The Combined Impacts of Ocean Acidification and Copper on the Physiology of European Sea Bass (*Dicentrarchus labrax*) and Shore Crabs (*Carcinus maenas*)

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Thesis Abstract

The following thesis explores the physiological effects on European sea bass (*Dicentrarchus labrax*) and shore crabs (*Carcinus maenas*) resulting from the dissolution of anthropogenic carbon dioxide (CO_2) into seawater: known as ocean acidification. It assesses how ocean acidification, characterised by elevated seawater pCO_2 (1200 µatm) and lowered pH (~7.7), affect the internal chemistry of these animals through the homeostatic process of acid-base regulation. Control conditions used for comparison were close to current ocean average values for CO_2 (~400 µatm) and pH (8.2). The proficiency and magnitude of these compensatory mechanisms was explored. Both sea bass and shore crabs were found to be highly effective acid-base regulators and employed the same strategy to compensate the hypercapnia-induced respiratory acidosis: namely an elevation of extracellular bicarbonate (HCO_3^-).

It then considers how these regulatory mechanisms both affect, and are affected by, simultaneous exposure to a ubiquitous coastal metal contaminant, copper. Evidence for a hitherto undocumented protective effect of elevated HCO_3^- against copper-induced DNA damage was found to be afforded to both sea bass and shore crab cells. DNA damage was used as a sensitive toxicity marker and blood cells were used as proxies for other internal tissues. Erythrocytes exposed *in vitro* (2 h) to copper (45 µg/L) showed significant DNA damage under control [HCO_3^-] (6 mM) but were completely protected when exposed under high [HCO_3^-] (12 mM). A similar protective effect was apparent in crabs under *in vivo* exposure (14 d) to 10 µg/L waterborne copper. Conversely, during exposure to higher waterborne copper concentrations (sea bass: 80 µg/L, shore crabs: 40 µg/L), animals showed a severe or total inhibition of acid-base regulatory ability in the face of simultaneously elevated seawater CO_2 (1200 µatm).

The downstream effects of longer-term (28 d) exposure to high CO_2 and copper, both individually and in combination was assessed. Food conversion efficiency (FCE), growth and copper accumulation were quantified in juvenile sea bass as economically relevant endpoints. Growth and FCE remained unaffected by either stressor and copper was not accumulated in the muscle tissue: pertinent to human consumption. As a bi-product of this longer term study assessment of gut calcium carbonate production rates in these animals was possible, providing some of the first evidence

of excretion rates in fish fed on naturally high calcium diets. A directly proportional influence of feeding rate on gut carbonate excretion rates as a result of increased dietary calcium was observed, and novel evidence provided of the proportional contribution of dietary and seawater calcium to excreted carbonate. Both findings have considerable application to global models of fish contribution to the oceanic carbon cycle.

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INTRODUCTION

1. Ocean Acidification

1.1 Global perspective

The last 250 years has seen the rapid proliferation of industry, in combination with the advent of both automotive and aeronautic transport. The almost complete reliance of such industries on fossil fuels as a source of readily available energy has had a profound effect on the composition of the atmosphere and the oceans. This industrial revolution, in combination with widespread deforestation to meet the space and food demands of the growing global population has resulted in net anthropogenic carbon dioxide (CO_2) emissions at unprecedented rates.

Atmospheric CO_2 is perhaps most often considered for its influence on global temperatures due to the greenhouse effect, whilst its impact on ocean chemistry is more often overlooked by the media and wider population.

1.2 Chemistry of Ocean Acidification

Given that 71 % of the earth's surface is covered by water, it is no great surprise that vast amounts of the CO_2 released into the atmosphere will enter the oceans and other bodies of water. CO_2 is absorbed by the oceans because gas exchange occurs between ocean surface water and the atmosphere as the two strive to reach equilibrium (Archer & Rahmstorf, 2010). Therefore as atmospheric levels of CO_2 increase, seawater pCO_2 will consequently also rise.

Prior to the industrial revolution estimates suggest that the influx of CO_2 (70.6 Gt/C/yr) was not substantially different from the efflux (70 Gt/C/yr) (Solomon et al., 2007). However, anthropogenic emissions now impose a considerably increased influx of CO_2 to these systems, meaning the oceans are a net sink for carbon. It is estimated that the oceans have absorbed around 25% of all anthropogenically released CO_2 since the year 1800 (Sabine et al., 2004).

The dissolution of CO_2 in seawater causes a decrease in pH as CO_2 reacts with water to form carbonic acid ($H_2CO_{3(aq)}$). This intermediate readily dissociates an H^+ ion, thereby reducing the water's pH: the inverse log of $[H^+]$ (Kleypas et al., 1999). This reduction in pH is termed 'ocean acidification' (OA). The equilibrium is represented by equation 1:

Equation 1: $CO_{2(aq)} + H_2O_{(l)} \leftrightarrow H_2CO_{3(aq)} \leftrightarrow HCO_3^-{}_{(aq)} + H^+_{(aq)}$

Besides reducing the pH, the dissolution of CO_2 results in an increase in concentrations of bicarbonate ions $[HCO_3^-]$ and dissolved inorganic carbon (DIC). Conversely it lowers the concentration of carbonates ions $[CO_3^{2-}]$ and the saturation states of the three primary biogenic carbonates employed by marine animals for production of shell and skeleton (aragonite, calcite and high magnesium calcite). As such the effects of OA on calcifying organisms has received a great deal of attention (Hoegh-Guldberg et al., 2007; Orr et al., 2005; Wood et al., 2008).

Proxy data from a variety of sources gives us reliable evidence that atmospheric CO_2 concentrations ranged between 200 and 280 µatm for the 400,000 yr prior to the industrial revolution (Feely et al., 2004). CO_2 levels have been carefully documented since the 1950's showing that the rate of atmospheric CO_2 rise is accelerating (Archer & Rahmstorf, 2010), and recently exceeded 400 µatm (NOAA, 2015).

The rate at which we are adding CO_2 into the atmosphere is of primary importance to ongoing and projected changes to ocean chemistry. Caldeira (2003) suggest that the time over which atmospheric CO_2 concentrations change is critical in determining the oceans ability to buffer potential changes in water chemistry. Given sufficient time carbonate minerals in the ocean are able to buffer the influence of the additional CO_2, lessening sensitivity to pH changes (Zeebe, 2012). As such, even under high partial pressures (>7500 µatm) the ocean pH is not thought to have dropped more than 0.6 units below the current pH of 8.0-8.2 for the last 300 Myr, with the possible exceptions of rapid changes following bolide impacts and methane degassing events (Caldeira, 2003).

Although current atmospheric CO_2 concentrations are only around 400 µatm, nowhere near the values found in the geological records, recent and predicted near-future increases are occurring over a vastly shorter time period. For this reason, pH changes are expected to be far more significant than might be expected based on modelled

atmosphere/ocean pH relationships from the past. More importantly, the rate of change will have severe implications on the ability of organisms to adapt or evolve to the changing marine environment.

Recent projections suggest that atmospheric pCO_2 concentrations may exceed 1200 µatm by the beginning of next century (IPCC, 2014), with consequent reductions in open ocean pH to as low as 7.73 (Bao et al., 2012).

It should be noted that environmental hypercapnia is not a phenomenon limited only to projected future changes to water chemistry resulting from climate change. High CO_2 conditions regularly occur in coastal and estuarine waters (Burnett, 1997) as well as intertidal rock pools (Truchot, 1986) as a result of respiratory oxygen (O_2) consumption and CO_2 release by aquatic organisms. As such the findings presented in the following chapters and much of the literature considered there-in can also be applied to fish and crustaceans experiencing such conditions as part of their current life history.

2. Ocean Acidification and Marine Animals

Ocean acidification is now recognised as a major threat to marine ecosystems across the planet (Fabry et al., 2008a). However, despite rapidly expanding research in this field many of the impacts on marine organisms are still not well understood (Dixson et al., 2010). Research on reduced seawater pH/high CO_2 has demonstrated a suite of effects on marine organisms including: acid-base regulation (Hayashi et al., 2004; Pörtner et al., 1998; Toews et al., 1983), calcification (Doney et al., 2009; Hoegh-Guldberg et al., 2007; Orr et al., 2005), metabolism (Franke, 2011; Pörtner et al., 2004; Pörtner et al., 2004; Small et al., 2010) , growth (Berge et al., 2006), immune function (Bibby et al., 2008), and behaviour (Dixson et al., 2010; Munday et al., 2009; Domenici et al,. 2011 .

However, as research in this field develops we are beginning to appreciate that there is no great consistency in the impacts that ocean acidification is having on any given physiological process (such as growth, reproduction, behaviour), across species, or even between the life-stages or the same species. Furthermore we are also realising that other environmental perturbations, such as salinity, temperature or the presence of contaminants, can drastically affect an animal's response to high CO_2.

Contradictory findings are increasingly common across much of the work investigating the impacts of OA on marine organisms. This may be a result of the complex interactions between physiological processes, each competing for the organisms energetic resources (Wood et al., 2008). For example the energetic cost of regulating internal acid-base balance in a high CO_2 environment could have knock-on effects for immune function, growth, reproduction and other important physiological functions and processes.

The complex and species-specific responses reported make it very difficult to generalise and therefore to predict the impacts that future elevations in seawater pCO_2 levels will have on a particular organism in a given context.

Debate also exists on whether there will even be significant ecological ramifications of ocean acidification. Hendriks et al. (2010) conducted a meta-analysis of the impacts of elevated pCO_2 on a diverse spectrum of marine biota. They suggested that organisms may be more resistant to the effects of ocean acidification than first thought (within the projected oceanic pCO_2 concentrations for the 21st century). It may be that existing temporal and spatial pH variability provides sufficient tolerance for near-future changes and that this will be further strengthened by acclimatization, migration, short and long term adaptation (Fabry et al., 2008; Hendriks et al., 2010)) . However, given the current rate of changes to surface water chemistry, many of these processes may be too slow to protect populations.

2.1 Research To-Date

A growing body of research suggests that the increases in seawater pCO_2 are often equally if not more important than decreases in pH when considering the impacts on the physiology of marine organisms. This is particularly true for those animals that can regulate their internal pH such as teleost fishes and most crustaceans. This is well evidenced by a comparative study by Ishimatsu et al. (2004) showing that CO_2-enriched seawater was significantly more toxic to silver sea bream (*Pagrus major*) than water acidified to the same extent by the addition of mineral acid (HCl).

As such, it is more prudent to talk about the effects of 'high CO_2', 'hypercapnia' or 'hypercarbia' rather than pH. Despite the rapidly expanding body of research, relatively little is known about the effects that rising seawater CO_2 and decreasing pH will have on marine fishes (Caldeira, 2003; Munday et al., 2009) and perhaps even less about

crabs. One of the problems is that a great deal of the earlier work done on high CO_2 did not consider near-future CO_2 levels. Prior to our characterization of ocean acidification and awareness of its potential impact on marine organisms, most work to study hypercapnia and physiology employed more extreme pCO_2 exposures: up to and above 1 % CO_2 (10,000 µatm). This limits the application of this work to understanding and predicting organism, population or ecosystem level effects of near-future CO_2 levels (Fabry 2008). This research does however have some application when considering the potential acute effects of more extreme hypercapnic conditions, for example leaks from deep carbon capture aquifers or even severely hypercapnic conditions resulting from poorly managed recirculating aquaculture facilities.

Very little research has been done on the lethality of extreme high CO_2 in teleosts. At extreme CO_2 partial pressures fish will become anaesthetized and will eventually die. Lethality is highly species dependent, but for our primary study species, European sea bass, the LC_{50} values are 68 and 62 matm for 48 and 120 hr exposures respectively (Grottum and Sigholt 1996).

The impacts of high CO_2 on teleost fish and crustaceans are considered below with a focus on physiological effects pertinent to the research presented in this thesis: namely acid-base regulation and the influence of high CO_2 on growth.

2.1.1 Acid-base Balance

One of the primary impacts that increased environmental CO_2 concentrations will have on fishes and crustaceans is the short term disruption of their internal acid-base status. Due to the close contact of seawater and blood across the large surface area of the gill, and the permeability of that gill to gases such as CO_2, their internal pCO_2 is directly influenced by external perturbations. As such, elevations in seawater pCO_2 would result in a respiratory acidosis of the intra- and extra- cellular fluids if left unabated. Fish and crustaceans acid-base regulate in order to compensate this respiratory acidosis. Due the relevance of this homeostatic mechanism the work contained within this thesis acid-base regulation is considered here in some detail.

2.1.1.1 Acid-base regulation

Acid-base regulation strives towards maintaining physiologically optimum internal pH, even when subjected to environmental changes (Claiborne, 1998). Both fish and crustaceans compensate for intracellular pH (pH_i) and extracellular pH (pH_e) perturbations through the dual approaches of: a) buffering using molecules already present in the tissues and, to a much greater extent, b) ion exchange between the animal and environment (Claiborne, 1998; Henry & Wheatly, 1992).

Buffering

Buffers are molecules that are able to accept or give up H^+ ions, thereby lessening any potential change in the pH of a solution. They represent an effective means of controlling transient acid-base disturbances. Buffers enable maintenance of pH_i/pH_e within a range suitable for continued functioning of vital processes such as metabolic activity during the period between the introduction of excess acid or base ions and their excretion (Heisler, 1986). These molecules can be broadly divided into non-bicarbonate buffers (Nbb) and bicarbonate buffers.

Non-bicarbonate buffers (Nbb): Non-bicarbonate buffers, such as proteins (including haemoglobin), amino acid residues (histidine, cysteine and $-NH_2$ terminal groups), as well as inorganic and organic phosphates, play a significant role in the maintenance of pH_i, and to a lesser degree pH_e, in fishes (Heisler, 1986). As fish cells typically contain higher levels of Nbbs, particularly protein residues and consequently are afforded a 10% better buffering capacity when compared to extracellular fluids (Claiborne et al., 2002). Unlike fish the respiratory pigment of crabs (haemocyanin) is not kept within blood cells, meaning the buffering capacity of these high molecular weight proteins can be utilized to maintain extracellular rather than intracellular pH (Henry & Wheatly, 1992; Truchot, 1983). Conversely this also means haemocyanin is not therefore protected from any perturbations in pH_e.

No changes in haemocyanin concentration were apparent under environmental perturbations in salinity and temperature (Lynch et al., 1973). This suggests that controlling concentration of these multifunctional proteins is not a strategy employed to alter haemocyanin buffering capacity.

Bicarbonate Buffers*:* Bicarbonate (HCO_3^-), in combination with H^+ ions, H_2O, CO_2, and an intermediate carbonic acid (H_2CO_3), form an effective buffering system through the reversible hydration/dehydration of CO_2 (Perry & Gilmour, 2006), see Equation 2.

Equation 2: $CO_{2(aq)} + H_2O_{(l)} \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^-{}_{(aq)} + H^+_{(aq)}$

The first part of this reaction is catalysed by carbonic anhydrase in both fish (review by Gilmour and Perry 2009) and crustaceans (review by Henry 1988).

Fish and crustaceans have a limited resource of HCO_3^- buffering capacity available to them in their intra- and extracellular compartments, due to the low pCO_2 of these fluids. This bicarbonate buffering system works passively in combination with Nbbs to assist in compensating the initial acidosis associated with the onset of elevated environmental pCO_2. Collectively, these buffers enable avoidance of large changes in pH prior to the initiation of transepithelial ion transport mechanisms across the gill.

In teleost fish and crustaceans there is some, albeit limited, scope for any excess CO_2, produced by the reduction of HCO_3^- by metabolic protons, to be excreted across the gills, by increasing the rate of ventilation. However, fundamentally both sets of animals face the same limitation in their capacity for reduction in their pCO_2 through ventilatory means. Oxygen uptake from water (which has 20-40 times lower O_2 content than air) necessitates a high ventilation rate which, in combination with the high solubility of CO_2 in water, means that CO_2 excretion across the gills is greatly facilitated. Therefore, unlike terrestrial animals, the pCO_2 in the blood/haemolymph of water-breathing animals is already low relative to the environment: typically around 1-4 mmHg (equivalent to 0.13-0.6 kPa) in fish (Heisler, 1986) and 1.5-4 mmHg (equivalent to 0.2-0.6 kPa) in crustaceans (Henry & Wheatly, 1992). This low blood pCO_2 means only small increases in CO_2 excretion are possible through hyperventilation (Burnett & Johansen, 1981; Claiborne et al., 2000). Due to these limitations, after initial buffering, fish and crustaceans must employ alternate (non-ventillatory) mechanisms to regulate their internal acid-base balance (Claiborne, 1998). This regulation is achieved through the transepithelial exchange of acid-base relevant ions with the environment.

Ion Exchange

Once fish and crustaceans saturate their endogenous buffering capacity and maximise their limited scope for ventilatory alterations, the role of counteracting high-CO_2-induced extracellular acidosis falls to ion transport mechanisms. The two main

methods by which this maintenance of pH_e is achieved are the uptake of HCO_3^- and the extrusion of acid, in the form of H^+ ions (Larsen et al., 1997; Michaelidis et al., 2007; Toews et al., 1983). In fish the intestine and kidneys play a small role in transport of acid-base relevant ions, but primarily (>90 %) this occurs across branchial epithelia of the gills (Claiborne et al., 2002). In crustaceans such exchanges were thought to occur primarily in posterior gills, with gas exchange occurring in the anterior. It's now thought that this may be an over simplification as anterior gills can play a role in ion transport and the specific sites acid-base related transport have not yet been localised (Freire et al., 2008).

Bicarbonate Accumulation: Many studies over the last four decades have emphasised the central role of accumulating extracellular bicarbonate in compensating for high-CO_2 induced acidosis. Heisler et al. (1975) showed that under environmental hypercapnia pH_e was recovered by accumulation of HCO_3^- in the extracellular fluid. This model was supported by Toews et al. (1983) who later showed compelling evidence for HCO_3^- accumulation as the primary means of extracellular acid-base regulation in the marine teleost *Conger conger*. More recently Wang et al. (1998) provided evidence that intracellular [HCO_3^-] elevation is also key in regulating pH_i. Some authors have proposed the direct uptake of HCO_3^- from the environment, but if this is the case, the mechanism(s) by which this occurs are not yet understood. Early studies linked HCO_3^- transport across the gill with concomitant equimolar exchange of counter-anions, namely Cl^- (Dejours, 1969), but there is no direct evidence of inward HCO_3^- flux. Most recently Esbaugh et al. (2012) demonstrated that gulf toadfish (*Opsanus beta*) exposed to hypercapnia in HCO_3^- free seawater showed a compromised ability to elevate plasma HCO_3^- following 24 h exposure. At first these results suggest that branchial HCO_3^- uptake rather than acid excretion was the primary means of acid-base regulation. However plasma pCO_2 in the hypercapnic low-HCO_3^- seawater treatment did not appear elevated at this time-point, and plasma pH was not significantly different from control animals. This suggests there was no respiratory acidosis for which compensation via HCO_3^- was necessary.

Truchot (1979) provides clear evidence that shore crabs also utilize elevations in extracellular HCO_3^- concentration to metabolically compensate respiratory acidosis during exposure to hypercapnia. The author proposed that these changes may be achieved in much the same way as in marine teleosts: by mediation of acid or base

transfers across the gill and by exchanges of HCO_3^- between tissue compartments and the haemolymph. But again there is no direct evidence of direct branchial HCO_3^- uptake from the environment.

Current models of acid-base regulation in fish suggest that Cl^-/HCO_3^- transporters do play a vital role in HCO_3^- accumulation, but not through direct uptake from the environment (*see below*). Claiborne et al. (1997) suggests that rather than direct uptake of HCO_3^- from the environment, elevation of extracellular [HCO_3^-] is achieved via the carbonic-anhydrase-catalysed hydration of CO_2 in the gill epithelial cells (Fig. 1). The resulting HCO_3^- is transported into the plasma via basolateral Cl^-/HCO_3^- exchangers and the H^+ ions are excreted into the seawater via apical Na^+/H^+ exchangers. Additionally the apical exchange of outward HCO_3^- for inward Cl^- is halted or diminished in an effort to accumulate further HCO_3^-.

In blue crabs the inhibition of CA using acetazolamide caused a reduction in extracellular Cl^- and Na^+ concentrations and a resultant metabolic alkalosis: and increase in pH and [HCO_3^-] at constant pCO_2 (Henry & Cameron, 1983). This is consistent with an acid-base regulatory model for crabs similar to the one described by Claiborne et al. (1997) whereby Cl^- and Na^+ influx is coupled with HCO_3^- and H^+ which are provided by the CA-catalysed hydration of CO_2 in the gill.

Proton Extrusion: Hydrogen ion extrusion as well as HCO_3^- accumulation appears to play an important role in the acid-base regulatory strategies of both these groups of animals. The majority of H^+ ion extrusion occurs across the apical surface of the gills of both teleosts and crustaceans.

Freshwater species can employ V-type H^+-ATPases to pump H^+ ion out across the gill providing a negative intracellular potential favourable for inward flux of Na^+ ions through sodium channels (Hwang et al., 2011; Kumai & Perry, 2012). In marine species the high Na^+ content of the external medium makes electroneutral exchange of Na^+ for H^+ more energetically favourable so H^+extrusion is achieved by Na^+/H^+ exchangers (NHE) (Maetz, 1971). Crustaceans also employ NHEs although these are thought to be localised to the basolateral membrane, with the additional presence of apical electrogenic $2Na^+/1H^+$ exchangers for proton extrusion at the gill surface (Shetlar & Towle, 1989).

These exchangers are energetically favourable to SW adapted animals as the high external salt concentration allows the passive movement of Na^+ ions inward down a concentration gradient. This influx enables H^+ ions to diffuse outward across the apical epithelium without the need for ATP-fuelled hydrolysis (Claiborne, 1998). Naturally this increased Na^+ uptake adds to the burden already faced by hypo-osmotic marine fish, however the imposition to osmoregulatory homeostasis is subsequently compensated (McDonald et al., 1982). This is achieved by the removal of Na^+ ions from the gill epithelial cells, both into the extracellular space and back across the apical membrane into the seawater. The former is achieved by Na^+/K^+-ATPase (NKA) transporters on the basolateral membrane. K^+-channels are then the route for K^+ ions to diffuse back into the extracellular fluid to help restore electroneutrality. This model is supported by the co-localization of Na^+/K^+-ATPase with NHEs in interlamellar gill cells (Catches et al., 2006) but not by evidence from Deigweiher et al. (2008) who observed the transient down regulation of NHEs in the first few days of exposure to high CO_2.

Overview: In summary, acid-base regulation is of great importance to both teleosts and crustaceans alike. It is linked to, and disturbed by, a number of other factors not least of which is the regulation of internal osmolality through the electroneutral exchange of ions. Most teleost fish and crustaceans examined to date employ very similar approaches to regulating their acid-base status when challenged with environmental hypercapnia. What we must acknowledge however, is that despite the remarkably conserved use of many ion-transporters, particularly those also involved in osmoregulation, we cannot say that all fish or all crustaceans possess the same transporter make-up. Furthermore that the cellular and subcellular locations of these transporters differs greatly between species and under differing environmental stimuli (Evans et al., 2005). As such the broad application of findings between acid-base regulating animals may be limited to ‘macro’ impacts such as elevated extracellular bicarbonate or changing energy allocation, rather than, for example, up or down regulation of genes for specific ion transporters. That said, the relatively conserved nature of their acid-base regulatory strategies make them interesting targets for comparative work focussed around these physiological changes under high CO_2 conditions (Chapters 2-4).

The energetic cost of such regulatory processes is not well known. To put it in some context, osmoregulation in seawater fish likely accounts for between 6 and 15 % of

the resting oxygen consumption (Kirschner, 1993). This would be further elevated under osmo- and acid-base regulatory perturbations induced by changes to external seawater under ocean acidification. Conversely later work to assess the same demand found no change in oxygen consumption rate and suggest energy requirements may be diverted from other processes (Kidder et al., 2006). As such, increases in dependence on these regulatory mechanisms could incur energetic costs that may have downstream effects on slow processes like growth (*Chapter 5*) and reproduction.

2.1.2 Growth

In the past growth in fish was typically only reported at CO_2 concentrations far exceeding those likely to be encountered under ocean acidification. Body size (measured over 43-70 d) was unaffected by increases in ambient pCO_2 up to values of 15,000 µatm, and growth tended only to decrease upwards of 26,000 µatm (Fivelstad et al., 1998; Fivelstad et al., 1999; Fivelstad et al., 2003; Foss et al., 2003). However, more recent work, particularly on early life stages, has reported detrimental ffects on embryonic growth under near-future pCO_2 conditions (~1000 µatm; Baumann et al., 2012), suggesting they may be more susceptible. However, these affects are not conserved across species (Munday et al., 2009; Munday et al., 2011). In fact Munday et al. (2009) reported increased growth in larvae in CO_2-acidified conditions. Still relatively little research been done on the potential effects of near-future high CO_2 concentrations on growth in fishes. This may be owing to their ability to regulate their internal pH against external perturbation. However, the limited information available suggests that early life stages may be differentially susceptible to ocean acidification, presenting potential bottlenecks in population success, despite greater tolerance in adults.

Information on growth in crabs under ocean acidification relevant CO_2 conditions is equally sparse. However there is limited evidence to suggest they are able to maintain normal growth rates and feeding behaviours in CO_2 exposure up to 1250 µatm (Appelhans et al., 2012).

2.1.3 Olfaction and Behaviour

Although not assessed within the remit of the thesis, consideration of the potential effect of ocean acidification on behaviour, particularly relating to olfaction (Dixson et

al., 2010; Dixson et al., 2009), auditory discrimination (Simpson et al., 2011), lateralization (Domenici et al., 2011) and predatory-prey interaction (Ferrari et al., 2011), has direct relevance to the findings presented. Recent observations of behavioural changes and sensory detriment in economically and ecologically important reef fishes under near future high CO_2 levels (as low as 600 µatm) have been attributed to acid-base regulatory mechanisms. These include reduced olfactory ability including predator avoidance (Dixson et al., 2010), prey detection (Cripps et al., 2011), habitat selection (Dixson et al., 2009) and decreases in favourable auditory discrimination (Simpson et al., 2011). Nilsson et al. (2012) attributes these disruptions to normal sensory function to altered neurotransmission as a result of elevated HCO_3^- accumulated as part of the acid-base regulatory strategy. The proposed mechanism by which such disruption occurs focuses on changes to the ionic gradients across neuronal cell membranes that express the main inhibitory receptor within the vertebrate brain: GABA-A. During acid-base regulation (increase and decrease in extracellular [HCO_3^-] and [Cl^-], respectively), stimulation of this receptor by GABA may induce excitation (rather than the usual inhibition) of nerve transmission, with subsequent shifts in processing of the above range of sensory information. The result being altered (usually detrimental) behaviours with clear potential for deleterious population and ecological consequences.

One of the salient trends in the literature on high CO_2 with respect to physiological perturbation in marine animals is the recurring acknowledgement that co-stressors differentially affect the responses, and/or that high CO_2 modulates the impact to other stressors. This highlights a clear need to better understand how co-stressors will modify animals' responses to high CO_2, such as acid-base regulation, and how such responses may in turn modify their reactions to such co-stressors (Aze et al., 2014). Alongside salinity and temperature, chemical stressors represent an excellent target for such multi-stressor approaches, as a ubiquitous group of contaminants whose impacts on marine biota are very often effected by changing water chemistry.

3. Chemical Stressors

The introduction of chemical contaminants into the ocean occurs predominantly through continental run-off or atmospheric deposition. As this run-off is the primary means by which pollutants enter the marine environment, coastal areas and estuaries

typically have the highest concentrations of these compounds. The prevalence of different contaminants varies greatly across geographical areas (Bewers & Yeats, 1989). Historically a large portion of work assessing the physiological effects of chemical stressors has focussed on freshwater species due to the higher concentrations of contaminants in these water systems. For the same reason, when considering these effects on marine species it is prudent to assess effects coastal or estuarine species.

Within the vast array of chemical contaminants entering water systems, toxic metals represent a major cause of harm to aquatic organisms. For this reason metals like copper, mercury, lead, zinc, chromium, cadmium and silver have all been widely studied. The physiological impacts of toxic metals differ with species, life stage, concentration and water chemistry. Among the most ubiquitous and potentially toxic of these metals, is copper. Copper is a particularly relevant chemical stressor to consider in the context of ocean acidification due the impact that pH can have on its speciation and as a result its potential for toxicity

3.1 Copper

3.1.2 Speciation and Toxicity

Water chemistry has a significant bearing on the bioavailability of copper to organisms. Copper speciation can determine how, where and how much the copper is taken up by the organism.

Copper has three ionic states Cu (solid), Cu^{+} (cuprous ion) and Cu^{2+} (cupric ion). To be biologically available, copper must be present in a soluble (ionic) form (Flemming, 1989). In seawater, inorganic speciation is dominated by copper carbonates, $CuCO_3$ and $Cu(CO_3)_2^{2-}$ (Grosell, 2012).

The speciation of copper is determined by its environment: primarily by pH, water hardness (Ca^{2+} and Mg^{2+} ions), alkalinity (HCO_3^{-} and CO_3^{2-} ions), and the presence and type of organic material. These influences determine whether copper is ionic, complexed or precipitated in any given medium (Flemming, 1989). Free ionic copper, typically Cu^{2+}, is generally considered the most bioavailable to aquatic organisms and the most toxic. Changes to ocean chemistry as a result of future increases in dissolution of CO_2 are predicted to have a profound effect on the relative proportion of

copper present as Cu^{2+}. Decreasing pH will increase the solubility of copper; a reduction in the concentration of CO_3^{2-} ions will reduce the formation of copper-carbonates; and increases in H^+ binding to organic molecules will reduce their ability to complex copper (Millero, 2009). The combined effect of all of these is predicted to be a doubling of the concentration of free Cu^{2+} before the end of the century, with clear potential for increased bioavailability and toxicity to organisms (Millero, 2009).

