy from another process. Diagram 8.1, adapted from Sibly & Calow (1986) outlines the costs of living, and demonstrates that if an increase to the cost of maintenance is not met by additional energy input, this cost will entail a decrease in energy to activity or tissue synthesis (P); this is known as the Principle of Allocation (Sibly & Calow 1986). Diagram 8.2 expands further on P to illustrate this cost may then be in terms of growth, reproduction or defence, and a reduction in P will reduce fitness.



= point where change to resource allocation may occur.

* = see Diagram 2 for further detail of resource allocation

Diagram 8.1. The Costs of Living (adapted from Sibly & Calow 1986)



U_1 = proportion of resources allocated to growth

U_2 = proportion of resources allocated to defence

Diagram 8.2. Allocation of resources (adapted from Sibly & Calow 1986)

The Principle of Allocation (Sibly & Calow 1986) sets out the conflicts and trade offs of energy allocation, and this is the basis for the new model presented here, the proposed mechanism of response to ocean acidification; the energy limitation model (hereafter referred to as ELM). Physiological processes within an organism are integrated and the increased energetic cost of pH_e (part of the cost of maintenance) has knock-on effects to other physiological processes. If and how an organism is able to obtain, and then allocate the energy to maintain pH_e is key to a species' survival *via* both direct and indirect mechanisms. The observations and principles presented here as part of the ELM are not novel and in each case the original work is cited. What is novel is linking together each of the mechanisms identified by previous ocean acidification studies with the increased energetic cost of acid-base balance under hypercapnia using the Principle of Allocation.

The scope for choice in where the trade offs in energy allocation occurs to 'fund' the increased cost of maintenance explains the differences in response to ocean acidification recorded between species to date. ELM ties together these responses with an underlying mechanism of energy limitation.

Case Study 1: *Amphiura filiformis*

The infaunal ophiuroid *Amphiura filiformis* responded to the ascribed increase in the cost of maintaining pH_e by metabolic upregulation and by utilising the energy within arm muscle resulting in muscle wastage (Chapter 2). As an echinoderm, *A. filiformis* has poor internal ion regulation capacity and falls within the taxa that utilise internal dissolution of their calcium carbonate skeleton to passively buffer pH_e. This is consistent with the view that the energy input to maintain pH_e in passively buffering invertebrates primarily goes to calcification to replace skeletal material lost through dissolution, and ensure there is a

continued supply of bicarbonate to regulate pH_e. After a 40 day exposure to pH levels as low as 6.8, *A. filiformis* survived and showed no change in the calcium carbonate proportion of their skeleton (Chapter 2), suggesting mesoskeleton $CaCO_3$ lost through dissolution had been fully replaced. If this $CaCO_3$ had not been replaced the ratio of organic material to $CaCO_3$ would have increased, and that was not reflected in the data. This is an important point, for the initial result that a taxa known for poor regulatory capacity can survive pH as low as 6.8 (albeit with muscle wastage consequences which are discussed below) appears counterintuitive; surely regulation must be key for maintaining pH_e? But the passive buffering mechanism merely requires a supply of bicarbonate to maintain pHe. *A. filiformis* does not require a highly developed ion regulation capacity to maintain pH_e. It merely requires the capacity to upregulate biogenic $CaCO_3$ production, and this is a function of energy availability and the ability to calcify faster than dissolution occurs, with the rate of dissolution dependant on the pH of the seawater. Concomitant upregulation of metabolism at lowered pH reflects the knock-on increase in energy requirement of up-regulating calcification. However, for *A. filiformis* the cost of this maintenance does not appear to be met by increased energy intake alone (Chapter 2). The nature of this burrow dwelling species' lifestyle necessitates continual ventilation of its burrow by arm movement, a process which also collects vital food, *via* suspension feeding. The increased oxygen demand of metabolic upregulation and increased food requirement (in response to the demands of calcification/bicarbonate production) further exacerbates the necessity of arm movement even when energy output outweighs input. In addition this fossorial lifestyle requires functioning, full length arms to supply the oxygen and food to the organism; this is reflected by increased rates of arm regeneration and functional recovery at lowered pH (Chapter 3) which in itself is an energetically costly process. Thus, the infaunal lifestyle of *A. filiformis* requires energy expenditure for survival and at lowered pH this combined with pH_e maintenance results in an energy deficit. This deficit is

