Acid-base regulation in the sea urchin *Parechinus angulosus* during CO$_2$-induced seawater acidification

Alexei Dyer

Submitted in partial fulfilment of a BSc (Hons) degree

Department of Biological Sciences

Faculty of Science

University of Cape Town

October 2013
The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.
Declaration

1. I know that plagiarism is wrong. Plagiarism is to use another’s work and pretend that it is one’s own.
2. I have used the …………………………… convention for citation and referencing. Each contribution to, and quotation in, this essay/report/project/……………… from the work(s) of other people has been attributed, and has been cited and referenced.
3. This essay/report/project/……………… is my own work.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature ______________________________
Ocean acidification is predicted to have adverse effects on the physiologies of marine organisms, particularly those that produce calcified structures. Extracellular homeostasis is considered to be critical to mediating the effects of ocean acidification. Due to their low metabolic rates and weak ability to regulate ion exchange, sea urchins are thought to be particularly weak acid-base regulators. Recent findings showing species-specific capacities for extracellular pH regulation however suggest that species currently exposed to natural CO$_2$ elevations, such as upwelling events, may have a higher capacity to tolerate elevated CO$_2$. The sea urchin *Parechinus angulosus* currently experiences natural CO$_2$ variations within the Benguela upwelling system and is therefore predicted to possess the capacity to compensate moderate acid-base disturbances. Urchins were submitted to control (8.0), intermediate (7.7) and low (7.4) seawater pH treatments for 14 days to investigate the capacity to regulate extracellular acid-base status. Extracellular pH changes induced by exposure to intermediate (pH 7.7) seawater acidification were fully compensated through the accumulation of approximately 2.0 mmol l$^{-1}$ of bicarbonate. The bicarbonate accumulation was only sufficient to partially compensate extracellular acid-base status during exposure to low (7.4) seawater pH. Results from acute (24 hour) exposure to low (7.4) seawater pH reveal that bicarbonate accumulation, despite being evident within 24 hours, is not sufficient to compensate extracellular pH. This study provides further support that sea urchins exposed to natural CO$_2$ variability possess a limited capacity to regulate extracellular acid-base disturbances. *P.angulosus* may therefore already be adapted to deal with a moderate reduction in seawater pH to 7.7, but lacks the iono-regulatory capacity to accumulate sufficient bicarbonate to deal with a reduction of seawater pH to 7.3. Long-term studies are needed to assess the role of acid-base regulation as a mediator of broader physiological tolerance to ocean acidification, and its consequences at the level of the whole organism.
# Table of Contents

1. Introduction .......................................................................................................................... 1

2. Methods .................................................................................................................................. 4

2.1. Urchin collection and acclimation ............................................................................... 4

2.2. Seawater acidification ................................................................................................... 4

2.3. Medium-term exposure (14 days) ................................................................................. 5

2.4. Acute exposure (24 hours) ............................................................................................ 5

2.5. Seawater carbonate system parameters ...................................................................... 5

2.6. Coelomic fluid sampling ............................................................................................... 6

2.7. Calculation of coelomic fluid acid-base parameters .................................................. 7

2.8. Statistical analysis ......................................................................................................... 7

3. Results ................................................................................................................................... 9

3.1. Extracellular acid-base response during medium-term exposure (14 days) .......... 9

3.2. Extracellular acid-base response during acute exposure (24 hours) ................. 11

4. Discussion ............................................................................................................................ 13

5. Conclusion .......................................................................................................................... 15

6. References ........................................................................................................................... 18

7. Appendix ................................................................................................................................ 21
1. Introduction

Atmospheric carbon dioxide (CO₂) concentrations have increased from 280 ppm to 380 ppm since the start of the industrial revolution (Orr et al., 2005), but have reached 400ppm in May 2013. During this time the ocean, a major sink for CO₂, has absorbed almost a third of all anthropogenic CO₂ emissions (Sabine et al., 2004). While this uptake of CO₂ has mitigated some of the immediate effects on the earth’s atmosphere, it has triggered changes to the oceanic carbonate system which are predicted to have unprecedented consequences for the health and function of marine ecosystems (The Royal Society, 2005).

As CO₂ dissolves in seawater to form carbonic acid (H₂CO₃), its subsequent dissociation into bicarbonate (HCO₃⁻) and hydrogen ions (H⁺) decreases seawater pH, a process referred to as ocean acidification (Caldeira and Wickett, 2003). Despite natural fluctuations in atmospheric CO₂, the ocean’s pH has however remained relatively stable. This is due to the capacity of the oceanic carbonate buffering system to neutralise the addition of H⁺ and maintain a chemical equilibrium (Zeebe & Wolf-Gladrow, 2001):

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+ \leftrightarrow \text{CO}_3^{2-} + 2\text{H}^+ \tag{1}
\]