3.1.2 Introduction to Copper

Copper enters the marine environment naturally through the erosion of mineralized rocks at a rate of 325,000 t/yr (Clark, 2001). However, often concentrations of biologically available Cu rise in localized areas as a result of anthropogenic inputs, typically in rivers, lakes and coastal zones. The solubility of copper means that, even if it does not enter these systems directly, it is leached from terrestrial sources into waterways. Typical copper concentrations in harbours around the southern UK have been measured at >5 µg/L but anthropogenic inputs can lead to vastly higher concentrations: most severe in areas associated with copper mining such as the Fal Estuary, Cornwall (Pirrie et al., 2003) where water concentrations were up to 100 µg/L towards the end of the period mines were active in that area (Bryan & Gibbs, 1983). In more extreme cases copper contamination can reach upward of 200 mg/L in freshwater systems directly downstream of mines (EPA, 2007).

Anthropogenic sources of copper release are diverse, including: smelting, mining, industrial processes (plating, steelworks, refineries), domestic waste, sludge dumping, electroplating, pesticides, fungicides, antifouling paints, wood preservers, ceramic glaze and glass paint, textiles, animal feeds fertilizers, and molluscicides (Water UK 2001; Clark, 2001).

There is a great deal of literature on the toxicological effects of copper on freshwater fish. At least in part due to the fact that fish are excellent indicators of metal contamination in aquatic systems, occupying different trophic levels, with diverse size, age and habitat (Gabbianelli et al., 2003). However, in comparison with studies on freshwater fish, far less research has been conducted on marine species. Due to the potential for elevated levels of copper in coastal and estuarine environments, there is

a need to better understand the effects copper has on marine species that subsist in these waters during their life cycle (Dang et al., 2009).

Copper is not considered to be a novel threat with respect to toxicological research. However, there is a clear need to address the potential for altered toxicological impacts of these types of stressors in combination with changing water parameters, such as those imposed by climate change.

3.1.3 Effects on Fish and Crustaceans

Copper is an essential micronutrient vital to plants and animals, playing a key role in physiological systems as a component of enzymes, acting as a structural cofactor and redox catalyst (Flemming 1989). These enzymes are involved in cellular metabolism, signal transduction, blood clotting, energy production (e.g. cytochrome oxidase), oxygen transport (e.g. haemocyanin) and peptide hormone maturation (Santos et al., 2010).

However, as concentrations exceed those required for physiological functioning copper becomes increasingly toxic. When copper levels within a cell are significantly elevated the once useful redox properties of copper result in the formation of harmful reactive oxygen species (ROS) (Bopp et al., 2008). These properties also cause copper to bind readily to the histidine, cysteine, and methionine sites of proteins, potentially leading to conformational changes and dysfunction (Grosell, 2012). The primary means of cellular toxicity are DNA damage, oxidative stress, lipid peroxidation and altered calcium homeostasis (Stohs & Bagchi, 1995; Valko et al., 2005), see Fig. 2. Whole organism effects include, detriment to olfaction in fish (Baldwin et al., 2003; Baldwin et al., 2011; Green et al., 2010; Hansen et al., 1999; Julliard et al., 1996), behaviour in fish (Hansen et al., 1999; O'Gara et al., 2004), swimming performance in fish (Beaumont et al., 1995; Waiwood & Beamish, 1978), as well as impaired function of a number of ionoregulatory processes in fish and crustaceans (Bjerregaard & Vislie, 1986; Thurberg et al., 1973): acid-base regulation (Larsen et al., 1997; Wang et al., 1998), osmoregulation (Grosell et al., 2004a; Hansen et al., 1993), nitrogenous waste excretion (De Boeck et al., 2007; Grosell et al., 2004b).

Due to the obvious physiological importance of balancing essentiality with toxicity, coupled with the variability of environmental concentrations, effective copper

homeostasis is vital. As such, organisms such as fish and crustaceans have sophisticated mechanisms for uptake, transport, detoxification, storage and excretion (Olsson et al., 1998; Rtal et al., 1996; Rtal & Truchot, 1996). However high concentrations of copper and/or chronic exposure can overwhelm these systems and result in varying degrees of physiological dysfunction. Some of the primary impacts of copper on fish and crustacean physiology are considered below, with particular focus on those relevance to this thesis.

3.1.3.1 Osmoregulation

Acute copper toxicity (and mortality) as a result of waterborne copper exposure is associated with osmoregulatory disturbances in both seawater (Grosell et al., 2004) and freshwater (Grosell et al., 2002) fish.

Marine fish are hypo-osmotic with respect to their aquatic environments. As such they must continually drink seawater and excrete Na^+ and Cl^- ions from the gills against the diffusive gradient in order to maintain optimum plasma and cellular salt balances (Zimmer et al., 2012). This osmoregulatory strategy is often disrupted in marine fish under exposure to waterborne copper. Grosell et al. (2004b) demonstrated that both acute (96h) and prolonged (30 days) copper exposures (762 µg/L) induced an osmoregulatory disturbance in the marine teleost the Gulf toadfish (*Opsanus beta*) and that a failure in effective Na^+ and Cl^- regulation this was the major cause of acute toxicity in these fish. This regulatory failure led to increased plasma [Na^+], [Cl^-] and osmolality. Additionally they noted an inhibition of drinking rate in the copper exposed fish, consistent with previous work done using silver (Grosell et al., 1999; Hogstrand et al., 1999).

Larsen et al. (1997) exposed Atlantic cod (*Gadus morhua*) to ~400 µg/L copper and noted a large and progressive elevation in plasma osmolality from 325 to 417 mOs*m* within 48 h due to elevations of plasma [Na^+] and [Cl^-]. These copper-induced disturbances in osmoregulation may be caused by either an inhibition of Na^+ and Cl^- excretion or an inhibition of water transport. Given that Na^+/K^+-ATPase is responsible for both Na^+ and Cl^- excretion at the gills and water transport in the intestine, inhibition of this enzyme by copper could explain both scenarios for osmoregulatory disturbance (Blanchard & Grosell, 2006). This is supported by Stagg and Shuttleworth (1982) who showed that exposure of gill homogenates from European flounder (*Platichthys flesus*)

to copper resulted in a dose dependent reduction in Na^+/K^+-ATPase activity. This was further supported by De Boeck et al. (2007) who found that under copper exposure, reduced Na^+/K^+-ATPase activity was correlated with increases in plasma [Na^+] and [Cl^-].

Grosell et al. (2004) highlight a lack of direct evidence for this enzymatic inhibition in copper-exposed seawater fish and that changes in gill permeability may be a more likely cause. Subsequent studies have shown that Na^+ balance disruption often occurs without the inhibition of the Na^+/K^+-ATPase transporters (Blanchard & Grosell, 2006; Grosell et al., 2004), suggesting that other mechanisms of osmoregulation disruption are occurring.

Whatever the mechanism, as a result of the disruption to normal osmoregulatory function, copper toxicity is significantly modulated by salinity. Copper–induced osmoregulatory disturbances appear most pronounced at high or low salinities, where fish are most reliant on such regulation, compared with media closer to isotonic with the extracellular fluid (Blanchard & Grosell, 2006; Wilson & Taylor, 1993).

Osmoregulatory function in crabs is also affected by waterborne copper exposure. However, unlike marine fish, shore crabs, are typically close to isosmotic (or slightly hyperosmotic) to their surrounding medium. The osmolality at which shore crabs maintain their haemolymph above the environment is dependent on the ambient salinity such that they are more hyperosmotic at lower salinities and approach isosmotic in media of around 900 mOsm/kg: equivalent to a salinity of 30 (Thurberg et al., 1973). As such, decreases in osmoregulatory function results in the loss, not gain, of Na^+ and Cl^- ions, at least at lower salinities. Thurberg et al. (1973) found that under progressive elevations in copper exposure (0-40 mg/L), shore crabs increasingly lost ions to their environment, equilibrating to isosmotic at the highest copper concentrations. This effect was most pronounced under lower salinities where the animals are comparatively more hyperosmotic to their environment and therefore more reliant on osmoregulatory mechanisms. Bjerregaard and Vislie (1986) found similar osmoregulatory dysfunction under 1 mg/L Cu, manifesting as a reduction in Na^+, Cl^-, and K^+ ions from the haemolymph in water with a salinity of 14 (400 mOsm/kg).

3.1.3.2 Acid-base Status

In seawater, Larsen (1997) showed that Atlantic cod (*G. morhua*) exposed to copper (~400 µg/L) were subject to a metabolic acidosis whereby plasma [HCO_3^-] decreased but pCO_2 remained constant. Similarly, De Boeck et al. (2007) reported a transient metabolic acidosis in spiny dogfish (*S. acanthias)* under exposure to 500 µg/L Cu, and a persistent acidosis at 1000 µg/L and 1500 µg/L Cu. In freshwater tilipia (*Oreochromis mossambicus*) Pelgrom et al. (1995) also report a plasma acidosis at their highest Cu concentration (200 µg/L).

Conversely, Pilgaard et al. (1994) found that 500 µg/L Cu induced a transient metabolic alkalosis in freshwater rainbow trout (*O. mykiss*) which was recovered after a few days. Wang et al. (1998) also found that 600 µg/L Cu produced a metabolic alkalosis in the same species. Taken together these studies demonstrate a clear impact of copper on acid-base balance but that the direction of acid-base disturbance does not consistently correlate with copper concentration, salinity or species.

Effects on acid-base regulation are typically attributed to inhibition of branchial ion transporters such as Cl^-/HCO_3^- (Grosell, 2012), in much the same way as is osmoregulatory dysfunction (Laurén & McDonald, 1985; Pelgrom et al., 1995; Reid & McDonald, 1988) and/or interaction between copper and carbonic anhydrase (CA). The inhibition of acid-base regulation in fishes exposed to Cu and hypercapnia (Larsen et al., 1997; Wang et al., 1998) is similar to that of fish exposed to hypercapnia and CA inhibitors (Georgalis et al., 2006). This lends further support to the potential importance of CA inhibition in acid-balance dysregulation under copper exposure (Fig. 1). Zimmer et al. (2012) added weight to this idea by reporting significant inhibition of CA in euryhaline guppy (*Poecilia vivipara*) under 20 µg/L Cu in both fresh and seawater.

In shore crabs, copper exposures of between 500-2000 µg/L Cu in seawater (salinity = 33) induced a non-lactic metabolic acidosis with no marked effect on the ionic composition of the haemolymph (Boitel & Truchot, 1989). The onset of this acidosis was partly limited by hypocapnia, possibly due to hyperventilation, and was recovered following 20 d exposure. A metabolic acidosis was also reported by Weeks et al. (1993) under 750 µg/L Cu and varying salinities (10-30). The causes of this metabolic acidosis are not well understood but it should be noted that a) osmoregulatory

disturbances were not reported (Boitel & Truchot, 1989), suggesting inhibition of ion transporters may not be the causes, and b) there is some evidence to suggest copper may also inhibit CA activity in crabs: at least *in vitro* (Vitale et al., 1999).

3.1.3.4. Cellular Stress and DNA Damage

Oxidative stress is widely accepted as one of the primary impacts that copper has on organisms under excessive cellular concentrations (Grosell et al., 2004) and occurs as a result of imbalances between the production of oxidizing agents and the concentration/production of anti-oxidants. Copper causes oxidative stress directly and indirectly (Fig. 2) by: production of free radicals, including but not limited to, reactive oxygen species (ROS) (Freedman et al., 1989; Grosell, 2012; Stohs & Bagchi, 1995; Valko et al., 2005) and the inhibition of antioxidant enzymes (Hansen et al., 2007; Sampaio et al., 2008; Sanchez et al., 2005).

Oxidative stress occurs rapidly when intracellular copper concentrations are elevated. Depending on the intensity of ROS production this stress can be combated by enzymatic and non-enzymatic antioxidant mechanisms. However, during severe or prolonged exposures the production of ROS can exceed the capacity of these defence mechanisms leading to the oxidation of biological molecules, not least of which includes the formation of DNA adducts.

Gabbianelli et al. (2003) exposed gilthead sea bream (*Sparus aurata*) to ~100 µg/L Cu^{2+} over a period of 20 days. Assessment of the erythrocytes using comet assay (*see Box 1*) post-exposure revealed a considerable increase in susceptibility to DNA damage in copper exposed animals. This was measured as a significant increase in the three main parameters upon which comet assay can be judged: tail length, tail intensity and tail moment. Santos et al. (2010) exposed sticklebacks (*Gasterosteus aculeatus*) to varying concentrations of copper (3.2 -128 µg/L) over a 4 day period and found a dose-dependent relationship between exposure severity and resulting DNA damage (comet assay). Bopp et al. (2008) also reported significant induction of DNA damage in gill cells of rainbow trout (*O. mykiss*) exposed *in vitro* to 63.5 and 159 µg/L Cu, but no lipid peroxidation (TBARS). This provides support for the view that DNA damage may be among the most sensitive indicators of intracellular oxidative damage.

With increasing intracellular damage, cells may become dysfunctional or non-viable and may succumb to necrosis or initiate apoptosis (programmed cell death). Vazzana et al. (2010) exposed European sea bass (*D. Labrax*) to varying concentrations of copper (0.1 µM - 1 mM) to assess the impact on red blood cell viability. They found a dose-time-dependent response in which both longer exposure durations and higher copper concentrations produced more profound reductions in cell viability, suggesting potential for cytotoxic as well as genotoxic effects under copper exposure. Copper exposure has also been reported to induce apoptosis in gill epithelial cells of Mozambique tilapia (*Oreochromis mossambicus*; Li et al. 1998), olfactory epithelial cells of rainbow trout (*O. mykiss*; Julliard et al., 1996) *in vivo*, as well as hepatic cells of rainbow trout (*O. mykiss;* Feng et al., 2003; Krumschnabel et al., 2005; Nawaz et al., 2006) *in vitro*. However, such findings are not consistent and similar effects were not apparent in comparable studies. Bury et al. (1998) reported necrosis but little apparent apoptosis following 24 h incubation of talipia (*O. mossambicus*) gill chloride cells in 50 and 100 µM $CuSO_4$. Similarly, Monteiro et al. (2009) found no biomarkers for apoptosis in gills of Nile talipia (*O. niloticus*) exposed to copper *in vivo* (40 and 400 µg/L). More research on the potential for Cu-induced apoptosis in both external and internal tissues is needed in order to elucidate potential mechanisms for such effects and better explain inconsistencies in these findings.

Despite its clear potential for mechanistically similar modes of toxicity, very little work has considered the effects of copper on DNA damage in crabs. In possibly the only papers to do this Pan et al. (2011) describes a dose-dependent induction of DNA damage in haemocytes, gill and hepatopancreas cells of marine crabs (*Charybdis japonica*) exposed to copper between 10-100 µg/L Cu, as measured by the comet assay. Similar dose and time-dependent induction of DNA damage was also reported in the more widely studied and economically important white shrimp (*Litopenaeus vannamei*) exposed to cadmium. This is further suggestive of induction of DNA damage being a useful biomarker for assessing metal toxicity in crustaceans as well as fish.

Box 1: Comet Assay

The comet assay, also known as single cell gel electrophoresis, is sensitive and cost effective method of assessing DNA damage in cells. It was developed by Östling & Johansson (1984) and then later modified by Singh et al. (1988). The assay entails the encapsulation of cells in agarose on microscope slides followed by lysis of those cells and electrophoresis of the DNA.

Following encapsulation in agarose, the cells are lysed in a concentrated salt solution containing a detergent (Triton-X) at alkaline pH (10). During this lysis stage the detergent dissolves the cell membrane and the salt disrupts cytoplasmic and nucleoplasmic constituents. These constituents subsequently diffuse into the agarose leaving only the DNA nucleoid. During this stage DNA becomes cleaved at sites where single strand breaks have occurred.

The slides are then subjected to an electric field, causing a size-dependent migration of DNA fragments into the surrounding agarose. Slides are then neutralised using a buffer solution and viewed under a fluorescence microscope using a suitable DNA binding stain: typically ethidium bromide or SYBR safe. Assessment of DNA damage is most often achieved by analytical software that measures the extent of the migration of the DNA fragments. Cells are described in terms of 'head' and 'tail' whereby the head represents the largely undamaged nucleoid and the tail represents fragmented DNA resulting from damage. As such the percentage of DNA in the tail (%tDNA) is one of the most widely used indicators of the extent of DNA damage, with more DNA in the tail being representative of more DNA damage.

3.1.3.5 Growth

Growth inhibition under sublethal copper exposure typically occurs anywhere between 20 and 80 % of the 96 h LC_{50} for a given species (Hansen et al., 2002; McGeer et al., 2002). Ali et al. (2003) exposed Nile tilapia (*O. niloticus*) to varying copper concentrations (0 - 0.5 mg/L) in order to assess the sublethal effects of chronic exposure. They found that weight gain, specific growth rate and condition factor all decreased during the exposure, displaying an inverse correlation with increases in copper concentration. Food intake was significantly decreased and food conversion ratio increased in copper exposed fish. Both protein efficiency and net protein retention

also reduced significantly and were negatively correlated with the copper concentration of the water. This pointed to a copper-induced detriment to the utilization and assimilation of proteins. Reductions in growth rate were also reported in later work by Kim and Kang (2004) who subjected juvenile rockfish (*Sebastes schlegeli*) to chronic elevations in water copper concentration of increasing severity (0- 500 mg/kg). Their results demonstrated that, for this species, the weight and length specific daily growth rates were negatively correlated with copper concentration. Parveen and Javed (2010) assessed the impact of sublethal copper concentrations (19.44 mg/L) on Indian carp (*Catla catla*) over a 90 day exposure. They found that under chronic exposure there was a significant and variable impact on body weight, fork and total length as well as condition factor, feed intake, food conversion ratio and length-weight relationship when compared with controls. In a study by De Boeck et al. (1997) juvenile carp (*Cyprinus carpio*) were exposed for 28 d to sublethal copper concentrations between 0.2 µM and 0.8 µM. The highest copper concentration resulted in an immediate reduction in food intake and caused a reduction in growth rate when compared with controls. Under 0.55 µM copper conditions, growth rates were also depressed despite maintenance of normal food consumption. At the lowest concentration, 0.2 µM, copper placed an increased metabolic demand on the fish presenting the challenge of meeting increased food demand.

There are a number of proposed explanations for the commonly observed copper-induced reductions in fish growth rates. Reduction in growth is often attributed to increased metabolic load due to the increased cost of compensating for Cu-induced physiological dysregulation (acid-base balance, osmoregulation, oxidative stress). Furthermore, the stress- response of the fish causes a release of hormones such as adrenaline, nor-adrenaline and cortisol. These hormones can produce a secondary stress response which can induce a suite of metabolic changes including depletion of muscle glycogen reserves, reduction in protein biosynthesis, hyperglycemia, hyperlacticemia, with the possible addition of muscle protein catabolism (De Boeck et al., 1997). The suggestion is that these catabolic effects, in conjunction with reduced food consumption may have a profound effect on energy stores such that growth rates are reduced.

The effects of copper on growth on shore crabs is not assessed within this thesis but it is worthy of mention that inconsistent impacts of waterborne copper have been

reported on growth in other crab species. Under 50 µg/L Cu the intermolt phase of juvenile blue crab (*Callinectes similis*) was shortened resulting in increased growth rates compared with controls (Neff & Anderson, 1977), whereas reductions in growth rate were reported in larval stages of the estuarine mud crab (*Rhithropanopeus harrisii*).

4. Physiological Overlaps: High CO_2 and Copper

On top of the clear potential for changing carbonate chemistry to fundamentally alter the bioavailability, and therefore toxicity, of copper there are a number of physiological overlaps between to two stressors. Both high CO_2 and copper have the potential to cause dysfunction in acid-base and/or osmo- regulation, with very little information known on the combined effects of these stressors on such physiologically important mechanisms. Furthermore both high CO_2 and copper have been implicated in the inhibition of growth in fishes, albeit often at higher CO_2 partial pressures than predicted for near future ocean chemistry. As such, there exists the potential for additive, antagonistic or synergistic interaction between these stressors, possibly resulting in markedly lower effect concentrations. The few papers to assess these two stressors in combination are considered here:

Larsen et al. (1997) exposed Atlantic cod (*G. morhau*) to high CO_2 (9,900 µatm) and copper (~400 µg/L), both separately and in combination. They found that cod kept under high CO_2 conditions displayed a typical transient extracellular respiratory acidosis, recovered within 12-24 h through a chloride-mediated net retention of HCO_3^- ions. This is consistent with acid-base regulatory mechanism employed by teleost fish in previous studies looking at high CO_2 exposures (*see 2.1.1.1*). However, fish in the combined exposure showed a reduced ability to recover this acidosis, with extracellular pH remaining depressed by 0.3 pH units after 72 hours. This was evidence of a copper-induced inhibition of the fish's acid-base regulatory strategy. As per Grosell et al. (2004), fish in the copper only exposure showed osmoregulatory disturbance manifesting as a large and progressive increase in plasma $[Na^+]$ and $[Cl^-]$. They also noted a metabolic acidosis in the copper exposed group.

However, during the combined copper and high-CO_2 exposure there was a decreased level of Na^+ and Cl^- retention when compared with copper-only groups. This suggested that the elevation in ambient CO_2 was in some way having a protective effect against

copper-induced osmoregulatory disturbances. More interesting still was the apparent reduction in copper toxicity under hypercapnic conditions. Copper induced mortality was 58 % under normocapnia, but was reduced to zero under high CO_2, suggestive of some undescribed protective mechanism.

Wang et al. (1998) provided good evidence to support these findings in freshwater fish. They exposed catheterised rainbow trout (*O. mykiss*) to copper (600 µg/L), under normocapnic and high CO_2 (7895 µatm) conditions in order to assess the impact of copper on the ability of the trout to regulate their acid-base status. Fish exposed to high-CO_2 alone showed a short-term extracellular acidosis which was fully compensated for within 24 hours through an accumulation in HCO_3^- ions from 8.1 mM to 24.4 mM. Consistent with Larsen et al. (1997), this ability to fully compensate for acid-base imbalances was no longer present during combined copper and high CO_2 exposures. Extracellular HCO_3^- concentrations in fish under combined exposure only reached an intermediate average of 16.3 mM, and pH remained acidotic throughout. This work, like that of Larsen et al. (1997), supports the view that acid-base regulation is sensitive to disruption under elevated ambient copper concentrations.

Work on bivalves has highlighted clear effects of high CO_2 on both uptake and intracellular stress under waterborne copper exposure, as well as further potential for a protective effects. Götze et al. (2014) showed that elevated pCO_2 (800 and 1500 µatm) caused an increase in the bioaccumulation of Cu in mantle tissue in both Eastern oyster (*Grassostrea virginica*) and hard shell clam (*Mercenaria mercenaria*). However, the inhibition of mRNA production for ubiquitin and tumour suppressor p53 by Cu seen under normocapnia was reversed under high CO_2 exposure in both species. Cellular energy deficiency, as evidenced by decreased glycogen, ATP and ADP, was noted in clams, although only under moderate hypercapnia (800 µatm). Ivanina et al. (2013) reported that uptake of Cu by the mantle cells of hard clams *in vitro* was also significantly increased by hypercapnia (1.52 and 3.01 kpa) but that Cu-mediated production of ROS was mitigated, affording the cells an apparent protective effect.

In summary, very little research exists on the combined impacts of high CO_2 and copper. However, those studies that do highlight interesting interactive effects, particularly with respect to toxicity as well as acid-base and osmo-regulation. Some even suggest that the two stressors may act antagonistically or in a protective manner,

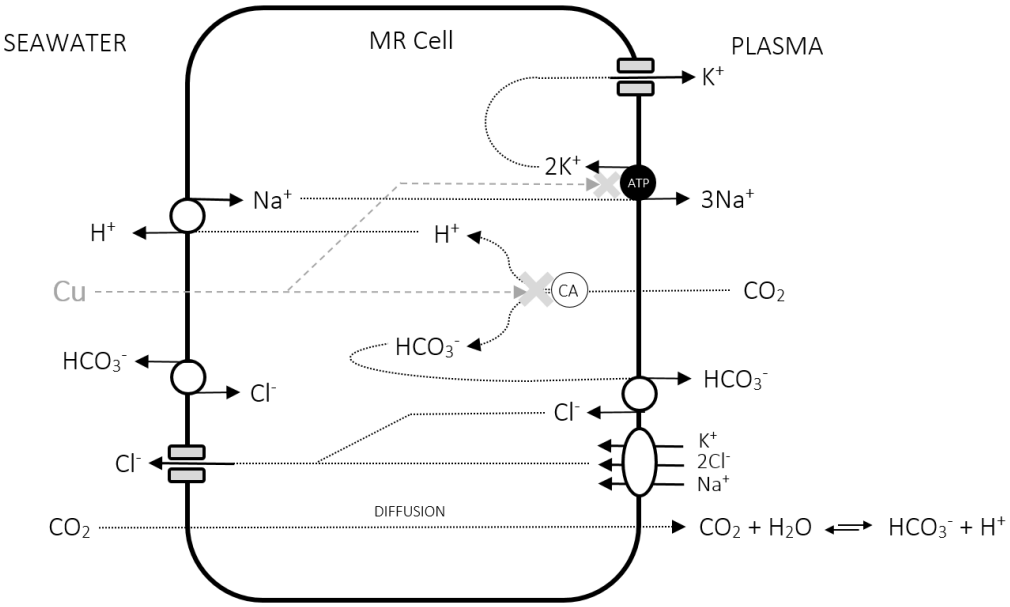
with high CO_2 apparently lessening the physiological impact of copper exposure in multiple instances.

5. Introducing the Thesis

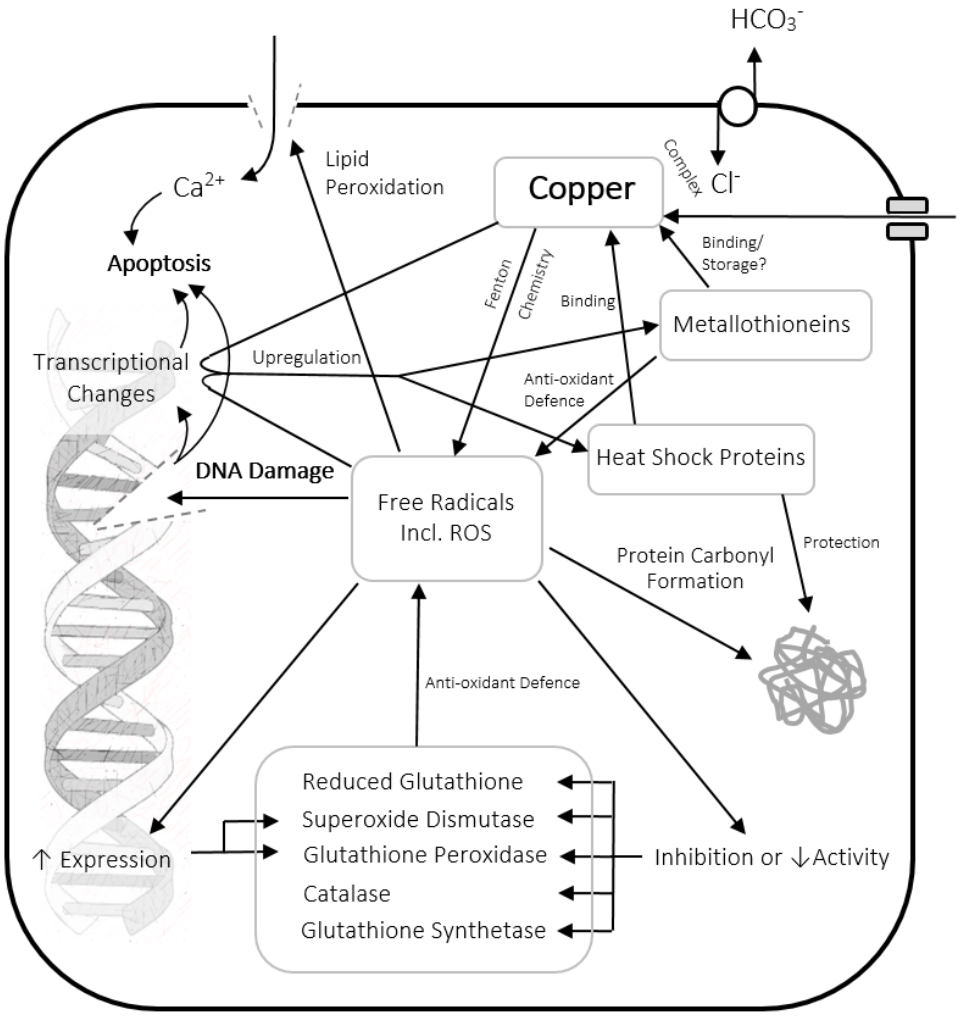
There is very limited amount of literature on the combined impacts of high CO_2 and copper and clear potential for important and undescribed interactive effects between these two stressors. Copper represents a ubiquitous stressor that will likely be subject to considerable changes in toxicity under ocean acidification and one with clear overlaps in physiological disturbances with high CO_2.

As such the aim of this project was to better understand the impacts of these stressors on processes pertinent to the above reported physiological effects, for example acid-base regulation. Furthermore, to assess the potential harm to the chosen model species at both a cellular and a whole organism level. In the following series of experiments sensitive biomarkers for cellular stress were employed, such as DNA damage on blood cells to assess the former, and indicators of condition and energetic cost such as growth and food conversion efficiency to assess the latter. Aside from the main species of interest, European sea bass (*Dicentrarchus labrax*), impacts on shore crabs (*Carcinus maenas*) were assessed in order to test hypotheses pertaining to acid-base regulation in an unrelated species that employs many of the same mechanisms with respect to ion homeostasis. Within this experiment the aim was not to seek to induce a physiological response under artificially extreme conditions but to assess the aforementioned effects using pCO_2 levels predicted for near-future climate change scenarios (1200 µatm) as well copper exposures that were environmentally realistic (10-80 µg/L). The following data chapter starts by first assessing the physiological responses of both sea bass and crabs to elevations in environmental pCO_2 in the absence of copper: namely their acid-base regulatory strategy.