met through an unsustainable means - muscle loss- which ultimately will result in mortality (Chapter 2). The energetic demands of pH_e maintenance in *A. filiformis* at lowered pH leads to an energy deficit under the ELM thus indicating long-term survival is unlikely.

The empirical data provided by *Amphiura filiformis* (Chapters 2 & 3) demonstrates the basic principle set out by the ELM; the increased cost of maintenance at lowered pH necessitates either an increase in energy intake, here increased metabolism, or a reallocation of resources, also demonstrated here by muscle loss. Without the framework of the ELM this muscle loss could be seen as another example of differing responses to ocean acidification between species, but the ELM places this muscle loss within the context of energy limitation and trade off.

Case study 2: Sea urchins

Sea urchins appear to be particularly poor at surviving ocean acidification. This vulnerability appears to be a function of insufficient upregulation of $CaCO_3$ production to replace the test lost through dissolution leading to incomplete maintenance of pH_e. The urchins *Hemicentrotus pulcherrimus* and *Echinometra mathaei* exhibited significant thinning of their calcium carbonate tests and mortality over long term exposure at a moderate pH decrease of 0.1 pH units (Shirayama & Thornton 2005), and *Psammechinus miliaris* were unable to fully compensate internal pH at even pH 7.44 (Miles et al 2007). It could be argued that the test thinning, and as such $CaCO_3$ production, was a consequence of incomplete pH_e regulation rather than its cause. Under the ELM hypothesis whereby insufficient $CaCO_3$ production was the cause of incomplete acid-base regulation this would of the energetic deficit compromises other functions and therefore survival. Clearly in these urchins, the energy deficit cannot be made up from elsewhere without a detrimental

effect to soft tissues and the maintenance of pH_e which results in mortality (Shirayama & Thornton 2005; Miles et al 2007).

The urchins described above have all been epibenthic species i.e. animals that live on or above the seafloor. However, when studies look at urchins that live within the sediment (infaunal) e.g. *Brissopsis lyrifera* and *Echinocardium cordatum* (Dashfield et al 2008; S. Widdicombe unpubl. data) the story is slightly different. *B. lyrifera* lives in muddy sediment which is shown to already be slightly hypercapnic, and thus experiences less of a pH change as a result of altered pH in the overlying seawater, (Dashfield et al 2008) while *E. cordatum* burrows in sand the interstitial waters of which generally reflect the seawater. Both species survived exposure to pH 7.2, and *B. lyrifera* survived at pH 6.8 where the sandy sediment counterpart *E. cordatum* suffered 100 % mortality (S. Widdicombe unpubl. data). Both species compensated pH_e over the long-term (4 months) however *B. lyrifera*, the species that lives in the low but stable pH muddy sediment, experienced less of a detrimental health effect than *E. cordatum* due to seawater pH manipulation (S. Widdicombe unpubl. data). The fossorial lifestyle of these urchins may influence their exposure to changes in seawater pH, and the low pH of muddy sediment suggests *B. lyrifera* has a pre existing adaptation to low pH conditions. The differing response of these two urchins demonstrates the determining role lifestyle can also have on a species' survival.

