The role of environmental variability in determining physiological responses in ecologically important bivalve species

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I certify that this thesis is entirely my own work and no part of it has been submitted for a degree or other qualification in this or another institution. I also certify that I have not collected data nor shared data with another candidate at Exeter University or elsewhere without specific authorisation.

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Abstract:

Climate change is causing alterations to marine ecosystems with ocean acidification (OA) and warming widely considered to be some of the most pervasive anthropogenic threats to global marine biodiversity. To date the majority of climate change studies using intertidal organisms neglect the inherent natural variability experienced within coastal ecosystems and the substantial periods of emersion encountered over daily tidal cycles, thus limiting our ability to extrapolate responses of coastal organisms to near-future conditions. This project investigates how a variable intertidal environment can influence acid-base responses to future climatic conditions in the ecologically and economically important bivalve species *Mytilus edulis* and *Perumytilus purpuratus*. Fieldwork is used to provide novel and much needed data on the natural environmental conditions experienced by two populations of *M. edulis* and one population of *P. purpuratus* along with the acid-base response over 12-hour tidal cycles. This data then helps to parameterise experimental work investigating the influence of size (37 – 50 mm and 60 – 79 mm shell length), temperature (7 °C, 13 °C, 20 °C, 28 °C) and low pH exposure (pH 7.7) as drivers of acid-base change during environmentally realistic (6 hour) immersion and emersion periods. I then investigate the potential for populations inhabiting upwelling regions to have an altered acid-base response to short-term exposures to elevated pCO_2 levels. Finally, I investigate if a variable pH/ pCO_2 regime will incur greater physiological costs than a stable pH/ pCO_2 regime over 14 days. My results add to further evidence suggesting mussels have a limited ability to regulate acid-base disturbances during both short- and medium- term exposures to elevated seawater pCO_2. In addition, populations inhabiting upwelling regions showed no significant difference in acid-base response suggesting a low adaptation potential in acid-base regulatory abilities. Furthermore, my results clearly demonstrate for the first time that size (shell length) and temperature are the predominant drivers of acid-base change during emersion and pH/ pCO_2 during immersion. Elevations in temperature had an increasing influence on acid-base balance, with size playing a crucial role in moderating these disturbances at increasingly higher temperatures. Perhaps most interesting, this project found a clear energetic cost of pH/ pCO_2 variability over 14 days, suggesting the use of stable pH values currently used in OA studies may not reliably inform predictions of future organism responses. With seawater pH/ pCO_2 variability expected to increase and

intensify in addition to warming, sea level rise and other climatic changes, this work highlights the need to use environmentally realistic scenarios that reflect natural variability in order to predict future outcomes for marine biota.

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Chapter 1: Introduction

1.1 Introduction to ocean acidification

Ocean acidification (OA), caused by the prolonged absorption of anthropogenic carbon dioxide (CO_2) emissions, is rapidly becoming recognised as a major threat to global marine biodiversity (Lovejoy & Hannah 2005; Doney et al. 2009; Kleypas & Yates 2009). Atmospheric CO_2 levels have remained relatively stable for over 400,000 years prior to the industrial revolution, oscillating between 200 - 280 µatm (Feely et al. 2004). It is only since the industrial era where the release of CO_2 from industrial and agricultural activities has caused the atmospheric CO_2 concentration to increase at a current rate of 2.73 µatm/yr^{-1}, reaching 400 µatm in 2016 (Dlugokencky & Tans 2016). The increase in atmospheric CO_2 is expected to continue under all IPCC emission pathways, with levels predicted to exceed 1000 µatm early into the next century under the business as usual RCP 8.5 scenario (Meinshausen et al. 2011; Bopp et al. 2013).

The concentration of atmospheric CO_2 is buffered from the absorption by oceanic and terrestrial sinks, which in total account for half of all CO_2 released. Approximately 20 % is stored within the terrestrial biosphere by photosynthetic CO_2 uptake with the remaining 30 % by oceanic uptake via air-sea gas exchange (Gattuso 1998; Sabine et al. 2004). Without this sink, atmospheric CO_2 levels are predicted to be at least 450 µatm today (Doney et al. 2009), playing a crucial role in moderating climate change.

Unlike the terrestrial biosphere, oceanic uptake of CO_2 is not benign and ultimately causes a reduction in seawater pH. This does not occur in isolation but as a result of an increase in hydrogen ions owing to $pH = -\log_{10}[H^+]$. In addition to other changes in carbonate chemistry this phenomenon is termed OA. Globally, the average open ocean pH has already decreased by approximately 0.1 units since the preindustrial era, corresponding to a 30 % increase in acidity (Royal Society 2005). This trend is set to continue with the average global ocean pH predicted to decrease by a further 0.3 – 0.4 units by the end of the century if atmospheric CO_2 concentrations reach 800 µatm (Orr et al. 2005; Doney et al. 2009). This would be equivalent to a 150 % increase in hydrogen ions and a 50

% decrease in carbonate ions, causing widespread concern for the future of marine ecosystems (Doney et al. 2009).

In addition to changes in open ocean chemistry, coastal areas containing over 90 % of marine species (WWF 2015) are likely to be significantly influenced by increases in atmospheric CO_2 and the resultant changes in carbonate chemistry. Although significantly less research has focused on the carbonate chemistry changes within coastal systems, recent studies suggests natural fluctuations in seawater pCO_2 can occur on daily through to annual timescales (Shim et al. 2007; Urbina et al. 2010; Leiva et al. 2015). This coincides with fluctuations in a range of abiotic factors such as temperature, salinity and oxygen levels. It is predicted these fluctuations are likely in increase in intensity and severity under near future climate change (IPCC 2014), potentially posing a significant physiological stress on coastal organisms. This unprecedented rate of change both within the open ocean and coastal ecosystems has not been experienced for the past 300 million years (Doney & Schimel 2007; Lüthi et al. 2008) and is therefore causing a concern for the future of marine ecosystems.

1.2 Ocean carbonate system

Global average open ocean pH is currently pH ~8.10 whereby 90 % of the inorganic carbon is bicarbonate ions (HCO_3^-), 9 % carbonate ions (CO_3^{2-}) and 1 % dissolved CO_2 (Doney et al. 2009). Elevations in atmospheric CO_2 levels can take approximately one year to equilibrate with surface water, triggering a series of reversible chemical reactions. Dissolved CO_2 reacts with seawater to form carbonic acid (H_2CO_3). This weak acid quickly dissociates to produce hydrogen (H^+) and HCO_3^- ions. The majority of these H^+ associate with CO_3^{2-} to form further HCO_3^- (Feely et al. 2009; Doney et al. 2009), limiting the reduction of surface ocean pH termed carbonate buffering. The absorption of additional CO_2 within the oceans therefore acts to increase aqueous CO_2, HCO_3^- and H^+ ion concentrations while reducing CO_3^{2-} concentration and ultimately pH.

The availability of CO_3^{2-} ions is historically reliant on the relatively slow process of geological erosion, therefore the increasing rate of anthropogenic CO_2 dissolving into global oceans is overwhelming the oceans natural buffering capacity. With CO_3^{2-} concentration diminishing, the ability of the oceans to absorb

atmospheric CO_2 in the long-term is dependent on the extent of $CaCO_3$ dissolution producing calcium (Ca^{2+}) and CO_3^{2-} ions. This primarily comes from the shells and skeletons of marine organisms, replacing the diminishing CO_3^{2-} and therefore increasing total alkalinity (Doney et al. 2009). The depth at which this dissolution occurs depends on the polymorph of $CaCO_3$ and the associated saturation horizon (the limit between under- and super- saturation) (Orr et al. 2005). Consequently shells formed using aragonite, a metastable form of $CaCO_3$, dissolve more readily than those formed using calcite, a more stable form of $CaCO_3$. These saturation horizons are not static within the water column but are also influenced by the increases in anthropogenic CO_2 uptake. Recent research reveals that anthropogenic CO_2 uptake has already caused aragonite saturation horizon's to shoal up to 200m in many regions (Caldeira & Wickett 2005; Orr et al. 2005), the shallowest occurring in the North Pacific (Feely et al. 2004). This trend is set to continue, especially within the Southern ocean where the aragonite saturation horizon is predicted to shoal from 120m up to the surface by 2100 (Orr et al. 2005). Although the dissolution of $CaCO_3$ helps to limit the reduction in oceanic pH, as the oceans become increasingly undersaturated with respect to $CaCO_3$ it will become more difficult for marine calcifying organisms to form biogenic $CaCO_3$ (Orr et al. 2005).

In comparison to the open ocean, carbonate chemistry within coastal systems is poorly understood, often limiting our ability to design OA experiments relevant to coastal organisms. Coastal systems are characterised by a number of natural fluctuations including pH/ pCO_2, temperature, salinity, oxygen and nitrogen concentration. These fluctuations can vary on a daily (tidal cycle), seasonal and/ or annual basis (Shim et al. 2007; Wootton et al. 2008; Urbina et al. 2010; Reum et al. 2014; Leiva et al. 2015) often depending on climatic and wave conditions (Denny & Wethey 2001). Recent field studies have observed pH/ pCO_2 fluctuations of up to one pH unit (Hinga 2002; Thomsen et al. 2013), exceeding stable open ocean pH predictions for the end of the century (Suzuki et al. 1995; Yates & Halley 2006; Price et al. 2012; Johnson et al. 2013; Reum et al. 2014). This is particularly evident within temperate regions where pH/ pCO_2 fluctuations are influenced by temperature, salinity, photosynthesis and respiration (Raven et al. 2005; Lee et al. 2006; Pörtner 2008).

Furthermore, several coastal regions are characterised by upwelling events which can carry oxygen-depleted and carbon-enriched water to the surface. Several field studies suggest these events are closely linked to strong southerly winds (Rixen et al. 2012; Thomsen et al. 2013) and can significantly increase pCO_2 exposure and reduce the aragonite saturation state to less than one (Feely et al. 2008). As OA progresses, pH/ pCO_2 variability is expected to intensify with seawater pCO_2 predicted to exceed >2000 µatm by the year 2100 in some regions (Melzner et al. 2013). The magnitude and combination of these fluctuations in addition to warming, sea level rise and other climatic changes have the potential to exacerbate intertidal extremes, creating one of the most physically harsh environments on earth (Newell 1979).

1.3 Our current understanding of the biological impacts

OA and the associated changes in carbonate chemistry are predicted to significantly influence the health and fitness of a vast number of marine organisms. To date, meta-analysis reveals approximately 63 % of echinoderms, 52 % of molluscs and 45 % of coral species may be negatively affected by increases in seawater pCO_2 predicted to occur by the end of the century (Wittmann & Pörtner 2013). The majority of this experimental work has focused on the effects of OA on biogenic calcification and growth rates of calcifying organisms (Gattuso & Buddemeier 2000; Langdon et al. 2000; Kroeker et al. 2010) as altered carbonate chemistry can directly affect the dissolution and deposition rates of $CaCO_3$. For species utilising the more soluble forms of $CaCO_3$ - aragonite and high-magnesium calcite - such as many corals, hard and soft clams, whelks, mussels, planktonic pteropods and urchins, changes in carbonate chemistry and pH are predicted to negatively influence organism performance (Morse et al. 2007). For example, field studies on the massive coral *Porites* on the Great Barrier Reef showed reductions in linear extension rate and skeletal density of ~16 % and ~6 % respectively, equivalent to a ~21 % reduction in growth rate (Cooper et al. 2008). This occurred on four reefs, in two near shore regions over the past 16 years since 1988. These results are in agreement with other laboratory and field studies reporting declines in calcification rate in a wide number of coral species (Langdon et al. 2000; Yates & Halley 2006; Hofmann et al. 2010)**.**

Reductions in calcification and growth rates have not only been observed in corals but other ecologically important species. For example, in the sea urchin *Echinometra mathaei* and the gastropod *Strombus luhuanus* reductions in growth by 21 % and 12 % respectively were observed over a 6 month exposure to an increase in seawater pCO_2 by 0.02 kPa (Shirayama & Thornton 2005). In addition, the intertidal mussel *Mytilus edulis* exhibited a decline in calcification rate by 25 % during exposure to pCO_2 levels of ~740 ppm (Gazeau et al. 2007) and a reduction in growth was observed at pH 7.3 in the mussel *Mytilus galloprovincialis* (Michaelidis et al. 2005). These reductions in mussel growth are likely to partially be a consequence of changes in acid-base status, reducing pH at the site of calcification resulting in the inner aragonite layer of the shells being exposed to fluid undersaturated with $CaCO_3$ (Heinemann et al. 2012). This coupled with the low concentration of free Ca^{2+} ions in the extrapallial fluid (Misogianes & Chasteen 1979) have the potential to reduce calcification rates and therefore growth in a wide range of marine invertebrate species. The increased understanding of the mechanisms behind reductions in growth and calcification accentuates the necessity to focus on the impacts of OA on other physiological processes and not just calcification.

Although the polymorph of $CaCO_3$ can increase the vulnerability of marine calcifying organisms to elevated seawater pCO_2, there are several mechanisms which enable the maintenance of physiologically processes under elevated pCO_2 levels. These include protective external organic layers and the regulation of pH at the site of calcification and within bodily fluids. These processes are therefore likely to play central roles in the sensitivity of many species. Protective external organic layers can separate the shell or skeleton from seawater undersaturated with respect to $CaCO_3$ reducing the possibility of shell dissolution. This can vary between total coverage of the shell or skeleton as seen in purple urchins, blue mussels and temperate corals to minimal coverage as seen in scallops, serpulid worms and periwinkles (Ries et al. 2009). The strategies among species can differ with a thick epicuticle surrounding the carapace in crustaceans, the formation of aragonite underneath layers of epithelial tissue in corals and epidermis and periostracum covering the shells of urchins and mussels respectively (Ries et al. 2009). These protective layers can act to reduce exposure of sensitive structures such as a $CaCO_3$ shell or skeleton to corrosive conditions experienced during increases in seawater pCO_2. Therefore the degree

of protection of the shell or skeleton with an external layer can be closely linked to the resilience of a species to elevated pCO_2.

In addition to protective external organic layers, the regulation of pH both at the site of calcification and within extra- and intra- cellular fluids can help to maintain physiologically processes and therefore enable normal functioning under elevated pCO_2. Increases in calcification despite reductions in seawater pH and the associated changes in carbonate chemistry can be attributed to either the direct utilisation of HCO_3^- as seen in coccolithophores (Iglesias-Rodriguez et al. 2008) or through the conversion of elevated dissolved inorganic carbon (DIC), predominantly in the form of HCO_3^-, being converted to CO_3^{2-}. The latter strategy has been observed in a number of calcifying organisms including scleractinian corals (Al-Horani et al. 2003; Cohen & McConnaughey 2003; McCulloch et al. 2012), coralline algae (McConnaughey & Whelan 1997; De Beer & Larkum 2001) and foraminifera (Rink et al. 1998) whereby pH at the site of calcification was shown to increase by up to two pH units above seawater along with increases in CO_3^{2-} concentration. As described by Ries et al. (2009), the mechanisms behind these increases vary between species and include proton channelling, light-induced proton-pumping, co- transporter proton-solute shuttling and CO_2 utilisation via photosynthesis (McConnaughey & Whelan 1997; De Beer & Larkum 2001; Al-Horani et al. 2003; Cohen & McConnaughey 2003). The ability of these mechanisms to maintain pH and therefore CO_3^{2-} concentration at the site of calcification is suggested to play a crucial role in the tolerance of a species to elevated pCO_2 (Crenshaw 1972; De Beer & Larkum 2001; Al-Horani et al. 2003).

A reduction in seawater pH and the subsequent changes in seawater carbonate chemistry have the potential to disrupt other physiological processes and behaviour by reducing both intracellular and extracellular pH. Elevations in seawater pCO_2 can directly cause CO_2 to diffuse into body fluids and tissues, increasing extracellular and intracellular pCO_2 and resulting in acidosis if left uncompensated. This is predominately seen in species dependent on a favourable tissue to environment CO_2 gradient and those without developed circulatory and ventilatory systems (Michaelidis et al. 2005). Buffering against acidosis is primarily achieved via the CO_2-bicarbonate system and/ or the non-bicarbonate buffering system (Melzner et al. 2009). The former is relatively inefficient, involving Na+/K+/-ATPase pumps actively transferring HCO_3^- from the

surrounding seawater across the gill epithelia (Lucu & Towle 2003; Santos et al. 2007). Over the longer term the non-bicarbonate buffering system uses amino acid side chains, N- terminal α-amino groups of proteins or organic/ inorganic phosphate groups to bind to respiratory protons (Melzner et al. 2009). These protons are subsequently expelled via active ion transport across specialised epithelia such as the gills, renal or digestive tissue, minimising pH changes under high seawater pCO_2 (Melzner et al. 2009). These processes are energetically costly, therefore the maintenance of acid-balance balance has the potential to shift energy budgets of cells, tissues and the whole organism (Seibel & Walsh 2003; Pörtner 2008).

For a species with a limited ability to regulate acid-base balance, little is known about how reductions in extracellular pH may affect pH sensitive pathways and other physiological processes. To date, declines in extracellular pH have been linked to metabolic depression in a number of marine invertebrates (Pörtner et al. 2004; Michaelidis et al. 2005; Fabry et al. 2008). The mechanisms behind metabolic depression have rarely been identified, however recent studies reject the influence of hypoxia and suggest hypercapnia alone is responsible owing to the maintenance of pO_2 levels within the haemolymph (Pörtner et al. 1998; Michaelidis et al. 2005). Although reductions in intracellular pH can inhibit the activity of enzymes involved in metabolism (Somero 1985) and can promote the transition of glycolytic enzymes to more inactive forms (Brooks & Storey 1997), recent research reveals metabolic depression can occur independently of reduced intracellular pH. This is highlighted in *Mytilus galloprovincalis* where significant reductions in oxygen consumption and the full restoration of intracellular pH were observed during exposure to elevated seawater pCO_2 (Michaelidis et al. 2005). Therefore the authors suggest extracellular pH may predominantly cause a reduction in oxygen consumption via the inhibition of net proton transport across the cell membrane (Pörtner et al. 2000; Michaelidis et al. 2005). While metabolic depression can be a beneficial strategy in response to short-term stress (Guppy & Withers 1999), prolonged periods of depression can lead to reduced growth and aerobic and locomotory capacity ultimately depressing ecological fitness (Langenbuch & Pörtner 2004; Melzner et al. 2009).

Changes in intracellular and extracellular acidosis as a result of hypercapnia can also stimulate ammonia excretion/ passive diffusion. This coupled with reduced

oxygen consumption can lower the oxygen-to-nitrogen (O:N) ratio, suggesting an increase in protein or amino acids such as asparagine and glutamine as the metabolic substrate (Michaelidis et al. 2005). This has been shown in at least two marine species to date, *Mytilus edulis* and *Sipunculus nudus* (Lindinger et al. 1984; Pörtner et al. 1998), and may indicate the net formation of metabolic bicarbonate potentially supporting pH regulation (Langenbuch & Pörtner 2002). Furthermore uncompensated acidosis has the potential to influence oxygen binding to pigments by directly altering the physiochemical conditions (Fabry et al. 2008). For pH sensitive respiratory pigments i.e. those with a high Bohr coefficient (Bohr et al. 1904), reductions in pH may affect the oxygen loading and unloading capacity, reducing the functional capacity of the respiratory pigments (Gutowska et al. 2010). Additionally, alterations in acid-base balance have been shown to alter synaptic transmission and the function of neurotransmitters or their receptors, potentially influencing organism behaviour (Reipschläger et al. 1997; Sinning & Hübner 2013). These examples demonstrate the importance of acid-base regulation in the physiological functioning of an organism and has therefore been suggested to play a central role in determining sensitivity to OA (Pörtner 2008).

Although the indirect effects of climate change stressors are relatively unknown, recent research suggests sublethal effects such as immune response, predator detection and sensory ability may also be altered under near future elevated pCO_2 conditions (Thompson et al. 2002; Sultan 2007; Munday et al. 2009). Exposure to elevated pCO_2 seawater has been shown to affect the immunological status of the mussel *Mytilus edulis* by reducing the immune response and suppressing levels of phagocytosis. This was attributed to elevated calcium concentration within the haemolymph owing to shell dissolution (Beesley et al. 2008; Bibby et al. 2008). These increases in calcium levels can disrupt cellular metabolism, function and signalling pathways for regulating haemocyte function (Massullo et al. 2006) which in turn can alter levels of phagocytosis (Humphries & Yoshino 2003), compromising immune response. Similarly, in the isopod *Idotea balthica*, exposure to high pCO_2 for 20 days reduced immunocompetence, resulting in a 60 % - 80 % reduction in immune response (Wood et al. 2014). These reductions in immune response can be exacerbated by disruptions in the electron transport chain leading to the increased production of reactive oxygen species and therefore increased oxidative stress or by an enhanced Fenton

reaction (Tomanek et al. 2011). Any reductions in physiological function and performance, albeit sublethal, have the potential in the long-term to have considerable consequences for invertebrate populations and communities.

The simultaneous changes in pH/ pCO_2, temperature and other climate stressors have the potential to alter energy budgets owing to the higher costs of metabolism and physiological performance. This is of a particular concern if reproductive condition and output are affected, potentially causing a bottleneck for species persistence and ecological success (Byrne 2011). For broadcast spawning marine invertebrates, gametes are fertilised externally, spending several months within the water column which can increase exposure to unfavourable conditions. Meta-analysis to date suggests significant differences amongst life stages (Dupont et al. 2010; Kroeker et al. 2010) and between taxonomic groups (Kurihara 2008). Hypercapnia can narcotise sperm (Brokaw 1990) ultimately reducing swimming speeds. However, the impact on fertilisation is predicted to be low and fairly robust to future OA and warming in many species (Havenhand et al. 2008; Byrne 2011). However, during developmental stages increased pCO_2 can reduce the ability of calcifying larvae to form shells and skeletons. This can result in smaller larvae with weaker shells impacting swimming and feeding efficiency and therefore increasing the potential of predation and shell damage (Allen 2008; Przeslawski et al. 2008; Soars et al. 2009). Any reduction in performance during the developmental stages can have direct negative consequences for adult populations and therefore marine communities (Harley et al. 2006; Przeslawski et al. 2008; Brierley & Kingsford 2009; Byrne 2011).

1.4 The role of environmental variability

The majority of experimental OA studies to date use predictions based on global open ocean averages, where pH and other carbonate parameters do not vary in time or space. However, as mentioned previously, this uniformity is uncharacteristic of coastal habitats limiting our ability to predict future responses of organisms living within these ecosystems. Exposure to pH/ pCO_2 fluctuations are likely to intensify under global climate change (IPCC 2014), therefore understanding the current responses to *in situ* variation is critical in aiding our understanding of the physiological responses to future variability.

Although the physiochemical conditions of the environment in which a coastal organism inhabits is often unknown, recent studies have investigated the influence of predicted pH/ pCO_2 variability. In mytilid mussels no difference in survivorship was observed between static and variable pH regimes. However, the decreased developmental rates observed in low pH treatments were alleviated during high-frequency pH oscillations (Frieder et al. 2014). Similarly, corals recruits benefitted from fluctuations in pH, growing on average 6 % - 19 % larger and exhibiting increased survivorship by 13 % – 18 % compared to those reared in static conditions (Dufault et al. 2012). In addition to pH/ pCO_2 oscillations, fluctuations in temperature by 5 °C resulted in increased hatchling and survival rates relative to those reared in constant temperatures in the mud-crab *Rhithropanopeus harrisii* (Costlow Jr & Bookhout 1971). The oscillations of pH/ pCO_2, temperature and other abiotic factors may therefore allow an organism to recover when exposed to favourable conditions, increasing species persistence.

The vast range of abiotic fluctuations currently experienced by intertidal organisms has raised the potential paradigm that these organisms may have an inherent tolerance to predicted pH/ pCO_2 and temperature variations in the future (Widdicombe & Spicer 2008; Melzner et al. 2009; Whiteley 2011). This may be possible through physiological mechanisms increasing phenotypic plasticity and therefore the capacity to cope in the future (Benedetti-cecchi et al. 2006; Johnson et al. 2014). However, some studies suggest these organisms are already living close to their physiological tolerances, even when situated well within their range limits (Sagarin & Somero 2006; Place et al. 2008; Beukema et al. 2009). Therefore during increases in intensity and frequency of fluctuations in the near future, these organisms may have minimal scope for adaptation. For example, fluctuations in abiotic factors were shown to intensify the effect of stressors in the calcifying coralline macro-alga *Arthrocardia corymbosa*, whereby fluctuations in pH acted additively to further reduce growth compared to static treatments (Cornwall et al. 2013). Increasing environmental variability may therefore push organisms beyond their physiological tipping points (Aguilera et al. 2013; Evans et al. 2013) significantly altering ecosystem function (Mumby et al. 2011).

There are however, areas that already experience elevated pCO_2 values predicted for the end of the century, such as within CO_2 seeps and upwelling

areas. For species with large geographic distributions encompassing these areas, it can be beneficial to investigate the physiological plasticity and/ or physiological and genetic adaptations which allow for species' persistence within these regions (Hochachka & Somero 2002; Sanford & Kelly 2011; Pespeni et al. 2013a). Within the California Current System (CCS), wind-driven upwelling events bring CO_2 enriched seawater to the surface (Checkley & Barth 2009). Analysis of gene expression between populations of the purple sea urchin *Strongylocentrotus purpuratus* along the CCS reveals that the changes in gene expression related to biomineralisation and metabolism are dependent on geographic location (Pespeni et al. 2013b). Those native to the Southern California Bight region characterised by cooler and resource abundant waters, exhibited consistently higher rates of gene expression and therefore scope for growth, resulting in a faster regrowth of spines. This is compared to the northern populations along the Oregon coast which experience warmer temperatures and higher pH values (Pespeni et al. 2013b). In a separate study using the same species, Pespeni et al. (2013c) correlated gene frequency with time spent at seawater below pH 7.7. The authors demonstrate that the genes relating to internal pH regulation and cellular stability at reduced pH such as a cytoskeletal associated protein, a vacuolar proton pump protein and a sterol carrier protein (Szászi et al. 2000; Pörtner 2008) were all significantly up-regulated the longer the population experienced low pH conditions (Pespeni et al. 2013c). In addition, purple sea urchin larvae have been shown to have a greater genetic variation for body size under elevated pCO_2 conditions, consistent with local adaptations to the carbonate chemistry conditions experienced in the field (Kelly et al. 2013). Similarly, within the Humboldt Current System along the Chilean and Peruvian coast, egg capsules from the gastropod *Concholepas concholepas* native to non-upwelling regions were more impacted by exposure to elevated pCO_2 than those frequently exposed to upwelling events (Vargas et al. 2015). Moreover, gastropods frequently exposed to higher pCO_2 values from upwelling events had a higher plasticity of their metabolic rate (Lardies et al. 2014). The capacity to adapt to periodic upwelling events, as shown in these studies, provides crucial insights into the potential for adaptations to occur over time in regards to climate change (Hofmann et al. 2014).

Furthermore, gastropods adapted to high seawater pCO_2 conditions at a shallow-water CO_2 seep were shown to have an altered metabolic energy demand when

compared to those found in coastal areas (Garilli et al. 2015). This was achieved by a reduction in whole organism size, which increases mass-specific energy consumption while significantly lowering whole metabolic rate, and thus allowing the maintenance of costly processes such as calcification (Garilli et al. 2015). This dwarfism or Lilliput effect has previously been considered in respect to past extinction events (Fraiser & Bottjer 2007; Twitchett 2007) and has only recently been applied to OA studies (Findlay et al. 2011; Garilli et al. 2015). This trade-off between energy demand and organism size may therefore generate physiological advantages in a rapidly changing ocean. Unfortunately, the ability to infer adaptive responses is often limited by our scarce understanding of the current environmental conditions experienced by intertidal invertebrates. These knowledge gaps therefore restrict our ability to inform predictions of future vulnerabilities to economically and ecologically important marine invertebrate species.