6. Figures



Chapter 1 Figure 1: Generalised schematic of a mitochondrial rich cell in the teleost gill; a model for acid-base regulation in seawater fishes, adapted from Claiborne et al. (1997). Grey represents the potential inhibition of copper of the enzymes carbonic anhydrase (CA), as per Zimmer et al. (2012) and Na^+/K^+-ATPase, as per Stagg and Shuttleworth (1982).



Chapter 1 Figure 2: Generalised effects of intracellular copper, produced from a chapter reviewing essentiality and toxicity of copper in fishes (Grosell, 2012).

7. References

Ali, A., Al-Ogaily, S. M., Al-Asgah, N. A., & Gropp, J. (2003). Effect of sublethal concentrations of copper on the growth performance of *Oreochromis niloticus*. *Journal of Applied Ichthyology, 19*(4), 183-188.

Appelhans, Y., Thomsen, J., Pansch, C., Melzner, F., & Wahl, M. (2012). Sour times: Seawater acidification effects on growth, feeding behaviour and acid‐base status of *Asterias rubens* and *Carcinus maenas*. *Marine Ecology Progress Series, 459*, 85-98.

Archer, D., & Rahmstorf, S. (2010). The climate crisis: An introductory guide to climate change. Cambridge University Press.

Aze, T., Barry, J., Bellerby, R. G., Brander, L., Byrne, M., Dupont, S., & Hattam, C. (2014). An updated synthesis of the impacts of ocean acidification on marine biodiversity *(CBD Technical Series 75)*.

Baldwin, D. H., Sandahl, J. F., Labenia, J. S., & Scholz, N. L. (2003). Sublethal effects of copper on coho salmon: impacts on nonoverlapping receptor pathways in the peripheral olfactory nervous system. *Environmental Toxicology and Chemistry, 22*(10), 2266-2274.

Baldwin, D. H., Tatara, C. P., & Scholz, N. L. (2011). Copper-induced olfactory toxicity in salmon and steelhead: Extrapolation across species and rearing environments. *Aquatic Toxicology, 101*(1), 295-297.

Bao, Y., Qiao, F., & Song, Z. (2012). Historical simulation and twenty-first century prediction of oceanic CO_2 sink and h change. *Acta Oceanologica Sinica, 31*(5), 87-97.

Baumann, H., Talmage, S. C., & Gobler, C. J. (2012). Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. *Nature Climate Change, 2*(1), 38-41.

Beaumont, M. W., Butler, P. J., & Taylor, E. W. (1995). Impairment of sustained swimming performance of brown trout (*Salmo trutta*) exposed to copper in soft acidic water. *Journal of Physiology-London, 483P*, 197-198.

Berge, J. A., Bjerkeng, B., Pettersen, O., Schaanning, M. T., & Øxnevad, S. (2006). Effects of increased sea water concentrations of CO_2 on growth of the bivalve *Mytilus edulis*. *Chemosphere, 62*(4), 681-687.

Bewers, J. M., & Yeats, P. A. (1989). Transport of river-derived trace metals through the coastal zone. *Netherlands Journal of Sea Research, 23*(4), 359-368.

Bibby, R., Widdicombe, S., Parry, H., Spicer, J., & Pipe, R. (2008). Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. *Aquatic Biology, 2*, 67–74.

Bjerregaard, P., & Vislie, T. (1986). Effect of copper on ion-and osmoregulation in the shore crab *Carcinus maenas*. *Marine Biology, 91*(1), 69-76.

Blanchard, J., & Grosell, M. (2006). Copper toxicity across salinities from freshwater to seawater in the euryhaline fish *Fundulus heteroclitus*: Is copper an ionoregulatory toxicant in high salinities? *Aquatic Toxicology, 80*(2), 131.

Boitel, F., & Truchot, J.-P. (1989). Effects of sublethal and lethal copper levels on hemolymph acid-base balance and ion concentrations in the shore crab *Carcinus maenas* kept in undiluted sea water. *Marine Biology, 103*(4), 495-501.

Bopp, S. K., Abicht, H. K., & Knauer, K. (2008). Copper-induced oxidative stress in rainbow trout gill cells. *Aquatic Toxicology, 86*(2), 197-204.

Bryan, G., & Gibbs, P. E. (1983). Heavy metals in the Fal estuary, Cornwall: A study of long term contamination by mining waste and its effects on estuarine organisms. *Occasional Publication of the Marine Biological Association 2*.

Burnett, L., & Johansen, K. (1981). The role of branchial ventilation in haemolymph acid-base changes in the shore crab *Carcinus maenas* during hypoxia. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 141*(4), 489-494.

Burnett, L., (1997). The challenges of living in hypoxic and hypercapnic aquatic environments. *American Zoologist, 37*(6), 633-640.

Bury, N. R., Jie, L., Flik, G., Lock, R. A., & Bonga, S. E. W. (1998). Cortisol protects against copper induced necrosis and promotes apoptosis in fish gill chloride cells *in vitro*. *Aquatic Toxicology, 40*(2), 193-202.

Caldeira, K., Wickett, M.E., (2003). Anthropogenic Carbon and pH. *Nature, 425*(6956), 365-372.

Catches, J. S., Burns, J. M., Edwards, S. L., & Claiborne, J. B. (2006). Na^+/H^+ antiporter, V-H^+-ATPase and Na^+/K^+-ATPase immunolocalization in a marine teleost (*Myoxocephalus octodecemspinosus*). *Journal of Experimental Biology, 209*(17), 3440-3447.

Claiborne, J., Compton - Mccullough, D., & Walton, J. (2000). Branchial acid - base transfers in the euryhaline oyster toadfish during exposure to dilute sea water. *Journal of Fish Biology, 56*(6), 1539-1544.

Claiborne, J. B. (1998). *The Physiology of Fishes* (D. H. Evans Ed.): CRC press.

Claiborne, J. B., Edwards, S. L., & Morrison-Shetlar, A. I. (2002). Acid–base regulation in fishes: cellular and molecular mechanisms. *Journal of Experimental Zoology, 293*(3), 302-319.

Claiborne, J. B., Perry, E., Bellows, S., & Campbell, J. (1997). Mechanisms of acid-base excretion across the gills of a marine fish. *Journal of Experimental Zoology, 279*, 509-520.

Clark, R. B. (2001). *Marine Pollution* (5th Ed. ed.): Oxford University Press.

Cripps, I. L., Munday, P. L., & McCormick, M. I. (2011). Ocean Acidification Affects Prey Detection by a Predatory Reef Fish. *PLoS ONE, 6*(7), e22736.

Dang, F., Zhong, H., & Wang, W.-X. (2009). Copper uptake kinetics and regulation in a marine fish after waterborne copper acclimation. *Aquatic Toxicology, 94*(3), 238-244.

De Boeck, G., Hattink, J., Franklin, N. M., Bucking, C. P., Wood, S., Walsh, P. J., & Wood, C. M. (2007). Copper toxicity in the spiny dogfish (*Squalus acanthias*): Urea loss contributes to the osmoregulatory disturbance. *Aquatic Toxicology, 84*(2), 133-141.

De Boeck, G., Vlaeminck, A., & Blust, R. (1997). Effects of sublethal copper exposure on copper accumulation, food consumption, growth, energy stores, and nucleic acid content in common carp. *Archives of Environmental Contamination and Toxicology, 33*(4), 415-422.

Deigweiher, K., Koschnick, N., Pörtner, H. O., & Lucassen, M. (2008). Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. *AJP - Regulatory, Integrative and Comparative Physiology, 295*(5), 1660-1670.

Dejours, P. (1969). Variations of CO_2 output of a fresh-water teleost upon change of the ionic composition of water. *The Journal of physiology, 202*(2), 113.

Dixson, D. L., Munday, P. L., & Jones, G. P. (2010). Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecology Letters, 13*(1), 68-75.

Doney, S. C., Fabry, V. J., Feely, R. A., & Kleypas, J. A. (2009). Ocean Acidification: The Other CO_2 Problem. *Annual Review of Marine Science, 1*(1), 169-192.

EPA. (2007). Aquatic Life Ambient Freshwater Quality Criteria–Copper. *Water*.

Esbaugh, A., Heuer, R., & Grosell, M. (2012). Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. *Journal of Comparative Physiology B, 182*(7), 921-934.

Evans, D. H., Piermarini, P. M., & Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Reviews, 85*(1), 97-177.

Fabry, V. J., Seibel, B. A., Feely, R. A., & Orr, J. C. (2008). Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES Journal of Marine Science: Journal du Conseil, 65*(3), 414-432.

Feely, R. A., Sabine, C. L., Lee, K., Berelson, W., Kleypas, J., Fabry, V. J., & Millero, F. J. (2004). Impact of anthropogenic co_2 on the $CaCO_3$ system in the oceans. *Science, 305*(5682), 362-366.

Feng, Q., Boone, A. N., & Vijayan, M. M. (2003). Copper impact on heat shock protein 70 expression and apoptosis in rainbow trout hepatocytes. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 135*(3), 345-355.

Fish, F. F. (1943). The anaesthesia of fish by high carbon-dioxide concentrations. *Transactions of the American Fisheries Society, 72*(1), 25-29.

Fivelstad, S., Haavik, H., Løvik, G., & Olsen, A. B. (1998). Sublethal effects and safe levels of carbon dioxide in seawater for Atlantic salmon postsmolts (*Salmo salar L.*): ion regulation and growth. *Aquaculture, 160*(3-4), 305-316.

Fivelstad, S., Olsen, A. B., Kløften, H., Ski, H., & Stefansson, S. (1999). Effects of carbon dioxide on Atlantic salmon (*Salmo salar L.*) smolts at constant pH in bicarbonate rich freshwater. *Aquaculture, 178*(1-2), 171-187.

Fivelstad, S., Olsen, A. B., sgård, T., Baeverfjord, G., Rasmussen, T., Vindheim, T., & Stefansson, S. (2003). Long-term sublethal effects of carbon dioxide on Atlantic salmon smolts (*Salmo salar L.*): Ion regulation, haematology, element composition, nephrocalcinosis and growth parameters. *Aquaculture, 215*(1-4), 301-319.

Flemming, C. A., Trevors, J.T. (1989). Copper toxicity and chemistry in the environment: A review. *Water, Air and Soil Pollution* (44), 143-158.

Foss, A., Røsnes, B., & Øiestad, V. (2003). Graded environmental hypercapnia in juvenile spotted wolffish (*Anarhichas minor Olafsen*): Effects on growth, food conversion efficiency and nephrocalcinosis. *Aquaculture, 220*(1), 607-617.

Franke, A., & Clemmesen, C. (2011). Effect of ocean acidification on early life stages of Atlantic herring (Clupea harengus L.). *Biogeosciences Discuss, 8*, 7097-7126.

Freedman, J. H., Ciriolo, M. R., & Peisach, J. (1989). The role of glutathione in copper metabolism and toxicity. *Journal of Biological Chemistry, 264*(10), 5598-5605.

Freire, C. A., Onken, H., & McNamara, J. C. (2008). A structure–function analysis of ion transport in crustacean gills and excretory organs. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 151*(3), 272-304.

Gabbianelli, R., Lupidi, G., Villarini, M., & Falcioni, G. (2003). DNA Damage induced by copper on erythrocytes of gilthead sea bream (*Sparus aurata*) and mollusk (*Scapharca inaequivalvis*). *Archives of Environmental Contamination and Toxicology, 45*(3), 350-356.

Georgalis, T., Perry, S. F., & Gilmour, K. M. (2006). The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology, 209*(3), 518-530.

Gilmour, K. M., & Perry, S. F. (2009). Carbonic anhydrase and acid-base regulation in fish. *Journal of Experimental Biology, 212*(11), 1647-1661.

Götze, S., Matoo, O. B., Beniash, E., Saborowski, R., & Sokolova, I. M. (2014). Interactive effects of CO_2 and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. *Aquatic Toxicology, 149*, 65-82.

Green, W. W., Mirza, R. S., Wood, C. M., & Pyle, G. G. (2010). Copper binding dynamics and olfactory impairment in Fathead Minnows (*Pimephales promelas*). *Environmental Science & Technology, 44*(4), 1431-1437.

Grosell, M. (2012). *Homeostasis and Toxicology of Essential Metals* (Vol. 31A).

Grosell, M., De Boeck, G., Johannsson, O., & Wood, C. M. (1999). The effects of silver on intestinal ion and acid-base regulation in the marine teleost fish, *Parophrys vetulus*. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 124*(3), 259-270.

Grosell, M., McDonald, M. D., Walsh, P. J., & Wood, C. M. (2004). Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) II: copper accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues. *Aquatic Toxicology, 68*(3), 263-275.

Grosell, M., McDonald, M. D., Wood, C. M., & Walsh, P. J. (2004). Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) I. Hydromineral balance and plasma nitrogenous waste products. *Aquatic Toxicology, 68*(3), 249-262.

Grosell, M., Nielsen, C., & Bianchini, A. (2002). Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 133*(1-2), 287-303.

Hansen, B. H., Rømma, S., Garmo, Ø. A., Pedersen, S. A., Olsvik, P. A., & Andersen, R. A. (2007). Induction and activity of oxidative stress-related proteins during waterborne Cd/Zn-exposure in brown trout (*Salmo trutta*). *Chemosphere, 67*(11), 2241-2249.

Hansen, H. J. M., Olsen, A. G., & Rosenkilde, P. (1993). The effect of Cu^{2+} on osmoregulation in rainbow trout (*Oncorhynchus mykiss*) assayed by changes in

plasma salinity and gill lipid metabolism. *Science of The Total Environment, 134, Supplement 2*(0), 899-905.

Hansen, J., Lipton, J., Welsh, P., Morris, J., Cacela, D., & Suedkamp, M. (2002). Relationship between exposure duration, tissue residues, growth, and mortality in rainbow trout (*Oncorhynchus mykiss*) juveniles sub-chronically exposed to copper. *Aquatic Toxicology, 58*(3), 175-188.

Hansen, J. A., Rose, J. D., Jenkins, R. A., Gerow, K. G., & Bergman, H. L. (1999). Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) exposed to copper: Neurophysiological and histological effects on the olfactory system. *Environmental Toxicology and Chemistry, 18*(9), 1979-1991.

Hansen, J. A., Woodward, D. F., Little, E. E., DeLonay, A. J., & Bergman, H. L. (1999). Behavioural avoidance: Possible mechanism for explaining abundanc and distribution of trout species in a metal-impacted river. *Environmental Toxicology and Chemistry, 18*(2), 313-317.

Hayashi, M., Kita, J., & Ishimatsu, A. (2004). Comparison of the acid-base responses to CO_2 and acidification in Japanese flounder (*Paralichthys olivaceus*). *Marine Pollution Bulletin, 49*(11-12), 1062-1065.

Heisler, N. (1986). *Acid-base regulation in fishes.* Amsterdam: Elsevier Science Publishers.

Heisler, N., Weitz, H., & Weitz, A. (1975). Hypercapnia and resultant bicarbonate transfer processes in an elasmobranch fish (*Scyliorhinus stellaris*). *Bulletin europeen de physiopathologie respiratoire, 12*(1), 77-86.

Hendriks, I. E., Duarte, C. M., & Álvarez, M. (2010). Vulnerability of marine biodiversity to ocean acidification: A meta-analysis. *Estuarine, Coastal and Shelf Science, 86*(2), 157-164.

Henry, R. P. (1988). Multiple functions of carbonic anhydrase in the crustacean gill. *Journal of Experimental Zoology, 248*(1), 19-24.

Henry, R. P., & Cameron, J. N. (1983). The role of carbonic anhydrase in respiration, ion regulation and acid-base balance in the aquatic crab *Calunectes sapidus* and the

terrestrial crab *Geocarcinus lateraus*. *Journal of Experimental Biology, 103*(1), 205-223.

Henry, R. P., & Wheatly, M. G. (1992). Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. *American Zoologist, 32*(3), 407-416.

Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Hatziolos, M. E. (2007). Coral reefs under rapid climate change and ocean acidification. *Science, 318*(5857), 1737-1742.

Hogstrand, C., Ferguson, E. A., Galvez, F., Shaw, J. R., Webb, N. A., & Wood, C. M. (1999). Physiology of acute silver toxicity in the starry flounder (*Platichthys stellatus*) in seawater. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 169*(7), 461-573.

Hwang, P.P., Lee, T.H., & Lin, L.Y. (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 301*(1), 28-47.

IPCC. (2013). The physical science basis. Working group i) contribution to the IPCC 5th assessment report – changes to the underlying scientific/technical assessment.

Ishimatsu, A., Kikkawa, T., Hayashi, M., Lee, K. S., & Kita, J. (2004). Effects of CO_2 on marine fish: Larvae and adults. *Journal of Oceanography, 60*(4), 731-741.

Ivanina, A. V., Beniash, E., Etzkorn, M., Meyers, T. B., Ringwood, A. H., & Sokolova, I. M. (2013). Short-term acute hypercapnia affects cellular responses to trace metals in the hard clams *Mercenaria mercenaria*. *Aquatic Toxicology, 140*, 123-133.

Julliard, A., Saucier, D., & Astic, L. (1996). Time-course of apoptosis in the olfactory epithelium of rainbow trout exposed to a low copper level. *Tissue and Cell, 28*(3), 367-377.

Kidder, G. W., Petersen, C. W., & Preston, R. L. (2006). Energetics of osmoregulation: I. Oxygen consumption by *Fundulus heteroclitus*. *Journal of Experimental Zoology Part A: Comparative Experimental Biology, 305*(4), 309-317.

Kim, S.G., & Kang, J.C. (2004). Effect of dietary copper exposure on accumulation, growth and hematological parameters of the juvenile rockfish, *Sebastes schlegeli*. *Marine Environmental Research, 58*(1), 65-82.

Kirschner, L. B. (1993). The energetics of osmotic regulation in ureotelic and hypo-osmotic fishes. *Journal of Experimental Zoology, 267*(1), 19-26.

Kleypas, J. A., Buddemeier, R. W., Archer, D., Gattuso, J.P., Langdon, C., & Opdyke, B. N. (1999). Geochemical Consequences of Increased Atmospheric Carbon Dioxide on Coral Reefs. *Science, 284*(5411), 118-120.

Krumschnabel, G., Manzl, C., Berger, C., & Hofer, B. (2005). Oxidative stress, mitochondrial permeability transition, and cell death in Cu-exposed trout hepatocytes. *Toxicology and applied pharmacology, 209*(1), 62-73.

Kumai, Y., & Perry, S. F. (2012). Mechanisms and regulation of Na^+ uptake by freshwater fish. *Respiratory Physiology & Neurobiology, 184*(3), 249-256.

Larsen, B. K., Pörtner, H. O., & Jensen, F. B. (1997). Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology, 128*(2), 337-346.

Larsen, B. K., Pörtner, H. O., & Jensen, F. B. (1997). Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology, 128*(2), 337-346.

Laurén, D., & McDonald, D. (1985). Effects of copper on branchial ionoregulation in the rainbow trout (*Salmo gairdneri*). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 155*(5), 635-644.

Li, J., Quabius, E., Bonga, S. W., Flik, G., & Lock, R. (1998). Effects of water-borne copper on branchial chloride cells and Na^+/K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). *Aquatic Toxicology, 43*(1), 1-11.

Lynch, M., Webb, K., & Van Engel, W. (1973). Variations in serum constituents of the blue crab, *Callinectes sapidus*: chloride and osmotic concentration. *Comparative Biochemistry and Physiology Part A: Physiology, 44*(3), 719-734.

Maetz, J. (1971). Fish Gills: Mechanisms of salt transfer in fresh water and sea water. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 262*(842), 209-249.

McDonald, D. G., Walker, R. L., Wilkes, P. R., & Wood, C. M. (1982). H^+ excretion in the marine teleost *Parophrys vetulus*. *Journal of Experimental Biology, 98*(1), 403-414.

McGeer, J. C., Szebedinszky, C., McDonald, D. G., & Wood, C. M. (2002). The role of dissolved organic carbon in moderating the bioavailability and toxicity of Cu to rainbow trout during chronic waterborne exposure. *Comparative Biochemistry and Physiology Part C: Toxicology& Pharmacology, 133*(1–2), 147-160.

Michaelidis, B., Spring, A., & Pörtner, H. (2007). Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish *Sparus aurata*. *Marine Biology, 150*(6), 1417-1429.

Millero, F. J. (2009). Effect of ocean acidification on the speciation of metals in seawater. *Oceanography, 22*(4), 72.

Monteiro, S. M., dos Santos, N. M., Calejo, M., Fontainhas-Fernandes, A., & Sousa, M. (2009). Copper toxicity in gills of the teleost fish, *Oreochromis niloticus*: Effects on apoptosis induction and cell proliferation. *Aquatic Toxicology, 94*(3), 219-228.

Munday, P. L., Dixson, D. L., Donelson, J. M., Jones, G. P., Pratchett, M. S., Devitsina, G. V., & Døving , K. B. (2009). Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proceedings of the National Academy of Sciences, 106*(6), 1848-1852.

Munday, P. L., Donelson, J. M., Dixson, D. L., & Endo, G. G. K. (2009). Effects of ocean acidification on the early life history of a tropical marine fish. *Proceedings of the Royal Society B: Biological Sciences, 276*(1671), 3275-3283.

Munday, P. L., Gagliano, M., Donelson, J. M., Dixson, D. L., & Thorrold, S. R. (2011). Ocean acidification does not affect the early life history development of a tropical marine fish. *Marine Ecology Progress Series, 423*, 211-221.

Nawaz, M., Manzl, C., Lacher, V., & Krumschnabel, G. (2006). Copper-induced stimulation of extracellular signal-regulated kinase in trout hepatocytes: the role of reactive oxygen species, Ca^{2+}, and cell energetics and the impact of extracellular

signal-regulated kinase signalling on apoptosis and necrosis. *Toxicological Sciences, 92*(2), 464-475.

Neff, J., & Anderson, J. (1977). The effects of copper (II) on molting and growth of juvenile lesser blue crabs *Callinectes similis*. *Pollutant Effects on Marine Organisms,* 155-165*.*

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sorensen, C., Watson, S.-A., & Munday, P. L. (2012). Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change, 2*(3), 201-204.

NOAA (2015). Trends in Carbon Dioxide. Retrieved 15.08.15, from http://www.esrl.noaa.gov/gmd/ccgg/trends/weekly.html

O'Gara, B. A., Bohannon, V. K., Teague, M. W., & Smeaton, M. B. (2004). Copper-induced changes in locomotor behaviours and neuronal physiology of the freshwater oligochaete, *Lumbriculus variegatus*. *Aquatic Toxicology, 69*(1), 51-66.

Olsson, P.-E., Kling, P., & Hogstrand, C. (1998). Mechanisms of heavy metal accumulation and toxicity in fish. *Metal metabolism in aquatic environments*, 321-350.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Yool, A. (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature, 437*(7059), 681-686.

Pan, L., Liu, N., Zhang, H., Wang, J., & Miao, J. (2011). Effects of heavy metal ions (Cu^{2+}, Pb^{2+} and Cd^{2+}) on DNA damage of the gills, haemocytes and hepatopancreas of marine crab, *Charybdis japonica*. *Journal of Ocean University of China, 10*(2), 177-184.

Parveen, A., & Javed, M. (2010). Effect of water-borne copper on the growth performance of fish *Catla catla*. *International Journal of Agriculture & Biology, 12*.

Pelgrom, S., Lock, R., Balm, P., & Bonga, S. W. (1995). Integrated physiological response of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure. *Aquatic Toxicology, 32*(4), 303-320.

Perry, S., & Gilmour, K. (2006). Acid–base balance and CO_2 excretion in fish: Unanswered questions and emerging models. *Respiratory Physiology & Neurobiology, 154*, 199-215.

Pirrie, D., Power, M. R., Rollinson, G., Camm, G. S., Hughes, S. H., Butcher, A. R., & Hughes, P. (2003). The spatial distribution and source of arsenic, copper, tin and zinc within the surface sediments of the Fal Estuary, Cornwall, UK. *Sedimentology, 50*(3), 579-595.

Pörtner, H., Langenbuch, M., & Reipschläger, A. (2004). Biological impact of elevated ocean co_2; concentrations: lessons from animal physiology and earth history. *Journal of Oceanography, 60*(4), 705-718.

Pörtner, H. O., Langenbuch, M., & Reipschlager, A. (2004). Biological impact of elevated ocean CO_2 concentrations: Lessons from animal physiology and earth history. *Journal of Oceanography, 60*(4), 705-718.

Pörtner, H. O., Reipschläger, A., & Heisler, N. (1998). Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level.

Reid, S., & McDonald, D. (1988). Effects of cadmium, copper, and low pH on ion fluxes in the rainbow trout, *Salmo gairdneri*. *Canadian Journal of Fisheries and Aquatic Sciences, 45*(2), 244-253.

Rtal, A., Nonnotte, L., & Truchot, J.-P. (1996). Detoxification of exogenous copper by binding to hemolymph proteins in the shore crab, *Carcinus maenas*. *Aquatic Toxicology, 36*(3–4), 239-252.

Rtal, A., & Truchot, J.-P. (1996). Haemolymph transport and tissue accumulation of exogenous copper in the shore crab, *Carcinus maenas*. *Marine Pollution Bulletin, 32*(11), 802-811.

Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., Rios, A. F. (2004). The oceanic sink for anthropogenic CO_2. *Science, 305*(5682), 367-371.

Sampaio, F. G., de Lima Boijink, C., Oba, E. T., dos Santos, L. R. B., Kalinin, A. L., & Rantin, F. T. (2008). Antioxidant defences and biochemical changes in pacu (*Piaractus mesopotamicus*) in response to single and combined copper and hypoxia exposure. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 147*(1), 43-51.

Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.-M., & Ait-Aissa, S. (2005). Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. *Environmental Toxicology and Pharmacology, 19*(1), 177-183.

Santos, E. M., Ball, J. S., Williams, T. D., Wu, H., Ortega, F., van Aerle, R., Tyler, C. R. (2010). Identifying health impacts of exposure to copper using transcriptomics and metabolomics in a fish model. *Environmental Science & Technology, 44*(2), 820-826.

Shetlar, R. E., & Towle, D. W. (1989). Electrogenic sodium-proton exchange in membrane vesicles from crab (*Carcinus maenas*) gill. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 257*(4), 924-931.

Simpson, S. D., Munday, P. L., Wittenrich, M. L., Manassa, R., Dixson, D. L., Gagliano, M., & Yan, H. Y. (2011). Ocean acidification erodes crucial auditory behaviour in a marine fish. *Biology Letters, 7*(6), 917-920.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low-levels of DNA damage in individual cells. *Experimental Cell Research, 175*(1), 184-191.

Small, D., Calosi, P., White, D., Spicer, J., & Widdicombe, S. (2010). Impact of medium-term exposure to CO_2 enriched seawater on the physiological functions of the velvet swimming crab *Necora puber. Aquatic Biology, 10*(1), 11-21.

Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K., Miller, H. (2007). The physical science basis. *Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change*, 235-337.

Stagg, R. M., & Shuttleworth, T. J. (1982). The effects of copper on ionic regulation by the gills of the seawater-adapted flounder (*Platichthys flesus L.*). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 149*(1), 83-90.

Stohs, S., & Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology and Medicine, 18*(2), 321-336.

Thurberg, F., Dawson, M., & Collier, R. (1973). Effects of copper and cadmium on osmoregulation and oxygen consumption in two species of estuarine crabs. *Marine Biology, 23*(3), 171-175.

Toews, D. P., Holeton, G. F., & Heisler, N. (1983). Regulation of the acid-base status during environmental hypercapnia in the marine teleost fish *Conger conger*. *Journal of Experimental Biology, 107*(1), 9-20.

Truchot, J.-P. (1986). Changes in the hemolymph acid-base state of the shore crab, *Carcinus maenas*, exposed to simulated tidepool conditions. *The Biological Bulletin, 170*(3), 506-518.

Truchot, J.-P. (1983). Regulation of acid-base balance. *The biology of Crustacea, 5*, 431-457.

Truchot, J.-P. (1979). Mechanisms of the compensation of blood respiratory acid - base disturbances in the shore crab, *Carcinus maenas* (L.). *Journal of Experimental Zoology, 210*(3), 407-416.

Valko, M., Morris, H., & Cronin, M. (2005). Metals, toxicity and oxidative stress. *Current medicinal chemistry, 12*(10), 1161-1208.

Vazzana, M., Salerno, G., Celi, M., Vizzini, A., Parrinello, D., Di Bella, M. L., & Arizza, V. (2010). Effect of *in vitro* exposure to cadmium and copper on sea bass blood cells. *Proceeding of the 18th ASPA Congress, Palerno* (Vol. 8).

Vitale, A., Monserrat, J., Castilho, P., & Rodriguez, E. (1999). Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 122*(1), 121-129.

Waiwood, K. G., & Beamish, F. W. H. (1978). Effects of copper, pH and hardness on the critical swimming performance of rainbow trout (*Salmo gairdneri* Richardson). *Water Research, 12*(8), 611-619.

Wang, T., Knudsen, P., Brauner, C., Busk, M., Vijayan, M., & Jensen, F. (1998). Copper exposure impairs intra-and extracellular acid-base regulation during hypercapnia in the fresh water rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B, 168*(8), 591-599.

Weeks, J. M., Jensen, F. B., & Depledge, M. H. (1993). Acid-base status, haemolymph composition and tissue copper accumulation in the shore crab *Carcinus maenas*

exposed to combined copper and salinity stress. *Marine ecology progress series. Oldendorf, 97*(1), 91-98.

Wilson, R. W., & Taylor, E. W. (1993). Differential responses to copper in rainbow trout (*Oncorhynchus mykiss*) acclimated to sea water and brackish water. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 163*(3), 239-246.

Wood, H. L., Spicer, J. I., & Widdicombe, S. (2008). Ocean acidification may increase calcification rates, but at a cost. *Proceedings of the Royal Society B: Biological Sciences, 275*(1644), 1767-1773.

Zeebe, R. E. (2012). History of seawater carbonate chemistry, atmospheric CO_2, and ocean acidification. *Annual Review of Earth and Planetary Sciences, 40*, 141-165.

Zimmer, A. M., Barcarolli, I. F., Wood, C. M., & Bianchini, A. (2012). Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. *Aquatic Toxicology, 122–123*(0), 172-180.