THE INFLUENCE OF LIFESTYLE

Habitat

Where a species lives and the ensuing impact this has on the cost of maintenance (defined as all processes necessary to maintain biomass in a viable state, Sibly & Calow 1986) are key to where the compromise in energy allocation is made. If a species lives in an environment that requires a large portion of energy for survival (maintenance costs, Diagram 8.1) in its habitat, when hypercapnia then increases the cost of pH_e regulation there is less energy available to be diverted from P or activity (Diagram 8.1) thus a conflict in energy allocation is more likely to arise between vital maintenance requirements. Consequently, where a species lives can be key to the determination of a species' ability to survive ocean acidification. This was illustrated by Case Study 1 (and Chapter 2), where the infaunal lifestyle of *Amphiura filiformis* entailed energetic demands to maintain survival (arm movement for burrow ventilation) resulted in an energy deficit. Conversely, the stability of a habitat, particularly for burrowing species, can provide protection from or a tolerance to changes in seawater pH, as shown by the survival of the urchin *Brissopsis lyrifera* at pH as low as 6.8 (S. Widdicombe unpubl. data). The following case studies further demonstrate the influence of lifestyle energy allocation.

Case study 3: *Ophiura ophiura*. An epibenthic species

The epibenthic ophiuroid *Ophiura ophiura* responded to the increased cost of pH_e regulation by metabolic upregulation, reducing energy output to movement (moving slower) and reducing arm regeneration rate (Chapter 5). Given that muscle wastage was

seen in the related ophiuroid *Amphiura filiformis* (Chapter 2) which has a similar biogeographical distribution, this may also be expected in *O. ophiura*. However no arm wastage was recorded in *O. ophiura* (Chapter 5); furthermore the reduction in arm regeneration rate is the complete opposite of *A. filiformis*. What may initially appear counter-intuitive begins to make some sense when considered within the context of the species' lifestyle. *O. ophiura* as an epibenthic species does not have the habitat constraints that require a large energy output, *via* arm movement, to obtain oxygen, which also means that the importance of arm regeneration is not as high as for *A. filiformis* either. While reduced motility and slower regeneration may have sublethal effects on fitness *via* les effective predator avoidance and food sensory capacity (Valentincic 1991), by redirecting energy from these non-vital functions, *O. ophiura* appears to balance energy input and output under low pH conditions, and in doing so facilitates species survival (Chapter 5).

Comparison of this temperate epibenthic species with a closely related (Paterson et al 1982) Arctic species of similar (epibenthic) lifestyle revealed the same response of slower arm regeneration and metabolic upregulation (Chapter 6). There are a number of important differences in the abiotic environment between these two species. *Ophiocten sericeum* is restricted to the Arctic Circle and as such experiences a temperature range of – 1.8 °C to 5 °C (Chapter 6), high rates of sedimentation due to the glacier scouring influenced riverine deposits, and highly seasonal food and light regimes due to sea ice (Chapter 6). By comparison *Ophiura ophiura* is a temperate species with a temperature range of 8 °C to 15 °C experienced within the Plymouth population alone (Chapter 5) the species temperature range is likely wider, and sedimentation rates are far lower and sporadic, linked to terrestrial run-off. These differences in the abiotic conditions and environmental variability experienced by these two species do not alter the common response (slower arm regeneration and metabolic upregulation) to seawater acidification. Thus underlining the

dominance of lifestyle and functional prioritisation of physiological and physical processes in determining where a species energy deficit is met. The difference between the energetic 'cost cutting' of *O. ophiura* (Chapter 5) and *O. sericeum* (Chapter 6) and the physical repercussions of unchanged energetic expenditure in *A. filiformis* (Chapter 2) reflects the difference in functional importance of the arms between these two species, and ultimately the importance of lifestyle and the basic energetic demands thereof.