The immediate capacity of seawater to buffer CO₂ accumulation and the associated H⁺ increase is however dependent on a limited supply of CO₃²⁻ which is not sufficiently replenished by weathering over short geological (< 1 million years) times scales (Pelejero et al., 2010). The rate at which anthropogenic CO₂ is predicted to be taken up by the oceans is 100 times greater than that experienced during the last glacial termination (Monnin et al., 2001; The Royal Society, 2005). The current rate of uptake has already overwhelmed the ocean’s capacity to maintain a chemical equilibrium and led to a reduction in average surface ocean pH of 0.1 units (Caldeira and Wickett, 2003). If current trends continue, the decrease in average surface ocean pH is predicted to reach 0.3-0.4 units by 2100 and up to 0.8 units by 2300 (Caldeira and Wickett, 2003, 2005; IPCC, 2007). Furthermore, as the partial pressure of carbon dioxide (pCO₂) in seawater increases and pH declines, many marine habitats are predicted to experience higher bicarbonate concentrations [HCO₃⁻] and lower carbonate concentrations (CO₃²⁻), leading to reduced saturation states of calcite (Ω_Ca) and aragonite (Ω_Ar) (Orr et al., 2005; Cao and Caldeira, 2008).

A unifying effect of elevated seawater pCO₂ experienced by both calcifiers and non-calcifiers is the combined hypercapnia (increased pCO₂) and acidosis (decreased pH) of extracellular fluids, which can disrupt acid-base homeostasis and consequently inhibit enzyme activity, protein synthesis and
calcification (Pörtner, 2008; Melzner et al., 2009). The capacity to regulate extracellular acid-base status, which relies on effective iono-regulation, is therefore considered critical to mediating the broader physiological responses to ocean acidification (Heisler 1989; Pörtner, 2008; Widdicombe and Spicer, 2008).

Marine organisms which precipitate calcium carbonate polymorphs such as calcite (e.g. coccolithophores, foraminifera), aragonite (e.g. corals, pteropods) or magnesium-bearing calcite (e.g. red coralline algae, echinoderms) are faced with the combined challenge of maintaining acid-base homeostasis and the precipitation of calcified structures, and are therefore considered to be particularly sensitive to the effects of ocean acidification (Orr et al., 2005; Fabry et al., 2008; Pörtner, 2008). The precipitation of calcium carbonate structures becomes thermodynamically less favourable as calcium carbonate saturation states decrease (The Royal Society, 2005), eventually promoting the dissolution of calcified structures if seawater becomes undersaturated with respect to calcium carbonate ($\Omega < 1$) (Findlay et al., 2009). Calcium carbonate polymorphs differ in their relative thermodynamic stability and thus have different saturation states. Calcite is the most thermodynamically stable polymorph, followed by aragonite and finally magnesium-calcite, which is considered to persist in a metastable state relative to that of pure calcite (Andersson et al., 2008; Pelejero et al., 2010). The relative vulnerability of calcifying taxa is therefore commonly inferred from the type of calcium carbonate they produce and the projected distributions of calcite and aragonite saturation states (Cao and Caldeira, 2008; Fabry et al., 2008; Orr et al., 2005a)

In most calcifying organisms, calcification occurs within isolated cellular compartments, whose carbonate saturation state is modulated by the transport of bicarbonate, calcium ions and protons across epithelial membranes (Carre, 2006; Pörtner, 2008). Calcified structures are also often not in direct contact with the surrounding seawater, and are instead covered by an epidermis that minimises direct exposure to the surrounding seawater (Ries et al., 2009). Seawater physiochemistry is therefore rather seen to have an indirect effect on calcification, subject to the ionic exchanges which occur across the extracellular-seawater interface (Pörtner, 2008).

Amongst calcifying organisms, sea urchins are thought to be particularly susceptible to the effects of ocean acidification due to their reliance on a magnesium-calcite skeleton and a limited capacity for iono-regulation. (Andersson et al., 2008; Pörtner, 2008; Melzner et al., 2009; McClintock et al., 2011). Recent studies however indicate that sea urchin species display a range of responses to ocean acidification. Ries et al. (2009) reported decreased calcification rates in the Pencil urchin *Eucidaris tribuloides* in response to declining aragonite saturation states. The same study however also observed elevated calcification rates in the Purple urchin *Arbacia punctulata*. Similarly, the capacity of sea urchins to regulate acid-base status during exposure to CO$_2$-acidified seawater appears to be species specific. A
limited capacity to buffer acidification-induced extracellular acidosis was reported in *Psammechinus miliaris* (Miles et al., 2007) and *Strongylocentrotus dröebachiensis* (Spicer et al., 2011) during 8 day and 5 day exposures to acidified seawater, respectively. When exposed to similar conditions for 10 days, *S. dröebachiensis* however accumulated a sufficient extracellular bicarbonate concentration to fully compensate acidification-induced respiratory acidosis (Stumpp et al. 2012), suggesting that sea urchins may be able to tolerate moderate hypercapnia, similar to that experienced during upwelling events. *In-situ* observations have also suggested that the distribution of two sea urchin species along natural CO$_2$ gradients may be linked to differing capacities to regulate extracellular acid-base status (Calosi et al., 2013).

The average surface ocean conditions predicted to occur by the end of the century under ocean acidification are already encountered near volcanic CO$_2$ vents (Calosi et al., 2013) and within coastal habitats during upwelling events and periods of high biological activity (Feely et al., 2008; Thomsen et al., 2010). It is uncertain whether organisms occupying these CO$_2$-enriched habitats are pre-adapted to tolerate future acidification, or whether they already exist close to their physiological limits. Habitats that currently experience natural CO$_2$ enrichment may therefore provide valuable insights into the responses of marine taxa to ocean acidification.