CHAPTER 2

Acid-base regulation in European sea bass (*Dicentrarchus labrax*) and shore crabs (*Carcinus maenas*)

CHAPTER 2

ACID-BASE REGULATION IN EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) AND SHORE CRABS (*CARCINUS MAENAS*)

1. Abstract

When challenged with hypercapnic environments (high pCO_2/low pH) fish and crustaceans must defend

their intra- and extra-cellular pH from acidosis to avoid detrimental physiological effects: a process known as acid-base regulation.

The following chapter examines the ability of European sea bass (*Dicentrarchus labrax*) and shore crabs (*Carcinus maenas*) to acid-base regulate their extracellular fluid under rapid-onset high pCO_2 seawater conditions. In these animals such regulation is achieved primarily by the elevation of their extracellular bicarbonate ion concentration [HCO_3^-], which compensates the acidifying effect of elevated blood pCO_2. Sea bass and shore crabs were exposed for 24 and 48 hours, respectively. Experimental conditions were chosen to represent early-next-century predictions for seawater pCO_2 (~1200 µatm).

The pCO_2 of the extracellular fluid rose immediately following the start of the exposure in both species, and achieved a steady state after 4 hours in sea bass and 1-2 hours in shore crabs. In shore crabs this rapid elevation in extracellular pCO_2 caused a marked acidosis. However, in sea bass an elevation in plasma [HCO_3^-] was so rapid (within 30 minutes) as to completely prevent an acidosis. The elevation of plasma [HCO_3^-] was sustained for the whole 48 hour experiment in sea bass, peaking at 8.9 mM (1.6-fold higher than pre-exposure values) and perfectly regulating blood pH throughout. By contrast a transient acidosis was seen during the first 24 hours in shore crabs despite an elevation in extracellular [HCO_3^-] of 2 mM during the 2 hours of exposure. No further increase was apparent until 12-24 hours when [HCO_3^-] began to rise again. Extracellular pH was returned to control values at 48 hours by which time a 1.6-fold increase in extracellular [HCO_3^-], from 6.8 to 11.0 mM, had occurred.

Both species were able to regulate their extracellular blood pH and did so by employment of the same primary strategy. Sea bass showed the most rapid regulation of blood pH observed in any fish species in response to elevated CO_2, by elevating extracellular [HCO_3^-] faster than the earliest time point at which a reliable blood sample could be obtained (30 min). How this acid-base regulation was achieved so swiftly is currently unknown but warrants further investigation. The response of shore crabs to acute environmental hypercapnia was a much slower (but more typical of aquatic animals studied previously) compensated respiratory acidosis via elevation of haemolymph [HCO_3^-] between 12 and 48 hours following the onset of high CO_2.

2. Introduction

Long before anthropogenically-driven ocean acidification fish and crustaceans developed ways of compensating endogenous acid-base disturbances. Carbon dioxide (which produces carbonic acid when dissolved in water) is constantly produced by respiration in mitochondria via the catabolism of proteins, fats and carbohydrates. Respiratory acidosis caused by elevated CO_2 is avoided by employment of a complex suite of cellular and molecular mechanisms. These strive towards maintaining intracellular and extracellular pH values most suited to physiological processes.

Many animals employ these same regulatory mechanisms when challenged with external environmental perturbations such as elevations in pCO_2 characteristic of ocean acidification. Elevations in the pCO_2 of the surrounding seawater causes the pCO_2 of the internal medium to rise, as the large surface of the gill is necessarily permeable to gases. Hydration of internalised CO_2 leads to the formation of carbonic acid in the same way as CO_2 from respiration. This would result in an intra- and extra-cellular acidosis if unabated. As such, the rise in seawater pCO_2 associated with anthropogenic emissions of CO_2 into the atmosphere has a direct bearing on the acid-base status of marine biota. Those animals that can, employ acid-base regulatory mechanisms to compensate this acidosis.

1.1 Why do animals acid-base regulate?

1.1.1 Enzyme function

Metabolic enzymes responsible for energy production have pH optima. Proper functionality of these proteins relies on an adequate rate of catalysis, a high degree of regulatory responsiveness and a correct balance between stability and lability. Intracellular conditions must be maintained within a narrow margin with respect to ionic strength, osmolyte composition and pH to facilitate effective and efficient functionality of these enzymes (Somero, 1986). Davis (1958) was the first to propose that the optimal intracellular pH (pH_i) was a state of neutrality. Within a pH region close to neutrality of water, ionisation of water soluble biosynthetic intermediaries is maximised with a consequent trapping of metabolites inside the cell (Rahn & Prakash, 1985).

Deviation from these conditions as a result of the accumulation of exogenous or endogenous acid-base relevant ions, will reduce metabolic performance (Heisler, 1986). Given that metabolic performance determines the amount of energy available to the animal for processes such as growth, reproduction and locomotion, maintaining optimal pH is vital for survival.

1.1.2 Oxygen transport

Additionally, the pH of an animal's internal fluid determines the affinity with which oxygen binds to the respiratory pigment. In crustaceans such as crabs where the respiratory pigment is not bound within a cell, this affinity will be determined by the extracellular pH (pH_e). In vertebrates the affinity of haemoglobin for oxygen is controlled by intracellular pH, which is obviously influenced by extracellular pH. The Bohr effect describes the relationship between pH and oxygen binding capacity. When oxygen binds or is released from the respiratory pigment, such as haemoglobin in fish (Riggs, 1988) or haemocyanin in crustaceans (Mangum, 1983), a conformational changes occur which results in alterations to its pK (Riggs, 1988). The result is that, at physiological pH, the binding of oxygen with these pigments causes protons to be released. The opposite is also true such that pigment molecules will take up protons when oxygen dissociates from them at lower pH (Riggs, 1988). Equally, changes in pH lead to a resultant affinity change. For example, lowering pH decreases the binding affinity between oxygen and the respiratory pigment, and vice versa. This relationship

is utilized in the delivery of oxygen to active tissues where CO_2 will be high (and therefore pH lower). This demand-based oxygen transport system cannot function effectively if animals are subject to large and persistent perturbations in pH_i/pH_e.

1.2 How do marine animals acid-base regulate?

Both fish and crustaceans compensate for pH_i /pH_e perturbations through the dual approaches of: a) buffering using molecules already present in the tissues, and to a much greater extent, b) ion exchange between the animal and environment (Claiborne, 1998; Henry & Wheatly, 1992). We considered these strategies in detail in Chapter 1, but focus here on the primary acid-base regulatory strategy and one most pertinent to the experiments presented here after: the accumulation of bicarbonate ions (HCO_3^-).

Once fish and crustaceans saturate their endogenous buffering capacity and maximise their limited scope for ventilatory alterations, the role of counteracting high-CO_2-induced extracellular acidosis falls to ion transport mechanisms. The main method by which maintenance of pH_e is achieved is the accumulation of HCO_3^- (Esbaugh et al., 2012; Larsen et al., 1997; Lloyd & White, 1967; Michaelidis et al., 2007; Toews et al., 1983).

The central role for accumulating extracellular HCO_3^- in compensating high-CO_2 induced acidosis is in little doubt and is has been found consistently in hypercapnic experiments in fish and crustaceans. What remains unclear is the precise origin of this accumulated HCO_3^-. Some of the earliest authors to describe the central role of HCO_3^- in acid-base regulation suggested it was taken up from the environment (Heisler et al., 1975; Lloyd & White, 1967; Toews et al., 1983) and this suggestion remains to-date (Esbaugh et al., 2012). The reversal of branchial Cl^-/HCO_3^- transporters was suggested as a mechanism by which HCO_3^- uptake might occur, in exchange for Cl^-.

Lloyd and White (1967) describe a significant elevation in plasma [HCO_3^-], coincident with a comparable drop in [Cl^-], under exposure to environmental high CO_2 (~17,000 µatm) in freshwater rainbow trout. In seawater Toews et al. (1983) found that conger eels (C*onger conger*) exposed to high CO_2 (10,000 µatm) showed a large and sustained increase in plasma HCO_3^- which appeared to be the major contributor in compensating blood pH following the initial acidosis. The author stated that the

considerable elevation in plasma [HCO_3^-] far exceeded that which could be attributed to extracellular non-bicarbonate buffering, pointing to an exogenous origin. This assertion was corroborated by a measured drop in the [HCO_3^-] of the experimental seawater. Furthermore the approximately equimolar drop in plasma [Cl^-] concentration follows an inversely proportional relationship over time when compared with the rise in plasma HCO_3^-, leading the author to conclude that Cl^-/HCO_3^- transporters were the primary means of HCO_3^- uptake and therefore acid-base regulation. Esbaugh et al. (2012) also proposed HCO_3^- was taken up from the environment when gulf toadfish (*Opsanus beta*) exposed the hypercapnia (1000 and 1900 µatm) in HCO_3^- free seawater showed a compromised ability to elevate plasma HCO_3^- following 24 h exposure. However, given that plasma pCO_2 was not elevated beyond control values in this treatment and pH remained unaffected it could be argued that there was in fact no acidosis in need of compensation. This would somewhat undermine the lack of elevated plasma HCO_3^- in the HCO_3^--free seawater condition as evidence for inhibited acid-base regulation.

Truchot (1979) found that shore crabs also utilize accumulation of extracellular HCO_3^- as a primary strategy for regulating acid-base status however no changes were observed in the extracellular Cl^- concentrations. This may be a result of experimental limitations in the ability to detect relatively minor changes in Cl^- (around 1-5 %) compared to 2-fold or more changes in HCO_3^- concentrations.

The lack of direct evidence for HCO_3^- led researchers to suggest alternative means by which animals may accumulate HCO_3^-. Rather than direct uptake from the environment, animals may utilize the carbonic anhydrase mediated hydration of CO_2 to endogenously produce HCO_3^- in the gill epithelium (Claiborne et al., 1997). This reaction produces HCO_3^-, transported basolaterally by Cl^-/HCO_3^- exchangers for extracellular buffering, and H^+ ions which are then excreted across the gill surface into the seawater via apical Na^+/H^+ exchangers. In addition the normal efflux of HCO_3^- for inward Cl^- movement is halted or diminished in an effort to accumulate further HCO_3^-. The result is an increase in extracellular [HCO_3^-], a decrease in [Cl^-], and the excretion of H^+ which would titrate HCO_3^- in the external medium giving the appearance of reduced ambient [HCO_3^-]. All of which are consistent with commonly reported findings.

Research to-date has been unable to elucidate which model (or both) holds true. However, regardless of the precise mechanism, the reliance on elevating extracellular HCO_3^- appears conserved across those acid-base regulating animals tested. This represents a considerable change in the acid-base chemistry of these animals' internal environment and in need of further consideration in the context of changing seawater pCO_2 conditions imposed by anthropogenic emissions.

Through the employment of electroneutral exchanges with Cl^- and Na^+, the movement of acid-base relevant ions is intimately linked with osmoregulation providing a further dimension to understanding potential physiological perturbations under high CO_2.

Little is known about the differential ability of groups of animals such as fish and crustaceans to acid-base regulate, nor the differences between individual species. Furthermore almost nothing is known about the energetic cost or effects of chronic high CO_2 exposure on other physiological functions.

Although most teleost fish and crustaceans examined to date employ very similar approaches to regulating their acid-base status when challenged with environmental hypercapnia, there seem to be differences in the utilization of, and dependence on, different configurations of ion-transporters. As such we must acknowledge that despite the remarkably conserved use of many such ion-transporters, particularly those also involved in osmoregulation, we cannot say that all fish or all crustaceans possess the same transporter make-up. Furthermore that the cellular and subcellular locations of these transporters differs greatly between species and under differing environmental stimuli (Evans et al., 2005). As such the broad application of findings between acid-base regulating animals may be limited to 'macro' impacts such as elevated extracellular HCO_3^- or changes to energy allocation, rather than, for example, up or down regulation of genes for specific ion transporters. In the present study such 'macro' effects on two essentially unrelated marine species, European sea bass (*Dicentrarchus labrax*) and shore crabs (*Carcinus maenas*) are considered.

1.3 Experimental Approach

The aim of the present study was to test the acid-base regulatory capability of the two test species under pCO_2 conditions predicted for the next century. Whilst almost entirely unrelated in taxonomical terms, marine teleosts and most crustaceans are

considered to employ the same strategies for acid-base regulation, as discussed above: namely the elevation of their extracellular [HCO_3^-]. This makes them interesting targets for comparative work, particularly with the aim of assessing potential tolerances across different marine taxa. The extent to which HCO_3^- was elevated and the time-course of the changes to the extracellular chemistry following onset of exposure to high CO_2 were considered. The experiments aimed to employ elevated environmental pCO_2 levels more realistic to near-future climate change scenarios than most previous work of this nature.

2. Methods

2.1 Animals

Young adult sea bass were transferred from Cefas, Lowestoft, UK in January, 2012. Animals were kept for 1 year in a 150 L stream tank fed by 1-micron-filtered, UV-sterilized Tropic Marin® artificial seawater (Nominal: 15 °C, pH 8.15, salinity = 35 ppt, pCO_2 = 400 µatm, total alkalinity (TA) ~ 2500 µmol/kgSW). Bass were fed with 1 %BW fish pellets (Skretting, Gamma Diamond) twice daily, three times a week. At the time of the experiment bass weighed 486.5 ± 41.8 g (mean ± SEM).

Adult shore crabs were collected from the Exe Estuary, Exeter, UK, in March, 2012. Animals were kept for five days in a 100 L holding tank, fed by the same quality of seawater detailed above, in order to acclimatise prior to experiment. Crabs were fed mussels (Tropical Marine Centre) to satiation upon arrival, but not for 3 days prior to the experiment, with the aim of minimising differences in starting nutritional state among individuals. At the time of the experiment crabs weighed 45.4 ± 0.99 g (mean ± SEM).

2.2 Experimental Design

For sea bass a repeated measures design was used to determine the effects of elevated pCO_2 on a number of blood acid-base parameters. Sea bass were acutely exposed to either control conditions (490 µatm), hereafter referred to as time = 0, or 0.5, 1, 2, 4, 8, or 24 hours of exposure to elevated pCO_2 (1295 µatm). N = 18, 6, 6, 6, 10 and 11, respectively.

For shore crabs, the volumes of haemolymph required for analysis necessitated an independent measures design. Crabs of mixed sex were acutely exposed to either control conditions (470 µatm), also referred to as time = 0, or 1, 2, 4, 8, 24 or 48 hours exposure to elevated pCO_2 (1230 µatm). N = 20, 8, 8, 8, 8, 8, 7 and 10, respectively

During all exposures animals were held in 20 L containers, fed by artificial seawater via gas impermeable Tygon ® tubing (R-3603) in a self-contained 500 L recirculation system. Photoperiod in the experimental room was 12:12 and water temperature was controlled at 15 °C.

2.3 Water Chemistry

Elevation of seawater pCO_2 was achieved via a two-pronged approach. Commercially available pH computers (Aqua Medic, Bissendorf, Germany) maintained water pH to an accuracy of 0.05 units via the computer-controlled release of pure CO_2 (BOC, ltd) into air-mixed header tanks. This enabled a rapid decrease in pH (increase in pCO_2) at the start of the exposure and maintenance with only minor fluctuations above and below the designated pH value. Additionally mass flow controllers (AALBORG, Mass Flow Controller GFC17) provided mixed gas of the target pCO_2 to each bucket via limewood air stones. Seawater pH, salinity and temperature were verified manually throughout the duration of the experiment using a handheld meters (Hanna Instruments HI8314 Membrane pH Meter with Radiometer Probe, YSI Inc. salinity, conductivity, temperature meter model 30/10). The pH meter was calibrated prior to each use using Radiometer Analytical buffer solutions (pH 7.00 and 10.00).

Samples were taken for subsequent analysis of dissolved inorganic carbon (DIC) and preserved using mercuric chloride ($HgCl_2$) up to 0.04 % (w/v), as per Dickson et al. (2007). Measurement of DIC was carried out using a custom-built automated DIC analyser system incorporating a Qubit Systems infra-red CO_2 analyser (Model S154). This system was based on that described by Friederich et al. (2002). Seawater samples were measured against Dickson standards (Kozyr, 2015). Data on pH, salinity and temperature in conjunction with DIC values enabled calculation of total alkalinity (TA) and pCO_2 using CO2sys (Pierrot, 2006). Values were derived using the NBS pH scale, K1 and K2 constants from Mehrbach et al. (1973), refitted by Dickson and Millero (1987), and dissociation constants for KSO_4 from Dickson (1990).

2.4 Acid base parameters

Attempts to cannulate sea bass from either the caudal artery, dorsal aorta or branchial arteries, were largely unsuccessful. This was due to relatively small diameter of the blood vessels in even the largest animals attempted (~600 g) and the difficulty in gaining access imposed by the structure of the bass vertebrae (caudal) and roof of the mouth (dorsal).

With cannulation not possible arterial blood was removed via a caudal puncture under anaesthesia (85 mg/L MS-222) and gill irrigation. This technique has been shown to produce blood gas and acid-base data identical to blood obtained from dorsal aorta catheters in unanaesthetised rainbow trout (Wilson & Cooper, in preparation), and so avoids the problems typically associated with gill collapse in air when sampling by caudal puncture. Anaesthesia was added to experimental tanks through a funnel inserted through the opaque lid in order to minimise stress to the animal. Dissolution of MS-222 during preparation of stock solutions can yield pH values as low as 2.8 (Ohr, 1976) due to the formation of methanesulfonic acid (Smith et al., 1999). For this reason MS-222 stocks are typically buffered using $NaHCO_3$ at a ratio of 1:2 (Popovic et al., 2012). In the present experiment however MS-222 stock solutions were titrated to treatment seawater pH using NaOH in order to match the water pCO_2. The drastically elevated pCO_2 that results from the use of $NaHCO_3$ as a buffer (Wilson and Urbina, unpublished) would compromise the water chemistry of the experimental tanks and therefore affect the animals' acid-base status.

After withdrawal, blood pH was immediately measured at controlled temperature (Hanna Instruments HI8314 Membrane pH Meter with Accumet Microprobe). Plasma samples were stored at 4° C in gas impermeable micro capillary tubes for analysis of total CO_2 (TCO_2). Measurement of TCO_2 was conducted within six hours of extraction from the animal using a Mettler Toledo 965D Carbon Dioxide analyser.

Extraction of haemolymph from shore crabs was achieved without catheterisation or anaesthetic. Crabs were not anaesthetised on ice, as is common, due to the effect this acute temperature change would have on haemolymph acid-base chemistry. Haemolymph extraction was conducted immediately upon emersion. Samples were discarded if extraction took longer than 60 seconds. Six-hundred microliters of prebranchial haemolymph was extracted from the infrabranchial sinus at the base of

the third and fourth walking legs of each animal (Truchot, 1973) using a 1 ml syringe and 21G needle. Haemolymph pH measurement and storage for TCO_2 then proceeded as above

2.5 Blood and Haemolymph Calculations

For sea bass blood acid-base variables were calculated using apparent pK (pK_{app}) values obtained from Boutilier et al. (1984) based on *in vitro* determinations in rainbow trout. pK_{app} is calculated by the rearrangement of the Henderson-Hasselbalch equation, as per equation 1:

Equation 1: $pK_{app} = pH - \log (TCO_2 / \alpha CO_2 * pCO_2) - 1$

Where αCO_2 is the CO_2 solubility coefficient for fish plasma at the given temperature: determined from equations given in the same chapter. For shore crab haemolymph pK_1 was calculated from in a same manner using a CO_2 solubility coefficient given in Truchot (1976).

2.6 Statistics

A one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to compare acid-base parameters between time points for both sea bass and shore crabs. The level of significance for all statistical analyses was $p<0.05$.

3. Results

3.1 Sea Bass

Experimental seawater was maintained at 490 µatm prior to the onset of high CO_2 exposure (T=O), before being successfully elevated to 1295 µatm for the duration of the high CO_2 exposure by the means outlined in *Methods: Acid-base Parameters* (see Appendix: Table 1 for full seawater chemistry).

A time-course of plasma pH, bicarbonate and pCO_2 was plotted to compare changes to these variables in the 24 hour period following elevation to high environmental CO_2. Plasma pCO_2 rose marginally (0.45 mmHg) immediately following onset of environmental high CO_2 and reached a significantly elevated steady state after 4 hours [ANOVA: $F_{(6,53)}=7.68$, $p<0.001$, Fig. 1a].

No significant decreases in blood pH were seen immediately following onset of high CO_2 or thereafter [ANOVA: $F_{(6,53)}$=2.08, p=0.07, Fig. 1b]. Plasma bicarbonate was significantly elevated within 30 minutes of exposure and remained elevated throughout the duration of the experiment, finally reaching a 1.6-fold increase from control [ANOVA: $F_{(6,53)}$=22.62, p<0.001, Fig. 1c].

The pH/bicarbonate/pCO_2 time-course (Fig.1) and Davenport diagram (Fig. 2) are that of a compensated respiratory acidosis in most respects, with rapid acid-base regulation prior to the first sample taken at 30 minutes. Changes in ventilation were not apparent, as evidenced by unchanged plasma pO_2 throughout the duration of the experiment [ANOVA: $F_{(6,53)}$=0.48, p=0.820, Fig. 1d].

3.2 Shore Crabs

Experimental seawater was maintained at 460 µatm prior to the onset of high CO_2 exposure, representing Control (T=0), before being successfully elevated to 1230 µatm for onset of the high CO_2 exposure by the means outlined in *Methods: Acid-base Parameters* (see Appendix: Table 2 for full seawater chemistry).

A time-course of plasma pH, bicarbonate and pCO_2 (Fig. 4) and Davenport diagram (Fig. 5) were plotted to compare changes to these variables in the 48 hour period following onset of high CO_2 A rapid and sustained increase in the haemolymph pCO_2 was seen immediately following onset of the exposure, reaching equilibrium between 1-2 hours (Fig. 4a). This elevated pCO_2 appeared to cause a transient extracellular acidosis during the first 24 hours (Fig. 4b) despite a rapid elevation in [HCO_3^-] of 2 mM during the 2 hours of exposure (Fig. 4c). Changes in haemolymph [HCO_3^-] showed a biphasic response with a rapid elevation to plateau by 2 hours but no further increase until 12-24 hours when both [HCO_3^-] and pH begin to rise again. The haemolymph pH was not fully restored to control values until 48 hours of high CO_2 exposure by which time a 1.6-fold increase in extracellular [HCO_3^-] from 6.8 to 11.0 mM had occurred. In summary, the response of shore crabs to acute environmental hypercapnia is one of a typical respiratory acidosis, compensated for an elevation of haemolymph bicarbonate between 12 and 48 hours following the onset of high CO_2.

4. Discussion

4.1 Acid-base Regulation in sea bass

Both sea bass and shore crabs were able to maintain the pH of their extracellular fluid when challenged with environmental hypercapnia that would otherwise result in acidosis. The large increase in bicarbonate seen in both sets of animals was coincident with maintenance of, or increases in, pH towards pre-exposure values. This strongly suggests that both species employed similar overall acid-base regulatory strategies, however the extent to which other mechanisms were involved were not be quantified in the present study: namely H^+ extrusion rates.

In sea bass, acute exposure to predicted next-century (IPCC, 2014) partial pressures of CO_2 (1295 µatm) led to an increase in extracellular plasma pCO_2 reaching an elevated steady state 4 hours after the onset of exposure. This is somewhat at odds with the generally accepted view that the pCO_2 of intra and extracellular fluids should come into near-immediate equilibration with the external environment. However similar pCO_2 equilibration profiles have been seen in other acid-base regulating marine animals (Pane & Barry, 2007). In the present study the respiratory acidosis expected in teleost fishes, as per Heisler et al. (1975) and Toews et al. (1983), appeared to be compensated prior to the first time-point at 30 minutes. The maintenance of extracellular pH observed in sea bass in the present study, despite a nearly 3-fold elevation in environmental pCO_2 (from 490 to 1295 µatm), appears to be achieved primarily through a rapid and sustained elevation of plasma HCO_3^- concentration; increasing 1.6 fold from 5.5 to 8.9 mM. This is consistent with previous studies conducted on both fresh water and seawater teleost fishes in which accumulation of HCO_3^- ions and excretion of H^+ ions were the primary means of extracellular acid-base homeostasis under environmental hypercapnia (Heisler et al., 1975; Lloyd & White, 1967; Toews et al., 1983). Although early work on acid-base regulation considered much higher concentrations of CO_2 (typically >10,000 µatm), recent work by Esbaugh et al. (2012) demonstrated a comparable fold increase in plasma bicarbonate of Gulf Toadfish (*Opsanus beta*) under a similar exposure: increasing from 380 to 1000 µatm for 24 hours. Comparison of the two datasets highlight interspecies variability in the equilibria of extracellular acid-base chemistry. For example in Esbaugh et al., (2012) the mean pH_e of control fish was ~7.7 (*Opsanus*

beta) compared with 7.92 (sea bass, present study), presumably related to differences in plasma [HCO_3^-]; ~3.5 mM compared with 5.5 mM respectively, as plasma pCO_2 values were similar (~1.7 and 1.4 mmHg respectively).

Sea bass were particularly effective in their extracellular pH regulation compared with previously assessed teleosts (Esbaugh et al., 2012; Michaelidis et al., 2007; Toews et al., 1983) and the shore crabs in the present study. So effective were they that a transient acidosis was not observed immediately following the onset of environmental hypercapnia, if one occurred at all. A complete lack of acid-base response to the elevation in external seawater CO_2 was ruled out due to the significant elevations in both plasma pCO_2 and HCO_3^-, confirming that they were indeed compensating for an acid-base perturbation.

The lack of measurable transient acidosis may be a function of: the sea bass possessing particularly efficient and effective acid-base regulating capability, the ability of the animals' buffering capacity to minimise acute disturbances, and the relatively low exposure pCO_2 of the present study when compared with much of the early work on acid-base regulation on teleosts (often >10,000 µatm). Plasma bicarbonate concentrations in sea bass (5.5 mM) kept under control conditions were within the normal range for fish in comparable seawater carbonate chemistry at 15 °C (3-11 mM; Norbert Heisler, 1984). They were somewhat higher than other species tested previously, for example Gulf toadfish (*Opsanus beta*), ~3.2 mM (Esbaugh et al., 2012), comparable to others: conger eels, (*Conger conger*), ~5 mM and sea bream (*Sparus aurata*), ~7 mM (Michaelidis et al., 2007; Toews et al., 1983), and lower than some: Atlantic cod (*Gadus morhua*), ~10 mM. In this context, the initial HCO_3^- concentrations of the sea bass in the present study were not remarkably high, and therefore was not likely attributable to the apparent lack of transient acidosis. The rapid (<30 min) compensation, or complete avoidance of, acidosis is therefore due to effective initial buffering by the non-bicarbonate buffering capacity of sea bass (not quantified in this study) and the exceptional rate at which extracellular HCO_3^- was accumulated.

Further work might aim to improve early-experiment time-point resolution in order to establish whether a very brief transient acidosis had occurred (e.g. within the first 30

min). Catheterisation of the dorsal aorta or caudal artery would assist this approach. Attempts to do so within the present study proved difficult (see Methods).

4.2 Acid-base Regulation in shore crabs

Shore crabs demonstrated a compensated respiratory acidosis, achieved by a large increase in the HCO_3^- content of their extracellular fluid. The elevation in extracellular HCO_3^- appeared to be biphasic in nature. This was characterised by a small but immediate increase in HCO_3^- coincident with the onset of acidosis, followed by a larger increase compensating the acidosis and returning pH to control values. One explanation of this biphasic response is the production of HCO_3^- ions by the hydration of CO_2 upon its diffusion into the haemolymph. This would elevate the haemolymph [HCO_3^-] but not to a sufficient degree to buffer the respiratory acidosis. The subsequent elevations in [HCO_3^-] were likely driven by either direct uptake via Cl^-/HCO_3^- exchangers or by the carbonic anhydrase mediated hydration of CO_2 (*see Introduction and Chapter 1 for discussion of the opposing models*). Regardless of the mechanism, the result in both instances is a net accumulation of HCO_3^- and excretion of H^+. This is consistent with Truchot (1979) who found that during exposure to environmental hypercapnia (~6800 µatm), shore crabs were able to effectively regulate their acid base-status by accumulation of extracellular HCO_3^- and apparent excretion of H^+ ions. These findings support a model for acid-base regulation in crabs, comparable to that of teleosts, driven by accumulation of HCO_3^- and excretion of H^+. The present study is also consistent with studies conducted on Dungeness crab (*Cancer magister*) who demonstrated the ability to near completely regulate their acid-base status during exposure to hypercapnia (~10,000 µatm; Pane & Barry, 2007). As per the present study these crabs underwent a transient acidosis immediately following the onset of hypercapnia, compensated for by significant elevations in extracellular HCO_3^-.

4.3 Effects of Chronic Acid-base Regulation

Aside from the benefits of a stabilisation in extracellular pH during hypercapnia, we must consider other potential physiological effects of chronic elevations in pCO_2 and HCO_3^- to fish and crustaceans, under long-term hypercapnia. For example, what are the costs, energetically or otherwise, associated with this regulation.

The first effect of exposure to a high CO_2 environment is an elevation in intra/extracellular pCO_2. Some studies have shown evidence for increased ventilation under hypercapnic conditions when O_2 was not limited (Burleson & Smatresk, 2000). However, given the fact that changes in ventilation are of limited benefit for reducing extracellular pCO_2, observations of increased ventilation under hypercapnia are usually attributed to maintenance of O_2 uptake in the face of a Bohr shift associated with a transient acidosis (McKenzie et al., 2002).

If energy supply is limited in the face of environmental stress, energy is conserved by reducing ATP-consuming processes such as protein biosynthesis, with considerable physiological implications, not least of which may be whole organism growth rates. Langenbuch and Pörtner (2003) reported significant drops in protein biosynthesis within fish hepatocytes under hypercapnia-induced acidosis, although these effects are likely transient, in parallel to the compensation of acidosis.