Level of activity

The review of responses of marine organisms to ocean acidification by Fabry et al (2008) includes a useful table summarising the published responses to ocean acidification arranged by phylum. Within the Mollusca every study on adults/juveniles reported reduced growth or net calcification as the principle response. The species which were reported to show reduced growth/ net calcification are all sessile, and as such are expected to have limited metabolic plasticity. The sessile lifestyle of these species invariably reduces the variability and overall energy output apportioned to activity in more mobile species such as ophiuroid brittlestars. The correlation between a sessile lifestyle and a trade off in growth rather than metabolic upregulation in hypercapnic conditions raises the question of whether the limited capacity of sessile species to upregulate metabolism plays a part in their ability to respond to the pH_e regulation demands of ocean acidification. This theory of the importance of metabolic plasticity is strengthened by within phyla variations in response to hypercapnia which correlate to metabolic plasticity. Pane & Barry (2007) suggested the observed difference in pH_e regulation between related deep and shallow water crab species may be a function of metabolism.

Case study 4: Mussels

The bivalve mussel genus *Mytilus* is reasonably well studied with respect to ocean acidification. One of the early investigations into the impacts of ocean acidification by Michaelidis et al (2005) on *Mytilus galloprovincialis* found reduced shell growth and protein degeneration. They also found that compensation of haemolymph acid-base through shell dissolution was incomplete. The closely-related *Mytilus edulis* also responded with reduced growth (Berge et al 2006), reduced net calcification (Gazeau et al 2007) and incomplete pH_e compensation (H Wood unpubl. data). All of these data indicate the response of mussels to ocean acidification is consistent with the ELM hypothesis; reduced shell growth is likely to be a product of increased effort on maintaining shell thickness in light of dissolution both internally for passive buffering and externally due to the undersaturated ($\Omega_{cal/ar}$) seawater. The reduction in net calcification seen by Gazeau et al (2007) was attributed to a reduction in calcification because the pattern of reduced net calcification was seen at 1000 ppm (here pH 7.9) before seawater became under saturated with respect to aragonite. However, given that mussels maintain pHe under hypercapnic conditions by internal shell dissolution (Berge et al 2006) the reduction in net calcification seen by Gazeau et al (2007) is more likely to be a result of increased dissolution exceeding calcification. This suggests that the passive buffering was insufficient is reflected by the incomplete compensation of pH_e in this species. The protein degradation, in the same manner as seen in the brittlestar *Amphiura filiformis* and proposed to also occur in urchins, further indicates the need for energy to regulate acid-base through the provision of bicarbonate has resulted in an ultimately fatal trade-off whereby soft tissue is used as an energy source. Michaelidis (et al 2005) suggested reduced growth may be related to metabolic depression recorded; protein synthesis is shown to be related to metabolism (Langenbuch & Pörtner 2002) however the occurrence of protein degradation in *M.*

galloprovincialis indicates something more than just inhibition of protein synthesis. In light of the ELM theory, the response of *M. galloprovincialis* strongly indicates this protein degradation is related to an energy deficit resulting from the prioritisation of $CaCO_3$ to maintain acid-base balance, *via* buffering.

METABOLIC DEPRESSION; A STONE IN THE WORKS?

The conflicting evidence with regards to metabolic response to ocean acidification warrants some discussion, particularly as some of the examples used in support of the ELM include metabolic upregulation yet other studies of ocean acidification, namely the aforementioned study of *Mytilus galloprovincialis* (Michaelidis et al 2005), report metabolic depression. The supposition that metabolic depression occurs as a response to ocean acidification is derived from the well established principle that low pH causes metabolic depression at the molecular level (see Guppy & Withers 1999 for review). However, this principle was also based on the concept that this was a short term (minutes / hours), rather than a long term (days / weeks / months) response (Langenbuch & Pörtner 2004) and several longer term experiments have shown metabolic upregulation (e.g. Walther et al 2009, Hauton et al 2009). Early evidence supporting ocean acidification induced metabolic depression was largely gathered from short term exposures to hypercapnia on non-calcifying invertebrates (e.g. the polychaete *Sipunculus nudus,* Reipschläger & Pörtner 1996; Langenbuch & Pörtner 2004) which have more complex pH_e buffering mechanisms not addressed by the ELM. Nevertheless, there have been some subsequent long term experiments where metabolic suppression was seen; juvenile *Mytilis galloprovincialis* (Michaelidis et al 2005) responded to lowered seawater pH with a metabolic rate decrease to 70% of the rate recorded prior to hypercapnia over a 90 day exposure. As previously mentioned, these mussels did not fully compensate for their acid-