For intertidal organisms, natural fluctuations in abiotic factors are coupled with alternating periods of emersion and immersion which have the potential to synergistically pose significant physiological stress (Waldbusser & Salisbury 2014). This is amplified for sessile species, such as intertidal mussels, which are unable to relocate to less exposed positions to avoid adverse conditions (Halpin et al. 2002). During emersion at low tide, intertidal mussels close their shell to prevent desiccation resulting in reduced access to oxygen (Coleman 1973a), their body temperature is completely dependent on air temperature and the effects of solar irradiance (Zandee et al. 1986; Hines et al. 2007) and they are unable to feed. However, during immersion mussels have access to food, dissolved oxygen and their body temperature remains relatively stable. These changes throughout a tidal cycle have been shown to influence physiological and behavioural changes such as metabolic rate (Zandee et al. 1986), oxygen consumption (Coleman 1973a), heart rate (Helmuth et al. 2010), valve opening (Widdows & Shick 1985; Shick et al. 1986), heat shock protein synthesis (Place et al. 2008; 2012; Dutton & Hofmann 2009) and intermediary metabolite cycles (Connor & Gracey 2012).

Upon emergence at low tide, mussels have been shown to use the tricarboxylic acid cycle and electron transport pathways to synthesise ATP under aerobic metabolism (Zandee et al. 1986). However, closure of the mussel valve traps

water within the mantle cavity, limiting the uptake and availability of oxygen. This restricts the ability of mussels to maintain aerobic metabolism and ultimately leads to hypoxia (Fan et al. 1991). At the onset of hypoxia metabolism switches from aerobic to anaerobic (Bayne et al. 1976; Brinkhoff et al. 1983; Babarro et al. 2007; Connor & Gracey 2011) which utilises fermentative pathways of glucose and aspartate producing succinate and alanine (Isani et al. 1995). Anaerobic metabolism in bivalves is significantly more efficient than the fermentation of lactate often seen in vertebrates, producing three times more ATP per glucose molecule (Hochachka & Somero 2002; Storey & Storey 2005). Consequently, less H^+ ions are released per ATP molecule (Hochachka & Somero 2002) assisting the prevention of acid-base disturbances during emersion.

During emersion sessile intertidal organisms can additionally experience extreme heat stress from rising body temperatures owing to solar irradiance, air temperature and convection heat exchanges (Helmuth 1998). This can increase the demand for ATP as heat stress related proteins are synthesised at the induction of gene expression (Gracey et al. 2008). In *M. edulis*, moderate heat stress has been shown to increase the emergence of protein chaperones, compared to extreme heat stress where genes targeting irreversible denatured proteins are synthesised (Gracey et al. 2008). This can increase the energetic demand in mussels by up to 25% (Hawkins & Bayne 1991) placing a significant demand on ATP production. If anaerobic metabolism is unable to generate enough ATP, increasing temperatures are likely to impose a metabolic deficit which has the potential to exacerbate metabolic acidosis. Although the physiological responses of *Mytilus* mussels to a tidal cycle are beginning to emerge, little is known about how current environmental factors interact or which drivers will influence acid-base response during both immersion and emersion phases.

1.5 Mussel ecology and physiology

Mussels are important intertidal species with a high economic and ecological value. In 2012 mussel production in the UK was worth approximately £27 million, harvesting just over 26 thousand tonnes (Ellis et al. 2012). Additionally, they have a high ecological and biogeochemical importance providing ecosystem services such as shelter from predation, habitat structure in the form of mussel beds, water purification by filtering phytoplankton and other particles and therefore increasing

the penetration of light and as a food source for several species (Asmus & Asmus 1991; Gutierrez et al. 2003; Gazeau et al. 2013). Mussels also have a large global distribution with species colonising areas from the deep sea up to the intertidal zone (Tunnicliffe et al. 2009), making them an ideal study species. Although historically abundant, reductions in natural populations owing to future climatic changes has the potential to alter food web dynamics, habitat structure and can potentially lead to an increase in job loss (Newell 2004).

To date the physiology, ecology and genetics of *Mytilus* mussels have been widely explored (Bayne et al. 1976; Gosling 1983; Seed & Suchanek 1992; Zippay & Helmuth 2012) with studies investigating the influence of OA (Berge et al. 2006; Bechmann et al. 2011), salinity (Bussell et al. 2008; Deschaseaux et al. 2011), wave exposure (Carrington 2002; Carrington et al. 2009; Wallin et al. 2011), food availability (Schneider et al. 2010) and chemical contamination (Luedeking & Koehler 2004; Apraiz et al. 2006; Hong et al. 2009). Although there is substantial knowledge of this genus, there are still significant knowledge gaps in our understanding of how these species' respond to current environmental variability and the possible local adaptations evolved from populations experiencing regular exposure to elevated pCO_2.

Despite the numerous reports on *Mytilus* mussels, significantly less is known about species from other genera, especially those native to non-European areas. This can be highlighted by *Perumytilus purpuratu*s distributed along the South American coast, whereby mussel beds can harbour up to 93 different taxa significantly influencing community structure and local biodiversity (Prado & Castilla 2006). Despite their ecological importance, very little is known about the physiology of this species especially the potential impacts of future climatic changes.

It has been proposed that the combination of highly expressed calcified structures, low levels of activity and the inability to regulate acid-base disturbances are key characteristics of sensitive taxa to increasing seawater pCO_2 (Wittmann & Pörtner 2013) suggesting mussels may be vulnerable to near-future OA conditions. Furthermore, very little is currently understood about the current environmental conditions experienced *in situ*, the influence of environmental variability on physiological responses and the drivers of acid-base disturbances during periods of immersion and emersion. Current physiological

studies suggest mussels are unable to regulate acid-base disturbances (Booth et al. 1984; Thomsen & Melzner 2010; Heinemann et al. 2012; Thomsen et al. 2013), however this has only been assessed during exposure to static pH/ pCO_2 conditions. Therefore it is still unclear how frequent fluctuations of abiotic factors may influence acid-base balance, which in turn has the potential to alter energy budgets, metabolism and other physiological processes. In addition, the majority of studies have yet to investigate the emersion phase of a tidal cycle which has the potential to pose significant physiological stress on these intertidal organisms. Therefore the already vast amount of research on mussel species' can provide the basis for further research into understanding how these important species may respond in the future.

1.6 Aims and objectives

This master's project aims to address some of these key questions in order to inform predictions of the future physiological responses of two key marine species'. The inclusion of a second mussel species allows for inter-species comparisons of physiological responses as well as the ability to infer the potential for any adaptive responses. *Perumytilus purpuratus* were chosen partially because of their large ecological importance and the relatively few physiological studies and in part because of their location. Situated along the Humboldt Current System on the Chilean coast, this unique system ensures these mussels experience a multitude of physiochemical conditions making the potential for local adaptation high in this region. This has already been shown in other species inhabiting these areas such as the gastropod *Concholepas concholepas* (Lardies et al. 2014; Vargas et al. 2015). Therefore if local adaptations are possible in mussels, it is likely that they will occur in this region.

Firstly I aim to investigate the current variability experienced by two populations of *Mytilus edulis* and one population of *Perumytilus purpuratus* and to determine the main drivers of acid-base change over a complete tidal cycle. These together will form Chapter 2 and test the hypothesis:

H1: pH/ pCO_2 is the main driver of acid-base change over a tidal cycle.

To test hypothesis 1 a combination of field and laboratory work was used. For two populations of the blue mussel *Mytilus edulis* located in the South West of England and one population of the Chilean mussel *Perumytilus purpuratus*, a

twelve-hour sampling period was used to determine the acid-base status and the seawater carbonate chemistry conditions over a complete tidal cycle. This was carried out over one year for both UK populations and throughout September and October 2015 for the Chilean population. The UK data were then used to parameterise laboratory work assessing the influence of pH/ pCO_2 exposure during immersion and aerial temperature and size (shell length) during emersion, as drivers of acid-base change during environmentally relevant periods of emersion and immersion.

In Chapter 3 I will go on to look at the potential for populations inhabiting upwelling regions and therefore regularly exposed to elevated pCO_2 conditions, to alter the acid-base response during short-term exposures to elevated pCO_2 levels. This forms the hypothesis:

H2: organisms living in habitats with variable pCO_2 will have a greater resilience to OA

To test this, two populations of *M. edulis* and three populations of *P. purpuratus* were exposed to short-term changes in seawater pH/ pCO_2. The three Chilean sites were chosen to represent different degrees of elevated seawater pCO_2 exposure; one with regular coastal variability, one within a seasonal upwelling region, and one within a seasonal upwelling region in addition to continuous riverine water discharge. Comparative analysis took place to investigate both intra- and inter- species differences and to investigate if living within an area exposed to elevated pCO_2 can alter the acid-base response of mussels during short-term changes in pH/ pCO_2.

Chapter 4 will follow with the investigation of the medium-term impacts of a variable compared to a static pH/ pCO_2 regime on the physiological responses of *M. edulis*. To do this, I will test the hypothesis:

H3: a variable pH/ pCO_2 regime will incur greater physiological costs than a stable pH/ pCO_2 regime.

The third hypothesis will be tested using one *M. edulis* population which was exposed to either a static or variable pH/ pCO_2 regime aimed to mimic current day and anthropogenic variability. Acid-base response along with metabolic rate and several health parameters were measured after 14 days.

This thesis will then conclude with chapter 5, discussing the combined findings of this Master's project from Chapters 2-4 and suggest the further implications of this work.

Chapter 2: Drivers of acid-base response over tidal cycles in the mussel *Mytilus edulis*: size and temperature matter more than pCO_2

2.1 Abstract

The majority of ocean acidification studies to date use stable open ocean pH values to predict the physiological responses of intertidal organisms to future climate scenarios, omitting the natural fluctuations of abiotic conditions. Fewer still account for the routine alternating periods of emersion and immersion during a daily tidal cycle. This study determines the carbonate chemistry parameters and the acid-base response of two populations of *Mytilus edulis* over a 12-hour tidal cycle throughout one year. These data were then used to parameterise experimental work investigating the influence of size (37 mm – 50 mm and 60 mm – 79 mm shell length), temperature (7 °C, 13 °C, 20 °C, 28 °C) and low pH exposure (pH 7.7) as drivers of acid-base change over environmentally realistic (6 hour) immersion and emersion periods. During 6 hours of emersion a significant increase in haemolymph pCO_2 and a reduction in pH was observed at 20 and 28 °C. In addition average change in haemolymph pH was greater in larger compared to smaller mussels with -0.7 pH units compared to -0.2 units respectively. During re-submersion recovery of all acid-base parameters occurred during the first 3 hours. Our results clearly show temperature and size are the central drivers of acid-base balance during emersion irrespective of prior immersion in low seawater pH conditions. However, during re-immersion pH/ pCO_2 exposure drives acid-base response independent of previous conditions experienced during emersion. This study provides new and much needed insight into the natural abiotic conditions experienced by two populations of *M. edulis* as well as determining the drivers of acid-base disturbances over a tidal cycle. This work therefore enhances our ability to understand the physiological responses of key marine biota to both current and future environmental changes.

Keywords: Ocean acidification, temperature, size, acid-base, emersion

2.2 Introduction

Coastal systems are characterised by natural fluctuations in abiotic conditions occurring on a daily (tidal), seasonal and annual basis (Shim et al. 2007; Wootton

et al. 2008). This is particularly evident within temperate regions where strong seasonal stratification in addition to upwelling events, temperature, salinity, photosynthesis and respiration can result in fluctuations of seawater pCO_2 (Raven et al. 2005; Lee et al. 2006; Pörtner 2008). This variability is predicted to intensify under increases in atmospheric CO_2, with ocean acidification (OA), warming and other climatic changes expected to exacerbate intertidal extremes (IPCC 2014). For intertidal organisms these fluctuations are coupled with alternating periods of emersion and immersion which collectively have the potential to pose significant physiological stress (Waldbusser & Salisbury 2014), affecting population distribution, fitness and growth rates (Boyce et al. 2006). Therefore understanding how an organism responds to natural environmental conditions is fundamental for informing predictions of future vulnerabilities.

To date the majority of studies investigating the impacts of future climate change suggest OA and temperature have the potential to impact physiology, growth and reproduction across a wide range of marine fauna (Pörtner et al. 2004; Portner & Knust 2007; Fabry et al. 2008; Doney et al. 2009; Sarà et al. 2011). However, for intertidal organisms OA and temperature changes will be combined with periods of emersion during a tidal cycle. During immersion at high tide intertidal organisms such as the common mussel *Mytilus edulis* have access to food, dissolved oxygen and their body temperature remains relatively stable, following that of the surrounding seawater. However, during periods of emersion at low tide, sessile organisms are unable to feed, have limited access to oxygen owing to the closure of valves to prevent desiccation (Coleman 1973b) and body temperature is completely dependent on air temperature and the effects of solar irradiance (Zandee et al. 1986; Hines et al. 2007). These tidal oscillations can therefore influence physiological and behavioural changes such as metabolic rate (Zandee et al. 1986), oxygen consumption (Coleman 1973a), heart rate (Helmuth et al. 2010), valve opening (Widdows & Shick 1985; Shick et al. 1986) and intermediary metabolite cycles (Connor & Gracey 2012). In addition, the closure of the mussel valve leading to a reduction in oxygen has been shown to shift metabolism to anaerobic pathways, which in turn may result in disturbances in acid-base balance. The ability to compensate these acid-base disturbances and maintain cellular homeostasis has been suggested to play an important role in the future survival and distribution of a given species (Pörtner & Farrell 2008; Calosi et al. 2013).

Maintaining cellular homeostasis is not solely dependent on environmental factors but also has the potential to be dependent on organism size. Reductions in size may be advantageous for organisms living in stressful habitats where there is an increased energetic cost. For example, those living within intertidal regions can experienced intermittent heat stress during emersion leading to the costly productions of heat stress proteins (Hawkins & Bayne 1991; Gracey et al. 2008). The change in size often termed dwarfism or the Lilliput effect can act to reduce total whole animal energy demand while increasing mass specific demand of essential processes, resulting in increases in metabolic rate (Garilli et al. 2015). Although this has principally been considered in respect to OA (Fraiser & Bottjer 2007) and past extinction events (Twitchett 2007), the physiological changes associated with smaller individuals may be advantageous in future warming and OA conditions.

The influence of OA on the acid-base status of marine fauna has principally been studied during prolonged exposure to stable future predicted pH/ pCO_2 values (e.g. Kurihara & Shirayama 2004; Shirayama & Thornton 2005; Gazeau et al. 2007). However little is known about the impacts of pH/ pCO_2 variability experienced by natural populations and how previous exposure to OA may influence acid-base balance during emersion. The continued absorption of increasing atmospheric CO_2 by the worlds' oceans has already led to a reduction in open ocean pH by 0.1 units and corresponding changes in carbonate chemistry (Orr et al. 2005; Cao & Caldeira 2008). In coastal areas, recent field observations have suggested fluctuations in pH/ pCO_2 can reach up to one pH unit (Hinga 2002), exceeding those predicted for the end of the century (Suzuki et al. 1995; Yates & Halley 2006; Price et al. 2012; Johnson et al. 2013). With seawater pH predicted to decrease by a further 0.3 - 0.4 units by the end of the century and fluctuations expected to intensify (IPCC 2014), OA may pose significant physiological stress on intertidal invertebrates.

A rise in seawater pCO_2 has previously been shown to induce extracellular acidosis in *M. edulis*, with no compensatory increase in bicarbonate ions (HCO_3^-) (Booth et al. 1984; Thomsen et al. 2010; 2013; Heinemann et al. 2012). The increase in HCO_3^- is often a product of active ion exchange, involving the Na^+/K^+/-ATPase pump actively transferring HCO_3^- from the surrounding seawater across the gill epithelia (Lucu & Towle 2003; Santos et al. 2007) facilitating the

compensation of extracellular acidosis. Without extracellular pH regulation, metabolic suppression can be induced leading to a reduction in growth rates when exposed to prolonged increases in seawater pCO_2 (Pörtner et al. 2004; Michaelidis et al. 2005; Fabry et al. 2008)**.** However, how a pre-exposure to OA conditions may influence the acid-base balance during emersion is still unknown.

Although *M. edulis* appears to be a weak acid-base regulator in response to stable elevated seawater pCO_2, little is understood of the current seawater conditions currently experienced by these, and other, intertidal species, in addition to how this may influence the magnitude of physiological and behavioural changes during both immersion and emersion. Field studies will therefore become increasingly important to measure the current environmental conditions experienced by test populations and the related biological changes in addition to identifying representative laboratory parameters. For example Thomsen et al. (2010) measured pH variability within the Kiel Fjord, an area inhabited by *M. edulis* and found these mussels were able to maintain growth and calcification rates at reduced pH values within the field. These data were then used to inform laboratory parameters to investigate the influence of food availability. Studies such as this are crucial in furthering our understanding of how intertidal species may respond to future changes.

In addition to changes in seawater pCO_2, extracellular acidosis may be induced via environmental temperature changes during emersion. Globally, average surface temperatures have increased by 0.7 °C (Hansen et al. 2006) and are projected to rise by up to 4 °C by the end of the century under the business as usual RCP 8.5 scenario (Pachauri & Meyer 2014) potentially posing a significant threat to intertidal organisms exposed for substantial periods of the day. Temperature alone has been shown to reduce extracellular pH by 0.06 to 0.115 units under a 4-5 °C increase in temperature in a number of crustaceans (Whiteley 1999). However, the influence of temperature during emersion in weak acid-base regulators, such as mussels, is still unknown. In addition, at higher temperatures the kinetics of metabolism can be affected altering O_2 supply and demand (Rahn 1966). This can lead to metabolic acidosis which in turn can be exacerbated by increases in environmental pCO_2 (Rastrick et al. 2014). These gradual average increases are likely to exacerbate summer maxima's and

increase solar irradiance which are not only likely to impact acid-base balance but also increase desiccation rates for the intertidal mussel.

Integrating periods of emersion into experimental design can help to provide a more holistic view of the threats facing intertidal organisms. Although limited to date, examples can be found in respect to metabolite cycles, ocean acidification and temperature. Evidence to date suggests environmental pCO_2 and increased temperature during emersion can reduce recovery speeds of extracellular acidosis in the velvet swimming crab *Necora puber* (Rastick et al. 2014). In addition, Paganini et al. (2014) suggests an interactive effect between the two environmental drivers whereby a reduction in respiration rate and an increase in heat tolerance was observed after 2.5 weeks in *Petrolisthes cinctipes.* A reduction in a species' physiological capacity as observed in these studies has been suggested to have the potential to impede a species ability to persist within the low-mid shore, ultimately influencing their bathymetric range in addition to the structure and diversity of intertidal assemblages (Rastrick et al. 2014). The inclusion of an emersion phase is therefore a vital next step to understanding the responses of intertidal organisms to environmental stress.

The combined effects of temperature and OA have been reported to influence the physiology of intertidal organisms (Keppel et al. 2014; Kroeker et al. 2014; Mackenzie et al. 2014; Wang et al. 2015), yet few have accounted for the routine acid-base disturbance following emersion and recovery during a daily tidal cycle. Owing to the complex nature of an intertidal environment very little is known about the current environmental conditions experienced by coastal organisms, therefore it can be valuable to incorporate field studies to aid our understanding of the current variability experienced in order to inform predictions of future responses to an additional climate signal. Here we couple laboratory and field studies to investigate the role of OA, size and temperature in driving acid-base responses in *M. edulis*, an economically and ecologically important bivalve species (Gutierrez et al. 2003; Ellis et al. 2012). We hypothesise that pH/ pCO_2 will be the main driver of acid-base change over a tidal cycle. To test this we first take field measurements to determine the current environmental variability experienced by two local populations of *M. edulis* and the acid-base response during tidal cycles throughout the year. We then use these measurements to help parameterise laboratory experiments to investigate the influence of OA,

temperature and size as drivers of acid-base disturbance during emersion to help inform predictions of the susceptibility of *M. edulis* to future climatic changes.

2.3 Materials and Methods

Field monitoring

Field site and determination of carbonate system parameters

Monitoring took place in November 2014 (Starcross only), April, July and September 2015 at Starcross, South Devon, a sheltered muddy estuary (50°37'03" N -3°26'56" W) and Port Gaverne, North Cornwall, an exposed rocky shore (50°35'38" N -4°49'26" W). Both sites contain large intertidal populations of *Mytilus edulis* experiencing semidiurnal tidal cycles.

Sampling took placed every 30 minutes over a 12 hour period to include a complete tidal cycle of one high and one low tide. Owing to the difficulty collecting mussels and accessing both beds during high tide, after the last sample at low tide or the previous low tide before sampling, mussels were collected and placed inside a 7 l mesh bag (Podsac) measuring 170 x 300mm. Except from the bottom, the bag was completely meshed ensuring maximum water flow to the mussels. Haemolymph samples taken throughout the immersion period were consistent suggesting the reduction in stocking density over time did not alter the haemolymph measurements. In addition haemolymph samples from the field were comparable to those taken in the laboratory at a similar pH/ pCO_2 level therefore, although a "bag effect" was not tested for, it is unlikely to have influenced these results.

At Port Gaverne this bag was then attached to a pulley system whereby the mussels remained upon the mussel bed and could be pulled up during each sampling point to allow collection. At Starcross, the flat muddy estuary meant a pulley system was not feasible but instead mussels within the mesh bag were brought closer to the shore and collected by wading during each sample. To differentiate between population differences and environmental influence, during the July sample at Port Gaverne, mussels from Starcross were transposed the previous day in a separate mesh bag and attached to the pulley system.

Similar difficulties reaching the mussel bed occurred during the collection of seawater samples at high tide. Therefore, for both sites seawater was collected from the surface above where the mussels were situated and assessed for pH, temperature, salinity and dissolved inorganic carbon (DIC). Seawater pH was measured using a Metrohm (826 pH mobile) pH NBS electrode calibrated prior to use. Salinity was measured at an accuracy of ±0.1 psu using a Mettler Toledo SG7 SevenGo pro conductivity meter. Temperature was additionally monitored using HOBO Pendant® Temperature/Light Data Loggers for the July and September samples at both sites. DIC samples were preserved on site with saturated $HgCl_2$ (Dickson et al. 2007) and analysed for DIC using a custom built system based on Friederich et al. (2002) following methodology from Lewis et al. (2013). Owing to practicalities of analysing DIC samples, only one was taken per time point. Five data outputs per sample were analysed using Logger Pro version 3.2 software with a measurement precision of ±3 µM. Total alkalinity and pCO_2 were calculated using CO2sys (Pierrot et al. 2006) using parameters selected by Findlay et al. (2013). K1 and K2 values were refitted by Dickson and Millero (1987) from Mehrbach (1973) and KSO_4 determined by Dickson (1990).

Acid-base measurements

For the acid-base measurements, mussels were collected every 30 minutes and haemolymph from the posterior abductor muscle was extracted using a 21 g needle and a 2 ml syringe at each sampling time-point. Immediately following extraction haemolymph pH was measured using a pH meter and transferred to 100 µl glass micro-hematocrit capillary tubes sealed with paraffin oil and hemato-sealTM capillary tube sealant (Fisher). Samples were then placed on ice and taken back to the laboratory for subsequent analysis of total CO_2 using a Corning 965 CO_2 analyser (Corning Ltd., UK) calibrated with a 10 mM $NaHCO_3$ solution. Haemolymph samples consist primarily of HCO_3^- and CO_3^{2-} with a very small percentage in the form of gaseous CO_2 (1- 2 %). Therefore the sealing of air-tight capillary tubes in addition to being placed on ice is likely to prohibit the diffusion of gaseous CO_2 from the capillary tubes. If however, some or all of the gaseous CO_2 leaks out, the small overall percentage is unlikely to significantly alter the results. Acid-base parameters were then calculated using a modified version of the Henderson-Hasselbalch equation using the first dissociation constant (pK) for

carbonic acid and solubility constant (αCO_2) for carbon dioxide derived from Truchot (1976).

Laboratory experiments- acid-base response to emersion

Collection of animals

Two size classes of adult *Mytilus edulis* (37 - 50 mm and 60 - 79 mm shell length) were collected from Starcross. Mussels left for 24 hours in aerated artificial seawater (©Tropic marine, salinity 32- a value representative of that naturally experienced), before being scrubbed clean and barnacles removed. Mussels were then held in a recirculating system of artificial seawater (pH 8.10, salinity 32, at 15 ± 0.5°C, photoperiod 12:12) for a minimum of 7 day before experimentation and fed 5000 cells ml^{-1} of dried *Isochrysis* Instant Phyto (ZM Systems) daily.

Seawater manipulation

Artificial seawater used for all experiments was filtered to 1 µm and then acidified via the manipulation of seawater CO_2. To reach the target pH of pH 7.70 a computerised control system (Aqua Medic, Germany) was used to control seawater pH, where by gaseous CO_2 was injected into the seawater through a solenoid valve until pH reached the target level. Seawater pH was additionally monitored with a pH meter and aerated to maintain oxygen levels close to 100% saturation. Seawater samples were collected for assessment of pH and DIC as described above.

Acid-base measurements

To understand the role of size (shell length), temperature and exposure to elevated seawater pCO_2 before emersion on the acid-base response of *M. edulis* we measured haemolymph pH, pCO_2 and bicarbonate concentration over 6 hours of emersion followed by 6 hours of recovery in treatment seawater (either pH 8.10 or pH 7.70). Mussels from Starcross were categorised into two size classes, small (37 – 50 mm, N = 78) and large (60 – 79 mm, N = 78), half of which were exposed to seawater at either pH 8.10 or pH 7.70 for one week at 13°C. Mussels were subsequently emersed in a temperature controlled room at either 7 °C, 13 °C, 20 °C or 28 °C (± 0.5 °C) for 6 hours. Following emersion mussels were then placed back into the treatment seawater to investigate recovery over

the following 6 hours. Every 45 minutes during emersion (N = 3) and every hour during recovery (N = 4) mussel haemolymph was extracted for assessment of acid-base response as described above.

Statistical analysis

Data are presented as mean ± standard error (SE) and tested for normality using the Shapiro-Wilk Test. For all laboratory work, linear model regression analysis (LM) was used to determine the effects of size, temperature and exposure to elevated seawater pCO_2 before emersion on acid-base parameters using R studio version 3.02.

2.4 Results

Field monitoring

Carbonate system parameters

The seawater carbonate chemistry during submersion of a mussel bed over 12 hours at Starcross and Port Gaverne are summarised in Tables 2.1 to 2.7. Surface water pCO_2 of Port Gaverne was almost always lower than present day average open ocean pCO_2 values (~400 µatm) (Dlugokencky & Tans 2016). For example seawater pCO_2 values varied between 243 ⌈atm and 334 ⌈atm in April compared to 276 ⌈atm and 1912⌈atm in July. No clear seasonal patterns were observed. Additionally, pH varied between pH 7.42 and pH 8.28 and salinity varied between 20.7 and 32.6 over all three sampling points.

Mussel beds situated within the Exe estuary at Starcross also experienced no clear seasonal variations in surface water pCO_2, with values lower than present day averages (~400 µatm) (Dlugokencky & Tans 2016). Here habitat pCO_2 varied between 142.3 ⌈atm to 647.1 ⌈atm, pH varied between pH 7.86 to pH 8.36 and salinity varied between 22.8 and 33.0 throughout the year. For both sites seawater pCO_2 values higher than the present day average (>400 ⌈atm) (Dlugokencky & Tans 2016) only occurred on 7 out of 35 and 5 out of 55 occasions for Port Gaverne and Starcross mussels respectively, suggesting this variability may be brief and infrequent. There was however, large variability from both sites in the seawater temperature (Tables 2.1 – 2.7).

Acid-base measurements

Haemolymph samples were taken every 30 minutes over 12 hours to investigate the acid-base response during a tidal cycle. As both populations became exposed during low tide, haemolymph pCO_2 levels increased at an average rate of 428 ⌈atm per hour and 572 ⌈atm per hour for Port Gaverne and Starcross mussels respectively. With a limited ability to regulate bicarbonate concentration ($[HCO_3^-]$), haemolymph pH subsequently decreased during the emersion period. The magnitude of this acidosis appears to be larger in mussels from Starcross. This acidosis was reversed when the mussel beds became re-submerged (figure 2.1 and 2.2).