Michaelidis et al. (2007) found that, when exposed to long-term environmental hypercapnia (~5000 µatm), sea bream displayed shifts in the metabolic profile of their red and white muscle cells from aerobic to anaerobic pathways of substrate oxidation. This led to a rise in steady state levels of lactate in the blood and would have incurred an energetic cost to animals due to the greater cost of handling anaerobic end-products of ATP production compared with aerobic: likely exacerbating the cost of acid-base regulation.

Nilsson et al. (2012) proposed elevation in HCO_3^- during exposure to high CO_2 as the cause of neurotransmitter interference in the brains of fishes, leading to observed changes in behavioural and olfactory responses (Cripps et al., 2011; Dixson et al., 2010; Munday, 2015; Munday et al., 2014; Munday et al., 2009; Munday et al., 2013). Such detriment to important sensory ability and behaviours may have considerable impacts on survivorship. Consideration of species living near CO_2 seeps suggests that prolonged exposure over generations does not reverse these effects and that current success of such populations may only be due to the low predator numbers in such areas (Munday et al., 2014).

The impacts of chronically elevated blood HCO_3^- could also have the potential to compromise or alter a number of other physiological functions (Esbaugh et al., 2012). One example is the suggestion that elevated HCO_3^- might explain the increases in

rates of otolith growth in fish exposed to high CO_2 with largely unknown downstream effects for the animal. Conversely, some of the physiological impacts may be more beneficial to the organism. For example, work by the authors suggests that elevations in extracellular HCO_3^- may afford sea bass and shore crabs some protection from copper-induced DNA damage (see Chapters 3 and 4). Elevated HCO_3^- in pigs an rats exposed to combined hypoxia/hypercapnia has also been implicated in affording cells protection against free radical damage (Vesela & Wilhelm, 2002).

Chronic acid-base regulation could conceivably represent a considerable energetic cost to these organisms. Many of the ion transporters involved in the exchange of acid-base equivalents across the epithelia of the gills, blood cells and other regulatory organs like the kidneys and intestine incur an energetic cost in the form of ATP. Even transporters that make use of favourable ion gradients such as apical Na^+/H^+ or Cl^-/HCO_3^- exchangers, cause ion imbalances which must be corrected using ATP-dependent pumps such as Na^+-K^+-ATPase (Ahearn et al., 1999). Crabs are known to employ $2Na^+/H^+$ transporters for H^+ extrusion, possibly coupled with ATP- dependent H^+-ATPase proton pumps (Kimura et al., 1994). Pane and Barry (2007) found evidence for an apparent lack of acid-base regulatory ability in deep sea crabs faced with hypercapnia. They suggest that the energetic cost may be too high for animals such as these with metabolic rates much lower than their shallow water relatives.

Changes in the gene expression of ion transporters such as NHE2 and NHE3, isoforms of the Na^+/H^+ exchanger, have been documented under respiratory and metabolic acidosis (Claiborne, Edwards et al. 2002) although many of these changes have been on the scale of hours. Chronic exposure also gives rise to long term changes in gene expression. Deigweiher et al. (2008) measured the mRNA expression for a broad-spectrum of ion transporters associated with acid-base regulation in eelpout (*Zoarces viviparous*). The results showed marked temporal changes in expression. Production of some ion transporters showed transient reductions over the first few days before re-establishing control levels. Others such as Na^+/K^+-ATPase showed significant and sustained elevations within 24 hours and throughout the duration of the 42 day experiment. The cost of up-regulating these genes and the associated protein manufacture will be magnified by the ATP consumption by active pumps (such as NKA) at the point of application, i.e. ionocytes.

The energetic cost of long-term acid-base regulation under environmental hypercapnia has received disproportionately little attention for its impact potential and should be a focus of future work in this area. Changes in energy budget and resource allocation towards maintenance of stable internal pH will likely have negative effects on processes like growth and reproduction with potential consequences to survivorship and fecundity. Alternatively, in order to meet the increased energy demand, animals would have to consume more food. This would have implications for resource availability at a population and ecosystem level as well as potential behavioural changes such increased risk taking behaviour.

Animals were unfed throughout the duration of the present study. This was done in order to avoid any confounding affects associated with differing levels of consumption (both species) and to avoid regurgitation during anaesthesia and blood sampling (sea bass). Feeding also has the potential to greatly affect an animals' acid-base status. Immediately following ingestion, H^+ ions are secreted from the gastric glands of the stomach in order to promote pepsin digestion of protein. In order to maintain the acid-base status of the acid-secreting gastric gland cells, an equimolar amount of base in the form of HCO_3^-, must be removed and enters the extracellular fluid. This causes a transient metabolic alkalosis, the 'alkaline tide', which was originally identified in mammals (Niv & Fraser, 2002) but recently documented in teleost fish (Bucking & Wood, 2008). Furthermore, digestion of food requires energy, thus increasing metabolic rate and therefore potentially impacting the energy budget for homeostatic processes like acid-base regulation to an unknown degree.

4.4 Application of Findings and Limitations

Application of data from the present study to real world conditions raises a number of considerations: most of which pertain to the artificial nature of the experiment. The life history of these animals, particularly intertidal shore crabs present them with a highly variable environment with respect to salinity, temperature, pCO_2 and pO_2, all of which will have a considerable effect on their acid-base status. Given that shore crabs haemolymph is maintained hyperosmotic or isosmotic to the normal seawater (Thurberg et al., 1973) and in marine teleosts, such as sea bass, blood is hypo-osmotic (Krogh, 1939), osmoregulatory mechanisms are employed when exposed to changing salinity. Osmoregulation is intimately linked to acid-base regulation through the dual

applications of a number of ion transporters, most notably Na^+/H^+ and Cl^-/HCO_3^- exchangers. This means that fluctuating environmental salinity, associated perhaps with an estuarine environment, can cause acid-base disturbances (Henry & Wheatly, 1992) and may affect the way in which different ion transporters are employed to maximise energy efficiency. Euryhaline crabs (such a shore crabs) must compensate a metabolic alkalosis under decreased salinity and a metabolic acidosis under increased salinity (Whiteley et al., 2001). In teleost fish, increased salinity necessitates higher drinking rates and therefore higher excretion of HCO_3^- into the intestinal lumen to facilitate water reabsorption. This leads to a drop a pH and metabolic acidosis that must be compensated (Grosell & Genz, 2006).

Environmental pO_2 also affects acid-base status. Hypoxia, causes a respiratory alkalosis coincident with reductions in extracellular [HCO_3^-] and pCO_2 (Butler et al., 1979), with potential reductions in metabolic rate further exacerbating acid-base disturbances by reducing energy availability for acid-base regulatory machinery. However, particularly for crabs, hypercapnia will coincide with hypoxia in intertidal pools isolated from water influx where respiration lowers O_2 and elevates CO_2. Under these conditions the opposing alkalosis and reduction in haemolymph pCO_2 associated with hypoxia likely serve to counteract hypercapnia-induced acidosis and elevation in pCO_2. The net effect is thought to be a considerably reduced demand on acid-base regulatory machinery under combined hypoxia/hypercapnia and likely explains why crabs have not evolved more rapid acid-base regulation capacities (Truchot, 1986)

Fluctuations in temperature also induce changes to acid-base status, resulting in sustained changes to the pH of both blood and haemolymph which stay at approximately constant relative alkalinity: the difference between extracellular pH and neutral pH (Reeves, 1972). Furthermore, metabolic changes associated with both changing temperature and adaptation to different temperatures are likely to influence energy allocation to acid-base regulation. Pane and Barry (2007) reported a lack of acid-base regulatory capability in the deep-sea crab (*Chionoecetes tanneri*), suggesting the lower metabolic rate associated with their environment may slow the regulatory process to such a degree that it was not apparent within the duration of the experiment.

When considered all together the strong influence of these various seawater parameters shows us the complex nature of animals' acid-base responses to environmental changes. As such we must be careful when attempting to apply findings from simplified and artificial experiments to complex models under future climate change scenarios at the exclusion of cofactors like elevating sea temperatures and/or decreasing salinity. Multiple stressor studies aimed at assessing physiological impacts under predicted climate change scenarios may offer a more reliable means of predicting future effects on marine animals.

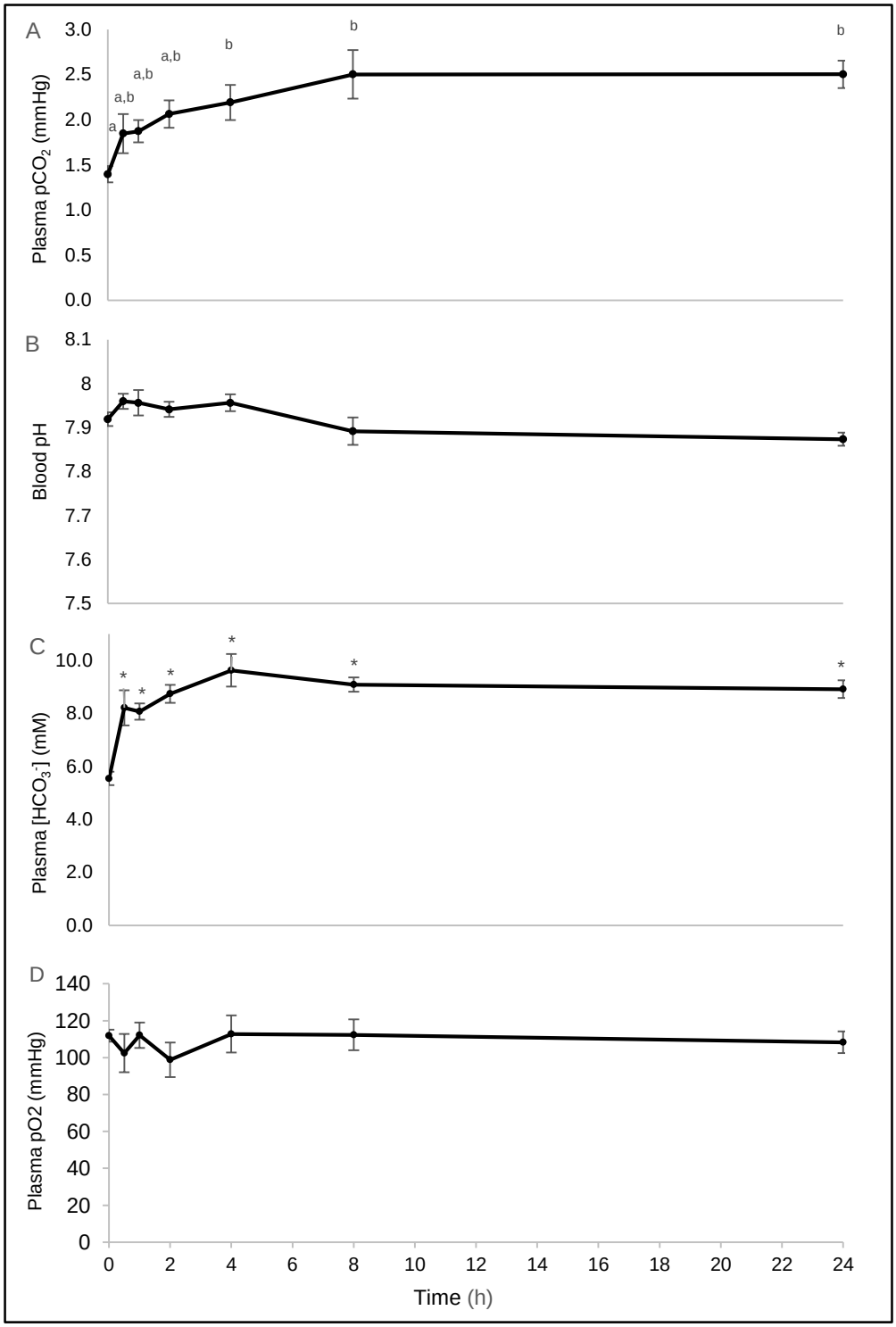
At the opposite end of the spectrum from the highly variable nature of shore crabs (intertidal) and juvenile sea bass (estuarine) habitats, are the deep ocean zones whose water chemistry shows very little variability. The animals inhabiting these zones are well adapted for their environment but may show little resistance to perturbations in seawater chemistry. Pane and Barry (2007) provided evidence to support such an idea when comparing shallow water crabs that were able to regulate their acid-base status effectively, with deep-sea crabs that showed no such regulatory ability. This idea has considerable implications for the vast array of marine species living in niches with low environmental variability, given that the rate of change in ocean chemistry may prevent adaptation.

4.5 Future Work

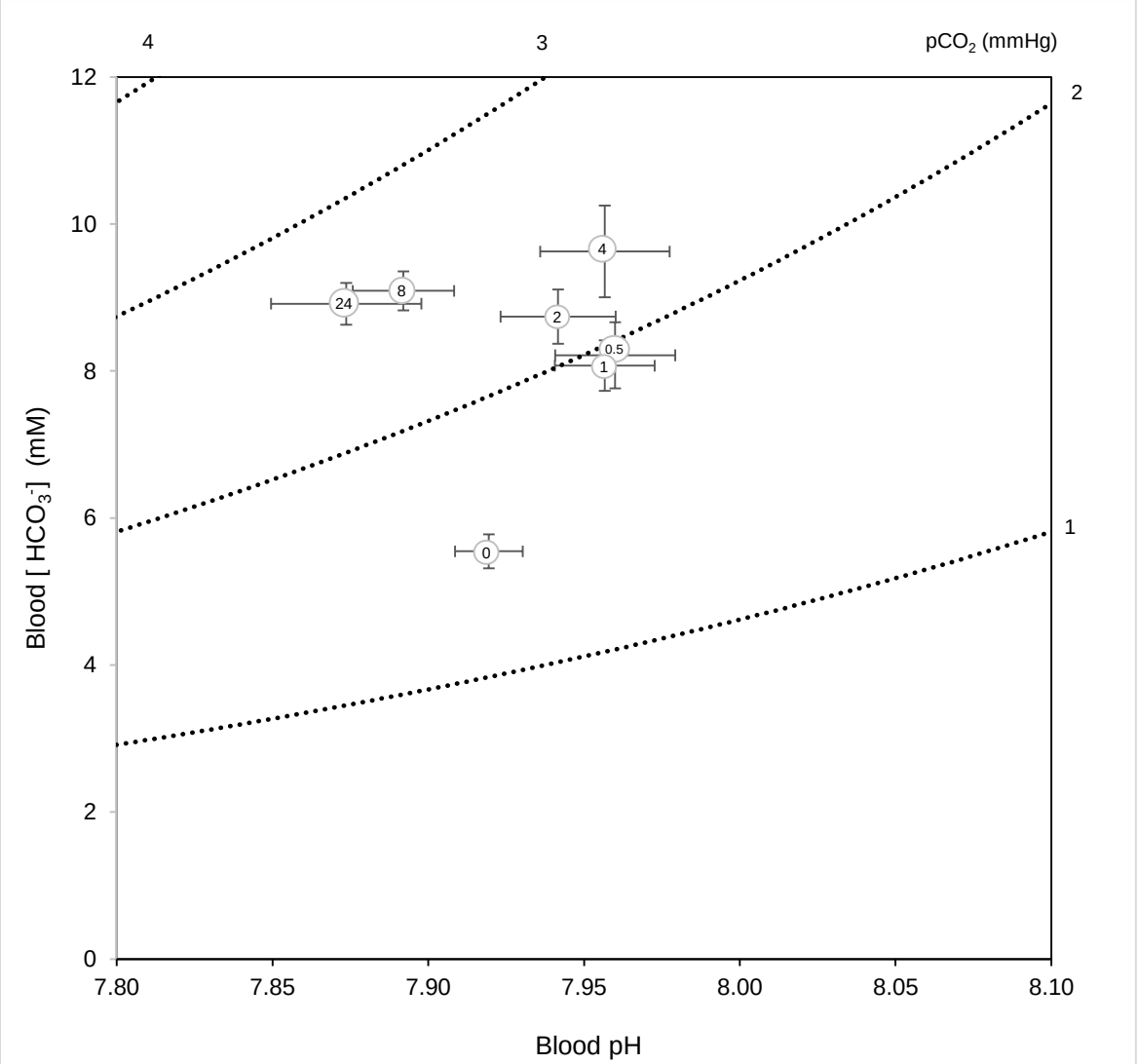
Future work should aim to elucidate the precise mechanisms of ion-transport between the extracellular fluid and the environment via the gill epithelial cells. This work might focus on establishing the source of the HCO_3^- ions accumulated to buffer extracellular fluid (endogenous or exogenous?). Manipulation of seawater [HCO_3^-] at a fixed pCO_2 and the assessment of any changes to the rate of acid-base regulation might help establish any role for endogenous HCO_3^-. The remit of such work, might include further characterisation of the role of CA in either or both models of acid-base regulation (*see Chapter 1: 2.1.1.1*). This might provide insight into ways in which inhibition of this enzyme, for example by chemical stressors such as copper, might influence an animal's ability to acid-base regulate, and therefore their tolerance to ocean acidification in contaminated environments. Work should aim towards better assessment of acid-base regulation under more environmentally realistic experimental conditions. Although the mechanisms will undoubtedly be the same when compared

with previous more extreme hypercapnia experiments, this type of work will provide us with a more realistic idea of the magnitude of effect we can expect on the acid-base chemistry of different species/taxa. An extension of this work would be to consider the acid-base regulatory ability of a wider range of species across different niches, particularly those inhabiting stable environments. Should a lack of regulatory ability be found in such species, the downstream cost to these organisms under a chronic acidotic state should be assessed. Conversely the energetics of acid-base regulation itself needs to be better understood. The energetic cost of regulatory mechanisms in the short-term as well as those employed over chronic exposures such as changes in gene regulation. The influence of food consumption on short-term acid-base regulation could be investigated to establish the influence of 'alkaline tide' on compensation of CO_2-induced acidosis. Closely linked to changes in energy allocation are a suite of other potential physiological changes, for example metabolic processes and protein biosynthesis as is suggested in the literature. Related to energy allocation is the potential for changes to metabolic rate or metabolic processes and any impact of a changing energy budget on processes such as growth (see Chapter 5) and reproduction. These ultimately have the potential for population and ecosystem level impacts. Finally, and posing arguably the most difficult question, is the assessment of the capacity for animals to adapt to chronic high CO_2. Some of the first work on this topic has been published recently and the implications are still somewhat unclear. On one hand a lab-based transgenerational experiment suggested that exposure of the parents to high CO_2 mediates to impact on juveniles of the second generation, reversing detrimental effects on length, weight, condition and survival (Miller et al., 2012). Whilst, conversely, a study examining juvenile reef fish from CO_2 seeps, showed the same behavioural and olfactory detriments previously found in lab-based single generation studies under artificially elevated CO_2 (Munday et al., 2014). These contradictory findings suggests continued and multigenerational exposure may reduce some but not all detrimental effects associated with high CO_2, and have only considered a few species. This is a key area of research in predicting the tolerance of, and physiological effects on, species in future high CO_2 oceans.

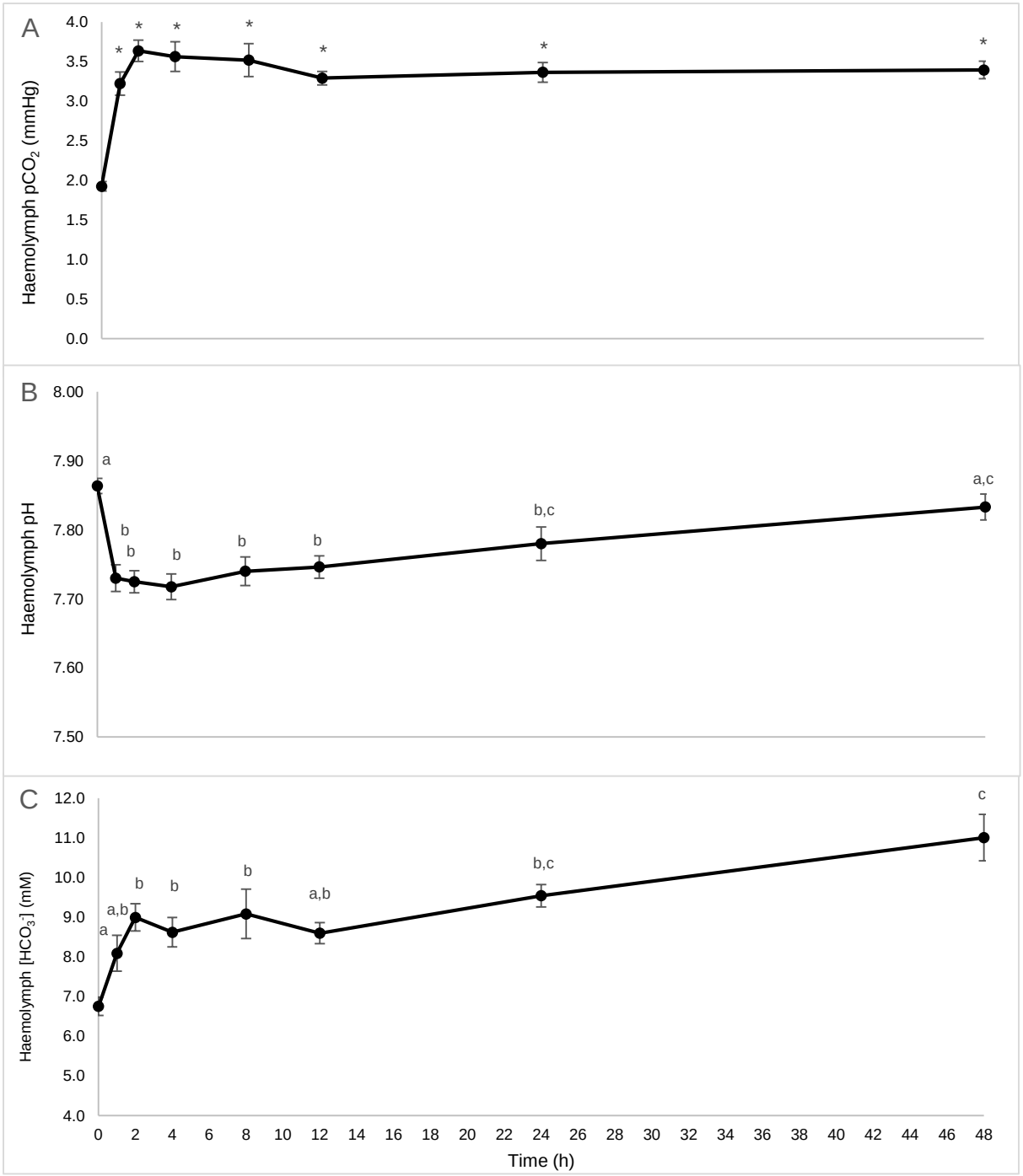
5. Figures



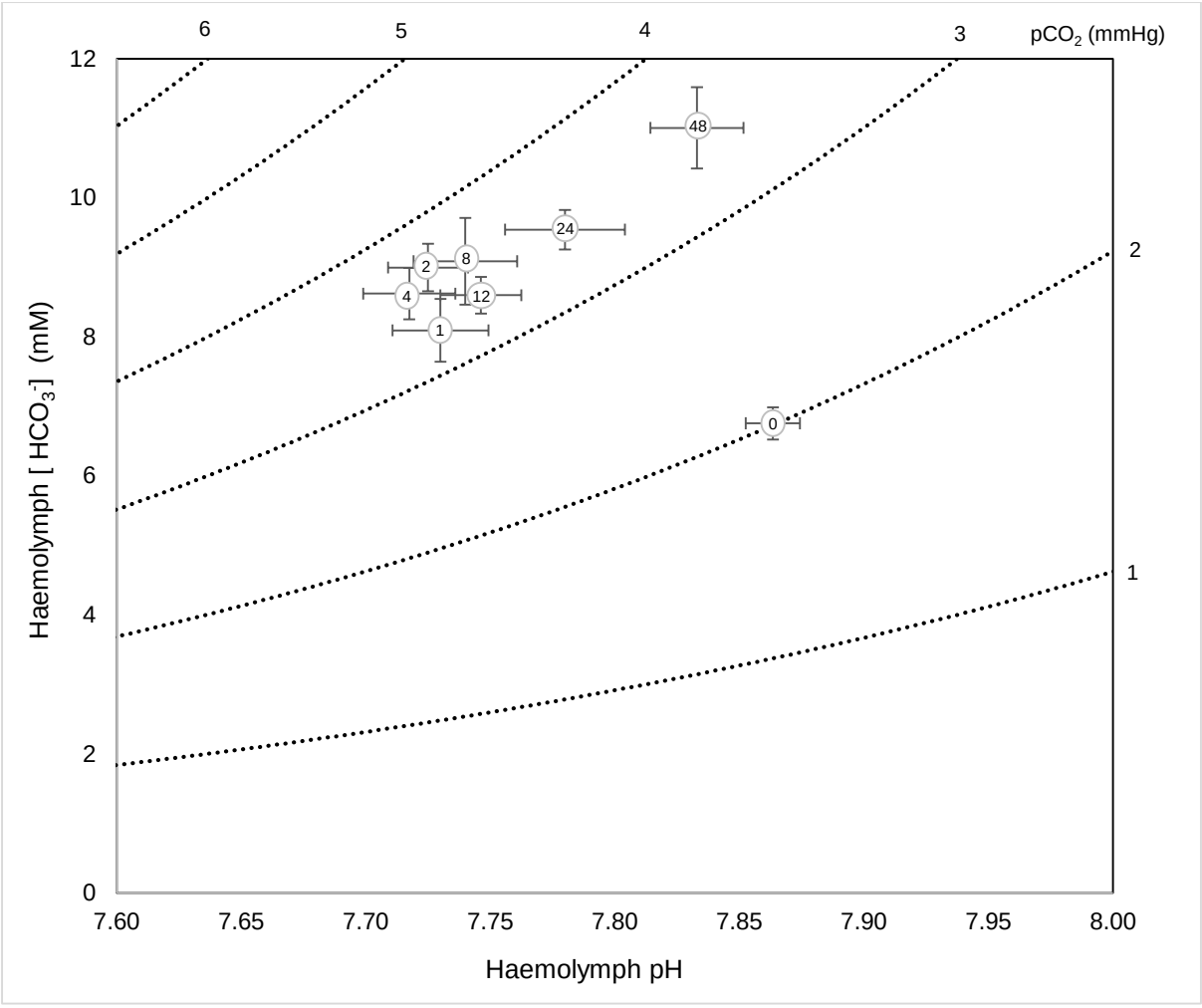
Chapter 2 Figure 1: Plasma pH, bicarbonate and pCO_2 concentrations of sea bass as a function of time following onset of environmental hypercapnia at 1295 µatm. Values represent mean ± SEM. N = 18, 6, 6, 6, 10 and 11 for 0, 0.5, 1, 2, 4, 8, and 24 hours respectively. HCO_3^-: A significant difference when compared to control (0 hours) is denoted by an asterisk (ANOVA, $p<0.05$). PCO_2: Subsets showing significant differences are labelled with letters (ANOVA, $p<0.05$).



Chapter 2 Figure 2: Time course of sea bass plasma acid-base parameters following the onset of environmental hypercapnia (1295 µatm) expressed as a pH/HCO_3^-/pCO_2 (Davenport) diagram. Circled numbers represent time in hours. Values represent mean ± SEM. N = 18, 6, 6, 6, 10 and 11 for 0, 0.5, 1, 2, 4, 8, and 24 hours respectively.



Chapter 2 Figure 3: Haemolymph pH, bicarbonate and pCO_2 concentrations of shore crabs as a function of time following onset of environmental hypercapnia at 1230 µatm. Values represent mean ± SEM. N = 20, 8, 8, 8, 8, 8, 7 and 10 for 0, 1, 2, 4, 8, 12, 24 and 48 hours respectively. Haemolymph pH and bicarbonate: Subsets showing significant differences are labelled with letters (ANOVA, $p<0.05$). PCO_2: A significant difference when compared to control (0 hours) is denoted by an asterisk (ANOVA, $p<0.05$).



Chapter 2 Figure 4: Time course of shore crab haemolymph acid-base parameters following the onset of environmental hypercapnia (1230 µatm) expressed as a pH/HCO_3^-/pCO_2 (Davenport) diagram. Circled numbers represent time in hours. Values represent mean ± SEM. N = 20, 8, 8, 8, 8, 8, 7 and 10 for 0, 1, 2, 4, 8, 12, 24 and 48 hours respectively.

6. Appendices

Table 1**:** Seawater carbonate chemistry for the two experimental treatments used in the sea bass acid-base regulation experiment. † calculated using CO2sys (Pierrot, 2006). Error shown as SEM.

Treatment	Temp. (°C)	pH_{NBS}	Salinity	TA (µmol/kg)†	TCO_2 (µmol/kg)	pCO_2 (µatm)†	HCO_3^- (µmol/kg)†	CO_3^{2-} (µmol/kg)†	ΩCa†	ΩAr†
Control	15.4 (± 0.02)	8.10 (± 0.00)	34.8 (± 0.02)	2396.8 (± 5.8)	2198.1 (± 5.42)	490.0 (± 4.1)	2031.1 (± 5.2)	148.9 (± 1.1)	3.6 (± 0.02)	2.3 (±0.02)
High CO2	15.4 (± 0.01)	7.71 (± 0.00)	34.8 (± 0.01)	2334.1 (± 10.6)	2287.1 (± 10.4)	1295.1 (± 7.3)	2174.7 (± 9.8)	64.6 (± 0.5)	1.5 (± 0.01)	1.0 (±0.01)

Table 2: Seawater carbonate chemistry for the two experimental treatments used in the shore crab acid-base regulation experiment. † calculated using CO2sys (Pierrot, 2006). Error shown as SEM.