The sea urchin *Parechinus angulosus* (Leske) inhabits the rocky intertidal and subtidal habitats along the west coast of South Africa, where it is exposed to seasonal upwelling of cold CO$_2$-enriched bottom waters of the Benguela current, as well as diurnal pH fluctuations within kelp forests and intertidal zones. In light of recent findings, which challenge the widespread view that sea urchins lack the ability to regulate acid-base balance, it is hypothesised that *P. angulosus* possesses the capacity to regulate moderate acid-base disturbances. To investigate the acid-base response of *P. angulosus* to CO$_2$-induced seawater acidification, the acid-base status of urchins was measured during medium-term (14 days) and acute (24 hours) exposure to CO$_2$-acidified seawater treatments. Experimental seawater treatments were chosen to reflect average surface ocean values predicted for the end of the century, and those likely to occur by the year 2300.
2. Methods

2.1. Urchin collection and acclimation

Adult sea urchins (P. angulosus) (test diameter: 5 – 7cm) were collected by hand during low tide from a temperate rocky shore in Sea Point, Cape Town, South Africa (33°55′15N, 18°22′51E) during May 2013. Urchins were returned to the Sea Point Research Aquarium, where they were acclimatised to laboratory conditions for 8-10 days in two 20 litre flow-through aquaria. The flow-through system was supplied with filtered local seawater (pH = 8.00, salinity = 35.0 ‰, T = 16.0°C). During the acclimation period urchins were fed fresh Ulva lactuca and kelp ad libitum to minimise pre-existing differences in condition, as feeding influences the buffer capacity of coelomic fluid (Collard et al., 2013; Stumpp et al., 2012). Urchins were monitored to ensure that all individuals were healthy and without injury.

2.2. Seawater acidification

Experimental exposure tanks were filled with equal volumes of filtered seawater, which was recirculated throughout the exposure period. As seawater temperature was not actively maintained, exposure tanks were filled 2-3 days before the start of the experimental period. Seawater temperature equilibrated with that of the surrounding air, and subsequently remained stable at 16 °C for the duration of the exposure period (Fig. A4b and Fig. A5b). Seawater carbonate chemistry and pH within individual exposure tanks was manipulated by introducing CO$_2$ gas as a fine stream of bubbles through porous diffuser stones. The supply of CO$_2$ stopped once the seawater pH had been reduced to the required level (control: pH = 8.0, intermediate: pH = 7.7 and low: pH = 7.4). This was achieved via automated feedback systems consisting of a pH electrode and controller set (medium-term: TUNZE Aquarientechnik, Germany; acute: Aqua Medic, Germany) coupled to a valve regulating the flow from a CO$_2$ cylinder. The accuracy of the pH controllers was confirmed by frequent pH measurements made using a handheld pH3110 multimeter (WTW, Germany) (Fig. A4a and Fig. A4b). pH meters were calibrated with Orion NBS buffers 4 and 7 (Thermoscientific).
2.3. Medium-term exposure (14 days)

Urchins were exposed to acidified seawater treatments (control: pH = 8.0, intermediate: pH = 7.7 and low: pH = 7.4) for a period of 14 days. Seawater within each 200 litre experimental aquarium (unreplicated) was circulated by a submersible 35W aquarium pump (SP103-2400, Guangdong Boyu, China). Additional aeration was provided through diffuser stones connected to a central air line. Eight urchins were randomly assigned to each exposure tank where they were kept separately within labelled sub-partitions of a previously-weathered PVC cage (Fig A1), thereby avoiding the need for tagging. Before being placed into exposure tanks (day 0), urchins were weighed and a coelomic fluid was sampled to establish the baseline acid-base status under ambient seawater conditions. Coelomic fluid was sampled again from each individual on days 4, 7, 11, and 14 of the exposure period. Urchins were fed equal portions of *Ulva latuca* and kelp a day after each sampling event. Uneaten food was removed a day before the next sampling event.

2.4. Acute exposure (24 hours)

The acute acid-base response of urchins exposed to acidified seawater treatments (control: pH = 8.0 and low: pH = 7.4) for 24 hours was investigated using a similar setup to that of the medium-term exposure. Seawater within each 20 litre experimental aquarium was circulated by a submersible 5W aquarium pump (JVP-101, Zhejiang Sunsun, China) and aerated using diffuser stones connected to a central air line. Six urchins were randomly assigned to each exposure tank and kept in individually labelled baskets. Each urchin was weighed and a coelomic fluid sample withdrawn, the time of which was recorded, before being transferred to the exposure tank. Coelomic fluid was sampled again from each individual after 1.5, 3, 6, and 24 hours of exposure (± 10 mins).

2.5. Seawater carbonate system parameters

Seawater carbonate parameters (pCO$_2$, [HCO$_3^-$], [CO$_3^{2-}$], Ω Ca, Ω Ar) (Table 1) were calculated from measured values of pH$_{NBS}$ and total alkalinity (TA) using the software package CO2SYS (Pierrot et al., 2006) with dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987), and [KSO$_4$] from Dickson (1990).