To investigate the differences between population responses, mussels from Starcross were transposed to Port Gaverne during the July sample (figure 2.1b, e and h). Both populations showed a mirrored response when immersed in seawater, reaching a maximum haemolymph pH of 7.67 and pH 7.68 for Port Gaverne (37 mm average shell length) and Starcross (63 mm average shell length) mussels respectively. However, during exposure at low tide larger mussels from Starcross exhibited a more pronounced extracellular acidosis, reaching a minimum of pH 6.91 compared to a minimum of pH 7.15 in the Port Gaverne population.

Laboratory experiments- acid-base response to emersion

Seawater carbonate chemistry

The seawater carbonate chemistry prior to the 6 hours of emersion and the following 6 hours of recovery (during re-immersion) are summarised in Table 2.8.

Acid-base measurements

Previous exposure to elevated seawater pCO_2 had no significant effect on haemolymph pCO_2, $[HCO_3^-]$ or pH during 6 hours of emersion (figure 2.3; LM for pCO_2 $F = 31.6_{(31, 405)}$, $P = 0.915$; for $[HCO_3^-]$ $F = 5.818_{(31, 405)}$, $P = 0.151$; for pH $F = 31.12_{(31, 405)}$, $P = 0.195$). Over the 6 hours of emersion, haemolymph pCO_2 levels significantly increased at 20 °C and 28 °C (LM for 20 °C and time $F = 31.6_{(31, 405)}$, $P = 0.045$; for 28 °C and time $F = 31.6_{(31, 405)}$, $P < 0.001$) with size also having a significant influence at 28 °C over time (LM for 28 °C, size and time

F = $31.6_{(31, 405)}$, P < 0.001). The increase in pCO_2 levels within the haemolymph did not necessarily translate to an increase in haemolymph [HCO_3^-]. Only at 13 °C and 20 °C did [HCO_3^-] significantly increase at an average rate of 0.07 mM over the 6 hours of emersion (LM for 13 °C F = $5.818_{(31, 405)}$, P < 0.001; for 20 °C F = $5.818_{(31, 405)}$, P < 0.001). With the absence or small accumulation of HCO_3^- ions, haemolymph pCO_2 levels caused a significant reduction in haemolymph pH at 20 °C and 28 °C (LM for 20 °C F = $31.12_{(31, 405)}$, P = 0.041; for 28 °C F = $31.12_{(31, 405)}$, P = 0.013), with temperature significantly influencing haemolymph pH over time at 28 °C (LM for 28 °C and time F = $31.12_{(31, 405)}$, P = 0.014). In addition, size had a significant influence on haemolymph pH over the emersion period (LM for size and time F = $31.12_{(31, 405)}$, P = 0.020), with larger mussels experiencing a greater average change of -0.7 pH units compared to -0.2 pH units recorded in the smaller mussels.

During recovery in treatment seawater (either pH 8.10 or 7.70 depending on previous exposure), time significantly influenced haemolymph pCO_2 levels, [HCO_3^-] and pH (figure 2.4; LM for pCO_2 F = $27.17_{(31,158)}$, P < 0.001; for [HCO_3^-] F = $29.25_{(31,158)}$, P < 0.001; for pH F = $16.35_{(31,158)}$, P < 0.001). Recovery of haemolymph pCO_2 to levels experienced before emersion primarily occurred within the first three hours of submersion in treatment seawater. The higher pCO_2 levels observed during emersion at 20 °C and 28 °C resulted in temperature during emersion having a significant influence on haemolymph pCO_2 recovery (LM for 20 °C F = $27.17_{(31,158)}$, P < 0.001; for 28 °C F = $27.17_{(31,158)}$, P < 0.001). In addition, smaller mussels exposed to 20 °C during emersion showed a reduction in haemolymph pCO_2 compared to larger mussels, together with time (LM for 20 °C and size F = $27.17_{(31,158)}$, P = 0.005; for 20 °C, size and time F = $27.17_{(31,158)}$, P = 0.024). A reduction in seawater pH during recovery also significantly increased haemolymph pCO_2 levels at 28 °C over the 6 hours (LM for 28 °C and time F = $27.17_{(31,158)}$, P < 0.001) as well as size of those exposed to 20 °C (LM for 20 °C and time F = $27.17_{(31,158)}$, P = 0.005).

Similar to haemolymph pCO_2 levels, the rate of recovery of [HCO_3^-] at 20 °C and 28 °C was significantly higher than during emersion at the lower two temperatures (LM for 20 °C F = $29.25_{(31,158)}$, P < 0.001; for 28 °C F = $29.25_{(31,158)}$, P = 0.022). This can primarily be attributed to the slightly higher increases in [HCO_3^-] at these

two temperatures during emersion together with quick recovery. A reduction in seawater pH and emersion at 20 °C and 28 °C resulted in significantly lower [HCO_3^-] concentrations (LM for 20 °C and seawater pH $F = 29.25_{(31,158)}$, $P = 0.025$; and for 28 °C and seawater pH $F = 29.25_{(31,158)}$, $P = 0.024$) in addition to time at 20 °C (LM for 20°C and time $F = 29.25_{(31,158)}$, $P = 0.004$). Size only influenced [HCO_3^-] at 28 °C over the 6 hours (LM for 28 °C and size $F = 29.25_{(31,158)}$, $P = 0.019$) with smaller mussels having a significantly lower [HCO_3^-].

Haemolymph pH was significantly lower in those exposed to 28 °C during emersion (LM for 28 °C $F = 16.35_{(31,158)}$, $P = 0.025$), in larger mussels (LM for size $F = 16.35_{(31,158)}$, $P = 0.025$), and those recovering in seawater at pH 7.70 (LM for seawater pH $F = 16.35_{(31,158)}$, $P = 0.043$) separately. A reduction in seawater pH during recovery and a larger size resulted in significantly lower haemolymph pH over time (LM for seawater pH and time $F = 16.35_{(31,158)}$, $P = 0.015$; for size and time $F = 16.35_{(31,158)}$, $P = 0.033$).

2.5 Discussion

By combining laboratory and field studies, our data provides new and much needed insight into the drivers of acid-base disturbances between alternating periods of emersion and immersion in the blue mussel *Mytilus edulis* from an intertidal habitat. Field studies from two sites in the South West of England demonstrate some of the variability in temperature, salinity and pH/ pCO_2 experienced by intertidal organisms, with no clear seasonal differences (tables 2.1 – 2.7, figures 2.1 and 2.2). Our sampling provides a snap shot of the variability experienced therefore it is likely with continuous sampling and during mid-winter and bloom periods that the conditions would differ. Our carbonate chemistry monitoring indicates mussels from the tidal estuary at Starcross experience fluctuations in seawater pCO_2, with values ranging from 142 µatm to 647 µatm over 12 hours. These fluctuations were markedly lower than those recorded in Port Gaverne, where pCO_2 values ranged from 238 ⌈atm to 1912 ⌈atm. These high and variable pCO_2 values have been suggested to be linked to southerly winds driving the upwelling of hypoxic and hypercapnic waters (Thomsen et al. 2013). Although this exposure to elevated pCO_2 was brief and infrequent, these measurements suggest mussels from Port Gaverne may already experience

seawater pCO_2 levels predicted for the end of the century in the open ocean under RCP 8.5 (Suzuki et al. 1995; Yates & Halley 2006; Price et al. 2012; Johnson et al. 2013).

As the frequency and intensity of pH/ pCO_2 fluctuations are expected to increase and intensify over the coming decades (IPCC 2014), it is essential to understand the influence of increasing pCO_2 on the acid-base balance of *M. edulis*. During both the field and laboratory studies, an increase in seawater pCO_2 directly resulted in an increase in haemolymph pCO_2. With no significant up-regulation of HCO_3^-, this increase led to a significant extracellular acidosis, supporting previous studies suggesting *M. edulis* are unable to regulate acid-base disturbances (Booth et al. 1984; Thomsen et al. 2010, 2013; Heinemann et al. 2012). The inability to compensate extracellular pH during exposure to elevated pCO_2, coupled with the subsequent reduction in extrapallial fluid pH (Thomsen et al. 2010; Heinemann et al. 2012), can potentially expose the inner aragonite layer of the shell with water undersaturated with $CaCO_3$, negatively impacting growth and calcification.

A greater reduction in haemolymph pH was observed in response to emersion both at low tide and within the laboratory, partially owing to uncompensated increases in haemolymph pCO_2. Although this has rarely been documented in bivalves, reductions in extracellular pH during periods of emersion have been observed in the acid-base regulating *Necora puber* by 0.3 - 0.6 pH units over 3 hours (Rastrick et al. 2014), and other crab species such as *Maja squinado* and *Cancer pagurus* (Taylor & Innes 1988; Whiteley 1999). The magnitude of the acidosis observed in this study differed in the field between the two populations, with larger mussels from Starcross accumulating more haemolymph pCO_2 over the emersion period. To differentiate between environmental factors experienced during the field observations and population differences, mussels from Starcross were transposed to Port Gaverne during the July sample. Again those from Starcross experienced a greater extracellular acidosis owing to a more pronounced increase in haemolymph pCO_2. The substantial difference in shell length (26 mm) between the two populations may suggest size is partially responsible for altering the physiological response triggered by emersion.

To differentiate between population and size differences, we investigated the acid-base response of small (37 - 50 mm) and large (60 - 79 mm) mussels from

the same population (Starcross) to an environmentally relevant emersion period (6 hours). In addition, we investigated the influence of air temperature and exposure to elevated seawater pCO_2 before emersion on acid-base balance. Over the emersion period, size and temperature appear to be the principal drivers of acid-base change in *M. edulis* (figure 2.3). Elevations in temperature had an increasing influence on acid-base balance, with size playing a crucial role in moderating these disturbances at increasingly higher temperatures. Acid-base disturbances during emersion were not significantly influenced by pre-exposure to elevated seawater pCO_2, however, reductions in seawater pH during re-immersion did significantly influence recovery (figure 2.4). In addition, size appeared to slightly but significantly influence recovery over the 6 hours, possibly owing to the greater acidosis experienced by larger mussels during the emersion period.

The combination of field and laboratory work used in this study suggests periods of emersion have the potential to pose a greater physiological stress on intertidal organisms. To date, studies have focused on physiological and behavioural changes in response to alternating periods of emersion and immersion (Marsden & Weatherhead 1998; Dowd & Somero 2012; Connor & Gracey 2012; Rastrick et al. 2014; Lockwood et al. 2015), however few have investigated acid-base responses. During immersion mussels use the tricarboxylic acid cycle and electron transport pathways to synthesise ATP under aerobic metabolism (Zandee et al. 1986). Upon emergence, mussel valves close restricting the uptake or availability of environmental oxygen limiting the ability to continue aerobic metabolism (Fan et al 1991). At the onset of hypoxia metabolism shifts from aerobic to anaerobic (Bayne et al. 1976; Brinkhoff et al. 1983; Babarro et al. 2007; Connor & Gracey 2011) as indicated by increases in the use of fermentative pathways of glucose and aspartate producing succinate and alanine (Isani et al. 1995). Anaerobic metabolism is often considered a determining factor in the resilience of bivalves to survive hypoxia during emersion (Hochachka 1986; Hochachka & Somero 2002) as three times more ATP can be produced per glucose molecule compared to the fermentation of lactate often seen in vertebrates (Hochachka & Somero 2002; Storey & Storey 2005). In addition, less H^+ ions are released per ATP molecule (Hochachka & Somero 2002), potentially helping to delay extracellular acidosis. The increase in H^+ ions resulting from metabolic acidosis may partially explain the reductions in extracellular pH seen

in all mussels emersed at 7 °C compared to the relatively small increases in haemolymph pCO_2.

Temperature was a primary driver determining acid-base balance throughout the emersion period. During anaerobic metabolism, increases in temperature can increase the demand for ATP by the induction of gene expression of heat stress related proteins (Gracey et al. 2008). In *Mytilus* mussels moderate heat stress has been linked to the induction of protein chaperones compared to extreme heat stress linked to the induction of genes targeting irreversible denatured proteins (Gracey et al. 2008). This can increase the demand for ATP, causing the cost of protein synthesis in relation to the energy budget to increase to approximately 25% for *M. edulis* (Hawkins & Bayne 1991). Although mussels are able minimise energy expenditure by reducing processes such as digestion, respiration and heart activities (Livingstone 1983; Zandee et al. 1986; Michaelidis & Storey 1991), if anaerobic metabolism is unable to produce enough ATP during emersion, increasing temperatures requiring increased production of heat stress proteins may result in cumulative stress by imposing a metabolic deficit. This may further exacerbate metabolic and therefore extracellular acidosis in mussels during emersion at higher temperatures as seen in this study.

As body temperature increases owing to solar irradiance, air temperature and convection heat exchanges (Helmuth 1998), metabolic acidosis can be intensified as a result of changes in the kinetics of aerobic metabolism. This can affect O_2 uptake and CO_2 excretion leading to a mismatch in O_2 supply and demand (Rahn 1966; Rastrick et al. 2014). In addition to metabolic proton production, extracellular acidosis can be influenced via increases in respiratory pCO_2. This diffuses into the extracellular fluid, hydrating and then dissociating to from H^+ ions and ultimately decreasing pH (Taylor & Innes 1988; Whiteley 1999; Rastrick et al. 2014). Respiration rate is heavily influenced by temperature as documented in several marine molluscs (Huang & Newell 2002; Martin et al. 2006) including *M. edulis* where respiration rate increased 5 fold with an increase in body temperature from 11°C to 18°C (Tagliarolo et al. 2012). The increases in haemolymph pCO_2 observed in larger mussels in this study may be attributed to increases in respiration rate at higher temperatures and therefore increasing respiratory pCO_2. This supports Rastrick et al. (2014), who observed both respiratory and metabolic acidosis over a 180 minute emersion period leading to

extracellular acidosis in the acid-base regulating velvet swimming crab *Necora puber*. The increases in respiratory pCO_2 leading to hypercapnia and respiratory acidosis can exacerbate metabolic acidosis which has the potential to affect aerobic scope and thermal tolerances, potentially altering species distribution (Pörtner & Farrell 2008; Calosi et al. 2013; Melzner et al. 2013).

During the 6 hour emersion period, size significantly moderated the extent of extracellular acidosis experienced. The influence of size on the acid-base response of intertidal species has yet to be determined. However, the ability of mussels to maintain aerobic metabolism before the onset of hypoxia may largely be determined by the quantity of water held within the mussel valves. This provides a suitable medium for respiratory exchanges, preventing the build-up of toxic end products which may accumulate to lethal levels (Gäde 1983; Brunetti et al. 1988; Marsden & Weatherhead 1998). Respiration rate has been shown to increase with body temperature up to 24°C in large *M. edulis* (43 – 66 mm), 6 °C higher than that of small mussels (24 – 26 mm) (Tagliarolo et al. 2012). The greater amount of water held within the mantle cavity in addition to a higher thermal threshold of respiration, may permit larger mussels to maintain aerobic respiration for longer, potentially increasing the likelihood of meeting the additional energy demand of thermal stress regulation. However, this comes at the cost of increased respiratory pCO_2 and consequentially greater extracellular acidosis. This is contrasting to the response of smaller mussels where the reduced amount of water held within the mantle cavity restricts aerobic metabolism.

Valve gaping has been observed in a number of intertidal mussels (Nicastro et al. 2010; Dowd & Somero 2012), and has been shown to allow the resumption of aerobic respiration in response to higher temperatures and hypoxia in the marine mussel *Geukensia demissa* (Fields et al. 2014). This acts to reduce body temperature via evaporative cooling as well as maintaining increased energy demands associated with increased heat stress (Lent 1968). However, a trade-off occurs as the majority of water held within the mussel cavity is lost, increasing the risk of desiccation. Without water in the mantle cavity, the gill tissue can lose physical support causing gill collapse (deFur & McMahon 1984; Fields et al. 2014). This can result in reductions in O_2 and CO_2 diffusion leading to additional respiratory acidosis as well as causing hypoxemia and metabolic acidosis

(Truchot 1975; Taylor & Innes 1988; Taylor & Whiteley 1989; Rastrick et al. 2014). Although in this study we did not monitor valve gaping, it is unlikely to have occurred in smaller mussels owing to the relatively small changes in haemolymph pCO_2 at higher temperatures and the sensitivity to an increased risk of desiccation and gill collapse. Instead smaller mussels may have gone into respiratory arrest, limiting the accumulation of respiratory pCO_2 and metabolic protons, resulting in minimal changes in acid-base balance. However, for larger mussels the increases in haemolymph pCO_2 and reductions in pH may be explained by both respiratory and metabolic acidosis increasing at higher temperatures, in addition to an increased metabolic demand at higher temperatures, with valve gaping facilitating the maintenance of aerobic respiration and therefore higher ATP production.

This study shows once mussels are re-immersed, seawater pCO_2/ pH is the primary driver of acid-base change, in agreement with earlier results. Upon re-immersion aerobic metabolism resumes with succinate and other anaerobic end products metabolised by the TCA cycle (Lockwood et al. 2015). In particular arginine kinase, generates phosphagen phosphoarginine which is able to rephosphorylate ADP during hypoxia without the generation of H^+, playing a central role in the recovery from anaerobsis (Grieshaber et al. 1994; Ellington 2001). In addition, the environmental conditions encountered during emersion can lead to a significant oxygen deficit in *M. edulis*, which has been showed to take up to 2 hours to be fully compensated via increases in oxygen consumption (Widdows & Shick 1985). In this study, *M. edulis* were able to recover quickly during re-immersion, with acid-base parameters recovering to pre-emersion levels within 3 hours.

Although there are an increasing number of studies investigating the responses of *Mytilus spp.* in the intertidal zone, few have accounted for the acid-base disturbances encountered during alternating periods of immersion and emersion, thus limiting our ability to understand the physiological responses of key marine biota to both current and future environmental changes. With seawater pH fluctuations expected to increase and intensify along with increases in air temperature, it is important to understand how organisms respond to current environmental conditions in order to predict responses to anthropogenic change. This study suggests size (shell length) and temperature are the central drivers of

acid-base balance during emersion at low tide irrespective of prior immersion in low seawater pH conditions. However, during re-immersion pH/ pCO_2 exposure drives acid-base response independent of previous conditions experienced during emersion. As a result of the additional stress posed by increasing air temperatures, smaller individuals may potentially be able to alleviate additional energetic demands by going into respiratory arrest during emersion, limiting the degree of extracellular acidosis and haemolymph pCO_2 accumulation. Although identifying the mechanisms behind these drivers was beyond the scope of this study, we highlight the importance of combining field and laboratory studies to assess the acid-base response of an ecologically and economically important intertidal species.

Table 2.1: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle in Port Gaverne, April 2015.

Time of day	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
08:00	12.8	8.14	32.4	2064.2	2300.8	309.2	1.881	170.7
08:30	11.5	8.16	32.6	2069.0	2307.1	293.0	1.886	171.1
09:00	11.1	8.10	32.6	2035.5	2237.2	333.7	1.875	145.9
09:30	11.3	8.21	30.7	2048.8	2296.6	259.3	1.859	178.9
10:00	12.1	8.14	28.6	2043.5	2245.9	313.6	1.880	150.5
10:30	15.4	8.14	32.4	2057.3	2316.1	311.1	1.859	186.7
11:00	17.3	8.13	28.5	2054.3	2293.2	330.2	1.865	177.9
11:30	19.6	8.14	26.5	2042.6	2289.1	328.9	1.845	186.1
17:00	21.6	8.23	25.3	2033.1	2339.5	265.7	1.793	231.6
17:30	18.3	8.21	29.4	2028.5	2328.9	265.4	1.800	219.7
18:00	17.8	8.24	28.7	2033.1	2341.6	247.7	1.798	226.5
18:30	16.0	8.16	30.5	2010.4	2267.0	293.6	1.814	186.0
19:00	16.2	8.11	31.1	1994.2	2228.2	328.7	1.813	169.5
19:30	15.3	8.23	30.3	1981.1	2268.0	242.5	1.766	206.1
20:00	17.2	8.22	26.9	1969.1	2236.5	256.3	1.762	197.3

Table 2.2: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle in Port Gaverne, July 2015.

Time of day	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
09:00	18.5	7.63	22.8	2086.3	2117.7	1176.1	1.991	53.5
14:30	26.2	8.10	20.8	2087.9	2320.8	406.0	1.889	187.0
15:00	23.5	8.22	20.7	2095.9	2373.3	299.4	1.866	220.2
15:30	22.3	8.17	21.2	2033.7	2271.5	326.0	1.836	187.6
16:00	23.9	8.14	22.4	2005.5	2248.7	343.0	1.805	189.4
16:30	23.0	8.12	22.8	2001.7	2230.2	357.1	1.813	177.8
17:00	22.3	8.22	23.8	2021.8	2312.2	276.4	1.791	222.1
17:30	21.8	8.19	24.2	1988.9	2257.3	291.6	1.775	204.1
18:00	21.6	8.15	24.8	1966.5	2214.0	316.4	1.769	187.3
18:30	20.3	8.08	24.7	1974.1	2175.5	376.8	1.807	154.7
19:00	20.2	8.16	24.8	1977.8	2220.1	309.0	1.784	183.5
19:30	19.4	8.01	24.8	2003.5	2170.1	451.9	1.857	131.2
20:00	18.4	8.08	24.1	2074.3	2264.6	395.5	1.910	149.7
20:30	17.9	7.42	23.9	2127.6	2105.2	1912.2	2.024	33.9
21:00	17.4	7.80	25.4	2057.9	2143.0	755.7	1.950	80.2

Table 2.3: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle in Port Gaverne, September 2015.

Time of day	Temperature (°C)	pH NBS	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
08:30	14.7	8.15	22.1	2111.4	2295.2	341.2	1.951	146.9
09:00	14.9	8.15	23.8	2130.9	2330.1	338.1	1.961	156.6
09:30	15.2	8.21	26.1	2135.5	2387.3	285.4	1.933	191.5
10:00	15.6	8.17	29.7	2124.4	2386.5	304.1	1.919	194.2
10:30	15.9	8.17	29.8	2145.7	2413.2	307.2	1.936	198.7
11:00	15.7	8.10	31.0	2060.9	2290.0	347.9	1.880	168.1
11:30	15.7	8.10	31.2	2056.6	2286.8	346.6	1.875	168.6
12:00	16.1	8.16	29.8	2062.1	2318.4	303.0	1.863	188.3
12:30	15.5	8.15	28.6	2055.9	2291.4	312.1	1.869	174.8
13:00	16.0	7.97	29.8	2055.0	2216.4	481.5	1.913	124.4
13:30	15.4	7.64	27.8	2074.6	2114.0	1073.4	1.978	55.7
19:00	15.4	8.28	24.1	2072.2	2342.0	237.6	1.857	205.7
19:30	15.1	8.24	26.7	2072.0	2339.7	255.2	1.862	200.2
20:00	15.1	8.20	27.5	2058.9	2309.6	278.2	1.861	186.7
20:30	15.1	8.11	27.6	2080.8	2287.0	351.2	1.911	156.2

Table 2.4: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle Starcross, November 2014.

Time of day	Temperature (°C)	pH NBS	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
07:00	11.6	8.19	26.8	2092.5	2305.8	287.7	1.919	160.9
07:30	11.4	8.16	25.4	2084.5	2271.4	312.4	1.927	143.8
08:00	11.6	8.00	23.3	1753.5	1854.2	395.3	1.655	81.0
13:00	12.5	7.94	24.6	1809.6	1904.5	467.1	1.711	78.5
13:30	12.6	7.90	23.7	1764.9	1843.8	505.5	1.675	68.5
14:00	11.9	7.92	23.1	1952.8	2036.8	534.4	1.853	75.9
14:30	11.9	8.25	23.3	1953.6	2161.1	240.4	1.786	157.3
15:00	12.2	8.24	22.8	1950.2	2151.2	247.7	1.786	153.3
15:30	12.3	8.25	29.6	2009.1	2276.2	232.9	1807	192.8
16:00	12.1	8.28	29.4	1991.4	2270.8	214.2	1.781	201.0
16:30	11.5	8.28	28.9	1809.0	2061.5	195.0	1.624	176.6
17:00	11.5	8.29	26.3	1917.0	2163.0	206.5	1.729	179.2
17:30	10.9	8.25	28.2	1984.2	2225.1	231.5	1.799	175.0
18:00	10.7	8.20	28.0	2051.4	2268.5	271.2	1.879	160.7
18:30	10.8	8.15	28.0	1957.2	2144.9	292.7	1.806	138.2
19:00	11.5	8.15	27.6	1838.1	2020.5	276.8	1.694	131.9

Table 2.5: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle in Starcross, April 2015.

Time of day	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
08:45	16.1	8.11	28.7	2215.5	2447.5	371.9	2.024	177.2
09:15	17.6	8.11	29.3	2172.1	2418.3	364.8	1.973	185.8
09:45	18.7	8.12	29.3	2225.9	2492.3	366.1	2.011	202.0
10:15	19.9	8.09	29.4	2235.9	2497.0	397.5	2.024	199.0
10:45	19.2	8.17	31.3	2070.0	2375.4	295.8	1.838	222.3
11:15	18.7	8.08	27.6	2077.1	2295.9	382.9	1.898	166.1
11:45	20.0	8.15	26.5	1875.6	2116.2	294.9	1.689	176.9
12:15	19.6	8.20	27.0	1972.2	2251.0	271.7	1.757	206.2
12:45	19.9	8.23	27.0	1979.4	2280.2	252.8	1.748	222.4
13:15	19.2	8.20	23.6	1977.6	2222.6	282.4	1.781	186.8
13:45	22.0	8.34	29.8	1957.7	2389.9	183.0	1.639	313.1
14:15	18.9	8.17	33.0	2093.6	2413.7	295.3	1.853	231.2
14:45	19.1	8.08	32.9	2075.5	2338.1	367.8	1.872	190.9

Table 2.6: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle in Starcross, July 2015.

Time of day	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
07:30	6.0	8.07	27.6	1997.8	2122.8	354.6	1.882	97.6
08:00	8.7	8.09	27.3	2032.7	2181.2	350.1	1.902	114.3
08:30	9.5	8.10	31.3	1971.2	2149.2	323.2	1.827	129.1
09:00	11.9	8.14	32.2	2087.6	2316.9	311.9	1.908	166.3
09:30	12.9	8.20	32.9	2107.8	2387.3	271.1	1.896	200.8
10:00	14.2	8.20	32.9	2065.2	2353.4	266.7	1.849	206.0
10:30	14.2	8.19	33.0	2067.5	2350.5	273.6	1.855	202.4
11:00	15.6	8.16	31.0	1992.4	2247.9	289.5	1.798	183.9
11:30	15.8	8.15	29.8	1937.8	2175.5	291.6	1.755	171.4
12:00	18.5	8.26	27.8	1900.7	2205.8	222.3	1.672	221.0
12:30	17.3	8.36	29.8	1876.8	2255.1	165.8	1.602	268.8
13:00	16.2	8.16	30.0	1953.0	2202.3	286.6	1.763	179.8
13:30	16.4	8.09	29.6	2086.3	2307.5	365.7	1.908	165.2

Table 2.7: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle in Starcross, September 2015.