Treatment	Temp. (°C)	pH_{NBS}	Salinity	TA (µmol/kg)†	TCO_2 (µmol/kg)	pCO_2 (µatm)†	HCO_3^- (µmol/kg)†	CO_3^{2-} (µmol/kg)†	ΩCa†	ΩAr†
Control	15.3 (± 0.1)	8.18 (± 0.00)	34.4 (± 0.1)	2697.6 (± 20.2)	2445.9 (± 17.1)	455.6 (± 4.3)	2237.1 (± 14.7)	191.9 (± 2.9)	4.6 (± 0.1)	3.0 (± 0.2)
High CO2	15.3 (± 0.1)	7.79 (± 0.00)	34.4 (± 0.1)	2697.6 (± 20.2)	2619.8 (± 20.2)	1226.5 (± 15.3)	2486.4 (± 19.3)	88.0 (± 0.8)	2.1 (± 0.1)	1.4 (± 0.1)

7. References

Ahearn, G. A., Duerr, J. M., Zhuang, Z., Brown, R. J., Aslamkhan, A., & Killebrew, D. A. (1999). Ion transport processes of crustacean epithelial cells. *Physiological and biochemical zoology, 72*(1), 1-18.

Boutilier, R. G., Heming, T. A., & Iwama, G. K. (1984). Appendix: physicochemical parameters for use in fish respiratory physiology. *Fish physiology, 10*, 403-430.

Bucking, C., & Wood, C. M. (2008). The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. *Journal of Experimental Biology, 211*(15), 2533-2541.

Burleson, M. L., & Smatresk, N. J. (2000). Branchial chemoreceptors mediate ventilatory responses to hypercapnic acidosis in channel catfish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 125*(3), 403-414.

Butler, P., Taylor, E., & Davison, W. (1979). The effect of long term, moderate hypoxia on acid-base balance, plasma catecholamines and possible anaerobic end products in the unrestrained dogfish *Scyliorhinus canicula*. *Journal of comparative physiology, 132*(4), 297-303.

Claiborne, J. B. (1998). *The Physiology of Fishes* (D. H. Evans Ed.): CRC press.

Claiborne, J. B., Perry, E., Bellows, S., & Campbell, J. (1997). Mechanisms of acid-base excretion across the gills of a marine fish. *Journal of Experimental Zoology, 279*, 509-520.

Cripps, I. L., Munday, P. L., & McCormick, M. I. (2011). Ocean acidification affects prey detection by a predatory reef fish. *PLoS ONE, 6*(7), 22736.

Davis, B. D. (1958). On the importance of being ionized. *Archives of Biochemistry and Biophysics, 78*(2), 497-509.

Deigweiher, K., Koschnick, N., Pörtner, H.-O., & Lucassen, M. (2008). Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. *AJP - Regulatory, Integrative and Comparative Physiology, 295*(5), 1660-1670.

Dickson, A., Sabine, C., & Christian, J. (2007). Guide to Best Practices for Ocean CO_2 Measurements.

Dickson, A. G. (1990). Standard potential of the reaction: and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K. *The Journal of Chemical Thermodynamics, 22*, 113-127.

Dickson, A. G., & Millero, F. J. (1987). A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Research, 34*, 1733-1743.

Dixson, D. L., Munday, P. L., & Jones, G. P. (Writers). (2010). Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues: Blackwell Publishing Ltd.

Esbaugh, A., Heuer, R., & Grosell, M. (2012). Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. *Journal of Comparative Physiology B, 182*(7), 921-934.

Evans, D. H., Piermarini, P. M., & Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Reviews, 85*(1), 97-177.

Friederich, G. E., Walz, P. M., Burczynski, M. G., & Chavez, F. P. (2002). Inorganic carbon in the central California upwelling system during the 1997-1999 El Nino-La Nina event. *Progress in Oceanography, 54*(1-4), 185-203.

Grosell, M., & Genz, J. (2006). Ouabain-sensitive bicarbonate secretion and acid absorption by the marine teleost fish intestine play a role in osmoregulation. *AJP - Regulatory, Integrative and Comparative Physiology, 291*(4), 1145-1156.

Heisler, N. (1984). Acid-base regulation in fishes. *Fish physiology, 10*(Part A), 315-401.

Heisler, N. (1986). *Acid-base regulation in fishes.* Elsevier Science Publishers.

Heisler, N., Weitz, H., & Weitz, A. (1975). Hypercapnia and resultant bicarbonate transfer processes in an elasmobranch fish (*Scyliorhinus stellaris*). *Bulletin europeen de physiopathologie respiratoire, 12*(1), 77-86.

Henry, R. P., & Wheatly, M. G. (1992). Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. *American Zoologist, 32*(3), 407-416.

IPCC. (2013). The Physical Science Basis. Working Group I Contribution to the IPCC 5th Assessment Report – Changes to the Underlying Scientific/Technical Assessment.

Kimura, C., Ahearn, G., Busquets-Turner, L., Haley, S., Nagao, C., & Couet, H. (1994). Immunolocalization of an antigen associated with the invertebrate electrogenic $2Na^+/1H^+$ antiporter. *The Journal of Experimental Biology, 189*(1), 85-104.

Kozyr, A. (2015). Information on Batches of CO_2 in Seawater Reference Material. Retrieved 16.08.15, from http://cdiac.ornl.gov/oceans/Dickson_CRM/batches.html

Krogh, A. (1939). *Osmotic regulation in aquatic animals.* Cambridge University Press.

Langenbuch, M., & Pörtner, H.-O. (2003). Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempi*) as a function of ambient CO_2: pH-dependent limitations of cellular protein biosynthesis? *Journal of Experimental Biology, 206*(22), 3895-3903.

Larsen, B. K., Pörtner, H. -O., & Jensen, F. B. (1997). Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology, 128*(2), 337-346.

Lloyd, R., & White, W. R. (1967). Effect of high concentration of carbon dioxide on the ionic composition of rainbow trout blood. *Nature, 216*(5122), 1341.

Mangum, C. P. (1983). Oxygen transport in the blood. *The biology of Crustacea, 5*, 373-429.

McKenzie, D., Taylor, E., Dalla Valle, A., & Steffensen, J. (2002). Tolerance of acute hypercapnic acidosis by the European eel (*Anguilla anguilla*). *Journal of Comparative Physiology B, 172*(4), 339-346.

Mehrbach, C., Culberson, C. H., Hawley, J. E., & Pytkowicz, R. M. (1973). Measurements of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnology and Oceanography, 18*(6), 897-906.

Michaelidis, B., Spring, A., & Pörtner, H.-O. (2007). Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish *Sparus aurata*. *Marine Biology, 150*(6), 1417-1429.

Miller, G. M., Watson, S. A., Donelson, J. M., McCormick, M. I., & Munday, P. L. (2012). Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. *Nature Climate Change, 2*(12), 858-861.

Munday, P. (2015). *Effects of Ocean Acidification on the Life History and Behaviour of Tropical Marine Fishes.* Paper presented at the 145th Annual Meeting of the American Fisheries Society.

Munday, P. L., Cheal, A. J., Dixson, D. L., Rummer, J. L., & Fabricius, K. E. (2014). Behavioural impairment in reef fishes caused by ocean acidification at CO_2 seeps. *Nature Climate Change, 4*(6), 487-492.

Munday, P. L., Dixson, D. L., Donelson, J. M., Jones, G. P., Pratchett, M. S., Devitsina, G. V., & Døving, K. B. (2009). Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proceedings of the National Academy of Sciences, 106*(6), 1848-1852.

Munday, P. L., Pratchett, M. S., Dixson, D. L., Donelson, J. M., Endo, G. G., Reynolds, A. D., & Knuckey, R. (2013). Elevated CO_2 affects the behaviour of an ecologically and economically important coral reef fish. *Marine Biology, 160*(8), 2137-2144.

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sorensen, C., Watson, S.-A., & Munday, P. L. (2012). Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change, 2*(3), 201-204.

Niv, Y., & Fraser, G. M. (2002). The alkaline tide phenomenon. *Journal of clinical gastroenterology, 35*(1), 5-8.

Ohr, E. A. (1976). Tricaine methanesulfonate-I. pH and its effects on anesthetic potency. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 54*(1), 13-17.

Pane, E. F., & Barry, J. P. (2007). Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. *Marine Ecology Progress Series, 334*, 1-9.

Pierrot, D. E., Wallace, D.W.R. (2006). MS Excel Program Developed for CO_2 System Calculations. *ORNL/CDIAC-105a*.

Rahn, H., & Prakash, O. (1985). *Acid-base regulation and body temperature* (Vol. 10): Springer Science & Business Media.

Reeves, R. B. (1972). An imidazole alphastat hypothesis for vertebrate acid-base regulation: tissue carbon dioxide content and body temperature in bullfrogs. *Respiration Physiology, 14*(1), 219-236.

Riggs, A. F. (1988). The Bohr Effect. *Annual review of physiology, 50*(1), 181-204.

Smith, D. A., Smith, S. A., & Holladay, S. D. (1999). Effect of previous exposure to tricaine methanesulfonate on time to anesthesia in hybrid tilapias. *Journal of Aquatic Animal Health, 11*(2), 183-186.

Somero, G. (1986). Protons, osmolytes, and fitness of internal milieu for protein function. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 251*(2), 197-213.

Thurberg, F., Dawson, M., & Collier, R. (1973). Effects of copper and cadmium on osmoregulation and oxygen consumption in two species of estuarine crabs. *Marine Biology, 23*(3), 171-175.

Toews, D. P., Holeton, G. F., & Heisler, N. (1983). Regulation of the acid-base status during environmental hypercapnia in the marine teleost fish Conger conger. *Journal of Experimental Biology, 107*(1), 9-20.

Topic-Popovic, N., Strunjak-Perovic, I., Coz-Rakovac, R., Barisic, J., Jadan, M., Persin Berakovic, A., & Sauerborn Klobucar, R. (2012). Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. *Journal of Applied Ichthyology, 28*(4), 553-564.

Truchot, J.-P. (1986). Changes in the hemolymph acid-base state of the shore crab, *Carcinus maenas*, exposed to simulated tidepool conditions. *The Biological Bulletin, 170*(3), 506-518.

Truchot, J.-P. (1973). Temperature and acid-base regulation in the shore crab *Carcinus maenas* (L.). *Respiration Physiology, 17*(1), 11-20.

Truchot, J.-P. (1976). Carbon dioxide combining properties of the blood of the shore crab *Carcinus maenas* (L): carbon dioxide solubility coefficient and carbonic acid dissociation constants. *The Journal of Experimental Biology, 64*(1), 45-57.

Truchot, J.-P. (1979). Mechanisms of the compensation of blood respiratory acid - base disturbances in the shore crab, *Carcinus maenas* (L.). *Journal of Experimental Zoology, 210*(3), 407-416.

Vesela, A., & Wilhelm, J. (2002). The role of carbon dioxide in free radical reactions in organism. *Physiological research, 51*(4), 335-340.

Whiteley, N., Scott, J., Breeze, S., & McCann, L. (2001). Effects of water salinity on acid-base balance in decapod crustaceans. *Journal of Experimental Biology, 204*(5), 1003-1011.

CHAPTER 3

Ocean Acidification and Copper: *In Vitro* and *In Vivo* Approaches to Assess Their Interactions in Sea Bass

CHAPTER 3

OCEAN ACIDIFICATION AND COPPER: *IN VITRO* AND *IN VIVO* APPROACHES TO ASSESS THEIR INTERACTIONS IN SEA BASS

1. Abstract

Anthropogenically released carbon dioxide (CO_2) in the atmosphere is readily absorbed by the oceans with profound effects on seawater carbonate chemistry: namely an elevation in the partial pressure of CO_2 (pCO_2) and concomitant decrease in pH (ocean acidification). Without compensation the intra- and extra- cellular fluids of fish would become acidotic as internal pCO_2 is obliged to increase in parallel with elevated external pCO_2. Teleost fish rapidly compensate such changes, primarily through the accumulation of bicarbonate ions (HCO_3^-) which buffers their internal pH to physiological norms in order to maintain function of physiological and biochemical processes. This study provides the first evidence that elevations in the HCO_3^- concentrations of the extracellular medium affords protection against copper-induced DNA damage in cells. We exposed erythrocytes extracted from European sea bass (*Dicentrarchus labrax*) to copper (Cu) in artificial salines designed to mimic the inorganic constituents of fish plasma. Cells exposed to Cu (45 µg/L) in salines with control [HCO_3^-] (6 mM) showed markedly increased levels of DNA damage. However, cells exposed to Cu under elevated [HCO_3^-] (12 mM), representative of sea bass acid-base regulating under near future ocean acidification scenarios, showed complete protection from Cu-induced DNA damage.

Assessment of this potential protective mechanism *in vivo* was more difficult due to the complex interaction of multiple responses of the organism to Cu exposure. Fish exposed to 60 µg/L Cu for 24 hours exhibited no significant increases in DNA damage in erythrocytes. Those exposed to 80 µg/L Cu for 14 days exhibited marginal increases in erythrocyte DNA damage, although only significantly so in the high CO_2 + Cu treatment when compared with controls. Changes to strong ion difference where indicative of inhibited acid-base regulation suggesting a reduction of elevated plasma HCO_3^- may explain why the protective effects seen *in vitro* where not observed.

Contrary to predictive models of Cu bioavailability under future carbonate chemistry scenarios, sea bass showed similar (if not higher) accumulation of Cu under

normocapnic conditions when compared with high CO_2. Differential accumulation of Cu in the hepatic tissue of animals from the four treatments was observed, with the highest concentrations resulting from Cu exposure under normocapnic conditions. Gill tissues showed no treatment effects on Cu accumulation after 24 hours. However gill concentrations were higher in all treatments after 14 days, possibly indicative of elevated Cu uptake to meet nutritional requirements.

2. Introduction

2.1 Ocean Acidification

Anthropogenic emissions have resulted in a substantial increase in the partial pressure of carbon dioxide (CO_2) since the start of the industrial revolution. In this time atmospheric pCO_2 has risen 40 %, from ~280 to ~400 µatm (Bao et al., 2012; NOAA, 2015; Solomon et al., 2007). Recent projections published as part of the Representative Concentration Pathways (RCP) database, suggest that atmospheric CO_2 concentrations may exceed 1200 µatm shortly into the next century (RCP 8.5) (IPCC, 2014).

Around 50 % of anthropogenic CO_2 emissions are absorbed by the oceans (Sabine et al., 2004) with the result being measurable changes to physical chemistry of seawater. Dissolution of CO_2 into seawater causes an increase in the partial pressure of CO_2 (pCO_2) and the resultant hydration reaction (via a carbonic acid intermediate) releases H^+ ions, causing a drop in pH: referred to as 'ocean acidification' (OA). Future elevations in global seawater pCO_2 are predicted to have significant and wide reaching effects on water breathing animals (Pörtner et al., 2004). Research on reduced seawater pH and high CO_2 has demonstrated a suite of effects on marine organisms including: acid-base regulation (Esbaugh et al., 2012; Masahiro Hayashi et al., 2004; Michaelidis et al., 2007; Pörtner et al., 1998; Toews et al., 1983), calcification (Doney et al., 2009; Hoegh-Guldberg et al., 2007; Orr et al., 2005), metabolism (Franke, 2011; Pörtner et al., 2004; Pörtner et al., 2004; Small et al., 2010) , growth (Berge et al., 2006), immune function (Bibby et al., 2008), behaviour (Dixson et al., 2010; Munday et al., 2009; Nilsson et al., 2012). Consequently there is a rapidly expanding body of research into the impacts of high CO_2 on a vast array of marine and freshwater

organisms considering effects from cellular mechanisms, to population and global models.

2.2 Copper

However ocean acidification is not occurring in an otherwise pristine environment. It is vital that we consider the potential impacts that increasing pCO_2 may have set against a background of other environmental stressors such as temperature, changing salinity, chemical stressors.

Copper is a well-documented, ubiquitous environmental contaminant whose impacts on biota are strongly determined by the physical and chemical properties of the medium in which it is dissolved. Copper toxicity and elevated environmental pCO_2 are naturally linked due to the well described influence that water pH has on the speciation of Cu, and as a consequence its bioavailability to aquatic organisms. Climate change-induced increases in seawater pCO_2 will increase the concentration of H^+ ions (reduce pH) and a decrease in the concentration of carbonate (CO_3^{2-}) ions. Current models suggest that both of these changes will result in an increased concentration of free Cu^{2+} ions (Millero et al., 2010), the most harmful and bioavailable form of copper (Chakoumakos et al., 1979). This has clear implications for increasing the potential harm to freshwater and marine biota caused by both legacy Cu deposits (Roberts et al., 2013) and ongoing influxes in the context of ocean acidification.

Furthermore Cu is known to exert a number of physiological impacts also observed in exposures to high CO_2 such as reduced growth rates, acid-base disturbance, osmoregulatory disturbance, reduced olfactory capability and enzyme inhibition (Adeyemi et al., 2012; Kim & Kang, 2004; Parveen & Javed, 2010; Wang et al., 1998), as well as its inhibition of carbonic anhydrase, a vital enzyme for acid-base regulation (Zimmer et al., 2012). These overlapping physiological effects suggest clear potential for interactions between the two stressors. As such Cu represents an environmentally relevant and mechanistically useful chemical stressor for work aimed at better understanding the combined impacts of ocean acidification and chemical stressors more generally.

Copper is an essential micronutrient in the metabolic pathways of both plants and animals. Copper is a constituent of a number of vital enzymes, acting as a structural cofactor and redox catalyst (Flemming 1989). However, Cu becomes increasingly

toxic when concentrations exceed those required for physiological functioning: typically above 10 to 50 times physiological requirements (Hall, 2002). When intracellular Cu levels overwhelm homeostatic mechanisms and rise beyond cellular needs the once useful redox properties of Cu result in the production of reactive oxygen species (ROS) creating an imbalance that leads to oxidative stress (Valko et al., 2006). Copper ions can also act more directly by non-specifically binding with biologically important molecules causing conformational changes and dysfunction (Gabbianelli et al., 2003). The net effects of Cu-induced cellular toxicity are: protein dysfunction, DNA damage (mitochondrial and nuclear), and lipid peroxidation (Valko et al., 2006). These can ultimately lead to structural damage, loss of cell integrity and reduced or total loss of cell function (Gabbianelli et al., 2003) if anti-oxidant defence, detoxification and damage repair mechanisms are overwhelmed. In the face of irreversible damage or dysfunction a cell may initiate programmed cell death (apoptosis) in order to safely remove itself from the population where it might otherwise result in pathological consequences. In addition to these more commonly cited effects, recent work on rats prone to Wilson's disease found that cellular Cu^{2+} accumulation resulted in the activation of acid sphingomyelinase (Asm) and subsequent release of ceramide (Brewer, 2007), a well-recognised apoptosis initiation signal (Taha et al., 2006; Woodcock, 2006). This represents a far more direct means by which Cu^{2+} may conceivably induce apoptosis in fish if the pathway is conserved.

In the present study DNA damage and measures of cytotoxicity such as apoptosis were employed to quantify the potentially harmful effects of Cu to the erythrocytes of European sea bass (*Dicentrarchus labrax*), both *in vitro* and *in vivo*. We consider how changes to the extracellular and intracellular environments (Wang et al., 1998) under high ambient pCO_2 may modulate the potential for cellular toxicity of internalised Cu. Furthermore, an assessment is made of how seawater chemistry determines tissue accumulation of Cu and the ionic status of plasma.

There exists clear potential for elevations in seawater pCO_2 to increase Cu toxicity to marine biota (Millero, 2009) but very limited literature to support this assertion so far (Lewis, 2015 unpub.). However examples are increasingly being found whereby modulations in the uptake or toxicity of trace metals under high CO_2 were not as predicted. Such findings appear complex as well as being species and metal dependent. For example, elevated ambient CO_2 was found to reduce cellular uptake

of Cd^{2+} in hard clams (*Mercenaria mercenaria*) (Ivanina et al., 2013). The inhibition of ubiquitin and tumour suppressor p53 mRNA production by Cd^{2+} and Cu^{2+} was reversed under high CO_2 in both the Eastern oyster (*Crassostrea virginica)* and hard shell clams. Acute exposure (48 h) of Atlantic cod (*Gadus morhua*) to Cu under high CO_2 drastically reduced lethality from 59 % to zero, when compared with exposure in control pCO_2 seawater (Larsen et al., 1997).

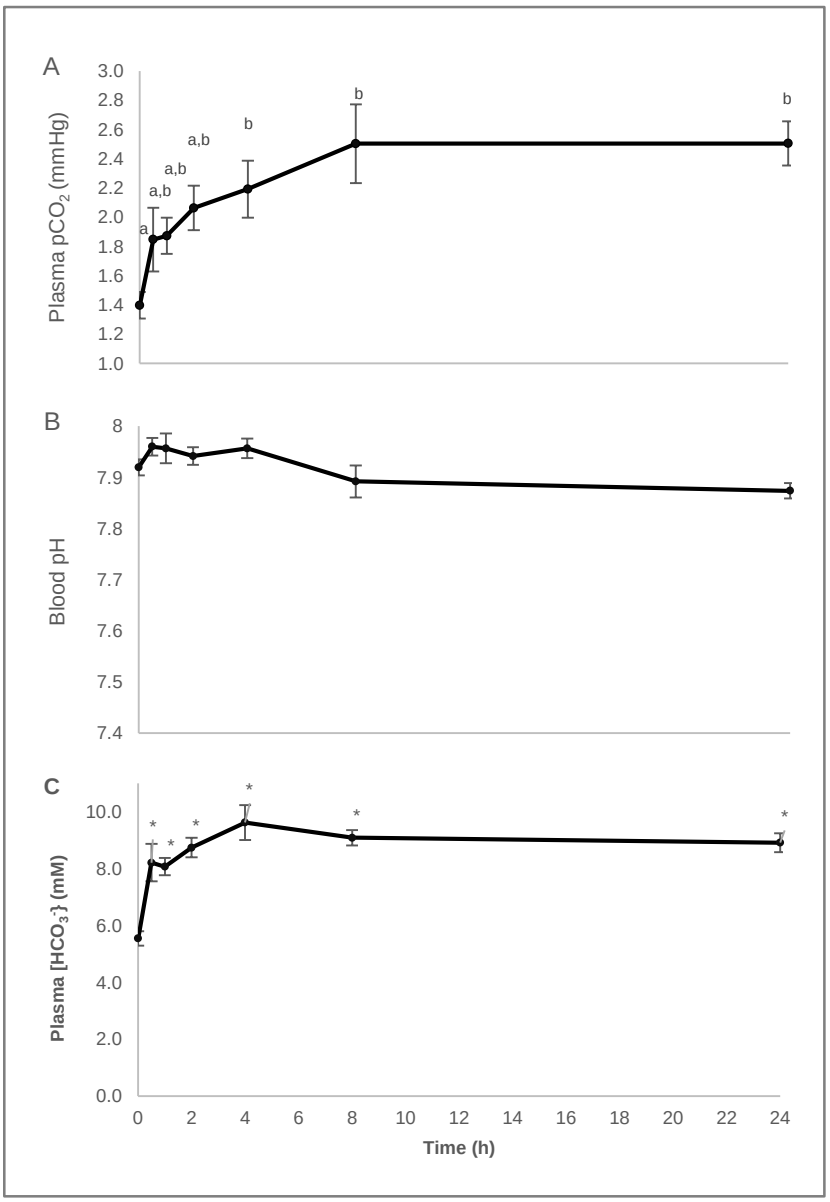
2.3 Experimental Overview

In the present study the hypothesis that exposure to simultaneous high CO_2 and Cu may modulate uptake and accumulation of Cu from the environment was tested, focussing on tissue copper analysis on those pertinent to uptake, transport and detoxification (gill, plasma and liver). Furthermore an assessment is made as to whether changes to extracellular chemistry under high CO_2, namely increases in bicarbonate (HCO_3^-) concentrations modulate the intracellular toxicity of Cu. Given the profound effect of seawater carbonate chemistry on Cu speciation and bioavailability, it stands to reason that such a relationship may also be in effect inside the animal. More specifically, that such changes in speciation will reduce the capacity for Cu to induce DNA damage and cytotoxicity in erythrocytes as a proxy for other internal tissues, both *in vitro* and *in vivo*.

A two-pronged approach to testing the aforementioned hypothesis was employed. First, the potential for a high HCO_3^- medium to protect erythrocytes from cytotoxic effects and DNA damage was tested *in vitro*. Second an assessment was made as to whether any effects were also found *in vivo*. This was done by constructing a 2 x 2 factorial experiment in which sea bass were exposed to high CO_2 and Cu, both separately and in combination. We also assessed the accumulation of Cu in gill, plasma and liver tissues, *in vivo* as well as determining the impacts of treatments on the fishes on plasma ionic status.

2.4 Chapter 2 Excerpt: *The role of bicarbonate in sea bass (D. labrax) acid-base regulation.*

When challenged with CO_2-induced acidosis as a result of seawater hypercapnia, the pH of extracellular fluids must be compensated in order to maintain effective physiological functioning. In Chapter 2 evidence was provided of the primary method by which teleost fish are known to maintain their extracellular pH in the face of environmental perturbations to CO_2: the accumulation of bicarbonate ions (HCO_3^-). These ions act to buffer the plasma, maintaining a physiologically optimum pH even under elevated internal and external pCO_2. In the test species, sea bass, this regulation occurred more rapidly (<30 mins) than previously recorded in fish and involved a 1.6-fold elevation in plasma [HCO_3^-].



Chapter 2 Figure 1: Plasma pCO_2, pH, and bicarbonate concentrations of sea bass as a function of time following onset of environmental hypercapnia (1200 µatm). Values represent mean ± SEM. For plasma bicarbonate significant differences compared with control (0 h) are labelled with asterisks (ANOVA, $p<0.05$). For plasma pCO_2 subsets showing significant differences are denoted by letters (ANOVA, $p<0.05$).

3. Methods

3.1 Animals

Juvenile sea bass were sourced from Ecloserie Marine de Gravelines, France, in August 2014. Animals were kept for 3 months in a 150 L stream tank fed by 1 micron filtered, UV sterilized Tropic Marin® artificial seawater (Nominal: 15° C, pH: 8.15, salinity: 35, pCO_2: 400 µatm, total alkalinity (TA): ~2000 µmol/kg SW). Bass were fed with approximately 2 % BW/d fish pellets (Skretting, Gamma Diamond) three times a week, and were allowed to exercise by swimming against a gently circulating current of water.

3.2 *in vitro* Experiment

3.2.1 Blood Sampling

Fish were anaesthetised under 135 µg/L Tricaine methanesulfonate (MS-222), upon which gills were irrigated with aerated seawater containing 85 µg/L MS-222 in order to maintain sedation and normal systemic gas and acid-base status during blood withdrawal. MS-222 stocks were adjusted to the experimental pH using 1 M NaOH. Two hundred microlitres of arterial blood were extracted into a heparinized (Monoparin®, CP Pharmaceuticals) syringe and 21G needle using the caudal puncture technique. Whole blood was kept on ice prior to beginning the *in vitro* exposure protocol, which was started no more than 30 minutes from the time of extraction.

3.2.2 Exposure

Artificial salines were produced that mimicked sea bass plasma with respect to inorganic constituents, based on Pavlidis et al. (1997) (*See Appendix: Tables 1 and 2*). The HCO_3^- and Cu concentrations of these salines were then manipulated.

Following extraction from the animal, red blood cells were rinsed using control saline before being suspended in 1 of 8 saline treatments: 1) Control, 2) High HCO_3^-, 3) Cu and 4) Cu + HCO_3^-, each equilibrated to either control or high pCO_2 by the addition of weak HCl (*see Appendix: Tables 1 and 2 for composition and carbonate chemistry*). PCO_2 and pH were found to have no observable effect on results so data were

grouped into the aforementioned 4 treatments (N=18, 14, 23, 23 respectively for comet assay and 11,12,11,10 respectively for flow cytometry). Treatments 1 and 3 had HCO_3^- concentrations (6 mM) consistent with the acid-base status of sea bass plasma under current day open ocean pCO_2 (400 µatm) (*See Introduction: Chapter 2 Excerpt*). Treatments 2 and 4 had elevated HCO_3^- concentrations (12 mM), consistent with the acid-base status of sea bass plasma under possible end-of-century climate change scenarios where seawater pCO_2 may exceed 1200 µatm (*See Introduction: Chapter 2 Excerpt*). Cu exposures were at 45 µg/L Cu (added as copper sulphate): optimised prior to the study in order to achieve sufficient DNA damage for comparison with non-copper treatments.

Red blood cells were incubated in sealed glass vials for 2 hours at the pre-experiment acclimation temperature of the animals, 15 °C. Following incubation cells were rinsed using control saline, upon which both comet assay and fluorescence-labelled flow cytometry were performed to assess DNA damage and cytotoxicity respectively.

3.2.3 Flow Cytometry

Red blood cells were suspended in 100 µl of control saline and stained with 5 µl of FITC-Annexin V (in in TE buffer containing 0.1% BSA and 0.1% NaN_3), Ethidium Homodimer III (in PBS) and Hoechst 33342 (in PBS), as per instructions (Promokine Apoptotic/Necrotic/Healthy Cell Detection Kit PK-CA707-30018). The simultaneous application of these fluorescent dyes enables the use of flow cytometry for rapid quantification of apoptotic, necrotic, and healthy cells respectively, as a percentage of the cell population.

Flow cytometry was conducted using a BD FACS Aria II Fluorescence Activated Cell Sorter (FACS) equipped with a 100 µm nozzle. Particle fluorescence was excited at 405, 488 and 633 nm and fluorescence intensity recorded using the appropriate detectors. A sheath fluid of control saline (Appendix: Tables 1 and 2) was used to suspend the cells for analysis instead of the more commonly used phosphate buffered saline so as not to minimise potential stress to the cells. Regular performance calibration was performed using BD FACS CS&T calibration software and beads (642412, BDBiosciences, U.S.A.). Fluorescence data indicative of positive staining (Annexin V, Ethidium Homodimer III, Hoechst 33342) for cytotoxic biomarkers, apotosis, necrosis and healthy cells respectively was graphically represented in the

form of dot plots and histograms. Areas of interest, known as gates, were assigned based on positive staining or lack thereof (control cells) in order to quantify the graphical output and describe the population in terms of percentage apoptotic, late apoptotic, necrotic or healthy cells. Ten thousand cells were assessed for each treatment.

3.2.4 Comet Assay

Unfrosted Thermo Scientific microscope slides were coated on one side with 1 % high melting point (HMP) agarose and allowed to set at room temperature overnight prior to commencing the Comet assay (Singh et al., 1988).