Seawater temperature and pH$_{NBS}$ was monitored using a pH3110 multimeter (WTW, Germany). Salinity was monitored using a salinity refractometer (S-10E, Atago, Japan). For measurements of total alkalinity (TA), 50ml seawater samples were collected during the medium-term experiment on the five
days where coelomic fluid was sampled, and three times over the course of the acute experiment. 25µl of 
HgCl$_2$ solution was immediately added to prevent biological activity during storage. Total alkalinity was 
measured using the spectrophotometric method developed by Sarazin et al. (1999): 500µl of a dye reagent 
prepared from known concentrations of methanoic acid, bromophenol-blue (BPB) and an ionic strength 
buffer (NaCl 0.7 mmol.l$^{-1}$) was added to each 500µl seawater sample. The mixture was thoroughly shaken 
to allow gaseous CO$_2$ to escape from the solution. Samples were then analysed at 590nm with a U-1900 
spectrophotometer (Hitachi, Japan). The calibration curve used to estimate the total alkalinity of seawater 
samples was generated from a dilution series of 3.13, 1.56, 0.78 and 0.39 mmol.l$^{-1}$ sodium bicarbonate 
diluted with artificial seawater (Fig A2).

The accumulation of nitrogenous waste products (NO$_2$, NO$_3$, NH$_3$ / NH$_4^+$) was monitored 
throughout both experiments using aquarium water quality test kits (Sera, Germany) (Fig. A3), to ensure 
that water quality remained suitable. Concentrations in the medium-term 200L exposure tanks never rose 
above 0.5 mg.l$^{-1}$ (NO$_2$), 10 mg.l$^{-1}$ ([NO$_3$]$^2-$) and 0.5 mg.l$^{-1}$ (NH$_3$ / NH$_4^+$). Nitrogenous products did not 
accumulate to detectable levels during the acute exposure.

2.6. Coelomic fluid sampling

Coelomic fluid samples (volume = 200µl) were taken from each individual at each sampling time, using a 
1ml syringe equipped with a 21G needle. This sample volume was chosen as it represented a small 
fraction of the total coelomic fluid volume, yet provided sufficient material for all analyses. The needle 
was inserted into the perivisceral coelomic space by piercing the peristomal membrane surrounding the 
jaw. To avoid damage to the gut or the water vascular system, the peristomal membrane was pierced to a 
deepth of less than 1cm between two rows of the water vascular system (sensu Stumpp et al., 2012) at an 
angle of approximately 20°. The sample was transferred into a 1.5 ml microcentrifuge tube (Eppendorf, 
Germany), after which two separate procedures were immediately carried out by two operators.

Total dissolved inorganic carbon (tCO$_2$) was determined by a carbon dioxide analyser (SBA-4, PP-
Systems) using methods adapted from Cameron (1971): a 20µl sub-sample of coelomic fluid was 
introduced into an acrylic acidification chamber via a gas-tight Hamilton syringe. Within the chamber, a 
magnetic stirrer bar mixed the sample with 0.1 mol.l$^{-1}$ sulphuric acid (H$_2$SO$_4$), causing bound HCO$_3^-$ to be 
released as gaseous CO$_2$. A 50ml/min nitrogen stream carried the CO$_2$ sample to an infrared analyser to 
order to determine the absorption signal intensity (Volts) of the sample. Calibration curves were 
generated for each sampling day from dilution series of 12.5, 6.25, 3.13, 1.56 and 0.78 mmol.l$^{-1}$ sodium bicarbonate diluted in distilled water.
The pH of the remaining perivisceral coelomic fluid (pH\textsubscript{cf}) was determined by immersing the tip of an Orion pH microelectrode coupled to an Orion 3-Star Plus benchtop pH meter. The pH meter was calibrated using AppliChem NBS biological precision buffers 10, 7 and 4. The remaining coelomic fluid was sealed in microcentrifuge tubes and stored at -20°C.

2.7. Calculation of coelomic fluid acid-base parameters

Partial pressure of carbon dioxide (pCO\textsubscript{2}) and bicarbonate concentration ([HCO\textsubscript{3}\textsuperscript{-}]) were calculated from the measured tCO\textsubscript{2} and pH\textsubscript{cf} values, using the Henderson-Hasselbach equation

\[ pCO_2 = tCO_2 \left( \alpha \left( 10^{(\text{pH} - pK')_1} + 1 \right) \right)^{-1} \tag{1} \]

\[ [HCO_3^-] = tCO_2 - \left( \alpha pCO_2 \right) \tag{2} \]

where \( \alpha \) is the solubility coefficient of CO\textsubscript{2} and pK’\textsubscript{1} is the first apparent dissociation constant of carbonic acid. Values for \( \alpha \) (0.330 mmol.l\textsuperscript{-1}kPa\textsuperscript{-1}) and pK’\textsubscript{1} (6.005) at approximately 19°C and 35 % salinity were estimated from nomograms designed for the shore crab \textit{Carcinus maenas} (Truchot, 1976). Davenport diagrams were plotted to depict the respiratory and non-respiratory processes contributing to changes in acid-base status.