Time of day	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
07:30	13.7	7.86	24.9	2065.3	2150.2	647.1	1.960	79.1
08:00	14.1	7.89	26.6	2019.7	2122.4	582.1	1.908	88.0
08:30	18.2	8.11	31.8	2028.2	2287.6	334.9	1.829	187.9
09:00	16.7	8.10	31.4	2015.8	2252.6	340.5	1.832	172.0
09:30	17.8	8.11	32.6	2004.3	2264.3	328.8	1.806	186.5
10:00	18.0	8.09	31.9	2014.4	2260.3	349.2	1.824	178.1
10:30	17.5	8.08	31.4	2017.7	2250.5	359.2	1.835	169.7
11:00	18.5	8.11	31.9	2079.4	2346.7	343.5	1.873	195.1
11:30	19.3	8.08	31.9	2157.0	2420.5	385.0	1.949	195.3
12:00	19.6	8.10	31.2	2143.8	2415.2	366.1	1.930	201.2
12:30	19.8	8.20	32.1	2091.8	2433.8	275.6	1.835	248.1
13:00	19.2	8.35	32.9	1609.5	1996.2	142.3	1.348	256.8
13:30	20.1	8.12	32.5	1724.9	1983.9	277.9	1.539	176.8

Table 2.8: Seawater carbonate chemistry from immersion prior to aerial exposure ([1]) and recovery ([2]) in treatment seawater. Mean ± SD.

pH Treatment	T (°C)	pH $_{NBS}$	S	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
8.10[1]	13.2± 0.0	8.10±0 .0	30.6± 0.0	2076.6± 75.8	2287± 81	342± 13	1.907± 0.070	156± 6
7.70[1]	13.2± 0.0	7.70±0 .0	30.6± 0.0	2145± 141.6	2204± 145	927± 52	2.042± 0.135	66± 5
8.10[2]	13.2± 0.0	8.10±0 .0	30.6± 0.0	2084.8± 116.2	2298± 124	341± 19	1.914± 0.107	157± 9
7.70[2]	13.2± 0.0	7.70±0 .0	30.6± 0.0	2144.2± 143.5	2201± 145	942± 65	2.041± 0.137	65± 4

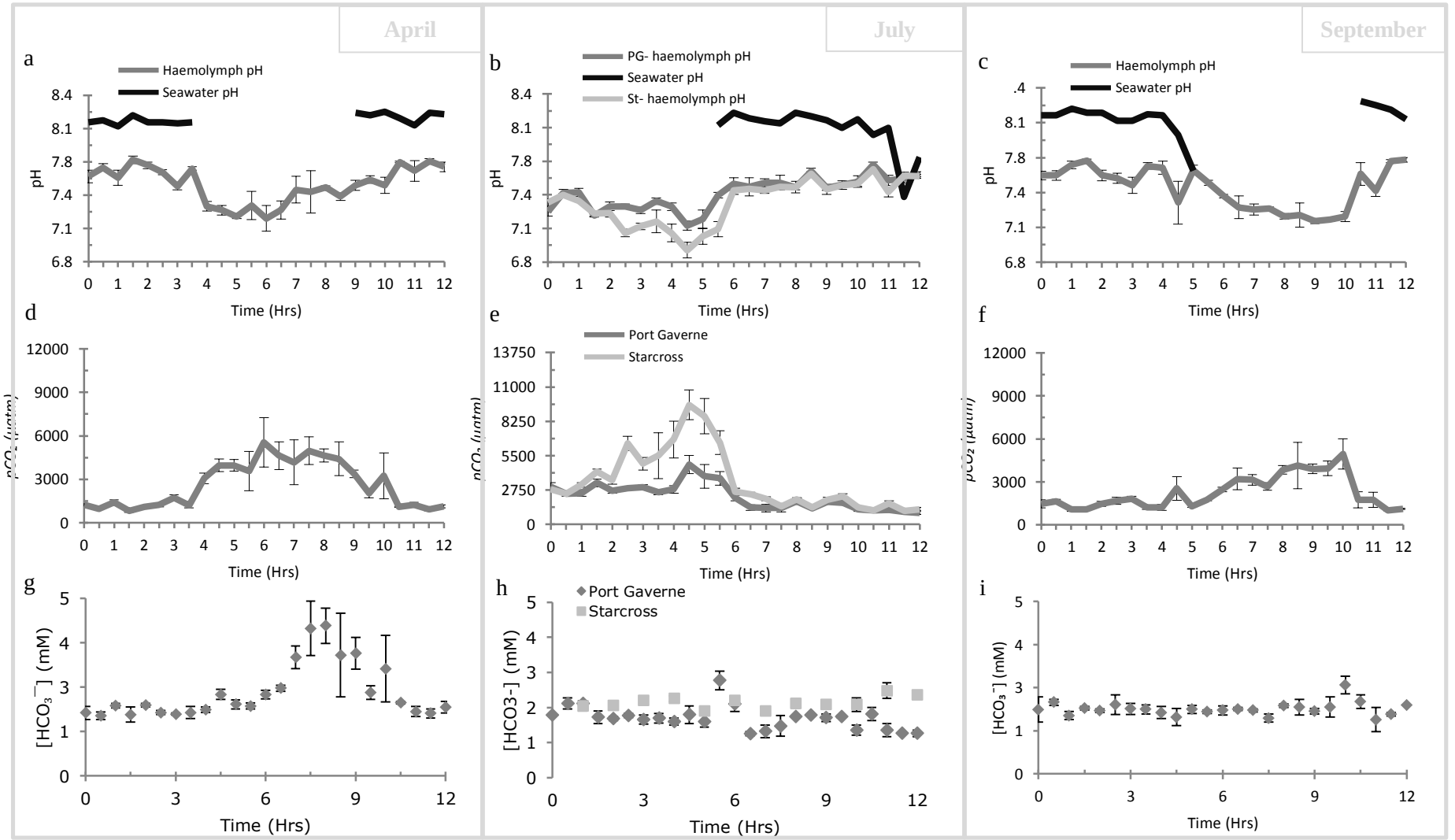

Figure 2.1: Seawater pH and acid-base parameters within the haemolymph of *Mytilus edulis* (n = 3) over a 12 hour field sample in Port Gaverne in April (a, d, g), July (b, e, h) and September (c, f, i) 2015.

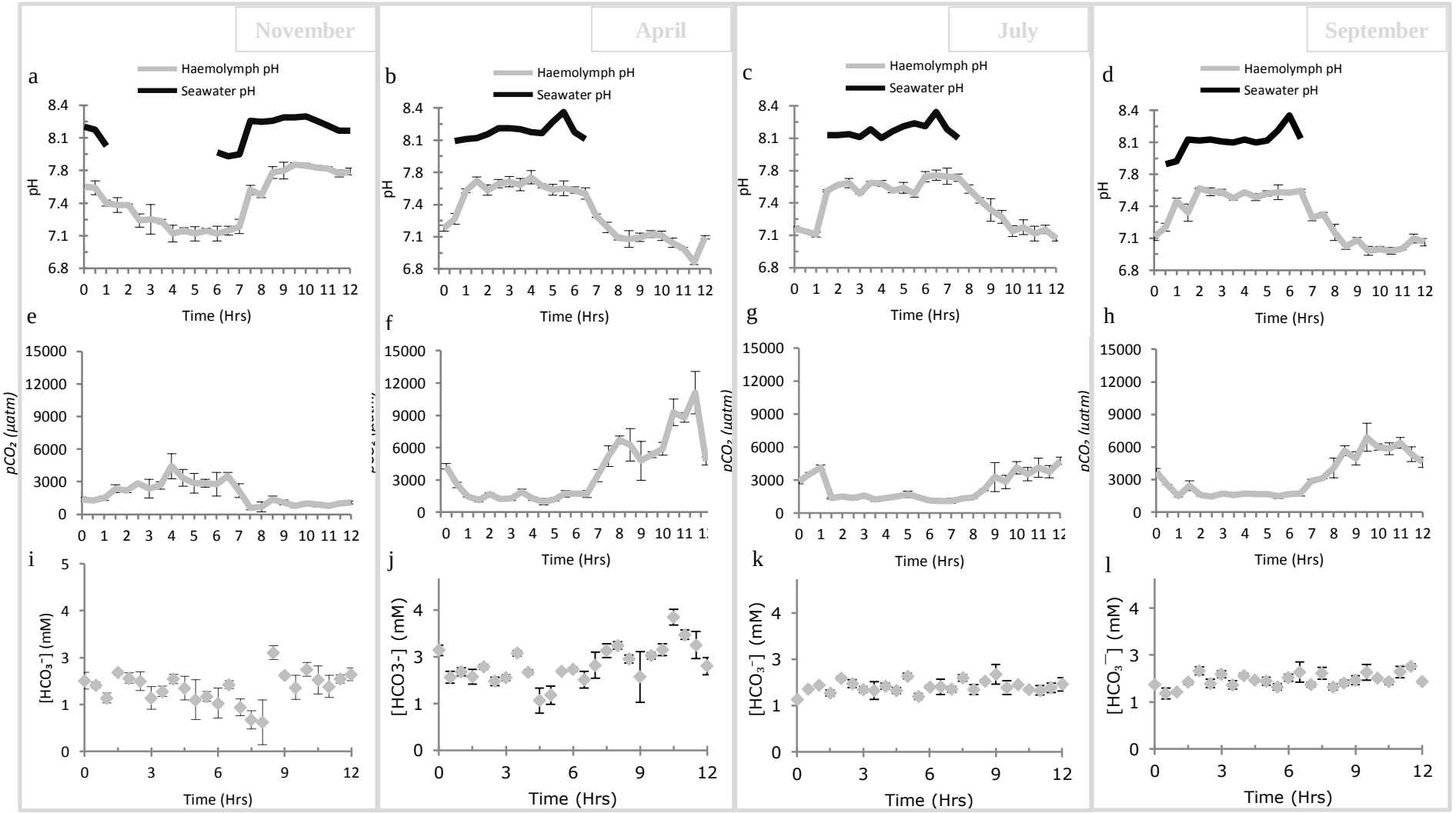

Figure 2.2: Seawater pH and acid-base parameters within the haemolymph of *Mytilus edulis* (n = 3) over a 12 hour field sample in Starcross in November 2014 (a, e, i), April (b, f, j), July (c, g, k) and September 2015 (d, h, l).

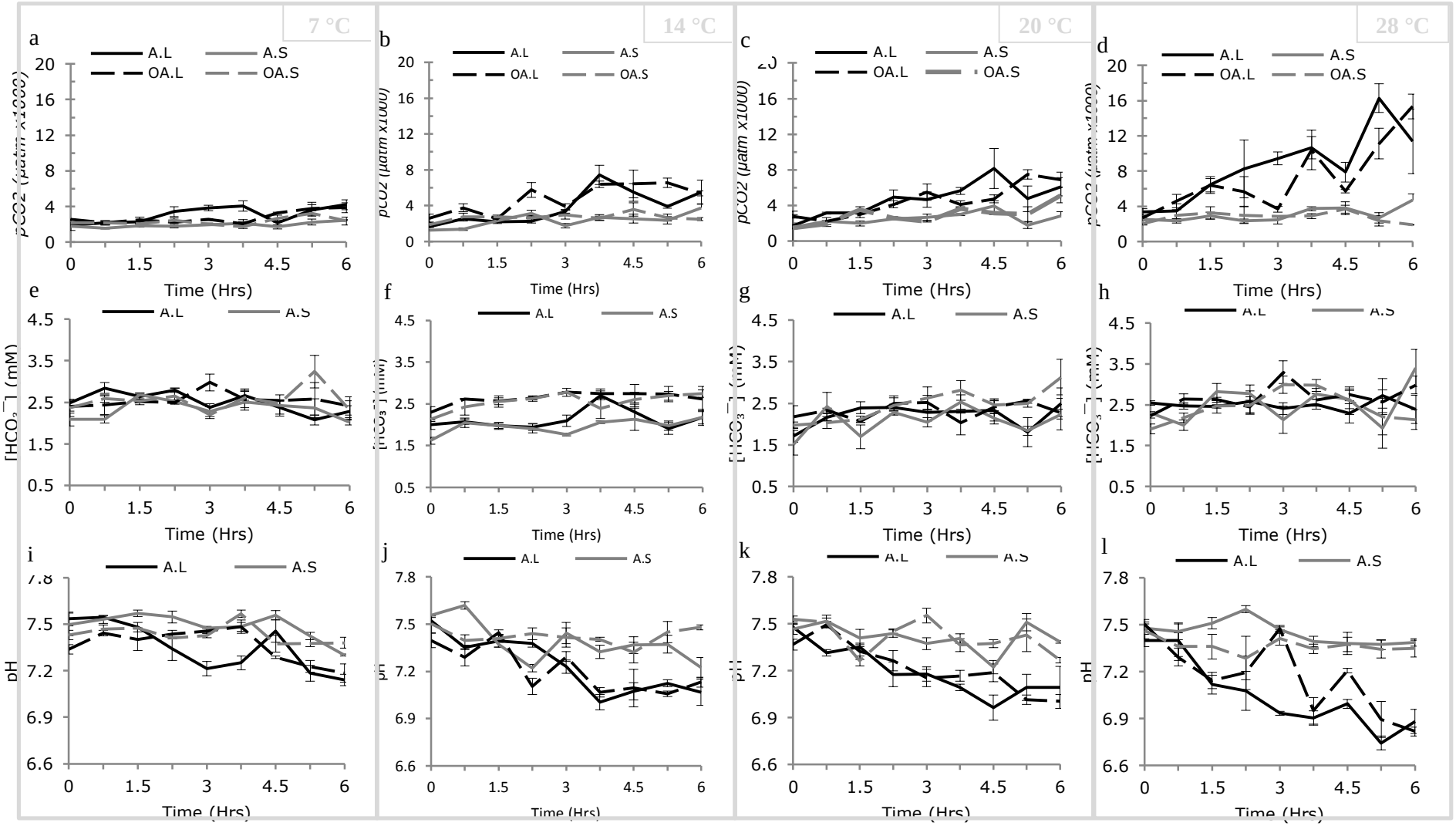

Figure 2.3: Acid-base parameters over 6 hours of emersion in *Mytilus edulis* (n = 3) at 4 temperatures; 7 °C (a, e, i), 14 °C (b, f, j), 20 °C (c, g, k), 28 °C (d, h, l). Ambient mussels were exposed to pH 8.10 and OA mussels to pH 7.70 for 1 week prior to emersion. A.L- ambient large, A.S- ambient small, OA.L- OA large, OA.S- OA small.

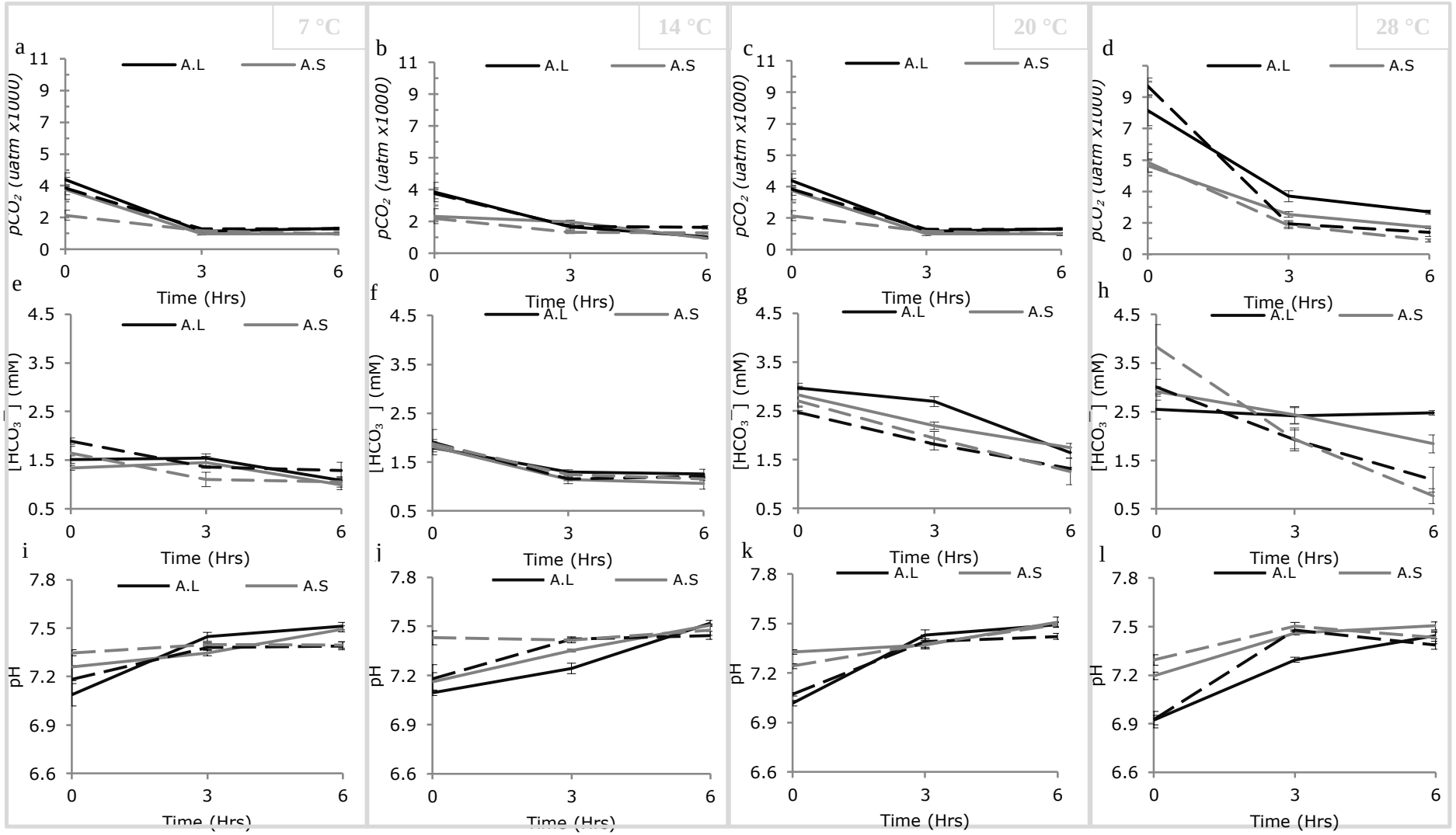

Figure 2.4: Acid-base parameters during recovery in *Mytilus edulis* (n = 4) in treatment seawater (either pH 8.10 or pH 7.70) after 6 hours of emersion at 7 °C (a, e, i), 14 °C (b, f, j), 20 °C (c, g, k), 28 °C (d, h, l). All seawater before and after emersion was 14°C.

Chapter 3: Population differences in the acid-base response of intertidal mussels during short-term changes in pCO_2

3.1 Abstract

The impacts of ocean acidification (OA) on populations and therefore community structure are likely to be substantially influenced by the adaptations and acclimatisation of local populations. By combining field and laboratory work, this study investigates if the regular exposure to elevated pCO_2 enhances the acid-base regulatory ability of the Chilean mussel *Perumytilus purpuratus*. We compare the acid-base response of three populations of *P. purpuratus* that naturally experience different seawater carbonate chemistry conditions with two populations of *Mytilus edulis*. First, the acid-base response and carbonate chemistry parameters experienced by a natural population of *P. purpuratus* during a 12-hour tidal cycle were determined. We then experimentally investigate the acid-base response of *P. purpuratus* and *M. edulis* to short-term changes (6 hour) in seawater pH/ pCO_2. Our results show haemolymph pCO_2 followed that of the seawater, increasing from ~400 µatm to a maximum of 5823 µatm over the 6 hours. With significant but no effective up-regulation of bicarbonate concentration, all populations experienced extracellular acidosis during short-term changes in seawater pCO_2. This acidosis was reversed following reductions in seawater pCO_2 providing further evidence that both species are relatively weak acid-base regulators. Moreover, our data reveals *P. purpuratus* living within upwelling regions do not appear to possess any adaptive mechanisms to enhance acid-base regulatory abilities under elevated seawater pCO_2 conditions. Interestingly, size appeared to be an important driver of acid-base change, with larger mussels experiencing a greater extracellular acidosis. Finally, our data indicates periods of emersion at low tide may cause a greater extracellular acidosis than exposure to elevated seawater pCO_2.

Keywords: Ocean acidification, upwelling, acid-base balance, adaptation, variability

3.2 Introduction

The increasing absorption of atmospheric carbon dioxide (CO_2) by the world's ocean is altering ocean carbonate chemistry, which has ultimately caused a reduction in open ocean pH by 0.1 pH units since the industrial revolution (Orr et al. 2005). This phenomenon is widely referred to as ocean acidification (OA). Under the IPCC business as usual scenario (RCP 8.5), atmospheric CO_2 levels are predicted to increase up to 1000 µatm by the end of the century (Meinshausen et al. 2011; Bopp et al. 2013). If attained, this would result in a reduction in average open ocean pH by up to 0.43 units, reaching pH 7.73 by the end of the century (Moss et al. 2010; Bao et al. 2012; Bopp et al. 2013; IPCC 2013). This substantial rate of change has not been experienced for at least 400,000 years (Doney & Schimel 2007) and has therefore being identified as one of the greatest threats to global marine biodiversity (Halpern et al. 2008; Wittmann & Pörtner 2013).

Recent research reveals the acute exposure to elevated pCO_2/ reduced pH may significantly impair physiological and behavioural functions, e.g. acid-base physiology, metabolism, calcification and energetic partitioning, in a wide range of marine species (Fabry et al. 2008; Widdicombe & Spicer 2008; Doney et al. 2009; Melzner et al. 2009; Munday et al. 2009; Kroeker et al. 2010). Owing to the complex nature of marine ecosystems, the majority of these studies investigate the effects of OA as a single stressor, using one or two fixed (i.e. stable) elevated pCO_2 levels in order to gain insight into the physiological responses of individuals. However, marine organisms will not experience OA in isolation but against a background of other environmental conditions. In addition, the effects of OA on populations and therefore community structure are likely to be substantially influenced by the adaptations and acclimatisation of local populations (Kuo & Sanford 2009). Therefore, the comparison of the physiological responses of species living in areas naturally enriched with CO_2, such as CO_2 seeps or upwelling areas can help to inform future predictions of the vulnerabilities of coastal organisms.

Although limited, the studies investigating organisms inhabiting upwelling areas raise the potential paradigm that organisms regularly exposed to variable or high pCO_2 may have a greater ability to cope with the changes in pCO_2 predicted to occur as OA progresses. For example, in purple sea urchins (*Strongylocentrotus*

purpuratus) inhabiting the California Current System, genes related to ion transportation, biomineralisation and lipid metabolism were all found to be highly correlated to the time spent living at pH <7.8 (Pespeni et al. 2013c). These larvae were also observed to have greater genetic variation for body size under elevated pCO_2 which was consistent with local adaptation to the carbonate conditions experienced in the field (Kelly et al. 2013). In addition, Vargas et al. (2015) demonstrated that egg capsules from the gastropod *Concholepas concholepas* collected from a site frequently exposed to upwelling events were less impacted by elevated pCO_2 than those from individuals inhabiting a less variable region. Moreover gastropods from upwelling sites had higher plasticity of their metabolic rate compared to the population not experiencing upwelling events (Lardies et al. 2014). To date, the limited field data on geographic variability of seawater pCO_2 levels restricts the ability to defer adaptive responses. However, these studies provide evidence to suggest that organisms living in areas regularly exposed to elevated pCO_2 can possess local adaptations.

Along the Chilean and Peruvian coast, the Eastern Boundary Upwelling System (EBUS) situated within the Humboldt Current System generates sporadic upwelling events (Aravena et al. 2014). In central Chile (35° - 37°) this produces one of the most biologically productive regions in the world, accounting for up to 50% of Chile's annual fishery landings (Arcos et al. 2001; FAO 2011). The EBUS is driven by a combination of equatorward surface winds and a shallow poleward undercurrent along the continental shelf (Sobarzo et al. 2001; Sobarzo et al. 2007). These upwelling favourable winds drive cold, low oxygenated and nutrient rich waters to the surface, predominately during austral spring and summer (Shaffer et al. 1997; 1999; Sobarzo et al. 2007; Torres et al. 2011). This process is not uniform with the frequency and intensity of upwelling events largely dependent on seasonal patterns of climate and the intensification of south-southwesterly winds, in addition to increases in sea level pressure and sea surface temperature predominately originating from warm El Niño Southern Oscillation (ENSO) events (Thiel et al. 2007; Escribano & Morales 2012). ENSO events remain largely unpredictable and can last for several years which in addition to complex coastal oceanography and changing seasonal climate patterns can lead to high temporal variability in upwelling events (Thiel et al. 2007; Sobarzo et al. 2007). Field estimates suggest the upwelling of CO_2 enriched waters can result in surface seawater pCO_2 levels reaching up to 1000 µatm,

equivalent to pH between 7.6 and 7.7 (Feely et al. 2008; Torres et al. 2011), thus exposing coastal organisms to conditions similar to those expected for OA in the open oceans by the end of the century (IPCC 2013).

Tolerance to elevated pCO_2 is often linked to acid-base regulatory capacities, as changes in extracellular pH has the potential to influence protein stability and enzyme function (Somero 1986), playing an important role in the ability to cope with elevated pCO_2 (Pörtner et al. 1998; Widdicombe & Spicer 2008; Whiteley 2011). An organism's ability to compensate acid-base disturbances and maintain cellular homeostasis to short-term exposures is often characterised by the capacity of the bicarbonate buffering system to up-regulate bicarbonate ions (HCO_3^-) (Melzner et al. 2009). This is seen in a number of invertebrate species whereby the Na^+/K^+/-ATPase pump actively transfers HCO_3^- from the surrounding seawater across the gill epithelia using active ion exchange (Lucu & Towle 2003; Santos et al. 2007).

The capacity to adjust acid-base balance is not solely based on regulatory systems but can also be influenced by environmental conditions. The high cost of ion transport can cause organisms to rely on a rich food supply to compensate for elevated energetic demands (Thomsen et al. 2013). For organisms with a limited food supply and therefore restricted available energy, this can be compensated via faster passive transport for extracellular pH regulation owing to the environmental conditions, as shown in Antarctic euechinoids (Collard et al. 2015). Owing to the cool environmental conditions, the authors propose these echinoids will be not be susceptible to OA in the future as seen in euechinoids studied in the tropics. Similarly, Briones et al. (2014) suggested the Chilean mussel *Perumytilus purpuratus* may have evolved under colder, high pCO_2 seawater. Therefore it may be possible that the colder seawater conditions increase the potential for passive ion transport assisting extracellular pH regulation. Although the potential for environmental conditions to influence acid-base regulatory capacity has been suggested, it is unknown how living in areas regularly exposed to elevated pCO_2 levels may alter the efficiency of acid-base regulation.

Bivalve molluscs are important components of coastal ecosystems providing important ecosystem services such as habitat structure, shelter from predation, water purification and a food source for several species (Asmus & Asmus 1991;

Gutierrez et al. 2003; Prado & Castilla 2006; Gazeau et al. 2013). In addition they have a high economic value, with world mussel production reaching over 15 million tons in 2013, worth an estimate $USD 21 million (FAO 2015). To our knowledge, no studies have investigated the influence of elevated pCO_2 on the acid-base regulatory abilities in the important Chilean mussel *Perumytilus purpuratus*, however the blue mussel *Mytilus edulis* is suggested to be unable to regulate acid-base balance owing to small or no compensatory increases in HCO_3^- when exposed to elevated seawater pCO_2 (Booth et al. 1984; Thomsen et al. 2010; 2013; Heinemann et al. 2012). However, these studies, like much experimental OA work, use stable pH/ pCO_2 values which are uncharacteristic of coastal habitats where abiotic factors fluctuate in time and space. Field studies are beginning to highlight the natural fluctuations of pH, temperature, salinity and oxygen occurring on daily, through to annual timescales in coastal systems (Shim et al. 2007; Wootton et al. 2008; Urbina et al. 2010; Leiva et al. 2015). Considering this, it is still unknown how these mussels respond to fluctuations in pH/ pCO_2 and if organisms living within upwelling areas processes adaptive mechanisms to cope with the elevated pCO_2. This may be essential as environmental variability increases in both magnitude and intensity under global climate change (IPCC 2013), potentially moving coastal organisms closer to their physiological limits and away from the range in which they are adapted (Menge & Sutherland 1987). For coastal organisms unable to compensated extracellular acidosis, this can lead to metabolic suppression, reductions in intracellular pH and a reduction in protein synthesis (Barnhart 1989; Reid et al. 1997; Michaelidis et al. 2005). Over the longer term this can result in reductions of growth and survival leading to the suggestion these species may be more vulnerable to future climatic changes. Therefore assessing the adaptation potential of organisms living in areas naturally enriched with pCO_2 may help to inform predictions of coastal organisms to future OA conditions.