base disturbance. It is well established that low extracellular pH (pH_e) is the cause of the metabolic suppression at lowered environmental pH (Pörtner & Reipschläger 1996, Pörtner et al 1998, Guppy & Withers 1999). It is proposed here that metabolic depression is not necessarily a long term response of benthic invertebrates to ocean acidification as previously suggested (see review by Fabry et al 2008), but rather a short term response in several species as a function of pH_e (Guppy & Withers 1999) and only a long term consequence of ocean acidification in species that are unable to fully compensate internal acid-base (and thus subject to long term pH_e) as demonstrated by Michaelidis et al (2005), or when several energetic requirements combine to reduce the ability of a species to compensate pH_e under hypercapnia.

Case Study 5: *Littorina littorea*

Bibby et al (2007) investigated some induced defences of the gastropod mollusc *Littorina littorea* to predator cues under normocapnic and hypercapnic conditions. In the absence of predator cues the gastropod showed no change to metabolic rate or shell thickness. However, under hypercapnia (low pH) the normocapnic response to predator cue of shell thickening with concomitant reduction in activity did not occur. Instead, increased activity and metabolic depression was seen. Unable to increase shell thickness, the gastropods modified their behaviour instead. The maintenance of shell thickness and metabolism at low pH (with no predator cue) indicates pHe is compensated by passive buffering, and that the shell lost *via* this process is successfully replaced by increased deposition of $CaCO_3$, thus suggesting the energetic demands of this are being met, potentially through the reduced (although not significant) decrease in activity. The addition of a predator cue however, brings an additional energetic requirement. Biogenic calcification is already upregulated to counterbalance passive buffering, and a lack of increase in shell thickness as

seen in normocapnic seawater suggests further calcification is not achievable, so instead avoidance behaviour is increased. However, this activity also requires energy. The ELM states that if the increased costs of pHe maintenance cannot be met pHe decreases, and this has been demonstrated to result in metabolic depression (Guppy & Withers 1999), which was seen in this example. Returning to the principle identified by the importance of the energy required for lifestyle, here we can extend this to include the importance of the energy required for survival. Predator presence is a high priority due to the obvious implications to fitness, and Bibby et al (2007) elegantly demonstrate that this temporally variable but essential energy output can have as great an impact on survival of ocean acidification as lifestyle.

Ellis et al (2009) also reported metabolic depression in the related *Littorina obtusata* combined with reduced activity and slower growth rates. However, this was in embryonic stages where energetics and regulatory capacity are far more variable. As Ellis et al (2009) stated, this was the first study to investigate larval heart function and environmental variables, and our understanding is not sufficient to attribute the response of larvae to the ELM.

WHERE DO THE UNCERTAINTIES OF ELM APPLICABILITY LIE?

The ELM by no means offers all the answers with regards to ocean acidification, as demonstrated by inconclusive application of the ELM to the embryonic data from Ellis et al (2009). In particular, there are notable areas where we are unable to evaluate the applicability of the ELM, either because there are not enough published data on the response of physiology to hypercapnia, (e.g. early lifestages, synergisms with other

environmental factors), or because the mechanisms of pH_e regulation are complex and fall outside our area of expertise (e.g. non-calcifying invertebrates).