2.8. Statistical analysis

For the medium and acute experiments, the interacting effects of pH treatment and exposure duration on the acid base parameters of coelomic fluid were tested using a mixed ANOVA, with pH treatment as the between-subjects factor and exposure duration as the repeated within-subjects factor. In addition to identifying whether effects were statistically significant, effect size statistics ($\eta^2$) appropriate to the repeated measures design (Bakeman, 2005) were calculated to infer biological significance. Cohen’s (1988) guidelines for interpreting effect sizes were followed, with < 0.02 considered to be a small, ~ 0.13 a moderate, and > 0.26 a large size effect. Significant main effects or interactions of pH treatment and exposure duration were followed by post-hoc tests (Tukey’s HSD) to identify where differences occurred.

Repeated measures designs benefit from more precise estimates of the treatment effect, by accounting for the variation associated with each individual (Cook and Ware, 1983). The effect of the repeated measures factor however needs to be interpreted with caution, as measurements made on the
same individual are inherently correlated with each other, leading to unequal covariance between pairs of levels within the repeated within-subjects factor. ANOVA employing conventional F-tests requires the assumption of multisample sphericity to be met (i.e., equal variance between all factor levels), as deviations from a spherical covariance structure inflate the Type I error rate (Keselman et al., 2001). Mauchly’s test of sphericity was applied to assess whether data violated the assumption of sphericity. Where the sphericity assumption was not met, F-tests were corrected using the Greenhouse-Geisser method. Greenhouse-Geisser correction adjusts the degrees of freedom by taking into account the departure from sphericity ($\eta^2$), providing a more conservative test of exposure duration and its interaction with pH treatment. Repeated measures ANOVA was conducted using the ‘ez’ package (4.2-0; Lawrence, 2013) in R version 3.0.1 (R Core Team, 2013).

Baseline measurements taken prior to exposure (time zero) were not included in the repeated measures ANOVA model. Instead, measures of baseline acid base status were tested to determine whether pre-existing differences existed between treatment groups. One-way ANOVA was used to test each baseline acid base parameter for significant differences between treatment groups in the medium-term experiment, after assessing that residuals were normally distributed and heteroscedastic (Fig A4.1–4.5). Within the acute experiment, Wilcoxon rank-sum tests, which are robust to deviations from normality, were used to compare baseline measurements of acid-base status and wet weight between treatment groups. Paired t-tests and Wilcoxon paired-sample tests were employed for pairwise comparisons within the long-term and acute exposures, respectively.

It should be noted that due to logistical constraints, the experiment was not replicated at the level at which the pH treatment was applied – that of the individual tank. The use of individual animals as experimental units in place of tank averages amounts to pseudoreplication (sensu Hurlbert, 1984). Statistical inferences are therefore not strictly possible, as the effect of pH treatment is confounded by the effect of individual tanks.
3. Results

The physiochemical parameters of seawater treatments are reported in Table 1. Comparable low (pH 7.4) and control (pH = 8.0) treatments were established in short and medium-term exposures. Total alkalinity (TA) and temperature were similar across all treatment levels. There were no significant difference in baseline acid-base parameters or initial wet weight between the treatment groups within the medium-term (min. \( F(2, 21) = 1.817, p = 0.187 \)) or acute exposures (min. \( W = 28, p = 0.132 \)) (Table A1). No mortalities were recorded during exposure treatments.

3.1. Extracellular acid-base response during medium-term exposure (14 days)

Fig.1 presents extracellular acid-base status of urchins after 0, 4, 7, 11 and 14 days of exposure to acidified seawater treatments (pH 7.7 and pH 7.4). Mauchly’s test indicated that the assumption of sphericity had been met for each of the measured acid base parameters (min. \( p = 0.122 \)), allowing uncorrected ANOVA results to be interpreted.
There was a large significant effect of pH treatment ($F_{(2, 21)} = 11.964, p < 0.0001, \eta^2_G = 0.421$) and a moderate, but significant effect of exposure duration ($F_{(3, 63)} = 5.625, p < 0.002, \eta^2_G = 0.089$) on coelomic fluid pH (pH$_{CF}$) (Fig. 1a,b). The pH$_{CF}$ of urchins exposed to the lowest pH treatment (pH 7.4) fell from 7.61 to 7.41 ($t_{(7)} = 6.121, p = 0.0005$) during the first four days of exposure (Fig. 1d). The differences between treatment groups at day 4 persisted for the duration of the exposure period, as indicated by the absence of a significant interaction ($F_{(3, 63)} = 1.203, p < 0.317, \eta^2_G = 0.040$). Following the initial acidification, pH$_{CF}$ in the lowest pH treatment group (pH 7.4) remained significantly lower (Tukey’s $p_{adj}$...
< 0.0001) than that of the control (pH 8.0) and intermediate (pH 7.7) treatment groups for the duration of the exposure period (Fig. 1a,d). Exposure to intermediate seawater acidification (pH 7.7) resulted in fully compensated pH\textsubscript{CF} - which remained similar to that of the control group (Tukey’s p\textsubscript{adj} = 0.519) (Fig. 1a).