The elongated nature of the Chilean coast makes for an ideal study location to investigate if organisms regularly experiencing elevated seawater pCO_2 have an altered acid-base response, as carbonate system parameters vary on regional and biogeographical scales (Torres et al. 2011; Mayol et al. 2012). This study focuses on three different geographic locations, Antofagasta Bay, Las Cruces and Dichato: two experiencing upwelling events and one without upwelling. The mussels collected within Antofagasta Bay in Northern Chile are situated within an

upwelling shadow (Castilla et al. 2002), therefore although the Antofagasta region typically experiences strong across-shore pH/ pCO_2 gradients, organisms situated within the bay are not affected by high pCO_2 waters (Lagos et al. 2008). In comparison the Las Cruces region is affected by both upwelling events and fresh water discharges from the Maipo river (Vargas et al. 2015). These non-seasonal riverine waters are often high in pCO_2 and DIC compared to oceanic waters (Salisbury et al. 2008; Duarte et al. 2013) which together with upwelling events can expose organisms to pCO_2 values of 712 µatm to 1067 µatm (Lagos et al. 2013; Ramajo et al. 2013). In Dichato, situated within Coliumo Bay, mussel populations also experience seasonal upwelling events (Ahumada & Chuecas 1979; Arcos & Wilson 1984) with pCO_2 values regularly exceeding 400 µatm (Torres et al. 2011). However the frequency and range of these variations is still relatively unknown. This combination of study sites allows the investigation of the inter- and intra- species responses from mussels regularly exposed to natural variability with populations experiencing seasonal upwelling events.

Our ability to predict the responses of key marine biota to future ocean conditions is also impaired by our limited understanding of an organism's physiological responses to both natural and anthropogenic environmental variability. Here we combine field and laboratory studies to investigate if regular exposure to elevated pCO_2 levels can enhance the acid-base regulatory ability of the Chilean mussel *Perumytilus purpuratus* and if there are any inter- and intra- species differences with regards to the acid-base responses of two *Mytilus edulis* populations. We hypothesis that organisms living in habitats with variable pCO_2 will have a greater resilience to OA than those living in habitats with a more stable pCO_2. Two of the Chilean populations experience seasonal upwelling events, one with the addition of riverine discharges, with one other population and both *M. edulis* populations without upwelling. First we determine the present variability experienced by one population of *P. purpuratus* and the acid-base response throughout a 12-hour tidal cycle. We then use laboratory studies to investigate the influence of pCO_2 variability experienced by natural populations, both with and without the presence of regular upwelling events, in determining acid-base responses to short-term changes in seawater pH/ pCO_2.

3.3 Materials and Methods

Field monitoring

Field site and determination of carbonate system parameters

Field sampling took place on three occasions between September and October 2015 in Dichato, Central Chile (36°32'S, 72°56'W), an area characterised by frequent upwelling events (Torres et al. 2011; Aravena et al. 2014). Dichato contains a large intertidal population of *Perumytilus purpuratus* forming beds on rocky zones experiencing semidiurnal tidal cycles.

Over a 12-hour period, encompassing one high and one low tide, seawater carbonate chemistry and mussel acid-base measurements were taken every 30 minutes from three different individual mussels for each time point. To enable samples to be collected at high tide whilst animals were submerged and inaccessible, mussels were collected and placed in a mesh bag on a pulley system beside the mussel bed and tied to an adjacent pier just prior to being submerged by the incoming tide. For each seawater sample at high tide (1 per time point), seawater was collected from above the mussel bed on the ocean surface and assessed for pH and temperature using a Metrohm 826 mobile and salinity using a Mettler Toledo SG7 SevenGo pro conductivity meter. Temperature was additionally monitored at one minute intervals using HOBO Pendant® Temperature/Light Data Loggers. Seawater samples were preserved on site with saturated $HgCl_2$ (Dickson et al. 2007) to allow for dissolved inorganic carbon (DIC) analysis. Methodology was followed from Lewis et al. (2013) using a custom built system (Friederich et al. 2002) and Logger Pro version 3.2 software. CO2sys (Pierrot et al. 2006) was then used to calculate total alkalinity and pCO_2 of each seawater sample.

Acid-base measurements

For the acid-base measurements, every 30 minutes haemolymph from the posterior abductor muscle was extracted (n = 3) using a 21 g needle and a 2 ml syringe and immediately measured for pH. Haemolymph samples were subsequently analysed for total CO_2 using a Corning 965 CO_2 analyser (Corning Ltd., UK) calibrated with a 10 mM $NaHCO_3$ solution. For the last two field samples, a problem with the total CO_2 analyser meant haemolymph was

transferred to 100 µl glass micro-hematocrit capillary tubes sealed with paraffin oil and hemato-sealTM capillary tube sealant (Fisher). Samples were then frozen and transported to Exeter, UK for analysis of total CO_2. This method was used to prevent the ~1-2 % of gaseous CO_2 diffusing out of the haemolymph sample. Although preliminary results suggested samples were unaffected (unpublished data), if diffusion did occur it is unlikely the small percentage would have significantly altered our results. To calculate HCO_3^- and pCO_2 within the haemolymph a modified version of the Henderson-Hasselbalch equation derived from Truchot (1976) was used.

Laboratory experiments- Short term responses to changes in seawater pH

Collection of animals

We then followed the acid-base response of *P. purpuratus* and *M. edulis* to investigate if the regular exposure to elevated pCO_2 levels can alter a mussel's acid-base response to short-term changes in seawater pCO_2. Adult *P. purpuratus* (20 - 35 mm shell length) were collected from three sites (Antofagasta, Las Cruces, Dichato, figure 3.1) along the Chilean coast and transported to Dichato (<24 hours) for experimentation. Two of the selected sites are characterised by frequent upwelling, Dichato and Las Cruces (33°30' S, 71°38'W) and one which does not experience upwelling, Antofagasta Bay (23°45´S, 70°26´W). All mussels were scrubbed clean and barnacles were removed before being held in glass aquaria containing aerated natural seawater (pH 8.11, salinity 28, temperature 13 ± 3 °C) which was changed every second day. Mussels were held for a minimum of 7 days prior to experimentation and fed 5000 cells ml^{-1} of a concentrated suspension of *Isochrysis* and *Pavlova sp.* daily.

In order to compare the responses of the Chilean upwelling mussels with responses in a different species that does not experience upwelling, we also collected adult *Mytilus edulis* from Starcross, a muddy estuary on the south coast of Devon, UK (50°37'03" N -3°26'56" W) and from Port Gaverne, an exposed rocky shore on the north coast of Cornwall, UK (50°35'38" N -4°49'26" W). All mussels were scrubbed clean and barnacles were removed before being left in aerated artificial seawater (©Tropic marine) for 24 hours. Mussels were subsequently transferred to a recirculating system (pH 8.10, salinity 32,

temperature 15 ± 0.5°C) and fed 5000 cells ml^{-1} of dried *Isochrysis* Instant Phyto (ZM Systems) daily. A minimum of 7 days was allowed for acclimation before experimentation.

Seawater manipulation and acid-base response

Seawater pH started between pH 8.12 and 8.23 (see tables 3.4 – 3.7) and was reduced at an average rate of 0.083 pH units h^{-1} for a total of 6 hours. Seawater pH was then increased at the same rate over a further 6 hours to investigate recovery. Natural seawater filtered to 1 µm was used for experimentation with *P. purpuratus* compared to artificial seawater (©Tropic marine) for *M. edulis*. A computerised control system (Aqua Medic, Germany) was used to acidify seawater through the injection of gaseous CO_2 when the seawater pH reached 0.05 units above the pre-programmed level. Oxygen levels were maintained close to 100% saturation via gentle aeration.

Every 30 minutes over the 6-hour sampling period, seawater pH measurements were taken using a pH meter (Metrohm 826 pH mobile) calibrated prior to use. Following pH determination, seawater samples were collected and preserved with saturated $HgCl_2$ (Dickson et al. 2007) and analysed for DIC as described above.

M. edulis (n = 6) and *P. purpuratus* (n = 3) were used for determination of acid-base response every 30 minutes for *M. edulis* and every hour for *P. purpuratus*, with all mussels only being used once. Haemolymph was extracted from the posterior abductor muscle as described above, and immediately measured for pH. For *M. edulis*, haemolymph samples were then transferred to 100 µl glass micro-hematocrit capillary tubes sealed with paraffin oil and hemato-sealTM capillary tube sealant (Fisher). These were subsequently placed on ice until analysis of total CO_2 as described above. For *P. purpuratus* haemolymph samples from the Antofagasta population and Lac Cruces recovery experiment were immediately analysed for TCO_2 after determination of pH. For the Las Cruces decrease in seawater pH and Dichato experiments, problems with the TCO_2 machine meant haemolymph samples were frozen in capillary tubes sealed with paraffin oil and hemato-sealTM capillary tube sealant (Fisher) and brought back to the UK for later analysis. Haemolymph pCO_2 and HCO_3^- were calculated

using a modified version of the Henderson-Hasselbalch equation from Truchot (1976) as described above.

Statistical analysis

All data was tested for normality using the Shapiro-Wilk Test and presented as mean ± standard error (SE). A one-way ANOVA was used to explore the influence of seawater pCO_2 on haemolymph pCO_2, HCO_3^- and pH followed by the Holm-Sidak post-hoc test. For data not normally distributed (all Starcross data, Antofagasta pH, Las Cruces pCO_2 and HCO_3^- and Dichato pCO_2) a one-way ANOVA on ranks (Kruskal-Wallis test) followed by Dunn's method post-hoc test was used. Data analysed was carried out in Sigma Plot version 12.0.

3.4 Results

Field monitoring

Carbonate system parameters

The seawater carbonate chemistry recorded during submersion of a mussel bed over 12 hours in Dichato, central Chile are summarised in Tables 3.1 – 3.3. During all three sampling occasions there was not an upwelling in progress, with seawater pCO_2 values being markedly lower than that of the surface global open ocean average of ~400 µatm (Dlugokencky & Tans 2016). Seawater pCO_2 did however fluctuate daily with values cycling between 198 µatm and 288 µatm on the 28th September compared to 147 µatm and 202 µatm on the 2nd October. Habitat pH varied between 8.08 and 8.41 over the two weeks and salinity between 26.1 and 28.3.

Acid-base measurements

Every 30 minutes during the 12 hour field sampling, haemolymph samples were taken to investigate the acid-base response throughout a tidal cycle over one high and one low tide (figure 3.2). As the mussel bed became exposed at low tide, haemolymph pCO_2 levels increased at an average rate of 694 µatm h^{-1}. For example on the 28th September haemolymph pCO_2 increased from 2670 µatm to 4271 µatm over a 6.5 hour period and from 1928 µatm to 2884 µatm over a 2

hour period on the 2nd October. This resulted in increases in bicarbonate concentration on the 28th September with haemolymph concentration increasing from 1.16 mM to 2.72 mM. Conversely, only slight changes in bicarbonate concentration were observed in the concluding two samples in October. Although bicarbonate concentration increased over the emersion period, no significant compensation was observed with regard to haemolymph pH, with decreases from pH 7.49 to pH 7.28, and a minimum pH of pH 6.89 on the 28th September sample, and from pH 7.23 to pH 7.05 on October 6th. This acidosis was reversed during high tide when the mussel bed became re-submerged (figure 3.2).

Laboratory experiments- short term responses to changes in seawater pH

Seawater chemistry

The seawater carbonate chemistry during a short-term (6 hour) exposure to changes in seawater pH was successfully manipulated to increase and then decrease over one pH unit (tables 3.4 – 3.10). Minimum pCO_2 values ranged between 220 µatm to 355 µatm with maximum pCO_2 values ranging between 2914 µatm to 4297 µatm. Across all experiments alkalinity values differed with the highest of 2554 µmol kg^{-1} at pH 8.17 during the Port Gaverne sample compared to a lowest starting alkalinity of 1965 µmol kg^{-1} at pH 8.22 during the Dichato and Las Cruces experiment.

Acid-base responses

Haemolymph pCO_2 significantly increased during an increase in seawater pCO_2 over 6 hours for all but one population tested. For this population, Antofagasta Bay, pCO_2 levels were significantly influenced by seawater pCO_2 but not linearly. For all other populations, haemolymph values ranged from a minimum of 408 µatm to a maximum of 5823 µatm which was subsequently reversed during a reduction in seawater pCO_2, showing a rapid recovery in haemolymph pCO_2 (*P. purpuratus* one-way ANOVA for Antofagasta $F_{13} = 45.914$, $P< 0.001$, figure 3.3a; and one-way ANOVA on ranks for Las Cruces $H_{13} = 48.361$, $P< 0.001$, figure 3.3b; for Dichato $H_{13} = 51.081$, $P< 0.001$, figure 3.3c; *M. edulis* one-way ANOVA on ranks for Starcross $H_{25} = 95.884$, $P< 0.001$, figure 3.4a; and one-way ANOVA for Port Gaverne $F_{12} = 9.833$, $P< 0.001$, figure 3.4b).

In response to changing seawater pCO_2, all populations showed altered bicarbonate concentration: significantly increasing with seawater pCO_2 and significantly decreasing during reductions in seawater pCO_2. However, the concentration and magnitude differed, with both *M. edulis* populations having a starting concentration over 2 mM compared to the highest starting concentration from *P. purpuratus* of 1.15 mM. In addition, the magnitude of change differed between populations, with those from Dichato (i.e. a site with frequent upwelling) experiencing a 61 % up-regulation of HCO_3^- compared to between 5 % and 24 % for all other populations (*P. purpuratus* one-way ANOVA for Antofagasta F_{13} = 45.914, P< 0.001, figure 3.3d; for Dichato F_{13} = 13.685, P< 0.001, figure 3.3f; and one-way ANOVA on ranks for Las Cruces H_{13} = 37.614, P< 0.001, figure 3.3e; *M. edulis* one-way ANOVA on ranks for Starcross H_{25} = 66.482, P< 0.001, figure 3.4c; and one-way ANOVA for Port Gaverne F_{12} = 2.718, P = 0.005, figure 3.4d).

Although bicarbonate concentration was significantly up-regulated in response to increases in seawater pCO_2, all populations experienced significant acidosis, with haemolymph pH decreasing as seawater pCO_2 increased. This haemolymph acidosis was reversed during reductions in seawater pCO_2 (*P. purpuratus* one-way ANOVA on ranks for Antofagasta H_{13} = 47.882, P< 0.001, figure 3.3g; one-way ANOVA for Las Cruces F_{13} = 79.231, P< 0.001, figure 3.3h; for Dichato F_{13} = 51.790, P< 0.001, figure 3.3i; *M. edulis* one-way ANOVA on ranks for Starcross H_{25} = 109.846, P< 0.001, figure 3.4e; and one-way ANOVA for Port Gaverne F_{12} = 12.015, P< 0.001, figure 3.4f).

3.5 Discussion

Previous research suggests marine mussels have a limited ability to regulate acid-base disturbances in response to stable elevated pCO_2 levels. Furthermore, our previous study suggests seawater chemistry is the primary driver of acid-base change during submersion. However, little is known about how mussels respond to natural environmental variability and the potential for local adaptations to influence acid-base response. Our field data collected from Dichato, central Chile, during Sept and Oct 2015 demonstrate the variability in abiotic conditions experienced by a local population of *P. purpuratus* (tables 3.1 – 3.3). Temperature fluctuations cycled on a daily basis but salinity levels remained

relatively stable. Carbonate chemistry monitoring found fluctuations of seawater pCO_2 ranged from 147 µatm to 298 µatm over 12 hours, levels much lower than those experienced on average in the open ocean today (Dlugokencky & Tans 2016). Upwelling centres have however been observed in central Chile with pCO_2 values regularly exceeding 400 µatm during the austral spring-summer (Torres et al. 2011). In particular, upwelling events have been recorded in Coliumo Bay where Dichato is situated and the adjacent Concepción Bay (Ahumada & Chuecas 1979; Arcos & Wilson 1984). Therefore, although our field sampling did not coincide with an upwelling event, it is likely organisms inhabiting this region are regularly exposed to seawater elevated with pCO_2.

During submersion of the mussel bed at high tide, the limited variability observed in seawater pCO_2 resulted in no observable changes in acid-base status. However, during emersion at low tide haemolymph pCO_2 levels increased, which with no observable up-regulation of HCO_3^-, led to a reduction in haemolymph pH. Currently studies investigating the acid-base response to emersion in mussels are scarce, however it has been suggested that the closure of the mussel valve can lead to hypoxia triggering a switch in metabolism from aerobic to anaerobic, possibly altering acid-base disturbances (Bayne et al. 1976; Babarro et al. 2007; Connor & Gracey 2011; Rastrick et al. 2014). This is coupled with changes in oxygen consumption (Coleman 1973a), heart rate (Helmuth et al. 2010), valve opening (Widdows & Shick 1985; Shick et al. 1986) and intermediary metabolite cycles (Connor & Gracey 2012). Although the mechanisms behind these changes were beyond the scope of this study, the extracellular acidosis observed suggests periods of emersion may have the potential to pose significant physiological stress on intertidal organisms.

Recent research suggests the potential for geographic location to influence physiological traits through adaptation to different environmental conditions (Kelly et al. 2013; Lardies et al. 2014; Vargas et al. 2015). Our results suggest the acid-base response of both *Perumytilus purpuratus* and *Mytilus edulis* are not significantly influenced by the natural environmental conditions experienced by a population (figures 3.3 and 3.4). Such that, pre-exposure to elevated pCO_2 levels from upwelling events is unlikely to significantly alter the acid-base response of *P. purpuratus* to short-term changes in seawater pCO_2 (figure 3.3). This is evidenced by the significant increase in haemolymph pCO_2 and therefore the

extracellular acidosis experienced by both populations of *P. purpuratus* regularly exposed to upwelling events during an increase in seawater pCO_2. Moreover, this response was similar to that observed in both *M*. *edulis* populations.

Even though the overall trends of acid-base disturbances were similar for all populations, including between species, there were noticeable differences in the extent of acidosis and regulatory ability. For mussels from Starcross, Antofagasta Bay, Las Cruces and Port Gaverne, increases in HCO_3^- concentration of 5 %, 6 %, 17 % and 24 % respectively were observed compared to a 61% increase from those originating from Dichato. A short-term accumulation of HCO_3^-, similar to that observed in this study, has been suggested to stabilise extracellular pH in a number of marine organisms (Pane & Barry 2007; Gutowska et al. 2010). For marine mussels, often considered unable to regulate acid-base disturbances, this HCO_3^- is likely to be a product of shell dissolution (Hall-Spencer et al. 2008; Melzner et al. 2011) or passive diffusion rather than of active ion transport. Dissolution of the exoskeleton can occur via increases in extracellular pCO_2 shifting the carbonate system towards reduced CO_3^{2-} concentrations. This subsequently reduces the aragonite saturation, producing progressively more corrosive conditions at the inner surface of the shell (Melzner et al. 2011), leading to increases in extracellular HCO_3^- and Ca^{2+}. Such dissolution has been indicated in *M. edulis* during long-term exposures (60 day) to seawater pH ranging from pH 6.5 - 7.8 (Beesley et al. 2008). The concentration of extracellular HCO_3^- was subsequently reversed during reductions in seawater pCO_2.

Despite differences in HCO_3^- accumulation, the increase in haemolymph pCO_2 with seawater pCO_2 resulted in significant extracellular acidosis in all populations except from mussels originating from Antofagasta Bay. Here the haemolymph pH recorded at the beginning of the 6 hour reduction in seawater pH was pH 7.19, a value of magnitude lower than recorded at the end of recovery (pH 7.57) and in the other two experiments using *P. purpuratus* (pH 7.79 and pH 7.85 for mussels from Las Cruces and Dichato respectively). Therefore we suggest a difficulty recording haemolymph pH may explain this result rather than an altered physiological response. The extracellular acidosis experienced in all other populations was immediately reversed during reductions in seawater pCO_2, with no observable differences between the two species. Few studies have investigated the influence of seawater pCO_2 on the acid-base status of *P.*

purpuratus, however these observations are in agreement with previous studies reporting *M. edulis* cannot actively avoid acid-base disturbances to stable pCO_2 values (Booth et al. 1984; Thomsen et al. 2010; 2013; Heinemann et al. 2012). This inability to compensate extracellular acid-base disturbances can result in reductions in extrapallial fluid pH (Thomsen et al. 2010; Heinemann et al. 2012) which in the longer term, has the potential to impact growth and calcification and therefore negatively influence individual fitness.

Most notably, the largest difference in acid-base response was observed between mussels from Starcross compared to all other populations. Here, increases in seawater pCO_2 resulted in a maximum haemolymph pCO_2 of 7301 µatm in mussels from Starcross compared to a maximum of 1160 µatm to 2812 µatm in all other populations. With no observable compensation in extracellular pH from the increases in HCO_3^- concentration, mussels from Starcross experienced a more pronounced extracellular acidosis.

Considering both populations of *M. edulis* are expected to experience similar environmental conditions and mussels are suggested to have limited acid-base regulatory mechanisms, we suggest the difference in acid-base response may be an indirect effect of organism size. Mussels from Port Gaverne (and all Chilean populations) are considerably smaller than those from Starcross, with an average shell length of 37 mm compared to 68 mm. A difference in organism size has been linked to changes in stress resistance (Sukhotin et al. 2003) and may therefore alter the behavioural response to a progressive stress, such as increasing seawater pCO_2. For larger mussels, this may result in valve gaping, allowing the maintenance of aerobic respiration and the accumulation of pCO_2 as a product of metabolism and from the diffusion from seawater resulting in a higher haemolymph pCO_2 over the 6 hours.

However, for smaller organisms this may result in the closing of the mussel valves, reducing the potential for extracellular fluids to be exposed to high pCO_2 seawater which in turn reduces the accumulation of extracellular pCO_2 from the seawater and therefore protecting extracellular pH. However, if the mussel valves close for an extended period of time, there is the potential for metabolic CO_2 to accumulate in addition to the onset of hypoxia limiting the ability to perform aerobic metabolism. For intertidal mussels, the onset of hypoxia during emersion has been shown to switch metabolism from aerobic to anaerobic, producing

succinate and alanine (Isani et al. 1995) and other end products which may have the potential to further alter acid-base balance (Rastrick et al. 2014). In this study the marginal changes in acid-base parameters during changes to seawater pCO_2 may suggest smaller mussels, such as those from Port Gaverne, may instead go into respiratory arrest after closing the mussel valves. This limits the extent of pCO_2 accumulation from metabolism to only passive diffusion from the seawater. This is supported by the maximum value of haemolymph pCO_2 of 2812 µatm in mussels from Port Gaverne, approximately equalling that of seawater after 5 hours (2647 µatm) permitting a lag time for diffusion.

In addition to potentially altering acid-base disturbances in response to changes in seawater pCO_2, reductions in size have been shown to help mitigate against increased energetic costs of OA by maintaining mass-specific rates of energy consumption allowing individuals to maintain calcification and partially repair shell dissolution (Garilli et al. 2015). Although the mechanisms behind these differences were beyond the scope of this study, future research investigating the potential for reductions in organism size to facilitate the survival of mussels in future predicted climatic conditions may provide crucial insight into the future of these ecologically and economically important species.

Overall the results from this study indicate mussel populations exposed to a range of environmental conditions have a similar acid-base response. Specifically, we found the regular exposure to elevated seawater pCO_2 is unlikely to alter the acid-base response of *P. purpuratus* to short-term changes in seawater pCO_2, suggestive of a limited capacity for acid-base regulatory mechanisms to adapt to changes in seawater pCO_2. Although evidence of changes in bicarbonate concentration was observed in all populations, increasing seawater pCO_2 resulted in a significant acidosis in all populations, adding to further evidence that both *M. edulis and P. purpuratus* are relatively weak acid-base regulators.

In agreement with our previous study investigating the acid-base response of *M. edulis* over a tidal cycle, extracellular acidosis appears to be greater not when immersed in high pCO_2 seawater, but during emersion at low tide. Therefore the alternating cycles of emersion and the environmental conditions faced during these periods may pose a greater physiological stress on an intertidal organism than high seawater pCO_2 encountered during upwelling events. This suggests the potential that being intertidal rather than subtidal may be more important in

determining acid-base responses in the future rather than inhabiting an upwelling/ non-upwelling region. Furthermore we provide evidence to suggest size is an important driver of acid-base change, with larger mussels experiencing a greater extracellular acidosis when compared to smaller mussels.

Overall this study highlights the possibility that size and conditions experienced during emersion may play an important role in determining acid-base responses in intertidal mussels, even in presence of OA and the strengthening of upwelling events. Although our data needs to be interpreted conservatively, we highlight the potential for using multiple population comparisons and areas naturally exposed to high pCO_2 seawater. Furthermore, we highlight key knowledge gaps in our understanding of the mechanisms behind differences in acid-base response and the longer-term response to fluctuations in pCO_2.

Table 3.1: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle in Dichato on 28.09.15.

Time of day	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
09:00	13.0	8.08	28.2	1605.6	1758.5	287.5	1.487	106.3
09:30	13.4	8.10	28.3	1604.2	1767.6	273.8	1.480	112.8
10:00	13.7	8.13	28.1	1592.0	1767.1	253.2	1.462	120.1
10:30	14.3	8.16	28.0	1588.2	1778.8	235.3	1.449	130.2
11:00	13.9	8.17	28.0	1658.3	1856.0	239.3	1.512	136.9
11:30	14.2	8.18	27.7	1731.7	1939.6	244.7	1.575	146.5
12:00	14.2	8.11	26.9	1657.0	1824.1	280.3	1.528	118.3
12:30	14.3	8.16	26.2	1525.8	1700.0	229.9	1.397	119.5
13:00	15.2	8.16	26.3	1658.5	1848.9	250.5	1.514	134.5
13:30	15.0	8.23	26.2	1613.8	1830.1	204.8	1.456	150.3
14:00	14.7	8.25	26.7	1645.5	1875.8	197.4	1.478	160.1

Table 3.2: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle in Dichato on 02.10.15.

Time of day	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
12:30	15.7	8.27	28.2	1757.3	2030.2	198.1	1.558	191.5
13:00	15.5	8.30	28.1	1787.3	2078.3	186.8	1.575	205.3
13:30	15.3	8.35	28.1	1788.2	2108.2	164.2	1.556	225.8
14:00	15.7	8.33	28.1	1829.0	2145.3	177.0	1.598	224.8
14:30	15.9	8.32	28.1	1832.7	2145.1	182.1	1.604	222.3
15:00	15.6	8.35	26.3	1805.4	2112.5	169.1	1.578	220.5
15:30	15.9	8.33	26.1	1808.7	2104.8	178.8	1.589	213.2
16:00	15.8	8.29	26.2	1844.5	2120.8	201.7	1.637	200.1
16:30	16.3	8.32	26.3	1759.0	2049.1	178.2	1.545	206.9
17:00	16.3	8.40	26.2	1786.2	2129.2	147.3	1.534	246.3
17:30	16.7	8.32	26.6	1869.6	2179.4	189.0	1.638	224.6
18:00	16.9	8.41	26.7	1835.3	2204.4	146.7	1.563	266.5

Table 3.3: Seawater carbonate chemistry during submersion of the mussel bed at high tide over a 12 hour tidal cycle in Dichato on 06.10.15.

Time of day	Temperature (°C)	pH NBS	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
08:00	14.3	8.18	28.2	1747.7	1961.1	245.9	1.588	150.2
08:30	14.7	8.23	28.1	1732.3	1971.6	215.6	1.556	167.4
09:00	14.8	8.22	28.1	1726.1	1960.5	220.4	1.553	163.9
09:30	15.1	8.17	26.4	1761.5	1964.2	259.2	1.606	145.8
10:00	14.5	8.12	26.3	1734.7	1909.0	288.3	1.597	125.9
10:30	15.6	8.11	26.2	1736.3	1913.0	297.5	1.597	128.0
11:00	14.9	8.16	28.1	1715.4	1920.7	254.5	1.561	144.1
11:30	15.3	8.23	28.0	1715.6	1957.6	214.2	1.539	168.9
12:00	15.1	8.26	28.1	1765.9	2028.0	204.1	1.574	184.2
19:30	14.4	8.16	26.2	1685.5	1871.9	254.0	1.543	132.5
20:00	14.0	8.23	26.2	1716.4	1934.0	217.0	1.553	154.3

Table 3.4: Seawater carbonate chemistry for the short-term acid-base response to a reduction in seawater pH for *Perumytilus purpuratus* from Antofagasta Bay.