Extracted blood cells were rinsed in control artificial saline, spun and suspended in 180 µl of 1 % low melting point (LMP) agarose (warmed to 45 °C in a water bath). This was split into two 75 µl aliquots that were then pipetted onto the HMP-coated slides upon which cover slips were placed in order to evenly spread the LMP-erythrocyte solution across the slide.

These slides were then left to cool at 4 °C for 20 minutes allowing the LMP to set. The Comet assay was then carried according to the protocol set out by Singh et al. (1988). Slides were soaked in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 µM Tris) at 4 °C for 1 hour in order to lyse the erythrocytes, during which all the cell contents (i.e. proteins, RNA, cytoplasmic/nucleoplasmic constituents) aside from DNA were liberated and then diffused into the agarose gel. The DNA unravels and fills the space in the agarose once occupied by the cell.

The slides were then placed in electrophoresis buffer for 40 minutes at 4 °C. The alkaline conditions of the electrophoresis buffer denatured the DNA double helix producing single strands. Finally the slides were subjected to electrophoresis in which a voltage (30 V) was applied across the solution for 30 minutes causing the migration of negatively charged DNA fragments. The slides were then placed between cloths soaked in neutralizing buffer prior to scoring. In order to visualise the DNA on a fluorescence microscope slides were first stained using SYBR-Safe dye (green). In order to score the slides on a fluorescence microscope they were first stained using 80 µl of dye solution (1:10^5, SYBR-Safe dye: $T_{10}E_1$ Buffer). Slides were then scored using a microscope (Zeiss Axiostar Plus) under 20X magnification and blue filter (HBO 50), connected via a mounted camera (Marlin, Allied Vision Technologies) to Comet

Assay IV (Perspective Instruments) analysis software. Cells are manually captured within an on-screen score area which was automatically analysed by Comet Assay IV software, calculating a number of end points (e.g. tail intensity). All slides were scored within 48 hours to prevent loss of slide quality through degradation. Percentage tail intensity, hereafter referred to as %tDNA is a representation of the how much DNA is present in the tail as a proportion of all the DNA in the cell. Comets with denser tails are formed when more DNA strand breaks have occurred. Therefore %tDNA is a direct indicator of the amount of DNA damage. Tail intensity is widely favoured for statistical analysis and recognised by many as the most suitable endpoint for comet assay, including the International Workshop on Genotoxicity Test Procedures (Burlinson et al., 2007)

3.3 *In vivo* Experiment

3.3.1 Animals

Sea bass (36 ± 2 g) were assigned randomly to one of the four treatments: 1) Control (400 µatm), 2) High CO_2 (1200 µatm), 3) Copper (24 h: 60 µg/L, 14 d: 80 µg/L), or 4) High CO_2 + Cu (See *Appendix*: *Tables 3 and 4 for detailed water chemistry, including measured Cu concentrations*). Two separate exposures were carried out lasting 24 hours and 14 days so as to assess any temporal changes in the toxicity response of the animals under the different treatments. Following each exposure, blood was extracted as per above method (3.2.1). Following extraction, blood was divided into two aliquots. From one, plasma was separated via centrifugation and diluted x401 in deionised water for measurement of ionic composition using Dionex (ICS-1000). These diluted samples were frozen until analysis could be performed. The second aliquot remained as whole blood and was kept on ice for no more than 2 hours prior to commencing comet assay during which time animals were dissected for tissue analysis of Cu concentration using ICP-MS. These tissues were frozen in liquid nitrogen (-198 °C) and stored at -80 °C.

3.3.2 Water Chemistry

Elevation of seawater pCO_2 was achieved via a two-pronged approach. Commercially available pH computers (Aqua Medic, Bissendorf, Germany) maintained water pH to

an accuracy of 0.05 units via the release of pure CO_2 into air-mixed header tanks. This enabled a rapid decrease in pH (increase in pCO_2) at the start of the exposure and maintenance with only minor fluctuations above and below the designated pH value. Additionally mass flow controllers (AALBORG, Mass Flow Controller GFC17) provided mixed gas of the target pCO_2 to each bucket via limewood air stones. Seawater pH, salinity and temperature were verified manually throughout the duration of the experiment using a handheld meters (Hanna Instruments HI8314 Membrane pH Meter- Radiometer Probe, YSI Inc. salinity, conductivity, temperature meter model 30/10). The pH meter was calibrated prior to each use using Radiometer Analytical buffer solutions (pH 7.00 and 10.00).

Dissolved Inorganic Carbon (DIC) samples were taken for subsequent analysis and preserved using mercuric chloride ($HgCl_2$) to 0.04 % of final volume, as per Dickson et al. (2007). Measurement of DIC was carried out using a custom-built automated acidification unit and Qubit Systems CO_2 analyser (Model S154). This system was based on a system described by Friederich et al. (2002). Seawater samples were measured against Dickson seawater standards. Data on pH, salinity and temperature in conjunction with DIC values enabled calculation of total alkalinity and pCO_2 using CO2sys (Pierrot, 2006). Values were derived using the NBS pH scale, K1 and K2 constants from Mehrbach et al. (1973), refitted by Dickson and Millero (1987), and dissociation constants for KSO_4 from Dickson (1990). *See Appendix for detailed water chemistry.*

3.3.3 Statistics

Comet assay data was measured as percentage tail intensity (%tDNA) and as such was transformed using a logarithmic function prior to analysis. In both *in vitro* and *in vivo* experiments, a large proportion of the data consisted of low values or zeros such that it was necessary to add one to each raw data point prior to transformation: $\log_{10}(\%tDNA+1)$. The mean is greatly influenced by extreme observations in tail intensity rendering it largely uninformative (Lovell & Omori, 2008). As such the 90^{th} percentile for each animal was used as a summary statistic and the mean of these calculated per treatment. Differences between treatments were determined using one-way ANOVA, followed by Tukey's post-hoc test. The results of these analyses were

accepted as significant if $p < 0.05$. All statistical analyses were carried out using SPSS version 20.0 and histograms were produced using Microsoft Excel 2013.
Chauvenet's criterion was applied to ICP-MS data sets in order to lessen the effect of a few extreme outliers on the mean tissue concentrations attributed. Six data points were removed from a total of 147 across the four tissues analysed.

4. Results

4.1 *In vitro* Results

4.1.1 DNA Damage

Following 2 hours of *in vitro* exposure, the comet tail intensities of cells from Cu only treatments were significantly higher (2.1-fold) than those in all other treatments (Fig. 1). Those cells exposed to Cu in conditions of elevated HCO_3^- showed significantly less DNA damage, comparable to both the control and bicarbonate only treatments [ANOVA: $F_{(3,74)}=9.49$, $p<0.001$]. The effects of pCO_2 and pH on copper-induced DNA damage were also explored and were found to have no significant effect: pH [ANOVA: $F_{(2,72)}=1.878$, $p=0.160$], pCO_2 [ANOVA: $F_{(,74)}=0.438$, $p=0.510$].

4.1.2 Flow Cytometry

The Cu only treatment also induced apoptosis in the greatest number of cells with 34% of the population being stained positive with FITC-Annexin V (Fig. 2). Cells exposed to control and high bicarbonate salines showed significantly lower levels of apoptosis at 3.4 and 5.1 % of the cell population respectively. The high bicarbonate + Cu treatment resulted in an intermediate number of apoptotic cells [ANOVA: $F_{(3,40)}=9.89$, $p<0.001$]. Staining for necrosis (Ethidium Homodimer III) revealed no significant differences between treatments [ANOVA: $F_{(3,40)}=0.767$, $p=0.519$]. Again, the effects of pCO_2 and pH on copper-induced DNA damage were explored and were found to have no significant effect: pH [ANOVA: $F_{(2,38)}=0.376$, $p=0.689$], pCO_2 [ANOVA: $F_{(1,40)}=583$, $p=0.450$].

4.2 *In vivo* Results

4.2.1 DNA Damage

Following 24 h exposure DNA damage in erythrocytes, as assessed by tail intensity, did not differ significantly from controls in any of the treatments. [ANOVA: $F_{(3,28)}$=1.81, p=0.17, Fig. 3]. However, mean damage was somewhat elevated in the high CO_2 + Cu treatment (56 % higher than copper only; 25 % higher than control and high CO_2).

Following 14 days exposure erythrocyte DNA damage was found to be significantly higher (88 %) in bass exposed to the high CO_2 + Cu treatment when compared with controls [ANOVA: $F_{(3,24)}$=3.97, p=0.019, Fig. 4]. DNA damage in the Cu only treatment was also considerably higher than both control and high CO_2 treatments (68 and 58 % respectively) although not significantly so. Grouping of treatments into copper and non-copper for more straightforward assessment of the impact of copper on DNA damage revealed a significant effect. Erythrocyte DNA damage was significantly higher in fish exposed to copper at 14 days [t(26)=-3.54, p=0.002].

4.2.2 Tissue Cu Accumulation

The copper concentrations measured in the plasma of animals following both 24 h and 14 d exposures revealed no significant differences between treatments [ANOVA: 24 h: $F_{(3,27)}$=2.125, p=0.120, Fig. 5, ANOVA: 14 d: $F_{(3,27)}$=1.799, p=0.174, Fig. 6]. However [Cu] did appear somewhat higher (21 %) in the high CO_2 + Cu treatment compared with other treatments after 24 hours. After 14 d both copper-exposed treatments showed higher concentrations (copper 50%: high CO_2 + Cu 25 %) compared with those not exposed to copper. Copper only treatment was found to have the highest concentration. After 14 d, grouping of treatments into ‘copper-exposed’ and ‘not copper exposed’ enabled statistical consideration of this trend and revealed copper-exposed animals to have significantly higher mean plasma [Cu] values (T-test: t(26)=-2.092, p<0.05). The same analysis revealed no such difference after only 24 h exposure (T-test: t(29)=-1.446, p=0.159).

After 24 hours exposure Cu accumulation in gill tissue was not apparent in any of the treatments with no significant differences found at this time point [ANOVA: $F_{(3,26)}$=0.810, p=0.500, Fig. 7]. Analysis of the same tissue after 14 d exposure also

yielded no difference between treatments, [ANOVA: $F_{(3,22)}$=1.21, p=0.331, Fig. 8], although mean Cu concentrations in the gills of animals from the Cu only treatments were around 2 times that of all other treatments. High variance and relatively low sample size (N=7-8) may account for lack of statistical difference.

Cu concentrations measured in the livers of animals following the 14 d exposure showed clear treatment effects. The highest Cu concentrations were found in the livers of animals from the Cu only treatment, with intermediate levels in both high CO_2 and high CO_2 + Cu treatments [ANOVA: $F_{(3,22)}$=5.44, p=0.006, Fig. 9].

4.2.3 Ionic Status

Strong ion difference (SID), simplified as $[Na^+]$ - $[Cl^-]$, was not affected significantly by treatment [ANOVA: $F_{(3,26)}$=1.947, p=0.147, Fig. 10], although small differences were suggestive of differential changes to plasma HCO_3^- concentrations under the four treatments (discussed below). These were not however measured directly.

Plasma $[Na^+]$ was significantly lower (~10 mM) in all other treatments when compared with controls [ANOVA: $F_{(3,26)}$=8.304, p<0.001, Fig. 11a]. The same trend was apparent in plasma $[Cl^-]$, although in this instance the copper only treatment was not as depressed (9 mM) as either of the high CO_2 treatments (12-13 mM) [ANOVA: $F_{(3,26)}$=5.879, p<0.01, Fig. 11b]. This difference (9 versus 12-13 mM) was similar in magnitude to the expected rise in plasma bicarbonate as a result of acid-base regulation during this level of hypercapnia.

5. Discussion

5.1 *In vitro*

Copper exposure induced increased levels of DNA damage in cells suspended in artificial salines with HCO_3^- concentrations comparable to sea bass plasma held under current seawater pCO_2 conditions, i.e. a plasma $[HCO_3^-]$ of 6 mM (*See Chapter 2 Excerpt in Introduction*). This damage was likely caused by the intracellular production of free radicals, including reactive oxygen species (ROS), and the subsequent oxidation of nucleotide bases resulting in both single and double strand breaks (Hayashi et al., 2006; Ueda et al., 1998; Yamamoto & Kawanishi, 1989). However, when the $[HCO_3^-]$ of the exposure medium was increased to 12 mM in order to mimic

acid-base regulating bass under ocean acidification (>1200 µatm), DNA damage was not significantly different from control values. This is evidence of a hitherto undocumented protective affect afforded to the DNA of erythrocytes by the elevated HCO_3^- environment surrounding the cell. We cannot ascertain from the present study the precise mechanism by which protection occurs but a number of possibilities are discussed below. Analysis of cytotoxic biomarkers using flow cytometry revealed significantly higher levels of apoptosis in populations exposed to Cu-containing saline when compared with control and high HCO_3^- treatments. Cells exposed to Cu under high HCO_3^- conditions showed a ~40 % lower occurrence of apoptosis than those exposed to Cu under control [HCO_3^-]. Although not statistically different, these data suggest a possible trend towards a milder protective effect afforded by the high HCO_3^- medium. Explanations for the less distinct protective effect of HCO_3^- with respect to Cu-induced cytotoxicity when compared with genotoxicity are as yet unexplained but may revolve around the site of action of Cu and/or HCO_3^- with regard to toxicity and protective interference, respectively. Many of the proposed mechanisms by which HCO_3^- protects cells affect the intracellular compartment (particular close to the nuclear DNA) and as such may not provide increased protection against Cu-induced loss of cell membrane integrity from extracellular copper, known to be causal in initiating apoptosis.

Work by the authors has provided evidence for a similar protective effect afforded to shore crabs (*C. maenas*) haemocytes under high CO_2 exposure (*Chapter 4*). In that study, the protective effect was not present at seawater Cu concentrations sufficient to prevent accumulation of extracellular HCO_3^- as a means of acid-base regulation. This lends considerable support to the suggested importance of chemical changes to the extracellular fluid during acid-base regulation as being key to modifying the toxicity of internalised Cu. Although not measured at a cellular level with regards DNA damage and cytotoxicity, there are other examples in the literature of a protective effect against Cu apparent under elevated environmental CO_2 conditions. For example high pCO_2 seawater (~10,000 µatm) was shown to drastically reduce acute (48 h) lethality in Atlantic cod exposed to Cu from 59 % mortality under normocapnic conditions to no mortality under high pCO_2 (Larsen et al., 1997).

Here a number mechanisms are postulated by which bicarbonate may afford protection to erythrocytes. Perhaps the simplest explanation is that HCO_3^- in the saline

medium serves to complex Cu ions in such a way as to decrease their entry into blood cells. This would significantly reduce the capacity for Cu-induced DNA damage and intracellular oxidative stress. Previous *in vitro* work considering the effects of ambient chemistry on cellular uptake of Cu provide evidence that speciation strongly determines Cu uptake. Erythrocytes from rainbow trout (*O. mykiss*) suspended in saline with the addition of 1 mM histidine showed reductions in Cu^{2+} uptake of 50 %, presumably due to the low permeability of the Cu-histidine complex (Bogdanova et al., 2002). The authors also showed that entry of Cu^{2+} ions into the cells was highly affected by the chloride concentration in the surrounding medium. The suggestion was that Cu^{2+} ions enter fish erythrocytes in complexation with negatively charged chloride and hydroxyl anions via the Cl^-/ HCO_3^- exchanger. This may provide a second explanation for the results of the present study. Given the importance of the Cl^-/ HCO_3^- exchanger in intracellular acid-base regulation there exists a clear potential for changes in the rate of cellular Cu entry under changing HCO_3^- concentrations. Increases in net efflux of Cl^- in exchange for HCO_3^- to compensate perturbations in intracellular pH may reduce chloride mediated entry of Cu^{2+} or assist in removal of intracellular Cu^{2+}. This is unlikely to explain the observed results however, as neither pH nor pCO_2 appeared to have any measurable effect on toxicity biomarkers independent of changes to [HCO_3^-].

A third explanation comes from the suggestion that HCO_3^- ions protect a key anti-oxidant, superoxide dismutase (SOD), from inactivation (Vesela & Wilhelm, 2002). In doing so HCO_3^- boosts one of the key defence mechanism against intracellular oxidative stress, such as that induced by Cu. By interfering with a histidine mediated reaction between SOD and hydroxyl radicals that would otherwise deactivate the enzyme, the presence of HCO_3^- allows reformation of the active form of the anti-oxidant enzyme (Vesela & Wilhelm, 2002). This is of further relevance to Cu exposures as Cu is known to catalyse the formation of hydroxyl radicals. However, this beneficial interference by HCO_3^- is not entirely without negative consequence as the reaction produces a carbonate radical. It could be argued however that a functioning anti-oxidant enzyme is of far greater benefit when weighed against the production of one radical. This HCO_3^- effect has be proposed by Vesela and Wilhelm (2002) as an explanation for the protective role of high CO_2 reported against free radical damage under hypoxic conditions in rats and piglets (Barth et al., 1998; Vannucci et al., 1997).

Furthermore, *in vitro* studies have suggested that in an aqueous medium CO_2 itself may be an effective scavenger of reactive nitrogen species, thus providing another means by which hypercapnia may assist in the prevention of oxidative stress.

Finally, oxidative stress causes dysregulation of iron metabolism which then increases concentrations of catalytic iron ions (Fe^{3+}) capable of producing free radicals via Fenton reactions. Bicarbonate plays a vital role in the stabilization of transferrin-iron complexes (Edeker et al., 1995), enabling the protein to protect cells from oxidative damage. Conversely, increases in H^+ ions (low pH) destabilizes this complex, putting cells at greater risk of oxidative stress from iron-catalysed production of free radicals. The divergent effects of these two ions may serve to explain the differential effects of hypercapnic acidosis (respiratory) compared with those of lactic acid (metabolic) on lipid peroxidation in rat brain tissue (Rehncrona et al., 1989).

The potential for reduced oxidative stress and maintenance of anti-oxidant activity afforded cells by HCO_3^- may explain the reduced levels of DNA damage measured in Cu exposed erythrocytes under high HCO_3^- concentrations in the present study. It may also serve to help explain reduced DNA damage in high CO_2 only treatments when compared with controls reported in other experiments (*Chapter 4*).

The fact that increased H^+ ion concentrations may serve to exacerbate intracellular oxidative stress under high CO_2 conditions may further explain the increased levels of DNA damage reported in acid-base conforming marine organisms, such as those in Lewis et al (*2015, submitted*). Not only are these animals subject to uncompensated or only partially compensated intra- and extra- cellular acidosis, but they are without the apparent protective effects of elevated HCO_3^-. Such influences may exacerbate an already increased rate of uptake due to increased bioavailability of Cu under low seawater pH.

5.2 *In vivo*

Unlike the *in vitro* exposure, there were no stark differences between treatments with respect to DNA damage following either 24 hour or 14 day exposures. DNA damage under Cu exposure alone after 14 days was similar to that seen in the closely related sea bream (*Sparus aurata*) under a comparable exposure regime (Cu ~100 µg/L for 14 days) (Gabbianelli et al., 2003) suggesting bass are similarly tolerant with respect

to Cu-induced erythrocyte intracellular toxicity. DNA damage was highest in the combined high CO_2 + Cu treatments following both experiment durations, although only significantly so when compared with controls after 14 days. Damage resulting from the Cu only exposure after 14 days was similarly high and therefore suggestive of the fact that some Cu-induced DNA damage may be occurring in both Cu treatments. This was supported by the direct statistical comparison of copper versus non-copper treatments. What was not evident in either exposure duration was the protective effect under high CO_2 predicted by the hypothesis due to elevated plasma HCO_3^- accumulated for regulation of acid-base status. One explanation may be the inhibition of normal acid-base regulatory responses by Cu. Although plasma pH and [HCO_3^-] could not be measured in the present study, some inferences can be made based on strong ion difference. Although strong ion difference was not statistically significantly affected by treatment, both the trends and differences in the mean values are suggestive of differential alterations to plasma [HCO_3^-] under the various treatments. Consider; the SID in the high CO_2 treatment was nearly 3 mM higher than in the control treatment. This is consistent with an elevation in plasma HCO_3^- typical of this animal's acid-base regulatory response to high seawater pCO_2. Furthermore it is similar in magnitude to plasma [HCO_3^-] elevations measured in previous work by the authors on the same species under the same pCO_2 conditions (*see Chapter 2 Excerpt in Introduction*): an elevation of 3.4 mM HCO_3^-. Strictly speaking the SID values for control and high CO_2 exposed fish cannot be directly compared without accounting for the influence of increased plasma pCO_2 (which slightly increases bicarbonate passively due to the hydration reaction, without its active accumulation). However at a given SID, pCO_2 has only a minimal bearing on HCO_3^-, suggesting it should be appropriate to use SID as an indicator of compensatory HCO_3^- accumulation despite the fact pCO_2 is elevated (Stewart, 1983). Applying the same approach to estimate specifically whether fish exposed to the combination of CO_2 and Cu could acid-base regulate as well as fish exposed to high CO_2 alone, the SID was about 5 mM lower in the combined treatment. This certainly suggests that the combined high CO_2 + Cu exposure prevented (or at least inhibited) the ability to compensate pH via accumulation of plasma bicarbonate. Such an inhibitory effect of Cu has been described in other studies by the author on shore crabs (*C. maenas*) at lower Cu concentrations (45 µg/L; Chapter 4). Studies by other authors have also reported inhibitory effects. Larsen (1997) found slow and incomplete acid-base regulation in

Atlantic cod (*G. morhua*) under more extreme Cu and CO_2 exposures (400 µg/L Cu, 9,900 µatm CO_2). Similar results were reported by Wang et al. (1998) under 600 µg/L Cu and ~7900 µatm CO_2. Decreased function of acid-base regulatory responses by Cu in fish are typically attributed to inhibition of carbonic anhydrase (CA; Christensen & Tucker, 1976; Vitale et al., 1999; Zimmer et al., 2012), a vital enzyme for the hydration of CO_2 to produce HCO_3^- for extracellular buffering (Georgalis et al., 2006; Gilmour & Perry, 2009). Preliminary research here found no inhibition of acid-base regulation under low Cu concentration (2.5 µg/L) (Fig. 12-14), suggesting acid-base inhibition is dose-dependent and the threshold lies between 2.5 and 60 µg/L.

Elevations in plasma [Na^+], [Cl^-] and osmolarity, typical of acute Cu exposure in seawater fish (Grosell et al., 2004; Larsen et al., 1997; Stagg & Shuttleworth, 1982) were not apparent in the present study following 24 hours exposure. In fact concentrations of both ions dropped (~10 mM) under copper exposure, as well as in both high CO_2 exposures. Depletion of chloride is frequently reported in conjunction with elevations in plasma [HCO_3^-], which was historically attributed to equimolar ion exchange across branchial membrane via Cl^-/HCO_3^- exchangers: although alternative models postulate other means (Claiborne et al., 1997). Changes in SID also support acid-base regulation as a causal factor in changes to ionic status under high CO_2. Interpretation of the cause of changes in ionic status under both copper exposures however is greatly complicated. Net SID changes are suggestive of a partially or fully inhibited acid-base response under combined copper and high CO_2 exposure possibly suggesting dysfunction of ion transporters and/or carbonic anhydrase. With respect to osmoregulation, copper induced dysfunction in marine fishes, which are hypo-osmotic to their environment, is associated with gains, not losses of Na^+ and Cl^-, making findings in the present study difficult to reconcile with existing literature.

5.2.1 Tissue Accumulation

Plasma: Plasma copper concentrations were not significantly affected by 24 h exposure to Cu, although slightly elevated (21 %) levels were measured in the high CO_2 + Cu treatment, compared with other groups. The lack of treatment effect is suggestive of a) minimal copper uptake at this time point, b) effective regulation of plasma copper levels (excretion/storage), or c) both. The fact that plasma copper concentrations measured after 14 d at higher copper exposure (80 µg/L) were not

many fold higher than controls, as was the case in the liver tissue, is further suggestive of effective regulatory mechanisms against a cumulative plasma Cu burden under prolonged exposure. Elevations in liver concentrations point to this organ as one sink for copper removed from circulating plasma. However, grouped comparison of 'copper-exposed' and 'not exposed' treatments revealed significantly higher plasma [Cu] in fish from the copper-exposed treatments. Plasma copper concentrations in the copper and high CO_2 + Cu treatments were 50 and 25 % higher, respectively, than animals not exposed to copper (controls and high CO_2 alone). This is highly suggestive of some increased plasma copper burden under prolonged waterborne exposure, despite the likely employment of homeostatic mechanisms to minimise such an effect. Although often reported as being under tight homeostatic control, there are many examples in the literature of plasma copper concentrations being affected by exposure to waterborne copper at concentrations similar to the present study. Data from clear nosed skate (*Raja eglanteria*) exposed to waterborne Cu (33 and 110 µg/L) for 7 days was suggestive of both a dose and time dependent increase in plasma [Cu] over the course of the study (~20 and 60 % respectively): albeit not statistically significant, possibly due to N values of only 5-6 (Grosell et al., 2003). Juvenile coho salmon (*Oncorhynchus kisutch*) exposed to 70 µg/L for 24 h and 14 d showed slight but again not significant (N=6) increases in plasma [Cu], ~15 and 18 % respectively (Buckley et al., 1982). Pelgrom et al. (1995) reported significantly increased plasma copper concentrations following 6 days exposure of tilapia (*Oreochromis mossambicus*) to 50, 100 and 200 µg/L Cu. Plasma measurements after 14 d in the present study suggest that waterborne copper does have an impact on plasma [Cu] concentrations and therefore has the potential to cause toxic effects on erythrocytes. Furthermore the data suggests that changes to plasma [Cu] as a result of waterborne copper are not differentially affected by high CO_2, as might be expected were rates of uptake increased due to increased bioavailability (Millero, 2009), and rates of excretion unaffected. We were unable to ascertain these uptake rates within the confines of the present study.

Gill: Copper accumulation at the gill is often associated with acute exposure whereas longer term studies suggest more variability in accumulation. Some suggest a form of acclimation response, often showing minimal differences between treatments (Bielmyer et al., 2005; McGeer et al., 2000), whereas others report clear elevations

post-exposure (Arellano et al., 1999; Buckley et al., 1982; Pilgaard et al., 1994) or progressive increases over time (Grosell et al., 2003). In the present study Cu exposed bass showed no differences in gill Cu accumulation when compared with control animals following 24 hours of exposure. As the two primary drivers of gill accumulation and whole organism copper uptake the observed low gill burden may be indicative of low apical uptake from the environment, high basolateral transfer of copper from the gill epithelial cells into the plasma, or both (Grosell et al., 2003).

Although the mean concentration of Cu measured in the gills of the Cu only treatment after 14 days exposure was nearly double that of all other treatments, this trend was not statistically supported. Gill tissue from animals in all treatments did however show markedly higher Cu concentrations when compared with 24 hours exposure. This was despite little or no difference in Cu concentrations between both sets of control and high CO_2 exposures (0.3-0.8 µg/L) and only a relatively small difference in the Cu exposure concentrations (60 and 80 µg/L). Given that the gills serve as a route of uptake (Grosell & Wood, 2002) it is perhaps unsurprising that prolonged exposure to Cu should lead to its accumulation in the gill tissue, as reported in previous studies (Arellano et al., 1999; Grosell et al., 2003; Playle et al., 1993). However this fails to explain the higher Cu concentrations in gill tissue from both control and high CO_2 treatments following 14 days when compared to 24 hours. Given that fish remained unfed throughout the 14 day experiment, we suggest that removal of dietary sources of Cu resulted in the modulation of Cu uptake across the gills in order to meet nutritional requirements. Kamunde et al. (2002) demonstrated that a Cu-deficient diet led to an increase in uptake of waterborne Cu. The lack of Cu availability via the diet in the present study may induce a similar response to receiving a Cu deficient diet suggesting an increase in Cu uptake across the gill was likely initiated. Even at low ambient Cu concentrations, the large volume of water passing over the gill offers a clear source of potential Cu assimilation (Grosell & Wood, 2002). This may account for the increases in gill Cu concentrations observed in control and high CO_2 after 14 days, although literature on starved vs fed fish has not previously shown such an effect (Segner, 1987).

Liver: In fish the liver is the primary tissue for both accumulation and detoxification (Roesijadi & Robinson, 1994). When faced with severe and/or chronic Cu exposure this bioaccumulation can lead to hepatic damage such as vacuolation and focal

necrosis, as is the case in many exposures using levels of Cu in the mg range (Baker, 1969; Figueiredo-Fernandes et al., 2007). However, previous work using Cu concentrations comparable to the present study suggested such levels were not sufficient to cause pathological damage to hepatic tissue (Segner & Braunbeck, 1990).

We found significantly elevated Cu concentrations in the livers of fish exposed to Cu under control pCO_2 conditions, compared to intermediate concentrations in both high CO_2 and high CO_2 + Cu treatments. Liver concentrations measured in control animals were vastly lower than all other treatments. Mean concentrations in copper exposed animals were comparable to those reported in a similar seawater exposure: Senegales sole (*Solea senegalensis*) following 7 days Cu (100 µg/L; Arellano et al., 1999).

Marked accumulation of Cu in the liver under seawater Cu exposure was as expected and consistent with a wealth of previous literature (Arellano et al., 1999; Buckley et al., 1982; Hamza et al., 1995). Equally so were the elevated levels in fish exposed to Cu under high CO_2, although the somewhat lower hepatic accumulation is interesting. This may be due to individual differences skewing mean values within a low sample size (6-7) or may be evidence of some biological process modulating Cu transport, detoxiification and excretory processes under high CO_2. The authors found evidence suggestive of a tissue depuration effect under high CO_2 such that concentrations of Cu in muscle tissue appeared reduced compared with control following 28 days exposure (*Chapter 5*). We speculate that this may be a result of elevated plasma [HCO_3^-] reducing the concentration of free copper, ultimately acting to draw intramuscular reserves into the plasma with the net effect being increased transport to, and ultimately excretion by, the liver. If such depuration were in effect under high CO_2, whatever the mechanism (discussed in Chapter 5), it stands to reason that hepatobiliary excretion of Cu would increase correspondingly to avoid elevated plasma loads (Grosell et al., 2001). This could conceivably account for elevated Cu concentrations in the livers of high CO_2 exposed fish when compared with controls.