Extracellular pCO\textsubscript{2} was characterised by a significant effect of pH treatment (F\textsubscript{(2, 21)} = 192.588, p < 0.0001, η\textsuperscript{2} = 0.871), but not exposure duration (F\textsubscript{(2, 21)} = 2.472, p < 0.074, η\textsuperscript{2} = 0.068). The lowest pH treatment elicited a doubling of extracellular pCO\textsubscript{2} over that of the control group (Tukey’s p\textsubscript{adj} < 0.0001). The intermediate pH treatment group (pH 7.7) maintained a small but significantly elevated extracellular pCO\textsubscript{2} relative to that of the control group (Tukey’s p\textsubscript{adj} < 0.0001). In both treatments, extracellular pCO\textsubscript{2} appeared to remain at an equilibrium elevated above seawater pCO\textsubscript{2}.

Extracellular [HCO\textsubscript{3}–] rose from 2.79 mmol l\textsuperscript{-1} on day 0 to maximum values of 5.19 mmol l\textsuperscript{-1} and 4.66 mmol l\textsuperscript{-1} after 11 days of exposure in the lowest (pH 7.4) and intermediate (pH 7.7) pH treatments, respectively (Fig 1c,f). There were significant differences in the accumulation of HCO\textsubscript{3}– between exposure treatments, as indicated by the significant interaction between pH treatment and exposure duration (F\textsubscript{(2, 21)} = 2.712, p = 0.021, η\textsuperscript{2} = 0.075). Post hoc tests conducted on the interaction revealed that [HCO\textsubscript{3}–] in the lowest pH treatment (pH 7.4) remained elevated over the control group on days 4, 7, 11 and 14 (max. Tukey’s p\textsubscript{adj} ≤ 0.001) (Fig. 1f). The accumulation of HCO\textsubscript{3}– in the intermediate pH treatment (pH 7.4) fluctuated over the course of the exposure period, with significant increases over controls only being recorded on days 4 and 11 (max. Tukey’s p\textsubscript{adj} = 0.035) (Fig. 1c).

3.2. Extracellular acid-base response during acute exposure (24 hours)

Fig. 2 presents the acute extracellular acid base response of urchins after 0, 1.5, 3, 6 and 24 hours of exposure to acidified seawater (pH 7.4). Mauchly’s test indicated that data violated the assumption of sphericity for pCO\textsubscript{2} and [HCO\textsubscript{3}–] (max. p = 0.018), while pH\textsubscript{CF} measurements satisfied the sphericity assumption (Mauchly’s p = 0.597).

During 1.5 hours of exposure to acidified seawater conditions, pH\textsubscript{CF} experienced a significant decrease from 7.60 to 7.27 (Wilcoxon paired-sample test: p = 0.032) (Fig. 2a). pH treatment had a large significant effect (F\textsubscript{(1, 10)} = 2.712, p < 0.0001, η\textsuperscript{2} = 0.723), maintaining the pH\textsubscript{CF} of exposed urchins below that of the control group for the duration of the exposure period (Fig. 2a). There was also an overall decrease in pH\textsubscript{CF} during the course of the exposure period (F\textsubscript{(1, 10)} = 5.451, GG\textsubscript{E} = 0.781, GG\textsubscript{adj} p = 0.009, η\textsuperscript{2} = 0.301) across both treatment groups, as indicated by the lack of a significant interaction (F\textsubscript{(1, 10)} = 0.081, GG\textsubscript{E} = 0.781, GG\textsubscript{adj} p = 0.944, η\textsuperscript{2} = 0.006).
An initial two-fold increase of extracellular pCO₂ (Wilcoxon paired-sample test: $p = 0.031$) in the acidified seawater treatment persisted for the full duration of the 24 hour exposure ($F_{(1, 10)} = 2.712, p < 0.0001, \eta^2_G = 0.803$) (Fig. 2b), without a significant interaction between pH treatment and exposure duration ($F_{(1, 10)} = 5.451, G\dot{\epsilon}_F = 0.781, G\dot{G}_{adj} p = 0.009, \eta^2_G = 0.301$).

Seawater pH treatment had a large significant effect on coelomic $[\text{HCO}_3^-]$ ($F_{(1, 10)} = 22.331, p < 0.0009, \eta^2_G = 0.507$), with the difference between treatment groups persisting for the full duration of the acute exposure, as indicated by the absence of a significant interaction ($F_{(1, 10)} = 1.855, G\dot{\epsilon}_F = 0.499, G\dot{G}_{adj}$)
p = 0.194, $\eta^2 = 0.091$). [HCO$_3^-$] in the control animals decreased from the pre-exposure level, once coelomic fluid sampling commenced. After 24 hours of exposure to ambient seawater (pH 8.0) and repeated coelomic fluid sampling, HCO$_3^-$ in the control group had decreased to a final concentration of 1.75 mmol l$^{-1}$. Coelomic [HCO$_3^-$] in urchins exposed to acidified seawater (pH 7.4) retained a similar concentration to the pre-exposure level (2.81 mmol l$^{-1}$).

4. Discussion

*P. angulosus* maintained a fully compensated extracellular pH during the 14 day exposure to moderately hypercapnic seawater conditions (pH 7.70, 1437 µatm pCO$_2$) through an extracellular bicarbonate accumulation of approximately 2.0 mmol l$^{-1}$ above control concentrations (Fig. 3a). Bicarbonate increased by a similar magnitude during a 14 day exposure to more strongly acidified seawater (pH 7.4, 3156 µatm pCO$_2$). Although there appeared to be some compensation of extracellular pH, it was not significant, and more importantly, had a small effect size (Fig 3b). On the basis of this study’s limited window of observation, compensation under more strongly acidified seawater conditions is incomplete. This conclusion may not generalise to longer exposure periods, however, as the accumulation of bicarbonate appears to be slow due to the poor capacity of echinoderms to undertake ionic exchange.