Time (hours)	Temperature (°C)	pH NBS	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
0.00	17.2	8.23	28.2	2039.6	2331.8	255.6	1.815	215.5
1.00	17.0	8.06	28.2	2064.3	2263.6	395.0	1.899	151.2
2.00	17.7	7.89	28.2	2174.4	2310.2	630.5	2.039	112.8
3.00	17.2	7.73	28.2	1985.5	2058.1	839.3	1.884	70.7
4.00	17.7	7.55	28.2	2009.8	2034.2	1295.4	1.915	48.4
5.00	17.3	7.37	28.2	2137.6	2110.8	2072.0	2.029	33.4
6.00	17.3	7.23	28.2	2182.4	2113.0	2893.9	2.053	24.5

Table 3.5: Seawater carbonate chemistry for the short-term acid-base response to a reduction in seawater pH for *Perumytilus purpuratus* from Dichato and Las Cruces.

Time (hours)	Temperature (°C)	pH NBS	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
0.00	14.9	8.22	28.4	1728.0	1965.9	220.1	1.554	165.9
1.00	15.8	8.03	28.4	1790.6	1952.8	366.2	1.658	118.4
2.00	15.8	7.88	28.4	1925.4	2038.4	565.4	1.812	91.6
3.00	16.1	7.73	28.4	1831.1	1897.0	768.0	1.739	63.0
4.00	16.3	7.54	28.4	1968.9	1986.4	1285.2	1.877	44.2
5.00	16.2	7.37	28.4	1982.0	1955.3	1904.0	1.881	29.8
6.00	15.9	7.32	28.4	2121.0	2076.1	2274.8	2.007	28.0

Table 3.6: Seawater carbonate chemistry for the short-term acid-base response to a reduction in seawater pH for *Mytilus edulis* from Starcross.

Time (hours)	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
0.0	13.5	8.12	32.3	2250.5	2495.5	355.4	2.054	182.5
0.5	13.5	8.06	32.3	2297.3	2512.8	420.5	2.117	163.8
1.0	13.4	7.98	32.3	2308.3	2484.0	513.2	2.150	137.9
1.5	13.5	7.90	32.3	2332.7	2474.8	629.0	2.190	117.3
2.0	13.6	7.81	32.3	2390.9	2499.7	799.2	2.261	98.8
2.5	13.5	7.72	32.3	2403.3	2477.7	991.8	2.282	80.7
3.0	13.5	7.64	32.3	2415.6	2464.6	1202.0	2.300	67.7
3.5	13.5	7.55	32.3	2381.5	2401.7	1458.8	2.269	54.3
4.0	13.5	7.45	32.3	2394.7	2384.0	1843.8	2.278	43.3
4.5	13.5	7.38	32.3	2432.6	2399.2	2194.7	2.308	37.3
5.0	13.5	7.24	32.3	2490.0	2407.5	3072.3	2.341	27.4
5.5	13.5	7.17	32.3	2447.4	2340.6	3523.4	2.285	22.8
6.0	13.5	7.12	32.3	2474.6	2346.5	3973.6	2.297	20.4

Table 3.7: Seawater carbonate chemistry for the short-term acid-base response to a reduction in seawater pH for *Mytilus edulis* from Port Gaverne.

Time (hours)	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
0.0	13.6	8.17	32.4	2275.5	2553.9	317.2	2.057	206.4
0.5	13.6	7.98	32.4	2389.5	2571.5	531.4	2.224	144.1
1.0	13.6	7.91	32.4	2373.4	2522.6	624.8	2.226	122.7
1.5	13.6	7.85	32.4	2369.3	2493.4	719.8	2.234	107.3
2.0	13.6	7.80	32.4	2443.0	2549.8	835.7	2.311	98.9
2.5	13.6	7.72	32.4	2490.0	2568.1	1028.2	2.365	84.2
3.0	13.6	7.65	32.4	2463.8	2517.4	1198.0	2.345	71.1
3.5	13.6	7.57	32.4	2477.9	2505.2	1449.9	2.361	59.5
4.0	13.6	7.49	32.4	2489.3	2490.9	1750.1	2.371	49.7
4.5	13.6	7.42	32.4	2575.1	2552.9	2123.0	2.448	43.7
5.0	13.6	7.33	32.4	2620.7	2566.8	2646.7	2.480	36.0
5.5	13.6	7.26	32.4	2607.7	2528.9	3078.9	2.456	30.3
6.0	13.6	7.17	32.4	2630.2	2515.4	3788.2	2.456	24.6

Table 3.8: Seawater carbonate chemistry for the short-term acid-base response to an increase in seawater pH for *Perumytilus purpuratus* from Antofagasta Bay and Las Cruces.

Time (hours)	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
0.00	17.1	7.23	28.4	2203.2	2132.8	2913.6	2.073	24.6
1.00	17.1	7.37	28.4	2335.7	2304.9	2258.0	2.217	36.4
2.00	17.6	7.55	28.4	2015.8	2040.4	1296.6	1.921	48.6
3.00	17.4	7.73	28.4	1981.6	2055.7	837.4	1.880	71.5
4.00	17.0	7.89	28.4	1938.1	2061.3	559.1	1.819	98.5
5.00	17.2	8.05	28.4	1760.4	1938.0	345.0	1.620	127.8
6.00	17.4	8.21	28.4	1880.2	2148.3	247.6	1.679	192.8

Table 3.9: Seawater carbonate chemistry for the short-term acid-base response to an increase in seawater pH for *Perumytilus purpuratus* from Dichato.

Time (hours)	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
0.00	16.5	7.21	28.4	2300.6	2217.6	3165.8	2.159	24.0
1.00	15.7	7.37	28.4	2361.0	2324.5	2260.0	2.241	34.9
2.00	15.6	7.54	28.4	2355.4	2369.5	1530.5	2.246	51.5
3.00	16.4	7.72	28.4	2119.4	2188.8	911.6	2.014	72.0
4.00	17.0	7.88	28.4	2131.4	2258.6	629.8	2.003	106.0
5.00	17.2	8.03	28.4	2094.1	2283.9	430.9	1.933	145.6
6.00	17.2	8.16	28.4	2117.5	2376.7	316.0	1.912	194.2

Table 3.10: Seawater carbonate chemistry for the short-term acid-base response to an increase in seawater pH for *Mytilus edulis* from Starcross.

Time (hours)	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
0.0	13.5	7.05	32.0	2297.5	2150.3	4296.8	2.111	15.8
0.5	13.5	7.13	32.0	2271.4	2157.6	3572.9	2.110	19.0
1.0	13.5	7.20	32.0	2230.1	2143.5	3009.4	2.088	22.1
1.5	13.5	7.27	32.0	2269.1	2204.2	2622.6	2.138	26.6
2.0	13.5	7.35	32.0	2249.8	2210.5	2174.5	2.132	31.9
2.5	13.5	7.45	32.0	2209.2	2200.1	1703.3	2.102	39.6
3.0	13.5	7.55	32.0	2204.9	2224.5	1352.5	2.101	49.9
3.5	13.5	7.65	32.0	2148.8	2197.6	1046.2	2.046	61.1
4.0	13.5	7.74	32.0	2159.7	2236.6	852.2	2.050	75.4
4.5	13.5	7.83	32.0	2128.5	2235.1	679.2	2.011	90.9
5.0	13.5	7.88	32.0	2131.8	2256.9	604.0	2.006	101.8
5.5	13.5	7.95	32.0	2103.4	2255.0	503.8	1.966	117.2
6.0	13.5	8.04	32.0	2087.9	2278.5	402.1	1.930	141.6

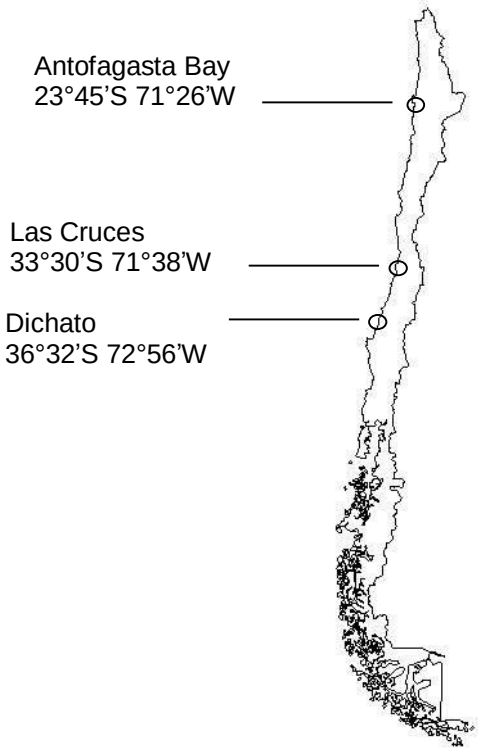

Figure 3.1: Study area within Chile showing the different geographic locations from which *Perumytilus purpuratus* were collected.

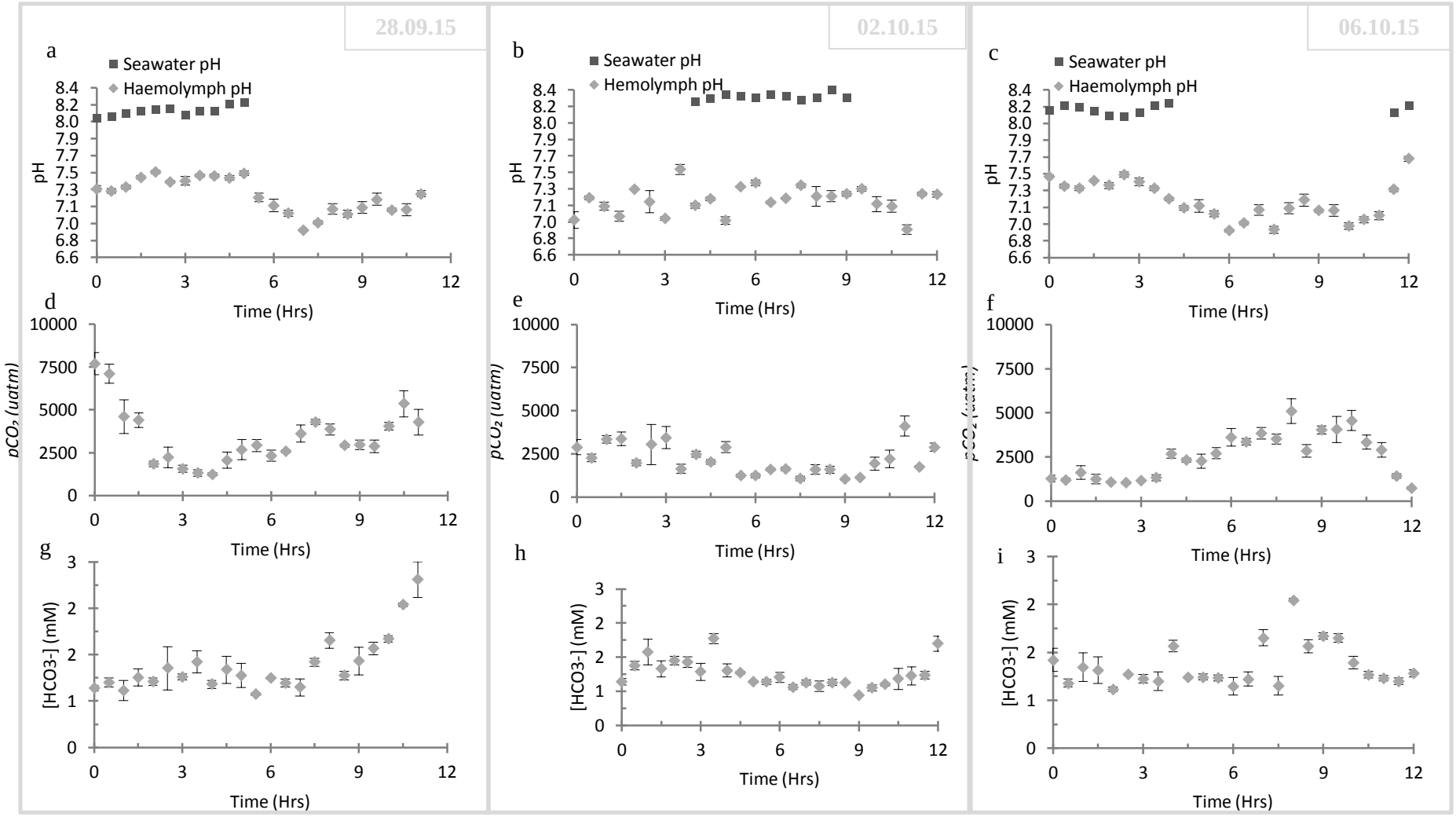

Figure 3.2: Seawater pH and acid-base parameters within the haemolymph of *Perumytilus purpuratus* over a 12 hour field sample in Dichato on 28.09.15 (a, d, g), 02.10.15 (b, e, h) and 06.10.15 (c, f, i).

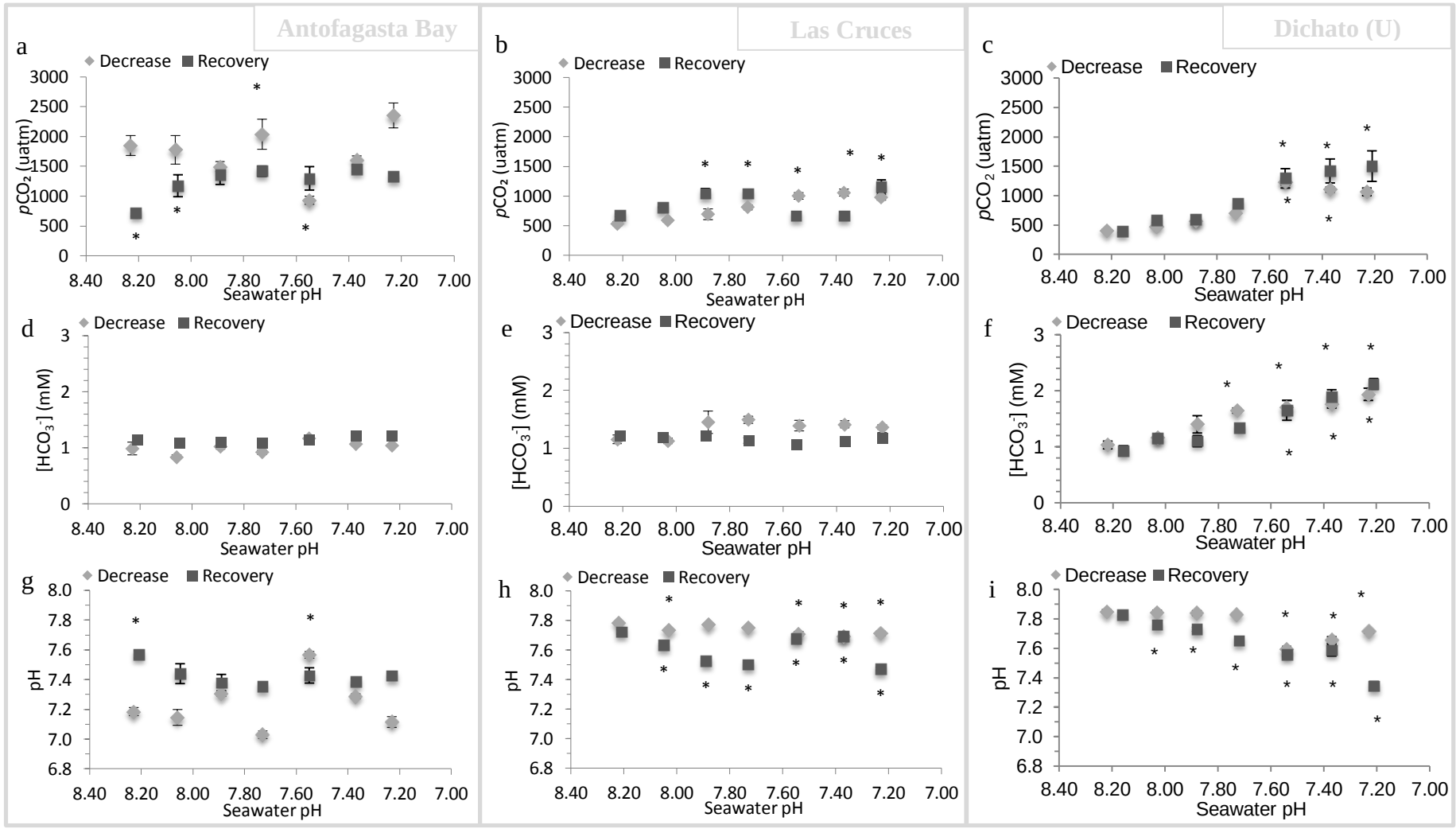

Figure 3.3: Acid-base parameters in the haemolymph of *Perumytilus purpuratus* from Antofagasta Bay (a, d, g), Lac Cruces (b, e, h) and Dichato (c, f, i) over a 6 hour gradual exposure to increasing or decreasing seawater pH. The pH/ pCO_2 variability experienced within each study location is summarised by: N- no upwelling, U- upwelling, U+R- upwelling and riverine input.

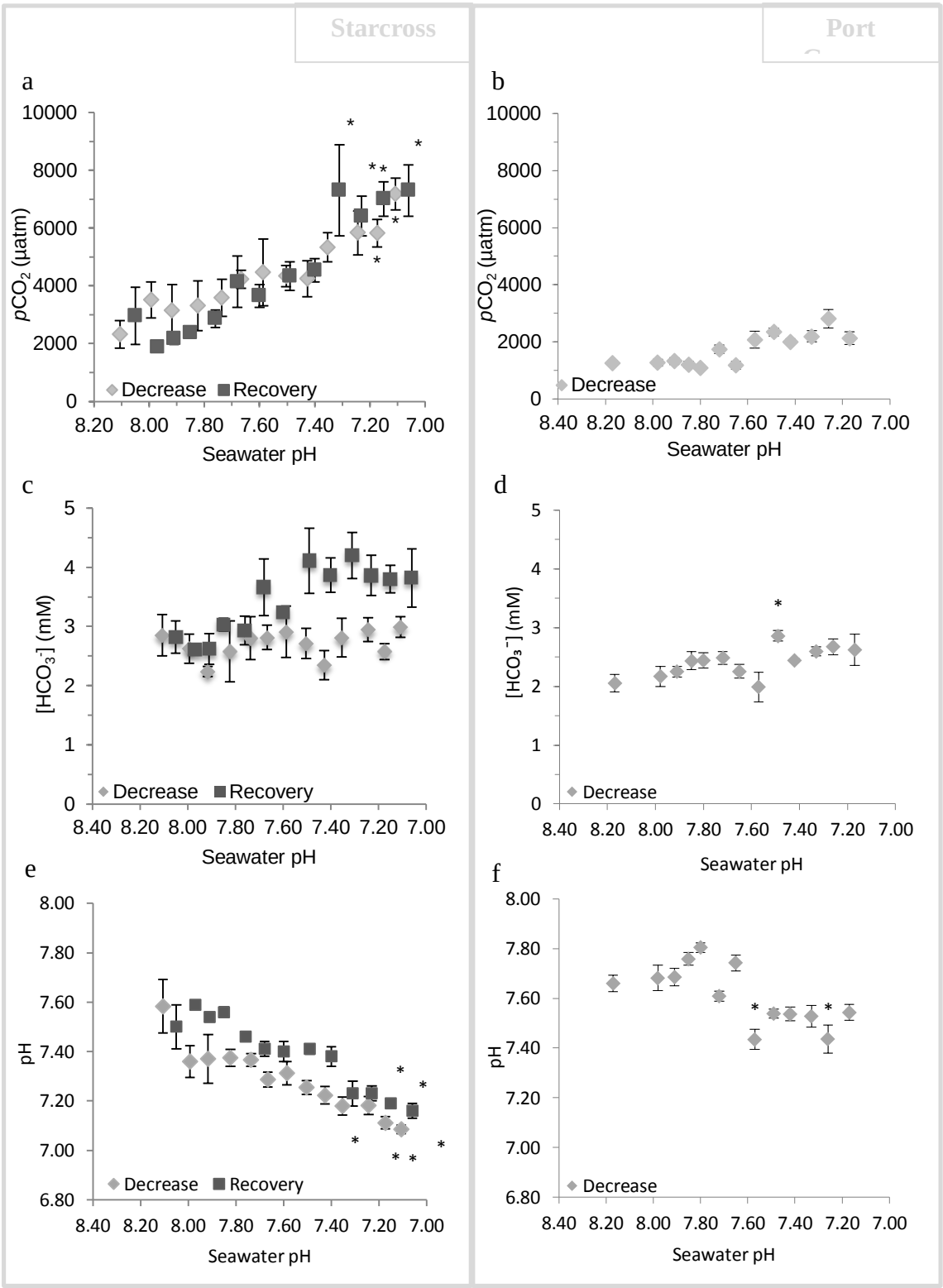

Figure 3.4: Acid-base parameters in the haemolymph of *Mytilus edulis* from Starcross (a, c, e) and Port Gaverne (b, d, f) over a 6 hour gradual exposure to increasing or decreasing seawater pH.

Chapter 4: Fluctuating seawater pCO_2 regimes are more energetically expensive than constant pCO_2 levels in the mussel *Mytilus edulis*

4.1 Abstract

The majority of ocean acidification studies to date use stable open ocean pH values to inform predictions of future species' vulnerabilities. Coastal organisms, however, experience fluctuations in a range of abiotic factors, thus rendering the responses to stable pH conditions inaccurate to represent current or future conditions. This study investigates the short-term response of *Mytilus edulis* to changes in pH/ pCO_2 and the medium-term consequences of a variable compared to a stable pH/ pCO_2 regime. First, adult *Mytilus edulis* were exposed to short-term (6 hour) changes in seawater pH/ pCO_2 to determine acid-base and metabolic responses. A separate group of mussels were then exposed to either a stable or fluctuating seawater pH regime over 14 days to represent current and future pH/ pCO_2 variability. Acid-base, metabolic and oxidative stress responses were measured. Our results clearly show *M. edulis* have a limited ability to regulate acid-base disturbances after both short- and medium- term exposures to elevated seawater pCO_2. More interestingly, after 14 days analysis of several physiological parameters showed an increased energetic cost to a variable seawater pH regime, with increases in metabolic rate, antioxidant enzyme activities and lipid peroxidation. These results demonstrate the extra costs of living in naturally variable environments compared to stable seawater conditions, which have the potential to negatively affect growth, survival and reproduction. With environmental variability expected to increase and intensify with future climatic change, we highlight the need to use environmental realistic OA scenarios to reliably inform future predictions.

Keywords: Ocean acidification, natural variability, acid-base balance, metabolism, oxidative stress, climate change.

4.2. Introduction

Atmospheric carbon dioxide (CO_2) levels are rising at a current rate of 2.73 ppm yr^{-1}, reaching 400 ppm in 2016 (Dlugokencky & Tans 2016), a rise of 100 ppm

since the industrial revolution. Recent projections from the Representative Concentration Pathways (RCP) database predict a continuing rise in atmospheric pCO_2, with levels predicted to exceed 1000 ppm early into the next century under the RCP 8.5 business as usual scenario (Meinshausen et al. 2011; Bopp et al. 2013). The accompanying absorption of atmospheric CO_2 by the oceans has led to a reduction in oceanic pH by 0.1 units and an alteration in carbonate chemistry (Orr et al. 2005; Cao & Caldeira 2008), termed ocean acidification (OA). This unprecedented rate of change has not been experienced for the past 300 million years (Doney & Schimel 2007; Lüthi et al. 2008) and has consequently led to OA being identified as a major threat to global marine biodiversity (Halpern et al. 2008; Doney et al. 2009; Kleypas & Yates 2009). There is now substantial evidence suggesting OA can impact the health and physiology of a wide range of marine invertebrate species (Fabry et al. 2008; Doney et al. 2009; Kroeker et al. 2010), with experimental OA negatively affecting over 50% of all molluscs tested to date (Wittmann & Pörtner 2013).

Whilst calcifying species are generally considered to be more susceptible to OA owing to the impact on $CaCO_3$ deposition (Gattuso & Buddemeier 2000; Kroeker et al. 2010), focus has now shifted to the impacts of OA on other physiological processes, in particular acid-base physiology and the energetic cost of ion regulation. Marine animals acutely exposed to elevated pCO_2 can experience an extracellular acidosis (Pörtner & Farrell 2008; Widdicombe & Spicer 2008), disturbing an organisms' acid-base balance. Tolerant taxa, such as teleost fish, are often characterised by higher activity levels and consequently higher metabolic rates and the ability to regulate these disturbances (Melzner et al. 2009; Whiteley et al. 2011). In the short-term, this is primarily achieved through the bicarbonate buffering system which acts to increase bicarbonate (HCO_3^-) concentration through active ion transport. This uses the Na^+/K^+/-ATPase pump to transfer HCO_3^- across the gill epithelia from the surrounding seawater (Lucu & Towle 2003; Santos et al. 2007). The ability to compensate extracellular pH and maintain cellular homeostasis has been suggested to play an important role in the future survival and distribution of a given species (Pörtner & Farrell 2008; P. Calosi et al. 2013) and is therefore considered to be a key determinant of an organisms' susceptibility to future OA conditions (Melzner et al. 2009).

Marine mussels, such as *Mytilus edulis*, provide a range of ecosystem services such as habitat structure, shelter and water purification as well as being a rich food source for both wildlife and humans (Asmus & Asmus 1991; Gutierrez et al. 2003; Gazeau et al. 2013), contributing £27 million to the UK economy in 2012 (Ellis et al. 2012). Despite their ecological and economical importance, little is known about the acid-base response of these species to environmental variability both in the short- and long- term. Previous studies investigating the acid-base response to elevated seawater pCO_2 in *M. edulis* reveal that hypercapnia induces extracellular acidosis (Booth et al. 1984; Thomsen et al. 2010; 2013; Heinemann et al. 2012) with no compensatory increase in HCO_3^-, suggesting a poor ability to acid-base regulate. If uncompensated, this reduction in extracellular pH has the potential to influence protein stability and enzyme function (Somero 1986), leading to the suggestion that these species may be more vulnerable to future elevated pCO_2 levels.

Over the longer term it is still unclear how the prolonged exposure to elevated pCO_2 and the consequential changes in acid-base balance may influence the health and physiology of marine mussels. To date, the few studies investigating the influence of stable elevated seawater pCO_2 have shown reductions in the immune function of *M. edulis* and the suppression of phagocytosis levels, an effect attributed to elevated calcium levels from the dissolution of the $CaCO_3$ shells (Beesley et al. 2008; Bibby et al. 2008). Elevated calcium levels can potentially cause a disruption to cellular metabolism and function as well as signalling pathways essential for regulating haemocyte function (Massullo et al. 2006) and may therefore alter levels of phagocytosis (Humphries & Yoshino 2003) compromising the immune response. The immune system can additionally be influenced by an increase in oxidative stress at elevated seawater pCO_2, by either the increased production of reactive oxygen species (ROS) as a result of disruptions in the electron transport chain or an enhanced Fenton reaction (Tomanek et al. 2011). These studies highlight the key knowledge gaps in understanding the impact of fluctuations of pCO_2 in key intertidal species.