Lifestage

In adults the allocation of energy can be crudely assigned to food acquisition and assimilation, maintenance (including pH_e regulation), growth and reproduction, a concept originally put forth by Sibly & Calow (1986) and shown in Diagrams 8.1 & 8.2. However, early life stages offer an entirely different and variable set of energy requirements; that juveniles often follow a sigmoidal growth curve (e.g. Sibly 1981) reflects the dynamic cost of growth over development. This changing energy allocation towards growth is further complicated in the many marine invertebrates that have indirect development where a large energy investment is seen during metamorphosis. This variability makes it harder to understand how, where and when energy limitation and trade offs may occur. Furthermore, the regulatory capacity of larvae and at earlier stage gametes is not fully understood. This also makes application of the ELM to larvae premature. This is not to say there hasn't been progress in documenting the vulnerabilities of early life stages to ocean acidification. Beginning with fertilisation, there is evidence fertilisation success is reduced by low pH seawater. The dominance of broadcast spawning amongst marine invertebrates inherently makes this stage vulnerable to a changing abiotic environment, for the protection afforded internally of pH_e regulation no longer applies. Gametes are bathed in hypercapnic water, and maintenance of intracellular pH (pH_i) is dependant on membrane permeability and ion exchange capacity. If pH_i is compromised in hypercapnic seawater, egg, sperm and zygotes all face disruption of cellular processes, many of which are pH dependant (Reipschläger & Pőrtner 1996). To date, reduced sperm swimming speed has been seen in the urchin *Heliocidaris erythrogramma* (Havenhand et al 2008, although c.f. Havenhand & Scheigel

2009), reduced fertilisation success in urchins (Kurihara & Shirayama 2004), reduced hatching success in copepods (Kurihara et al 2004) and reduced viability in a gastropod (Ellis et al 2009). In larvae, similar negative effects of ocean acidification dominate; slower development and abnormalities (Dupont et al 2008), increased mortality (Kurihara et al 2004) and altered response to thermal stress (O'Donnell et al 2009). The mechanisms behind these results in both fertilisation and larval development are poorly understood, and further research to identify *how* pH is causing these observed changes is necessary before the validity of ELM or any other encompassing mechanism can be considered.

Applicability of ELM to non-calcifying and symbiotic species

The primary reason that non-calcifying invertebrates have not been included in the ELM thus far is due to a lack of data. Understanding of the response of non-calcifying invertebrates is derived from the response of a limited number of species, in particular the intertidal *Sipunculus nudus* (Reipschläger & Pörtner 1996; Langenbuch & Pörtner 2002; Langenbuch & Pörtner 2004; Langenbuch et al 2006). Sipunculids use ion exchange to actively buffer pH_e (Pörtner et al 1998) rather than passive buffering of most calcified invertebrates, and this is the main grounds for the exclusion of non-calcifying invertebrates from the ELM at this stage. Supporting evidence for the ELM from this data is mixed; despite observations of reduced activity, incomplete compensation of pH_c and metabolic depression at lowered pH (Pörtner et al 1998), no decrease in protein content of the worms (which would be indicative of muscle loss) was seen prior to death, which occurred after 2 months at pH 7.04 (Langenbuch & Pörtner 2004). The second reason that non-calcifying invertebrates are not yet included within the ELM is that the energetic and physiological costs of pHe regulation under hypercapnia are notably lacking in the ocean acidification literature (but c.f. *Sipunculus nudus* above). This has been identified as an area of priority

for future study (Chapter 7); research of a greater variety of taxa, and at realistic pH scenarios, the sipunculid work was carried out at pH 7.05 and pH 6.55, is required to fully understand how these species respond to ocean acidification.

The second group to be excluded from the ELM contain those organisms with symbiotic relationships, primarily corals. The reliance of these species on bacteria or algae as a source of energy inherently complicates a response based on the requirement for more energy. Without an understanding of how the symbiotic algae/bacteria will respond to lowered pH seawater it would be premature to assume energy limitation within symbiotic reef building invertebrates. The investigations of the response of corals to ocean acidification to date have often looked at both increasing temperature and acidity (e.g. Hoegh-Guldberg et al 2007; Anthony et al 2008), as the bleaching (loss of symbionts) under temperature stress is a known threat facing reef systems (Sampayo et al 2008).