During a 5 day exposure to moderately acidified seawater, Spicer et al. (2011) observed a transient bicarbonate increase of approx. 1.0 mmol l$^{-1}$ in the extracellular fluid of *S. dröebachiensis* and concluded that acid-base compensation was incomplete, as had been observed in *P. miliaris* under a similar short...

![Figure 3: Davenport diagrams illustrating changes to bicarbonate and pH of coelomic fluid in sea urchins (P.angulosus) exposed to acidified seawater conditions for 14 days. Seawater pH treatments: (a) pH = 7.7 (filled squares), (b) pH 7.4 (filled triangles), control pH 8.0 (open circles). Points represent means ± SD at each time point (days). Isopleths show calculated pCO$_2$ (kPa) values for given pH and [HCO$_3^-$] combinations.](image-url)
term exposure (Miles et al., 2007). In both cases, the transient bicarbonate increase was attributed to possible shell dissolution associated with an in extracellular [Ca$^{2+}$] and [Mg$^{2+}$]. When subjected to comparable 10 and 45 day exposures it has however emerged that *S. dröebachiensis* possesses the ion-regulatory capacity to increase extracellular bicarbonate concentration by approx. 2.5 mmol l$^{-1}$ above control concentrations (Stumpp et al., 2012), and thereby maintained full extracellular pH compensation. This threshold is likely to be tied to a limited capacity to further regulate ionic exchange (Melzner et al., 2009) and is manifested in the partial compensation of extracellular pH during exposure to more strongly acidified seawater. Similarly, *P.angulosus* may have reached a bicarbonate threshold at approx. 2.0 mmol l$^{-1}$ above control concentrations, preventing further regulation of acid-base status under more strongly acidified seawater conditions. Whether this is the case would need to be established through longer exposure trials.

The immediate ability of *P.angulosus* to regulate extracellular acid-base balance during acute (24hr) exposure to CO$_2$-induced acidification appears to be limited, with extracellular pH remaining uncompensated. Interpretation of the acute exposure however needs to take into account the close proximity of repeated sampling events which may explain the unexpected acidosis in the control group. There appeared to be a metabolic component to this acidosis, as indicated by the Davenport diagram (Fig. 4). A small decreasing trend in extracellular pH in both control and acidified treatments (Fig. 2a) also points to acidification that is not linked to pCO$_2$. Although the concentration of organic acid metabolites such as lactate was not determined, they have been associated with metabolic acidosis in sea urchins (Catarino et al., 2012; Spicer et al., 2011). Despite this confounding factor, exposure to strongly acidified seawater was associated with a gradual accumulation of bicarbonate, relative to the decrease observed in the control group (Fig 2c).

An immediate accumulation of bicarbonate could indicate the passive short-term accumulation of bicarbonate sourced from the dissolution of the Mg-calcite test, as has been observed in sea urchins during exposure to acidified seawater (Miles et al., 2007; Spicer and Widdicombe, 2012; Spicer et al., 2011). Such dissolution events are thought to occur due to the direct exposure of the stereom (test) to conditions within the extracellular coelomic fluid (Holtmann et al., 2013), but appear to only partially contribute to extracellular pH compensation during short-lived periods of hypercapnic stress, such as emersion (Spicer et al., 1988). A more plausible alternative is that *P. angulosus* possesses effective iono-regulatory mechanisms that allow it to rapidly accumulate bicarbonate from the surrounding seawater. Such rapid uptake of bicarbonate has been observed in *P.lividus*, which occupies habitats surrounding natural CO$_2$ vents (Calosi et al., 2013), and is therefore expected to be exposed to periodic hypercapnia. Calosi et al. (2013) suggest this rapid accumulation of bicarbonate is not likely to be sustainable during prolonged exposure, due to the high energetic costs associated with iono-regulation.
Figure 4: Davenport diagram illustrating changes to extracellular bicarbonate and extracellular pH in sea urchins (*P. angulosus*) during an acute 24 hour exposure to acidified seawater conditions. Seawater pH treatments: pH = 7.4 (filled circles) and control pH 8.0 (open circles). Points represent means ± SD at each sampled time point (days). Isopleths show calculated pCO$_2$ (kPa) values for given pH and [HCO$_3^-$] combinations.

The absence of respiratory component is not surprising, as diffusion primarily occurs through the tube feet, which are not specialised for gas exchange in most sea urchin species (Collard et al., 2013). As extracellular CO$_2$ is evacuated passively, extracellular fluid pCO$_2$ must be elevated to maintain a positive diffusion gradient with seawater, consequently inducing respiratory acidosis. It has also been argued that sea urchins, which display the highest extracellular buffer capacity amongst echinoderms and the least effective gas exchange mechanisms, have evolved bicarbonate buffer systems as an alternative to the specialised respiratory structures found in other echinoderms (Collard et al., 2013). *P. angulosus’* relatively enhanced capacity to accumulate extracellular bicarbonate may therefore be linked to a stronger need to maintain a positive diffusion gradient with the surrounding seawater.