Similar to the above studies, the majority of experimental-OA studies to date use predictions based on global open ocean averages, where pH and other carbonate chemistry parameters do not vary in time and space. This uniformity is uncharacteristic of coastal habitats where natural fluctuations of pH, temperature,

salinity and oxygen can occur on a daily (tidal cycle), seasonal and annual basis (Shim et al. 2007; Wootton et al. 2008; Urbina et al. 2010; Leiva et al. 2015). Recent field observations have shown this environmental variability can result in current seawater pCO_2 values exceeding predictions for the end of the century (Suzuki et al. 1995; Yates & Halley 2006; Price et al. 2012; Johnson et al. 2013), with pH deviations of up to one unit (Hinga 2002; Thomsen et al. 2013). The magnitude of coastal fluctuations in seawater pH/ pCO_2 is predominately influenced by upwelling events, temperature, salinity, photosynthesis and respiration (Raven et al. 2005; Lee et al. 2006; Pörtner 2008). Given that the majority of mussel populations are either intertidal or subtidal within coastal habitats, experimental designs using stable seawater pH exposures do not accurately reflect the pH conditions that most mussel populations are likely to experience *in situ* under near-future OA. With pH fluctuations expected to intensify along with other global climatic changes (for example rises in sea level and temperature) (Harley et al. 2006; IPCC 2014), intertidal organisms are facing a suite of challenges which may synergistically pose significant physiological stress (Waldbusser & Salisbury 2014). Unfortunately, we currently do not understand, how mussels, or any other intertidal marine invertebrate will respond to the natural fluctuations of pCO_2 in either current or near-future pCO_2 conditions, nor how close these species are to their physiological limits.

Our ability to predict the responses of key marine biota to future ocean conditions is currently impaired by our limited understanding of an organism's physiological responses to both natural and anthropogenic environmental variability and their physiological thresholds in the face of increased OA on top of this variability. Here we investigate if a variable pH regime compared to static pH exposures can influence acid-base balance along with other health indicators in the common mussel *M. edulis*. Apart from being a weak acid-base regulator, very little is known about how this species responds to current and natural pH/ pCO_2 variability and the associated effects the amplitude of this variation can have on acid-base balance and consequently the health of individuals long term. We hypothesise a variable pH/ pCO_2 regime will incur greater physiological costs than a stable pH/ pCO_2 regime. To test this hypothesis we first investigate the acid-base and metabolic response of mussels to short-term changes in seawater pCO_2. We then investigate the longer-term consequences of a variable compared to a static pH/ pCO_2 regime on acid-base balance, metabolic response and other

health indicators over 14 days. With the potential to impact survival, reproduction, geographic distribution and the whole intertidal community structure, it is crucial to understand how organisms respond to current natural variability in order to accurately predict the ecological impacts of expected future increases in climate variability.

4.3. Materials and Methods

Collection of animals

Adult *Mytilus edulis* (55-73mm shell length) were collected from a muddy estuary at Starcross, Devon, UK (50°37'03" N 3°26'56" W). After 24 hours in gently aerated artificial seawater, all shells were scrubbed clean and barnacles removed before being transferred to a recirculating system of artificial seawater (©Tropic marine, pH 8.10, salinity 33, at 15 ± 0.5°C) with a photoperiod of 12:12 (L:D). Mussels were held for a minimum of 7 days prior to experimentation and fed 5000 cells ml^{-1} of dried *Isochrysis* Instant Phyto (ZM Systems) daily.

Seawater manipulation

Artificial seawater was acidified via the manipulation of seawater CO_2 using a computerised control system (Aqua Medic, Germany), whereby gaseous CO_2 was injected into seawater through a solenoid valve until pH reached +0.05 units of the desired level. Seawater pH was additionally monitored with a Metrohm 826 pH mobile. Gentle aeration maintained oxygen levels close to 100% saturation.

Seawater samples were collected during each sampling point for assessment of pH and dissolved inorganic carbon (DIC) (measured once per time point). Samples preserved with saturated $MgCl_2$ (Dickson et al. 2007) were analysed for DIC using a custom built system based on Friederich et al. (2002) and following methodology from Lewis et al. (2013). Data outputs were analysed using Logger Pro version 3.2 software with a measurement precision of ±3 µM. Total alkalinity and pCO_2 were calculated using CO2sys (Pierrot et al. 2006) using parameters proposed by Findlay et al. (2013). K1 and K2 values were refitted by Dickson and Millero (1987) from Mehrbach (1973) and KSO_4 determined by Dickson (1990).

Short term responses to changes in seawater pH

Acid-base response

Mussels were placed into tanks at a starting pH of 8.12 (pCO_2 = 355 µatm, salinity 32.3, temperature 13.5 ± 0.5 °C). The manipulation of gaseous CO_2 gradually reduced seawater pH by one unit over 6 hours before being increased by one unit over another 6 hours. Seawater samples for DIC analysis and acid-base measurements were taken every half an hour.

Six mussels were used for determination of acid-base response at each time point, with all mussels only being used once. Haemolymph from the posterior abductor muscle was extracted using a 21 g needle and a 2 ml syringe and immediately measured for pH. The haemolymph samples were then transferred to 100 µl glass micro-hematocrit capillary tubes sealed with paraffin oil and hemato-sealTM capillary tube sealant (Fisher) and placed on ice until subsequent analysis of total CO_2 using a Corning 965 CO_2 analyser (Corning Ltd., UK) calibrated with a 10 mM $NaHCO_3$ solution. Acid-base parameters were then calculated using a modified version of the Henderson-Hasselbalch equation using the first dissociation constant (pK) for carbonic acid and solubility constant (αCO_2) for carbon dioxide derived from Truchot (1976).

Metabolism

In a separate group of mussels (n= 12) metabolic rate was evaluated during a 6 hour decrease in seawater pH from pH 8.15 to 7.15 and an immediate (~ 10 minutes) recovery back to pH 8.15. After being starved for 3 days, mussels were transferred to individual glass beakers and left aerating and undisturbed to acclimate for the final 24 hours. Three blanks were included to assess any potential for bacterial respiration or diffusion. From a header tank where seawater was aerated and pH controlled, all chambers were simultaneously flushed through before each sampling point, starting at pH 8.15 and decreasing by approximately 0.12 units until pH 7.15 and a final measurement at pH 8.15. After five minutes the partial pressure of oxygen (pO_2) was assessed over a 30 minute period using a fibre-optic oxygen sensor (FireStingO2) and recorded by the Pyro-Science Fire Sting logger (v2.363 2012). During this period the oxygen concentration did not decrease below 80% saturation. Immediately following the final reading, mussels were removed and seawater weighed (Scout Pro 600g

(OHAUS)). Shell length and wet weight were recorded for each mussel before being placed into a drying oven for 48 hours at 60°C to determine shell and organic dry weight. MO_2 was calculated using organic dry weights for each mussel. The oxygen probe was calibrated with fully aerated water and a saturated sodium sulphite solution at the corresponding salinity equilibrated at 15 °C (Urbina et al. 2011).

Physiological responses to variable pCO_2

To investigate the role of seawater pH variability in determining OA response, mussels (n = 16 per treatment) were placed in to one of four treatments; 1) stable pH 8.1; 2) stable pH 7.7; 3) variable pH 8.1 ± 0.4; (N.B the maximum pH achieved by aeration was 8.20) and 4) variable pH 7.7 ± 0.4. pH 7.7 was targeted to represent near-future OA under IPCC RCP 8.5 (IPCC 2013; Meinshausen et al. 2011). Each exposure system consisted of a 150 l header tank and 1 l individual replicate tanks in self-contained re-circulation. Diall compact 24 hour timers were connected to the pH computers and set to replicate a semidiurnal tidal cycle (6 hours on, 6 hours off) to attain a fluctuating regime. Seawater was aerated and pH additionally monitored using a pH meter which recorded pH every 10 minutes. Mussels were fed 5000 cells ml^{-1} of dried *Isochrysis* on days 0 and 7 (after MO_2 measurements).

Oxygen consumption rates were measured after 7 and 14 days, as previously described above. Briefly, each mussel remained in its individual aquarium tank (to avoid any potential stress) with the water supply removed. After 5 minutes, pO_2 was determined over 2.5 hours and the flow re-established (day 7). Seawater was then removed and weighed before being immediately replaced (<1 minute). Succeeding haemolymph extraction and pO_2 measurements on day 14, wet weight and shell length were recorded for each mussel and dry weight quantified after drying for 48 hours at 60°C. MO_2 was calculated using organic dry weights for each mussel.

Acid-base parameters were measured after 14 days. For the oscillating treatments measurements were taken on the downwards phase at the mean pH value (either pH 8.10 or 7.70) to allow for direct comparisons with the static treatments. Haemolymph was extracted and analysed for acid-base status as

described above. The remaining haemolymph was either placed on ice or immediately frozen in liquid nitrogen for further analysis.

Lysosomal stability

The neutral red (NR) uptake assay was used to provide a measure of lysosomal stability of mussel haemocytes following the methodology from Cajaraville et al. (1996). Modified by using 0.2% NR solution and absorbance measured using a Tecan NanoQuant infinite M200PRO.

Oxidative stress

Superoxide dismutase (SOD) production was quantified using the nitroblue tetrazolium assay, following the methodology of van der Oost et al. (2005). Known SOD concentrations of 0-3 U ml^{-1} were used to allow interpolation of haemolymph SOD concentration. SOD activity was then normalised to protein content using Bradford protocol and BSA as standards (Bradford 1976).

Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) assay determines the relative level of oxidative degradation of lipids by reactive oxygen species (ROS) within the haemolymph (Camejo et al. 1999). Methodology followed Camejo et al. (1998) and normalized to protein as described above.

Statistical analysis

All data are presented as mean ± standard error (SE) and tested for normality using the Shapiro-Wilk Test. All data investigating the influence of short-term changes in seawater pH/ pCO_2 on haemolymph pH, pCO_2 and HCO_3^- concentration were not normally distributed therefore a one-way analysis of variance (ANOVA) on ranks (Kruskal-Wallis test) followed by Dunn's method post-hoc test was used. For the metabolic response to short-term changes in seawater pCO_2 a one-way repeated measures ANOVA was performed followed by the Holm-Sidak post-hoc test.

To investigate the implications of a variable and static pH/ pCO_2 regime a one-way ANOVA was used followed by the Holm-Sidak post-hoc test on all normal data. For data that failed the normality test (haemolymph HCO_3^- concentration, SOD concentration, NR uptake and metabolic rate) a one-way ANOVA on ranks

(Kruskal-Wallis test) followed by Dunn's method post-hoc test was used. All data analysis was carried out in Sigma Plot version 12.0.

4.3. Results

Seawater carbonate chemistry

The seawater carbonate chemistry for the short-term acid-base response to a reduction and increase in seawater pH/ pCO_2 and the metabolic response to a reduction in seawater pH are summarised in Table 4.1 and 4.2 respectively. The carbonate chemistry at Day 0 and 14 of the 14 day variable pCO_2 exposure are summarised in Table 4.3.

Short term responses to changes in seawater pH

Acid-base response and metabolism

As seawater pCO_2 levels increased from 355 µatm to 3974 µatm over the 6 hour experimental period, the pCO_2 of mussel haemolymph increased significantly by 3 fold (figure 4.1a; one-way ANOVA on ranks $F_{12,65} = 3.894$, $P< 0.001$). With no significant increase in haemolymph bicarbonate levels (figure 4.1b; one-way ANOVA on ranks $F_{12,65} = 0.588$, $P = 0.844$) this increase in haemolymph pCO_2 caused a 6.5% decrease in haemolymph pH from 7.58 to 7.09 (figure 4.1c; one-way ANOVA on ranks $F_{12,65} = 0.6993$, $P< 0.001$). The mussels quickly recovered from this acidosis in the following 6 h increase in seawater pH, with haemolymph pH increasing significantly to pH 7.4 (figure 4.1c; one-way ANOVA on ranks $F_{12,65} = 14.679$, $P<0.001$). Both haemolymph bicarbonate (fig 4.1b) and pCO_2 levels (fig 4.1a) also significantly decreased (one-way ANOVA on ranks for $[HCO_3^-]$ $F_{12.65} = 3.054$, $P= 0.02$; and for pCO_2 $F_{12,65} = 8.207$, $P = <0.001$) reaching values comparable to those in ambient (pH 8.10) seawater.

Similarly, respiration rate was also significantly affected by seawater pH (figure 4.1d; one-way repeated measures ANOVA $F_{8,88} = 3.21$, $P < 0.001$) decreasing by 29.8% over a one unit decrease in seawater pH, recovering immediately when exposed to ambient seawater.

Physiological responses to variable to pCO_2

Acid-base physiology

Similar to the response found during a short-term change in seawater pH, haemolymph pCO_2 levels increased by 42.9 % and 48.3 % in treatments pH 7.70 static and fluctuating respectively compared to both pH 8.10 treatments (figure 4.3a; one-way ANOVA $F = 63.424$, $P < 0.001$). Haemolymph bicarbonate levels were significantly higher in both the pH 7.70 treatments, and slightly but non-significantly higher in the fluctuating pH 8.10 treatment (figure 4.3b; one-way ANOVA on ranks $F = 4.032$, $P = 0.049$). High haemolymph pCO_2 values led to a significant acidosis in the haemolymph in both pH 7.70 treatments, decreasing by up to 0.19 units compared to the ambient treatments (figure 4.3c; one-way ANOVA $F = 70.882$, $P<0.001$).

Health parameters

A fluctuating pH regime was found to significantly influence several health parameters. The activity of the antioxidant enzyme SOD within the haemolymph significantly increased by 49.4 % and 44.4 % in the fluctuating pH 8.10 and pH 7.70 treatments respectively, when compared to the pH 8.10 static treatment (figure 4.4a; one-way ANOVA on ranks $F= 15.563$, $P< 0.001$). Neutral red uptake was also significantly higher in both fluctuating treatments, 40.7 % and 43.0 % in the pH 8.10 and pH 7.70 treatments respectively (Figure 4.4b; one-way ANOVA on ranks $F = 30.672$, $P < 0.001$). In both the pH 7.70 treatments, the level of lipid peroxidation increased significantly (Figure 4.4c; one-way ANOVA $F = 7.968$, $P= 0.007$) in addition to a slight but non-significant increase in the pH 8.10 fluctuating treatment (Figure 4.4c; one-way ANOVA $F = 0.000$, $P= 0.987$). Differing from the short-term effect of decreasing seawater pH, there was no significant difference in MO_2 between mussels held a pH 8.10 and pH 7.70 static after 14 days (Figure 4.4d; one-way ANOVA on ranks, $F = 0.76$, $P= 0.388$). However, a fluctuating pH regime significantly increased MO_2 by 38.5 % and 50.3 % in a fluctuating pH 8.10 and pH 7.70 respectively (Figure 4.4d; one-way ANOVA on ranks $F = 19.684$, $P< 0.001$).

4.4. Discussion

There are currently conflicting paradigms regarding the sensitivity of intertidal invertebrates to near-future OA. Some studies suggests that pre-exposure to the inherent variation experienced in intertidal environments has the potential to increase phenotypic plasticity of intertidal organisms, increasing their physiological capabilities to cope with change (Benedetti-cecchi et al. 2006; Johnson et al. 2014). However, an increasing number of studies are proposing the capacity to cope with current environmental conditions does not necessarily translate to a capacity to cope in the future. Intertidal organisms already living close to their physiological limits may have minimal scope for adaptation with increasing variability pushing organisms beyond their physiological tipping points (Aguilera et al. 2013; Evans et al. 2013). Both paradigms currently remain relatively under-tested for both laboratory and field-based studies. Our data clearly shows that exposure to a variable seawater carbonate chemistry elicits a very different set of physiological responses than exposures to static conditions for both ambient pCO_2 and OA exposures in blue mussels, *Mytilus edulis,* collected from an intertidal habitat.

Consistent with other physiological studies, our results show that *M. edulis* have a limited ability to regulate acid-base balance during both short-term (6 h) and medium term (14 day) changes in seawater pCO_2 (Booth et al. 1984; Thomsen et al. 2010; 2013; Heinemann et al. 2012). An increase in seawater pCO_2 caused a direct and immediate increase in haemolymph pCO_2, with levels reaching up to 1816 µatm in the medium term exposure and up to 7200 µatm at the highest seawater pCO_2 value in the short-term experiments. High extracellular pCO_2 values of 1000 µatm to 4000 µatm can facilitate the diffusive excretion of metabolic CO_2 via a steep CO_2 gradient (Melzner et al. 2009). This increase in extracellular pCO_2 has been observed in a number of marine organisms (Pörtner et al. 2004; Spicer et al. 2007) including *M. edulis* where extracellular pCO_2 increased linearly with seawater pCO_2 (Heinemann et al. 2012) up to the highest pCO_2 treatment of 2724 µatm.

The increase in haemolymph pCO_2, uncompensated via an increase in HCO_3^-, led to significant extracellular acidosis during both the short- and medium- term exposures. This acidosis was immediately reversed during reductions in seawater pCO_2. These observations are in agreement with previous studies

reporting *M. edulis* cannot actively avoid acid-base disturbances (Booth et al. 1984; Thomsen et al. 2010; 2013; Heinemann et al. 2012). The short-term extracellular accumulation of HCO_3^- is often reported as an efficient mechanism in stabilising extracellular pH in active marine ectotherms (Pane & Barry 2007; Gutowska et al. 2010). Although we saw an increasing trend in haemolymph HCO_3^- concentration after 14 days, no compensation was observed in extracellular pH. Reductions in extracellular pH can subsequently lead to similar reductions in extrapallial fluid (EPF) pH (Thomsen et al. 2010; Heinemann et al. 2012) resulting in the inner aragonite layer of the shell being exposed to a fluid undersaturated with $CaCO_3$. Despite the secretion of proteins binding to chitinous layers (Suzuki et al. 2009), the reductions of EPF pH in addition to the low free calcium concentration within the EPF (Misogianes & Chasteen 1979) have the potential to leave the site of calcification unprotected under higher pCO_2 levels. In the long-term the inability to compensate extracellular acid-base disturbances therefore has the potential to negatively impact upon growth and calcification under hypercapnia.

In addition to influencing the acid-base status of mussels over short-term exposures, increases in seawater pCO_2 also led to a linear reduction in metabolic rate, with an immediate recovery of oxygen consumption in ambient seawater (pH 8.20). This reduction in oxygen consumption could be driven by a number of mechanisms, such as metabolic suppression in response to the elevated pCO_2 or a reduction in enzyme efficiency under the acidosis conditions of the haemolymph. For example, the reduction of oxygen consumption owing to reductions in extracellular pH was suggested to be a result of an inhibition of net proton transport across the cell membrane in *M. galloprovincialis* (Pörtner et al. 2000; Michaelidis et al. 2005). The reduction of oxygen consumption eliciting metabolic depression in response to acid-base disturbances when exposed to hypercapnia has been suggested in a number of invertebrate taxa (Langenbuch & Pörtner 2002; Rosa & Seibel 2008) with similar decreases observed in adult *M. galloprovincialis* exposed to pH 7.3 for 5 hours (Michaelidis et al. 2005). In addition to the drop in extracellular pH, metabolism may also be influenced by reductions in intracellular pH by inhibiting the activity of enzymes involved (Somero 1985) and favouring the transition of some glycolytic enzymes to less active forms (Brooks & Storey 1997). A combination of these factors producing metabolic depression during a short-term increase in hypercapnia may therefore

be an adaptive strategy to cope under short-term stressful conditions as seen during tidal related exposures to a reduction in seawater pH.

Interestingly, the metabolic response of *M. edulis* to OA treatments over the 14 day exposure differed considerably. Here we observed a significant increase in metabolic rate by 39 % and 50 % in both fluctuating regimes of pH 8.10 and 7.70 respectively with no difference in O_2 consumption measured between the static 8.10 and 7.70 pH treatments. The difference in metabolic rate between the two pH 7.70 treatments may partly be explained via changes in energy demand associated with intracellular acid-base regulation. Although we did not measure intracellular pH in this study, other studies have shown this can be regulated close to control levels under OA stress (Zange et al. 1990; Michaelidis et al. 2005), requiring an enhanced energy driven active ion transport (Deigweiher et al. 2010). This additional energy demand through active ion transport regulating intracellular pH may therefore induce elevated metabolic rates. The increased metabolic demands of extracellular acid-base regulation have been shown to require 50% of metabolic demand for *Alcolapia grahami* living in an high alkaline environment (Wood et al. 2002), suggesting the regulation of intracellular pH also has the potential to increase metabolic demands. As shown in *M. galloprovincialis*, intracellular pH can take a few days to be fully compensated (Zange et al. 1990; Michaelidis et al. 2005) possibly explaining the immediate reduction in oxygen consumption during short-term exposures compared to increases and the maintenance of intracellular pH over a medium-term exposure. The continuous reduction in extracellular pH seen during the static pH 7.7 treatment may also alter the behaviour of ion transporters. This can reduce the rate at which Na^+/H^+- and Na^+- dependent Cl^-/HCO_3^- transporters can exchange H^+ ions, in turn reducing sodium being transported via Na^+/K^+-ATPase diminishing the energy requirements of acid-base regulation (Pörtner et al. 2000). The difference in metabolic rate between a fluctuating and static regime may therefore partially reflect the inability to obtain a steady state in ion transportation, although, testing this hypothesis was beyond the scope of this study.

An elevated stress response during a fluctuating seawater pH/ pCO_2 regime was observed for a number of the stress-related biomarkers within the haemolymph. There was no increase in the production of superoxide dismutase (SOD), an important antioxidant enzyme, in the static OA (pH 7.7) treatment, however there

were similar increases in SOD activity of 49 % and 44 % in the fluctuating pH 8.10 and pH 7.70 treatments, indicating higher levels of oxidative stress in fluctuating compared to stable pH regimes. This is in agreement with both Bibby et al. (2008) and Matozzo et al. (2013) who observed no significant change in SOD production in *M. edulis* and *M. galloprovincialis* respectively when exposed to static OA conditions.

Without the production of SOD catalysing the dismutation of superoxide anions, the antioxidant defence system can become overwhelmed by reactive oxygen species causing oxidative stress (Soldatov et al. 2007), as evidenced in this study. The use of antioxidant systems, such as SOD production and metallothioneins, can help to modulate the longer term damages from lipid peroxidation (Cherian & Chan 1993; Quig 1998) as measured as the production of thiobarbituric acid reactive substances (TBARS). In this study an increase in lipid peroxidation was seen in the OA static and both fluctuating treatments compared to the static ambient (pH 8.10) treatment after 14 days. This occurred despite the increase in SOD levels measured in both of the fluctuating treatments. The interaction of SOD activity and the level of lipid peroxidation has predominately been used in relation to metal toxicity exposure, with the intensification of the antioxidant system limiting the formation of malondialdehyde (measured in TBARS analysis) in *M. edulis* (Géret et al. 2002). This may partially explain the similarity between TBARS production in the fluctuating treatments and the static pH 7.70 treatment, whereby a static seawater pH treatment did not trigger an antioxidant response, which over the 14 days caused any oxidative stress to accumulate, resulting in an increase in lipid peroxidation.

In addition to SOD and TBARS production, neutral red (NR) uptake was also used to assess overall organism health (Lowe et al. 1995). The NR assay is based on the ability of viable cells to incorporate and retain the dye within the lysosomes, which are central to many biological processes. Our results showed no significant difference between NR uptake in both static pH treatments, however, a significant elevated response in both fluctuating treatments. This response to static OA exposures is dissimilar to Beesley et al. (2008) who found *M. edulis* exposed to seawater pH of 7.80, 7.60 and 6.80 for 60 days showed a significant reduction in NR retention. However, the authors' only state pH had an effect on NR retention and therefore it is difficult to compare NR uptake at a

similar seawater pH. The increased uptake and retention of NR in fluctuating treatments may again be explained via the up-regulation of antioxidant enzymes (SOD production) translating into an intensification of the immune system and therefore decreasing the permeability of the lysosome membrane. As the lysosomes store hydrolytic enzymes involved in intracellular degradation (Winston et al. 1996) this would prevent activation and possible release of these enzymes into the cytoplasm which can cause cytolytic damage (Bayne et al. 1985). This however does appear to come at an energetic cost as shown via the significant increase in metabolic rate under both fluctuating regimes. This may be negligible under conditions of high food availability, however, if food becomes limiting these physiological costs may divert energy away from reserves, with the potential to reduce general fitness, reproductive output and ultimately survival (Stoeckmann & Garton 2011).

Overall our study highlights the need to consider the natural environmental conditions experienced in order to reliably inform predictions of future responses to climatic changes. With seawater pH fluctuations expected to increase in intensity and frequency over the coming decades (IPCC 2014), it is import to understand how intertidal organisms respond to current variability in order to predict responses with an additional OA signal. Our data clearly shows different physiological responses of organisms exposed to a fluctuating compared to a static seawater pH/ pCO_2 regime. In agreement with other studies, we support the finding that *Mytilus edulis* are unable to regulate extracellular acid-base disturbances (Booth et al. 1984; Thomsen & Melzner 2010; 2013; Heinemann et al. 2012), with acid-base parameters following that of the seawater conditions over both short- and medium- term exposures. In addition, analysis of several health parameters suggests exposure to a fluctuating pH/ pCO_2 regime may be more stressful for intertidal organisms than static pH/ pCO_2 stimulating antioxidant systems. This in turn can limit long-term damage as well as enhance cell viability. However this comes at an energetic cost, indicated by significant increases in metabolic rate, which have the potential to negatively affect growth, survival and reproduction in a food limiting environment. Furthermore our results suggest a more careful examination of the predicted responses of *M. edulis* and other intertidal species are needed, in order to suitably predict organism responses to future OA conditions.

Table 4.1: Seawater carbonate chemistry for the short-term acid-base response to a reduction in seawater pH for *Mytilus edulis*.

Time (hours)	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
0.0	13.5	8.12	32.3	2250.5	2495.5	355.4	2.054	182.5
0.5	13.5	8.06	32.3	2297.3	2512.8	420.5	2.117	163.8
1.0	13.4	7.98	32.3	2308.3	2484.0	513.2	2.150	137.9
1.5	13.5	7.90	32.3	2332.7	2474.8	629.0	2.190	117.3
2.0	13.6	7.81	32.3	2390.9	2499.7	799.2	2.261	98.8
2.5	13.5	7.72	32.3	2403.3	2477.7	991.8	2.282	80.7
3.0	13.5	7.64	32.3	2415.6	2464.6	1202.0	2.300	67.7
3.5	13.5	7.55	32.3	2381.5	2401.7	1458.8	2.269	54.3
4.0	13.5	7.45	32.3	2394.7	2384.0	1843.8	2.278	43.3
4.5	13.5	7.38	32.3	2432.6	2399.2	2194.7	2.308	37.3
5.0	13.5	7.24	32.3	2490.0	2407.5	3072.3	2.341	27.4
5.5	13.5	7.17	32.3	2447.4	2340.6	3523.4	2.285	22.8
6.0	13.5	7.12	32.3	2474.6	2346.5	3973.6	2.297	20.4

Table 4.2: Seawater carbonate chemistry for the short-term acid-base response an increase in seawater pH for *Mytilus edulis*.