Synergisms with other climate change parameters

Increasing atmospheric levels of carbon dioxide may be causing ocean acidification, but other climate change factors will also affect the marine environment. Increasing atmospheric temperature is resulting in a concomitant increase in sea surface temperature, increased rainfall and ice melt will cause localised changes to salinity and likely result in anoxia at sites heavily influenced by river run off as a consequence of nutrient enrichment. For a realistic assessment of organism and ecosystem response to future environmental change, synergisms between changing ocean pH and climate change factors such as temperature, salinity and anoxia need to be considered.

Of the few multifactorial studies to date, temperature is most represented, and these preliminary data suggests the principles of the ELM hold true when temperature stress is added, with a greater occurrence of negative effects, most likely associated with an increased occurrence of energy deficits due to metabolic upregulation as a result of temperature rendering a further response to pH unachievable (e.g. *Ophiura ophiura,* Chapter 5). In addition, the crab *Cancer pagurus* was more sensitive to increased temperature at low pH (Metzger et al. 2007), sea urchin larvae showed increased temperature stress at low pH (O'Donnell et al. 2009), Anthony et al. (2008) found that acidification and warming acted synergistically to lower the thermal bleaching threshold of three reef building coral species, and the arctic brittlestar *Ophiocten sericeum* showed a greater impact on arm regeneration at combined low pH, high temperature conditions (Chapter 3). These preliminary results demonstrate exaggerated ELM at higher temperature, but as this overview shows, studies of both temperature and pH are few, and more research is needed to be confident of the mechanism involved.

Both salinity and anoxia have the potential to exacerbate the physiological stress of hypercapnia, and if these factors remain within viable thresholds then the additional energetic cost of maintenance, as demonstrated by the ELM, is likely to exaggerate the response to ocean acidification. However, the empirical evidence to assert this theory simply does not exist (but c.f. Egilsdottir et al 2009 on embryo development w.r.t. salinity and pH change). Therefore, the applicability of the ELM to synergistic pH and salinity/anoxia physiological effects cannot be assessed until more data become available.

SUMMARY

The premise of the energy limitation model (ELM) is the increased energetic cost of acid base (pH_e) regulation under hypercapnia, and the consequences and paths of meeting this increased energy requirement. As stated at the start of this chapter, the principles that form the ELM are not novel; acid-base balance has been recognised as an important basic response to ocean acidification (Widdicombe & Spicer 2008; Pörtner et al 2008), the additional costs of this regulation under hypercapnia highlighted by Ichimatsu et al (in press), the influence of metabolic capacity has been discussed by Pane & Barry (2007) and the short and long term viability of metabolic depression considered by Langenbuch & Pörtner (2004). However, the ELM brings together these principles into an overarching theoretical framework upon which it is possible to hang the responses of calcifying benthic invertebrates to ocean acidification. From this perspective this model appears to rationalise responses to ocean acidification published to date. A criticism may be that the ELM cannot explain all changes that occur with regard to ocean acidification e.g. the thickening of the gut lining in the urchin *Brissopsis lyrifera* (S. Widdicombe, unpubl. data). What the ELM does is rationalise the physiological changes that occur in the pursuit of pH_e maintenance. Where this is not achieved lowered pH_e will have a whole suite of impacts upon the organism, therefore, what is not covered by the ELM are the changes that occur when pH_e is not fully compensated, which can ultimately result in mortality. The model is not perfect; early lifestages are not yet able to be included, nor symbiotic and non-calcifying species. In that respect this chapter is a call to arms. More studies are needed to address the mechanisms not yet understood, to test the principles of the ELM with focused research, and a call for those with a greater understanding of the acid base regulation of non-calcifying species to consider whether the ELM is applicable to passive buffering species only, or extends to all benthic invertebrates. Notwithstanding these limitations, the

principle of ELM takes us closer to understanding where and why species may be vulnerable to ocean acidification.

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