5. Conclusion

The present study reveals that *P. angulosus* possesses the capacity to regulate acid-base balance during a 14 day exposure to moderate CO$_2$-induced acidification (pH 7.7), which is already occurring in seasonally CO$_2$-enriched habitats at present. Compared to most sea urchins studied to date, the acid-base physiology of *P. angulosus* appears to be more tolerant to the effects of ocean acidification. Because the maintenance of intracellular pH depends on the acid-base status of the extracellular fluid, *P. angulosus* is expected to be more likely to avoid many of the physiological costs induced by moderate hypercapnia.
(Melzner et al., 2009; Widdicombe and Spicer, 2008). Although the maintenance of extracellular pH may prevent metabolic depression, the costs associated with maintaining acid-base homeostasis result in an altered energy budget (Stumpp et al., 2012), and consequently a reduced investment in growth and reproduction (Pörtner, 2008; Wittmann and Pörtner, 2013). The effects of ocean acidification therefore need to be considered in terms of the whole organism. Future studies should investigate the role of acid-base regulation as a possible predictor of broader physiological tolerance to ocean acidification.
Acknowledgments

I would like to thank my supervisor, Mike Lucas, for his support and encouraging feedback, especially during the write up.

Thank you to my co-supervisor, Lutz Auerswald, for his assistance with the planning and execution of experiments, and for creating a great working environment.

Thanks go to Jarred Knapp for his technical insights and assistance during the experiment.

Nina Lester is thanked for her technical insights and valuable discussions.

Lastly, thank you to my family for their support and encouragement.
6. References


7. Appendix

Figure A1: Labelled sub-partitions of a previously-weathered PVC cage housing urchins for the duration of the medium-term (14 days) exposure to acidified seawater.

Figure A2: Calibration curve for the determination of seawater total alkalinity (TA). Equation for the estimation of total alkalinity (mmol.l\(^{-1}\)): \( \text{TA} = 5.157 \times \text{Abs} + 0.283 \)  
\( \text{R}^2 = 0.993 \)  
\( \lambda = 590\text{nm} \)
Figure 3: Information provided by Sera water quality test kits for the determination of nitrogenous waste.
Figure A4: Daily measurements of seawater (a) pH and (b) temperature over the course of the medium-term exposure (14 days) within three pH treatments.

Figure A5: Measurements of seawater (a) pH and (b) temperature over the course of the acute exposure (24 hr) within two pH treatments.

Figure A6: Measurements of seawater total alkalinity (TA) over the course of the medium-term exposure (14 days) within three pH treatments.
Fig A6: Diagnostic plots of pH standardised residuals measured from urchin coelomic fluid before the start of the (a) medium-term and (b) acute experiments. Plots identify whether data satisfy the assumptions of (i) heteroscedasticity and (ii, iii) normality, required for parametric tests.
Fig A7: Diagnostic plots of HCO$_3^-$ (µmol.l$^{-1}$) standardised residuals measured from urchin coelomic fluid before the start of the (a) medium-term and (b) acute experiments. Plots identify whether data satisfy the assumptions of (i) heteroscedasticity and (ii, iii) normality, required for parametric tests.

Fig A8: Diagnostic plots of pCO$_2$ (kpa) standardised residuals measured from urchin coelomic fluid before the start of the (a) medium-term and (b) acute experiments. Plots identify whether data satisfy the assumptions of (i) heteroscedasticity and (ii, iii) normality, required for parametric tests.
Fig A9: Diagnostic plots of tCO₂ (µmol.l⁻¹) standardised residuals measured from urchin coelomic fluid before the start of the (a) medium-term and (b) acute experiments. Plots identify whether data satisfy the assumptions of (i) heteroscedasticity and (ii, iii) normality, required for parametric tests.
Fig A10: Diagnostic plots of wet weight (g) standardised residuals measured from urchins before the start of the (a) medium-term and (b) acute exposure treatments. Plots identify whether data satisfy the assumptions of (i) heteroscedasticity and (ii, iii) normality, required for parametric tests.
Table A1: Comparison of baseline acid base parameters and wet weight (g) of *P. angulosus* between treatment groups prior to exposure to acidified seawater treatments in the short (24hrs) term and medium-term (14days) experiments.

<table>
<thead>
<tr>
<th></th>
<th>Acute Experiment (24hrs)</th>
<th>Medium-term Experiment (14 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W</td>
<td>p-value</td>
</tr>
<tr>
<td>coel pH</td>
<td>19</td>
<td>0.936</td>
</tr>
<tr>
<td>HCO3- (µmol/kg-SW)</td>
<td>28</td>
<td>0.132</td>
</tr>
<tr>
<td>pCO2 (kpa)</td>
<td>17</td>
<td>0.937</td>
</tr>
<tr>
<td>cCO2 (µmol/kg-SW)</td>
<td>28</td>
<td>0.132</td>
</tr>
<tr>
<td>Wet mass (g)</td>
<td>13</td>
<td>0.485</td>
</tr>
</tbody>
</table>