Time (hours)	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
0.0	13.5	7.05	32.0	2297.5	2150.3	4296.8	2.111	15.8
0.5	13.5	7.13	32.0	2271.4	2157.6	3572.9	2.110	19.0
1.0	13.5	7.2	32.0	2230.1	2143.5	3009.4	2.088	22.1
1.5	13.5	7.27	32.0	2269.1	2204.2	2622.6	2.138	26.6
2.0	13.5	7.35	32.0	2249.8	2210.5	2174.5	2.132	31.9
2.5	13.5	7.45	32.0	2209.2	2200.1	1703.3	2.102	39.6
3.0	13.5	7.55	32.0	2204.9	2224.5	1352.5	2.101	49.9
3.5	13.5	7.65	32.0	2148.8	2197.6	1046.2	2.046	61.1
4.0	13.5	7.74	32.0	2159.7	2236.6	852.2	2.050	75.4
4.5	13.5	7.83	32.0	2128.5	2235.1	679.2	2.011	90.9
5.0	13.5	7.88	32.0	2131.8	2256.9	604.0	2.006	101.8
5.5	13.5	7.95	32.0	2103.4	2255.0	503.8	1.966	117.2
6.0	13.5	8.04	32	2087.9	2278.5	402.1	1.930	141.6

Table 4.3: Seawater carbonate chemistry for the short-term metabolic response to an increase in seawater pH for Mytilus edulis.

pH treatment	Temperature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
8.15	12.9	8.15	31.6	2083.8	2322.3	306.1	1.898	173.4
8.06	12.9	8.06	31.6	2248.8	2451.7	412.3	2.078	154.3
7.90	12.9	7.90	31.6	2243.3	2374.8	605.3	2.110	108.5
7.80	12.9	7.80	31.6	2300.0	2395.7	786.9	2.179	89.0
7.70	12.9	7.70	31.6	2311.2	2373.3	999.8	2.199	71.3
7.56	12.9	7.56	31.6	2391.2	2409.9	1430.6	2.280	53.5
7.40	12.9	7.40	31.6	2409.3	2378.6	2075.3	2.288	37.2
7.29	12.9	7.29	31.6	2376.3	2311.0	2619.5	2.242	28.3
7.16	12.9	7.16	31.6	2453.5	2338.2	3603.3	2.286	21.4
8.20	12.9	8.20	31.6	2384.6	2677.6	309.3	2.152	220.6

Table 4.4: Seawater carbonate chemistry from the 14 day variable pCO_2 exposure at the day 0 ([1]) and day 14 ([2]). Values represent the mean ± SD.

pH treatment	Temp-erature (°C)	pH $_{NBS}$	Salinity	TCO_2	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (mM)	CO_3^{2-} (µmol kg^{-1})
pH 8.10 Static[1]	13.2	8.14	31.7	2144.7 ±8.9	2385.8 ± 9.6	323.2 ± 1.3	1.955 ± 0.008	177.1 ± 0.7
pH 8.10 Static[2]	13.2	8.11	30.1	1975.1 ±33.6	2177.2 ± 35.9	324.1 ± 5.5	1.815 ± 0.031	147.3 ± 2.5
pH 7.70 Static[1]	13.2	7.69	31.6	2275.3	2335.1	1009.4	2.165	69.4
pH 7.70 Static[2]	13.2	7.69	30.4	2081.3 ±120.9	2134.7 ± 122.3	929.5 ± 54.0	1.982± 0.115	61.6 ± 3.6
pH 8.10 Fluc[1]	13.2	7.68	32.1	2156.1	2213.1	976.6	2.052	65.1
pH 8.10 Fluc[2]	13.2	8.09	30.4	1969.4 ±9.1	2163.6 ± 9.7	338.7 ± 1.6	1.814 ± 0.008	141.7 ± 0.7
pH 7.70 Fluc[1]	13.1	7.29	32.1	2557.4	2488.3	2817.3	2.413	31.1
pH 7.70 Fluc[2]	13.2	7.70	30.0	2370.3 ±35.2	2428.8 ± 35.6	1036.6 ± 15.4	2.257 ± 0.034	71.1 ± 1.1

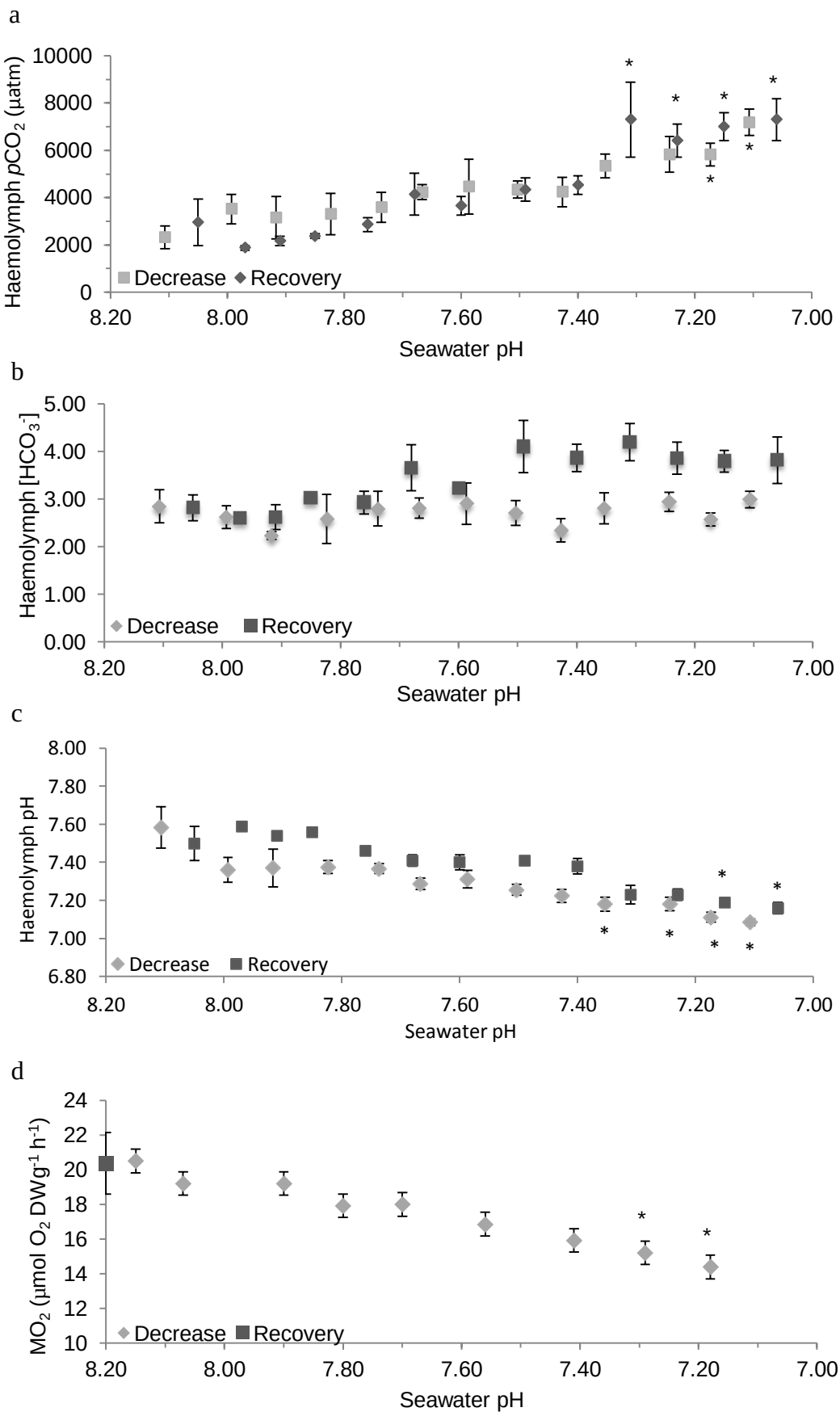

Figure 4.1: Acid-base parameters in the haemolymph (a, b, c) (n= 6 per time point) and metabolic rate (d) (n = 12) of *Mytilus edulis* over a 6 hour gradual exposure to increasing (a, b, c) or decreasing seawater pH (a, b, c, d).

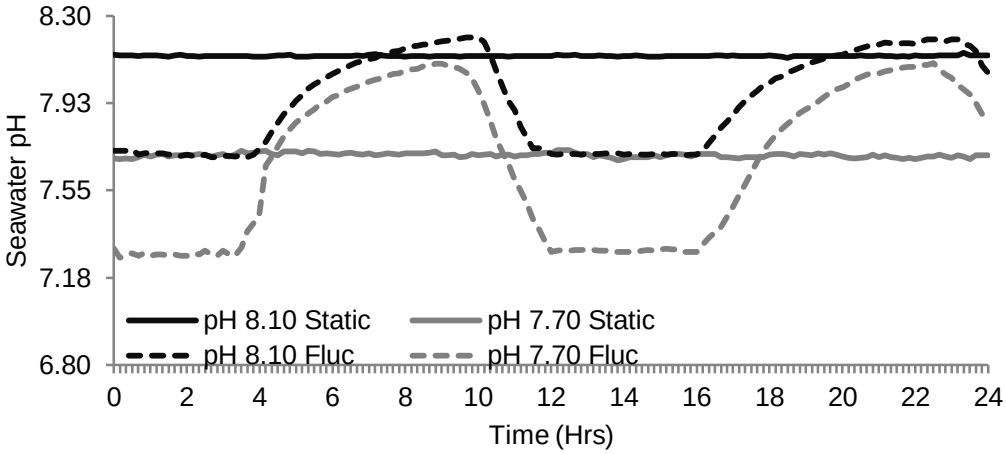

Figure 4.2: The mean seawater pH cycles for all four treatments over 24 hours.

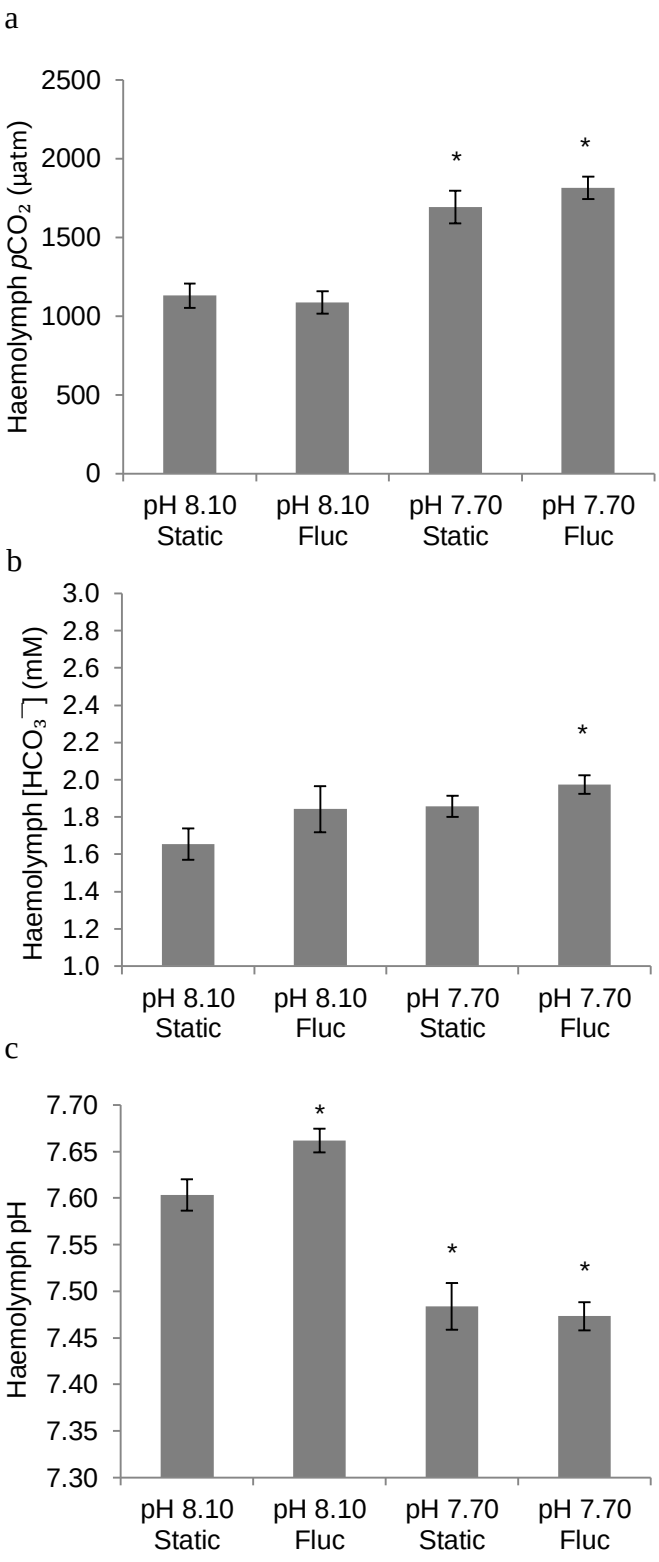

Figure 4.3: Acid-base parameters in the haemolymph of *Mytilus edulis* (n = 16) following a 14 day exposure to elevated pCO_2 with and without the addition of a cyclic seawater pH regime; (a) haemolymph pCO_2, (b) haemolymph bicarbonate concentration ($[HCO_3^-]$) and (c) haemolymph pH. * Represents significant differences from the static pH 8.10 treatment.

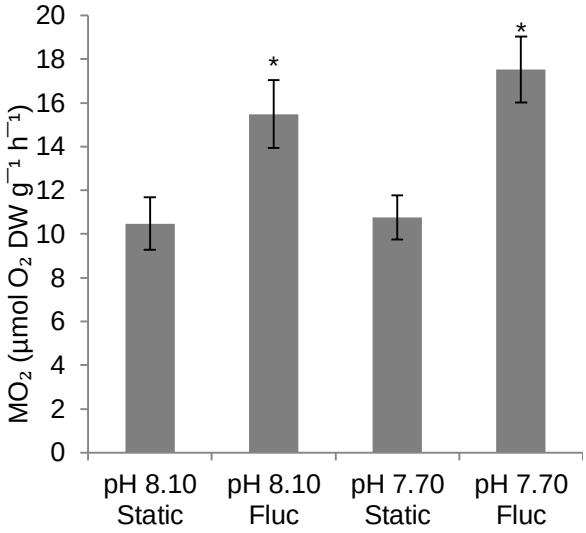

Figure 4.4: The metabolic rate of *Mytilus edulis* following a 14 day exposure to elevated pCO_2 with and without the addition of a cyclic seawater pH regime. * Represents significant differences from the static pH 8.10 treatment.

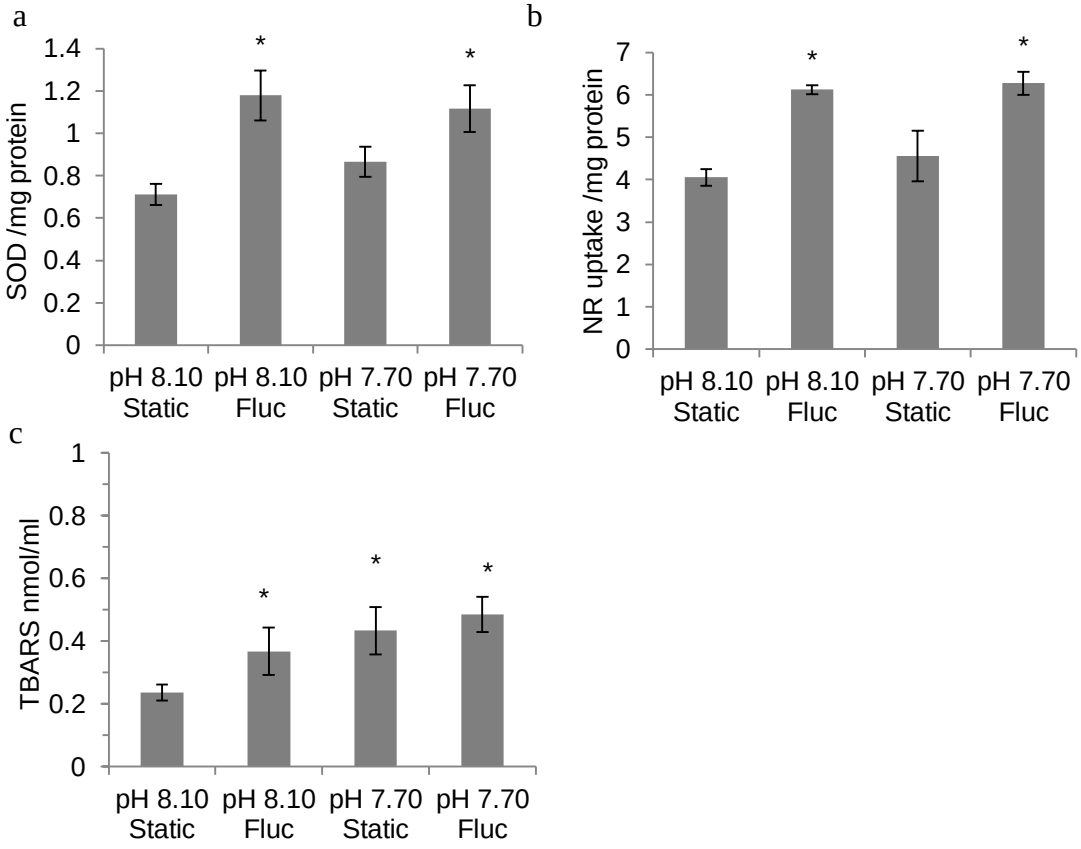

Figure 4.5: Health indicators in the haemolymph of *Mytilus edulis* following a 14 day exposure to elevated pCO_2 with and without the addition of a cyclic seawater pH regime; (a) activity of the anti-oxidant enzyme superoxide dismutase (SOD), (b) cell viability measured as neutral red uptake and (c) activity of Thiobarbituric Acid Reactive Substances (TBARS). * Represents significant differences from the static pH 8.10 treatment.

Chapter 5: Discussion

Global climate change is already causing widespread concern for the future of the world's oceans, as increased atmospheric carbon dioxide (CO_2) concentration can lead to a rise in sea level, surface and air temperatures and ocean acidification (OA) at a rate not experienced for at least 400,000 years (Doney & Schimel 2007; Lüthi et al. 2008; Doney et al. 2009; Kleypas & Yates 2009; Bopp et al. 2013). The majority of research to date has focused on the effects of OA and temperature which suggests future climate change has the potential to negatively impact physiology, growth and reproduction across a wide range of marine fauna (Pörtner et al. 2004; Portner & Knust 2007; Fabry et al. 2008; Sarà et al. 2011; Wittmann & Pörtner 2013). Owing to the novelty of this environmental change, the majority of research to date investigates the impacts of OA and temperature as single stressors. This helps to identify physiological and demographic changes within populations however, even with this understanding, the interaction of multiple stressors on both individual organisms and communities can be difficult to predict (Crain et al. 2008; Zippay & Helmuth 2012). Furthermore, the effects of climate change on community structure can often be dependent on local adaptations and acclimatisation of local populations (Kuo & Sanford 2009) which has the potential to indirectly influence trophic cascades and intra- and inter- species competition (Edwards & Richardson 2004; Pincebourde et al. 2008; Broitman et al. 2009).

The result of these challenges has led for a need to use an interdisciplinary approach incorporating field-based methods, statistical models and experimental work (Brown et al. 2011; Burrows et al. 2011). Moreover it can be beneficial to consider model species with high ecological importance where considerable information is known about their physiology, morphology and behaviour (Monaco & Helmuth 2011; Zippay & Helmuth 2012). This study focuses on two different mussel species; *Mytilus edulis* inhabiting the Southwest of England and *Perumytilus purpuratus* inhabiting the Chilean coast. Mussels are considered to be important ecosystem engineers as mussel beds increase habitat complexity and provide shelter from predation, promoting greater species richness (Arribas et al. 2014). In addition mussels provide vital ecosystem services such as water purification by filtering phytoplankton and other particles which act to increase the penetration of light as well as providing a food source for several species (Asmus

& Asmus 1991; Gutierrez et al. 2003; Gazeau et al. 2013). This additionally gives them a high economic importance with the UK alone harvesting just over 26,000 tonnes in 2012 which was worth an estimated £27 million (Ellis et al. 2012).

The overarching aim of this body of research was to investigate the role of natural environmental variability in determining physiological responses of mussels to future predicted OA. Although there is substantial knowledge of the *Mytilus* genus (Bayne et al. 1976; Gosling 1983; Seed & Suchanek 1992; Zippay & Helmuth 2012), there are still significant knowledge gaps in our understanding of the environmental variability currently experienced and how mussels respond to these fluctuations, which ultimately limits our ability to inform predictions of future outcomes. This project incorporates both field-based and experimental work to investigate the role natural variability may have on influencing physiological responses of important mussel species. I focused on the physiological impacts, primarily acid-base change, as the maintenance of cellular homeostasis can play an important role in the survival and distribution of a species (Pörtner & Farrell 2008; Piero Calosi et al. 2013). The aims were achieved by testing three hypotheses:

H1: pH/ pCO_2 is the main driver of acid-base change over a tidal cycle

H2: organisms living in habitats with variable pCO_2 will have a greater resilience to OA

H3: a variable pH/ pCO_2 regime will incur greater physiological costs than a stable pH/ pCO_2 regime.

To test hypothesis 1 I used field and laboratory work to highlight the current environmental variability experienced and the drivers of acid-base change during a complete tidal cycle. This has not previously been investigated using marine mussels, with the majority of studies neglecting the emersion phase. Although I did not sample in a bloom period or mid-winter, my seawater carbonate chemistry data reveals the differences in abiotic conditions experienced by different populations of *M. edulis*, with those from Port Gaverne periodically experiencing seawater pCO_2 levels predicted under future OA for the stable open ocean (Dlugokencky & Tans 2016). More interestingly, my results clearly demonstrate that size (shell length) and temperature are the predominant drivers of acid-base change during emersion. Irrespective of prior immersion in low seawater pH

conditions, elevations in temperature had an increasing influence on acid-base balance, with size playing a crucial role in moderating these disturbances at increasingly higher temperatures. These results therefore suggest that the conditions encountered during the emersion phase, not pH/ pCO_2 during immersion, may have the potential to cause greater physiological stress under increasing temperature extremes in the future. Furthermore during re-immersion, pH/ pCO_2 exposure drives the acid-base response independent of previous conditions experienced during emersion. We therefore reject our first hypothesis on the basis that pH/ pCO_2 appears to be the main driver of acid-base change during periods of immersion only, and not the main driver throughout the whole tidal cycle.

Following this work, I looked to test hypothesis 2 by investigating if the regular exposure to elevated seawater pCO_2 during upwelling events can alter the influence pCO_2 has on the acid-base response of mussels during immersion. Similar to the previous study, fieldwork from Dichato, central Chile reveals extracellular acidosis was greater for *P. purpuratus* during periods of emersion compared to immersion at high tide. Furthermore, my results demonstrate that the acid-base response of *P. purpuratus* and *M. edulis* are not influenced by the natural environmental conditions experienced. An increase in seawater pCO_2 caused an immediate increase in haemolymph pCO_2 resulting in significant extracellular acidosis in both populations of *P. purpuratus* regularly experiencing upwelling events and both *M. edulis* populations. The similarity in response suggests that the acid-base regulatory abilities of these marine mussels may have a limited capacity to adapt to changes in environmental conditions, such as the regular exposure to elevated seawater pCO_2. Most interesting, size appeared to influence the acid-base response to short-term changes in seawater pCO_2, with smaller mussels experiencing a reduced extracellular acidosis compared to larger mussels. This combined with my previous study adds to further evidence suggesting size is an important driver of acid-base change throughout a tidal cycle. I therefore reject my second hypothesis on the basis that the regular exposure to elevated seawater pCO_2 did not appear to influence the acid-base response of both *M. edulis* and *P. purpuratus* suggesting these species' may be vulnerable to future predicted OA. However, more research is needed to support this statement, in particular the acid-base response of other populations living in

elevated seawater pCO_2 conditions and an understanding of the mechanisms behind acid-base change in marine mussels.

So far my project has provided further evidence to suggest mussels, in particular *Mytilus edulis* and *Perumytilus purpuratus*, have a limited ability to regulate acid-base disturbances (Booth et al. 1984; Heinemann et al. 2012; Thomsen et al. 2010; 2013) over short-term changes in seawater pCO_2. However the possible cost of being unable to regulate these disturbances on both energy budgets and organism health is still unknown. Over 14 days, my data clearly shows that exposure to either a static or fluctuating pH/ pCO_2 regime can elicit different physiological responses in *M. edulis*. In support of my previous studies, *M. edulis* were unable to regulate acid-base balance to either a fluctuating or static pH/ pCO_2 regime. Although mussels do not appear to incur the energetic costs of acid-base regulation, a variable pH/ pCO_2 regime did significantly increase metabolic rate suggestive of higher energetic demands. This may be a result of the significant up-regulation of the antioxidant system as evidenced by an increase in superoxide dismutase (SOD) production. Surprisingly, these physiological responses were unaffected by the magnitude of pH/ pCO_2 fluctuation. These results therefore provide evidence to suggest a variable pH/ pCO_2 regime will incur greater physiological costs than a stable pH/ pCO_2 regime providing evidence to accept the third hypothesis. However, this is one of the first studies to investigate the impacts of a stable versus a variable pH/ pCO_2 regime therefore more research is needed to understand the longer-term impacts. In particular, to incorporate the emersion phase of a tidal cycle and to understand the influence of organism size.

The difference in response between static and variable pH/ pCO_2 regimes highlights the importance of experimental design when investigating the responses of marine biota to predicted future OA conditions. This suggests the current use of stable open ocean pH/ pCO_2 values may not accurately reflect potential outcomes in the future for coastal organisms living within a highly variable environment. However, environmental conditions are often location specific therefore the limited amount of field data restricts our ability to modify OA experimental design in addition to extrapolate any potential adaptation responses. This can be overcome either by snap shot data such as that collected during this study or through the deployment of pH sensors creating high

resolution time-series data. The methods used in this study crucially allow for the assessment of physiological responses increasing the ability to reliably extrapolate the results as well as provide realistic parameters for laboratory experiments. The culmination of these factors makes these methods labour intensive and therefore they may not always be suitable to combine with other experimental studies. An alternative has been used by Hofmann et al. (2011) whereby a pH sensor was deployed creating high resolution time-series data. While providing much needed coastal carbonate chemistry data, these sensors cannot provide data during the emersion phase of a tidal cycle making them of prominent use for subtidal studies.

Environmental variability is predicted to intensify under increases in atmospheric CO_2, with OA, warming and other climatic changes expected to exacerbate intertidal extremes (IPCC 2014). If mussels are unable to adapt to elevated pH/ pCO_2 and alter acid-base regulatory mechanisms, as suggested in this study, an increase in pH/ pCO_2 variability may therefore lead to a rise in energetic cost as climate change progresses. This may additionally be exacerbated at higher aerial temperatures during emersion as thermal stress related proteins are synthesised (Place et al. 2008). This therefore has the potential to move coastal organisms closer to pose significant physiological stress and push organisms closer to their physiological limits (Menge & Sutherland 1987).

This highlights the potential for a variable environment to be more energetically costly than a stable environment over a medium-term exposure, however the effects over the longer-term, especially with the inclusion of emersion cycles, and at population level are still largely unknown. However, in an environment where food is abundant, an increase in energetic cost is unlikely to significantly compromise organism performance (Thomsen et al. 2013). However, in a food-limiting environment a shift in the energy budget could result in modifications in mussel abundance and distribution, which could potentially have a cascading effect on food web dynamics. As suggested in this project, a trade-off between growth and organism size could be one possible solution to an increase in energetic cost. A reduction in organism size would reduce whole animal energy demand while increasing mass specific demand of essential processes (Garilli et al. 2015). This appears to moderate the acidosis experienced during both emersion at low tide and during short-term changes in seawater pCO_2.

Reductions in size however, are unlikely to be benign but could possibly alter the ecosystem services that these species' provide. For example, this could lead to a change in farming practices, such that more mussels will be kept subtidally.

Increased variability could also lead to alterations in range shifts or reduce the occurrence of intertidal mussel beds. Mussel distributions are often mosaics rather than latitudinal gradients (Helmuth et al. 2006) making future distributions difficult to predict. However a reduction in the vertical distribution of *Mytilus californianus* by 51% has already been observed over the last 50 years (Harley 2011), suggesting similar changes may be possible for other mussel species'. In addition to range shifts, as aerial temperatures increase there may be a pressure for intertidal mussels to reduce the time exposed at low tide and to consequently move towards the lower shore. This again would likely incur a trade-off against predation while reducing its potential as a food source, resulting in changes in food web and competition dynamics.

Overall this study accentuates the complex nature of intertidal habitats and the necessity to consider an organism's natural environmental conditions when designing future research. This study highlights key avenues for future research such as a need for more field data, an understanding of the mechanisms behind differences in acid-base balance and the role size can play in potentially moderating higher energetic costs. Furthermore our results suggest a more careful examination of the predicted responses of *M. edulis* and other intertidal species are needed, in order to reliably inform predictions of organism responses to future OA conditions.

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