6. Conclusions

Our results suggest that despite likely differences in Cu speciation in the seawater due to the differential carbonate chemistry imposed by high pCO_2 conditions (greater than 2-fold increase in Cu^{2+}; Millero, 2009), Cu appears to be similarly bioavailable in the two treatments. This is evidenced by the lack of statistical difference in accumulated

Cu in the plasma, gills and livers of fish from the 'Cu' and 'high CO_2 + Cu' treatments. This is at odds with predictive models of Cu speciation and bioavailability under future ocean acidification conditions (Millero, 2009) and literature suggesting low pH increases accumulation of Cu (Götze et al., 2014; Hans et al., 2014; Ivanina et al., 2013). In fact a trend towards higher accumulation in gill and liver tissues of Cu exposure under normocapnic conditions suggests Cu may be marginally less bioavailable under high CO_2, although this is unsubstantiated.

The *in vitro* approach to testing the hypothesis that bicarbonate could protect cells from Cu-induced damage was effective as a proof-of-concept. The strength of this approach was its simplicity and the removal of the complex physiology of the animal in order to answer a specific question. This work provides some of the first clear evidence of the protective influence of HCO_3^- against Cu-induced toxicity at a cellular level. Assessing the same hypothesis *in vivo* proved more complicated. Elevating environmental Cu concentrations did not have the predicted effect of increasing cellular DNA damage. This was likely due to the complex suite of defence and repair mechanisms employed by fish (and other organisms) to counteract the potentially harmful effects of waterborne Cu. As such acid-base regulation appeared inhibited at Cu concentrations insufficient to elicit a measurable increase in DNA damage compared with control, with the exception of the high CO_2 + Cu exposure at 14 days. As such extracellular HCO_3^- in Cu exposed animals under high CO_2 was likely not elevated, leaving us unable to effectively test the hypothesis. Furthermore, over the time periods assessed in the *in vivo* work, particularly when compared with the 2 h *in vitro* experiment, only the net effect on DNA damage is being considered, which will be a product of both damage and repair mechanisms. This is further complicated by the fact that copper is known to inhibit DNA damage repair mechanism (Whiteside et al., 2010).

It may however be a fair assumption, given the *in vitro* findings, to suggest that the protective effects of HCO_3^- are in operation under high CO_2 and lower ambient Cu conditions insufficient to prevent acid-base regulation: as per the pilot study (Fig. 12-14). Under these environmentally realistic Cu concentrations, elevated HCO_3^- resulting from the fish's acid-base regulatory strategy may serve to reduce the oxidative stress, production of free radicals and DNA damage associated with Cu exposure. Such protection may have downstream effects on the energetic cost of responding to

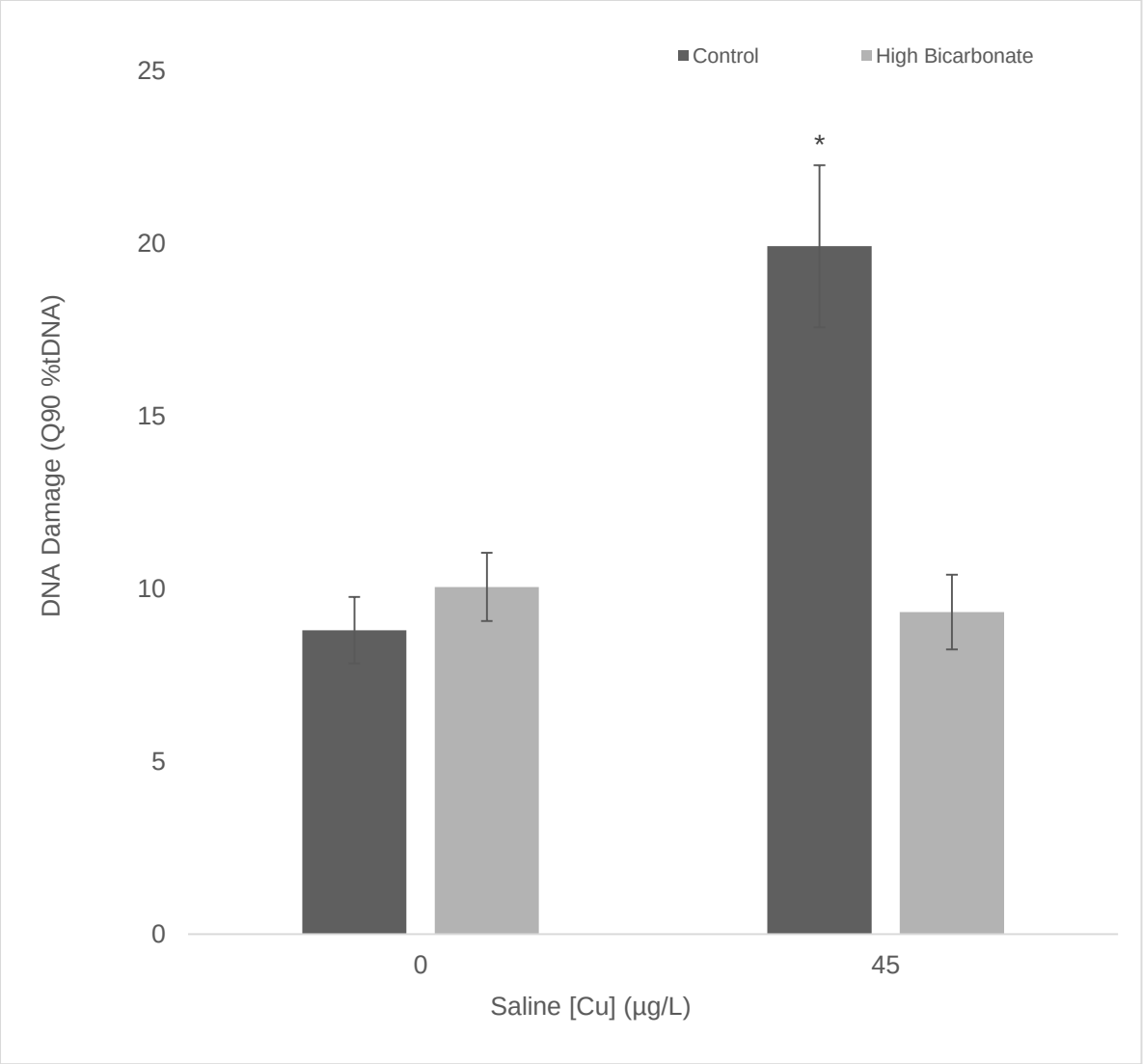
waterborne Cu, potentially affecting slow processes like growth (*See Chapter 5*) and reproduction. Broadly speaking, any reductions in the cost of coping with Cu exposure under high CO_2 conditions will likely have positive effects on both wild and farmed populations with respect to growth, reproduction and survivorship if only to a small degree. Conversely the present study shows some evidence of the potential for Cu to inhibit the normal acid-base regulatory responses of sea bass. This could have severe implications for the ability of these animals to respond to elevations in seawater pCO_2 under future climate scenarios in areas of moderate Cu contamination with potentially profound impacts on physiology resulting from uncompensated intra- and extra-cellular acidosis. Such changes to the chemistry of these fluids could most profoundly affect oxygen delivery (Riggs, 1988) and enzyme function (Somero, 1986) with a multitude of downstream effects, such as reduced protein biosynthesis (Langenbuch & Pörtner, 2003) and cardiac performance (Farrell et al., 1988). These impacts would have considerable consequences for the success of individuals and therefore of populations as a whole.

Future work should aim to more comprehensively assess whether the protective mechanism observed *in vitro* is also present *in vivo*. Such work might employ more sensitive markers of cellular toxicity/stress such as free radical formation, as well as more comprehensively characterization cell responses, for example, anti-oxidant responses and DNA repair mechanism. A different approach might be the artificial introduction of HCO_3^- into the plasma *in vivo* under high Cu concentrations that induce measurable cellular detriment but would otherwise prevent acid-base regulation. Furthermore this work might aim to elucidate the precise mechanism by which HCO_3^- conveys protection on erythrocytes and whether this protection is afforded to other cells within the organism. Importantly it should also consider whether such mechanisms are specific to Cu or if similar protection is present against toxicity induced by other ubiquitous metal stressors such as Cd, Cr, Cu, Fe, Pb and Zn, whose speciation (Millero, 2009), and therefore potential for toxicity, are also predicted to change considerably in future high CO_2 conditions.

7. Figures

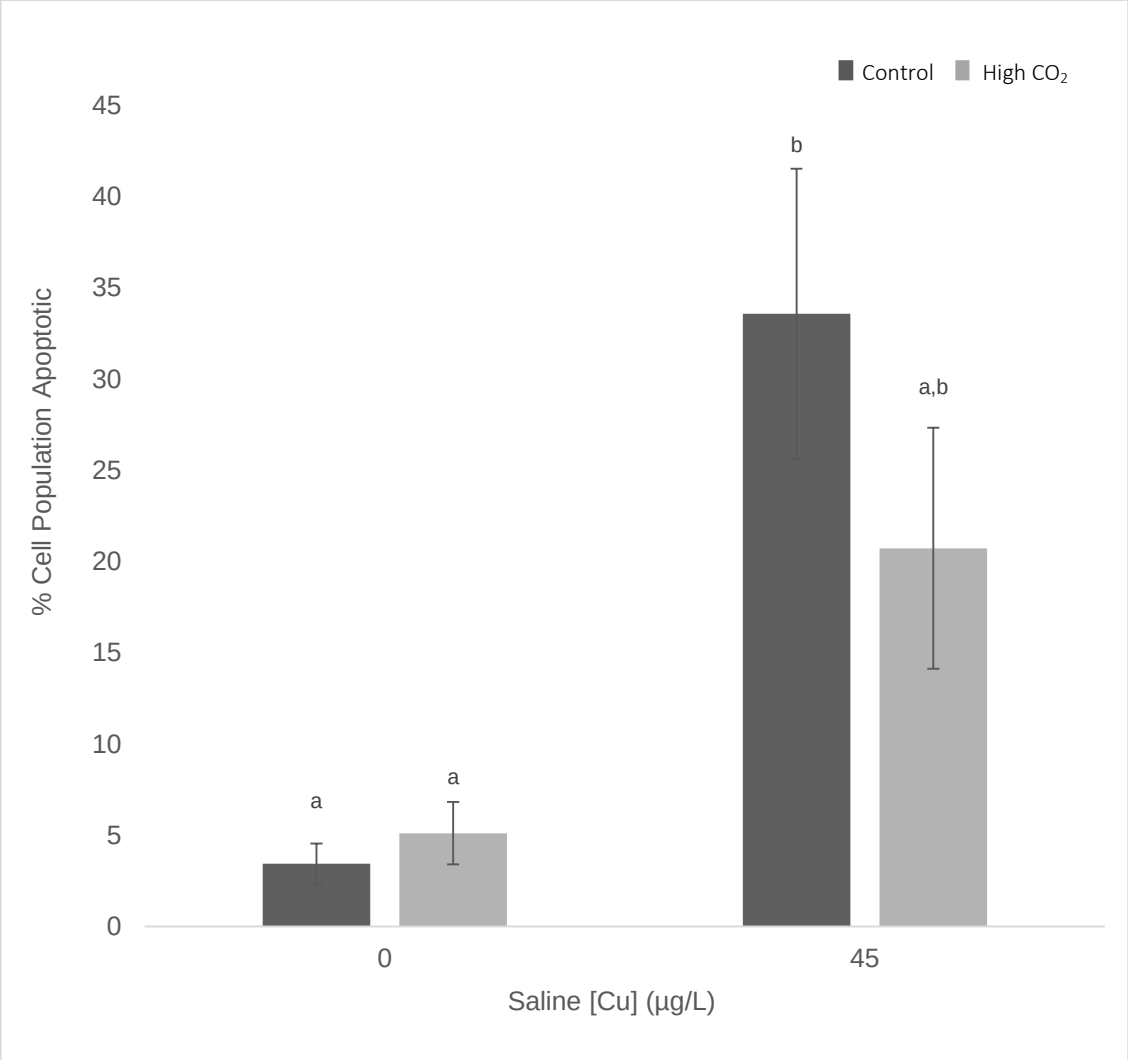
7.1 *In vitro*

7.1.1 DNA Damage



Chapter 3 Figure 1: DNA damage in red blood cells of *D. labrax* following 2 hour *in vitro* exposure in artificial saline as measured by comet assay. Values represented treatment means for Q90 of log transformed percentage tail intensity (log%tDNA) ± SEM. N=18, 14, 23, 23. Significant differences are denoted by an asterisk (ANOVA, $p<0.001$).

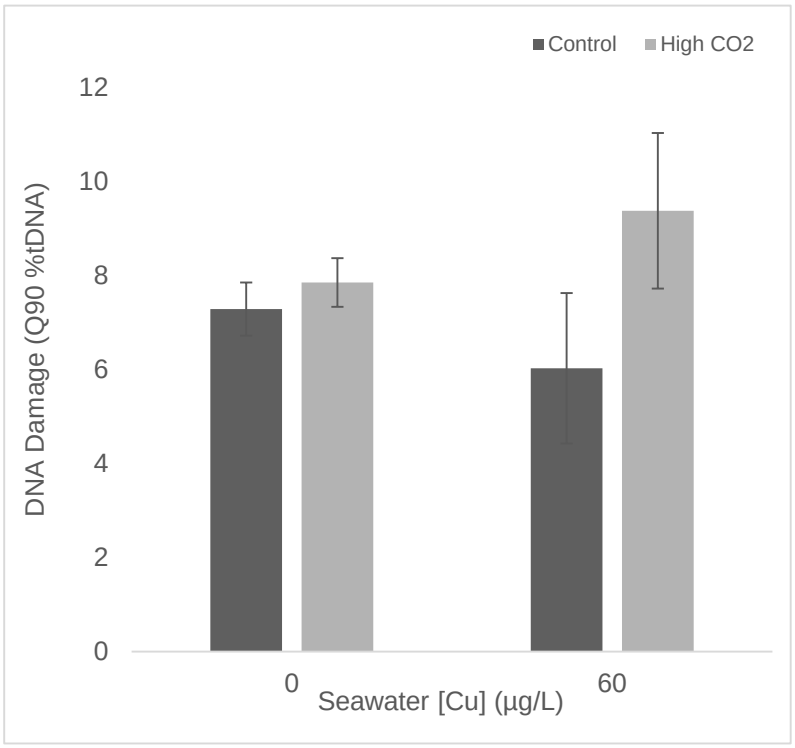
7.1.2 Cytotoxicity



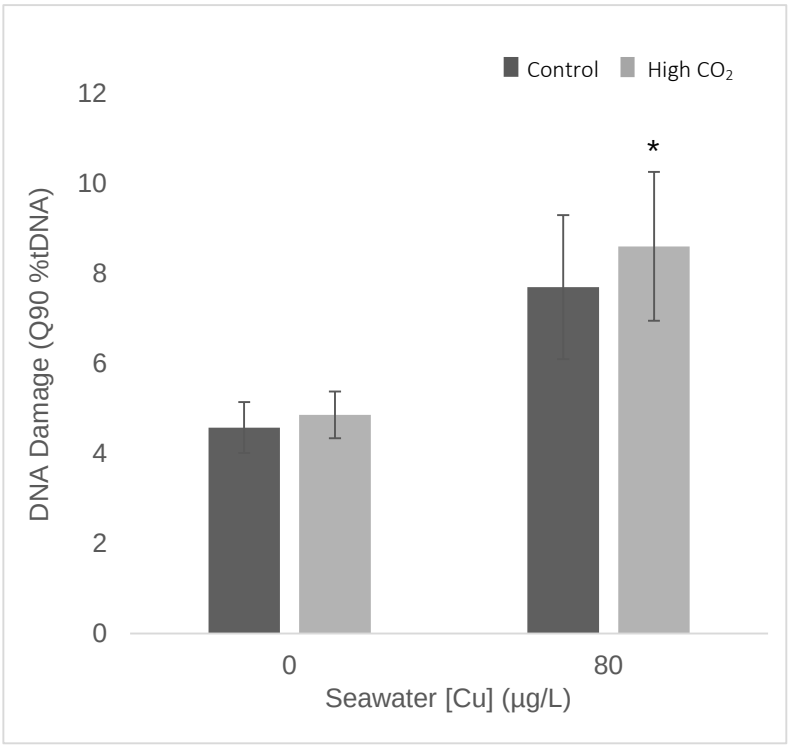
Chapter 3 Figure 2: Percentage *D. labrax* red blood cell population showing positive staining for apoptosis of following 2 hour *in vitro* exposure in artificial saline, as measured by fluorescence-labelled flow cytometry using FITC-Annexin V. Values represent mean ± SEM. N= 11,12,11,10. Significant differences are denoted by letters (ANOVA, $p<0.05$, Tukey's post-hoc test).

7.2 *In vivo*

7.2.1 DNA Damage

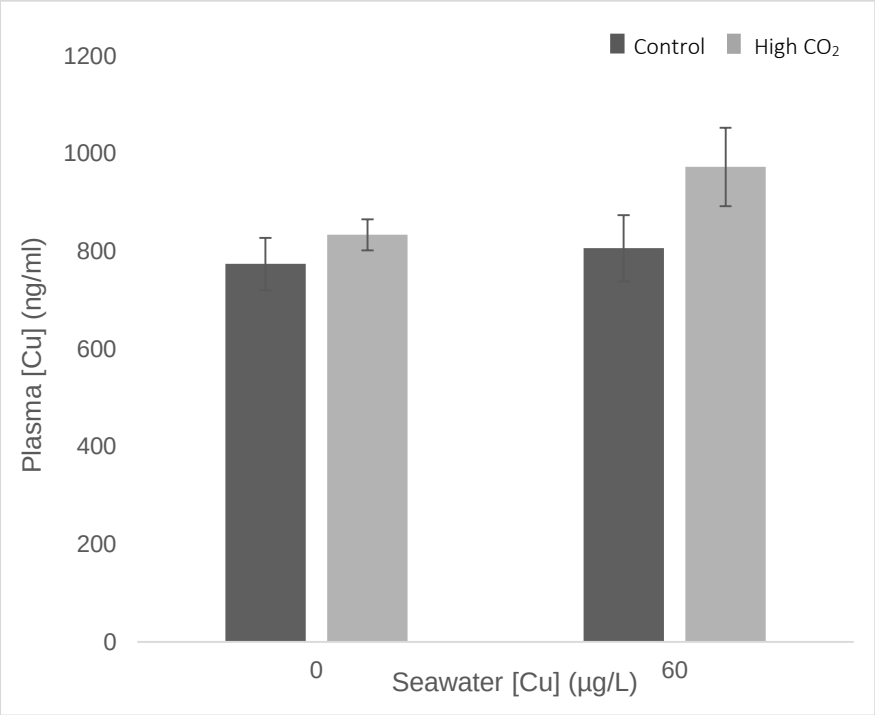


Chapter 3 Figure 3: DNA damage in red blood cells of *D. labrax* following 24 hour *in vivo* exposure, as measured by comet assay. Values represent treatment means for Q90 of log transformed percentage tail intensity (log%tDNA) ± SEM. N=8

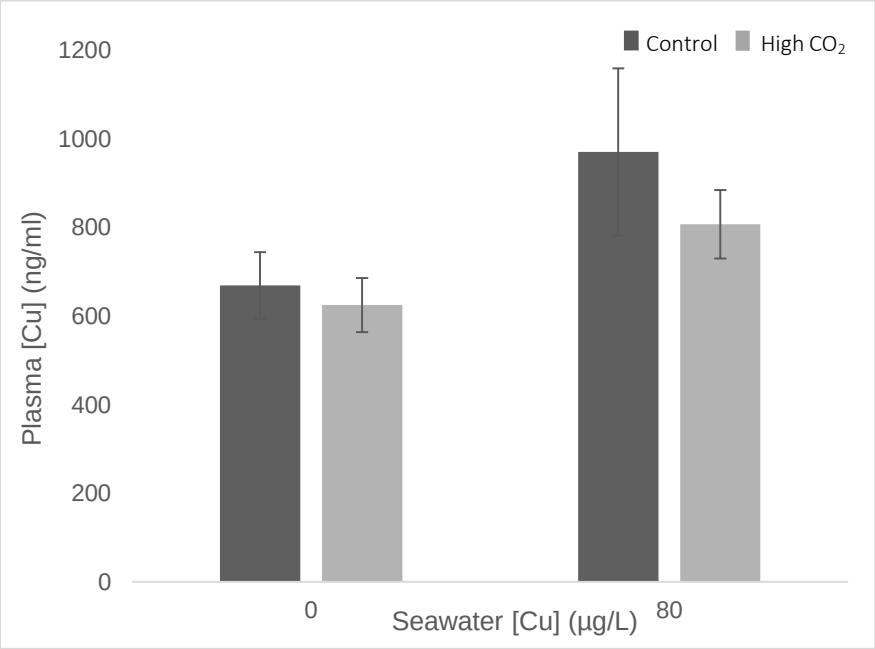


Chapter 3 Figure 4: DNA damage in red blood cells of *D. labrax* following 14 day *in vivo* exposure, as measured by comet assay. Values represent treatment means for Q90 of log transformed percentage tail intensity (log%tDNA) ± SEM. N=7, 6, 7, 8. Significant difference from control denoted by asterisk (ANOVA, $p<0.05$, Tukey's post-hoc test).

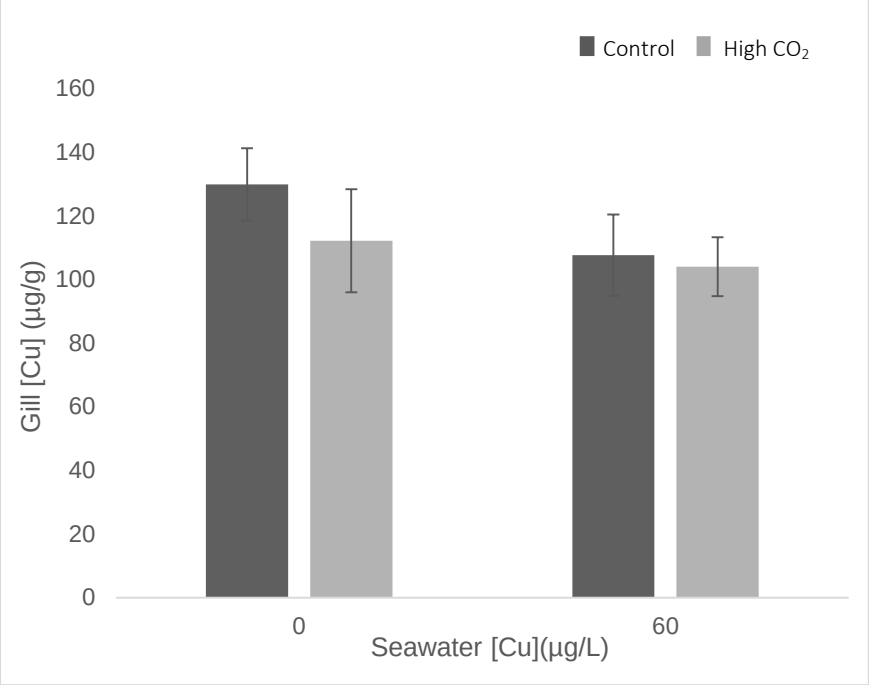
7.2.2 Tissue Cu Concentrations



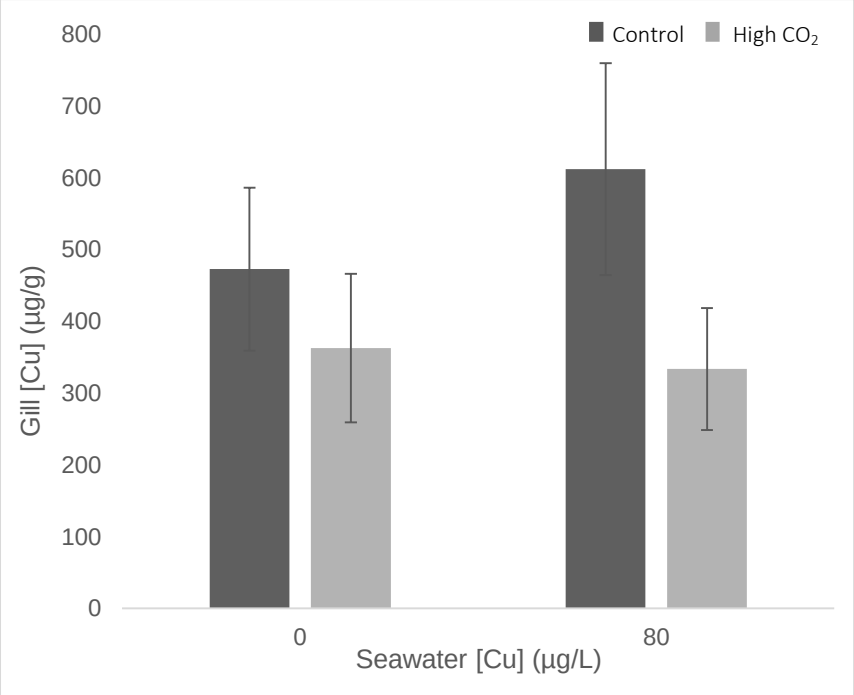
Chapter 3 Figure 6: Total Cu concentrations measured in plasma of *D. labrax* after 24 h exposure. Value represent Mean ± SEM. N=8,8,7,8



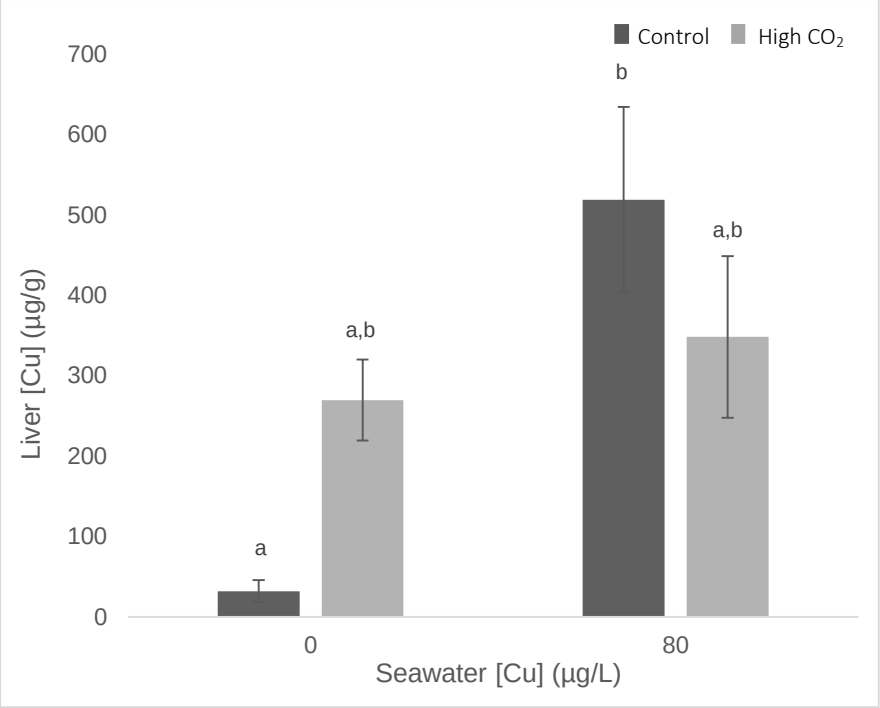
Chapter 3 Figure 8: Total Cu concentrations measured in plasma of *D. labrax* after 14 d exposure. Value represent Mean ± SEM. N=8,7,8,9



Chapter 3 Figure 9: Total Cu concentrations measured in gill tissue of *D. labrax* after 24 h exposure. Value represent Mean ± SEM. N=8,8,7,7.

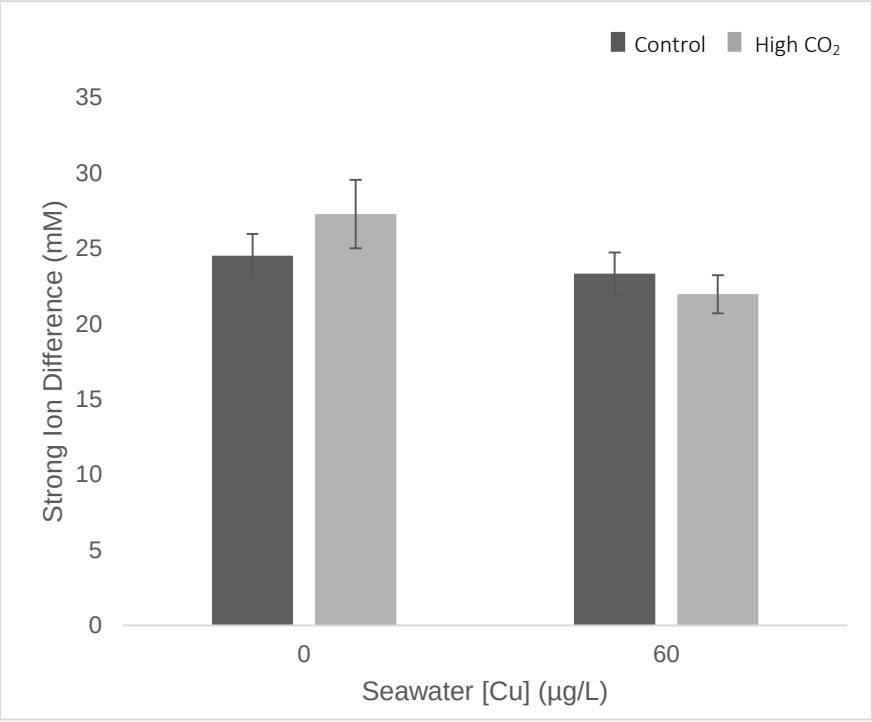


Chapter 3 Figure 11: Total Cu concentrations measured in gill tissue of *D. labrax* after 14 d exposure. Value represent Mean ± SEM. N=7,6,5,8.

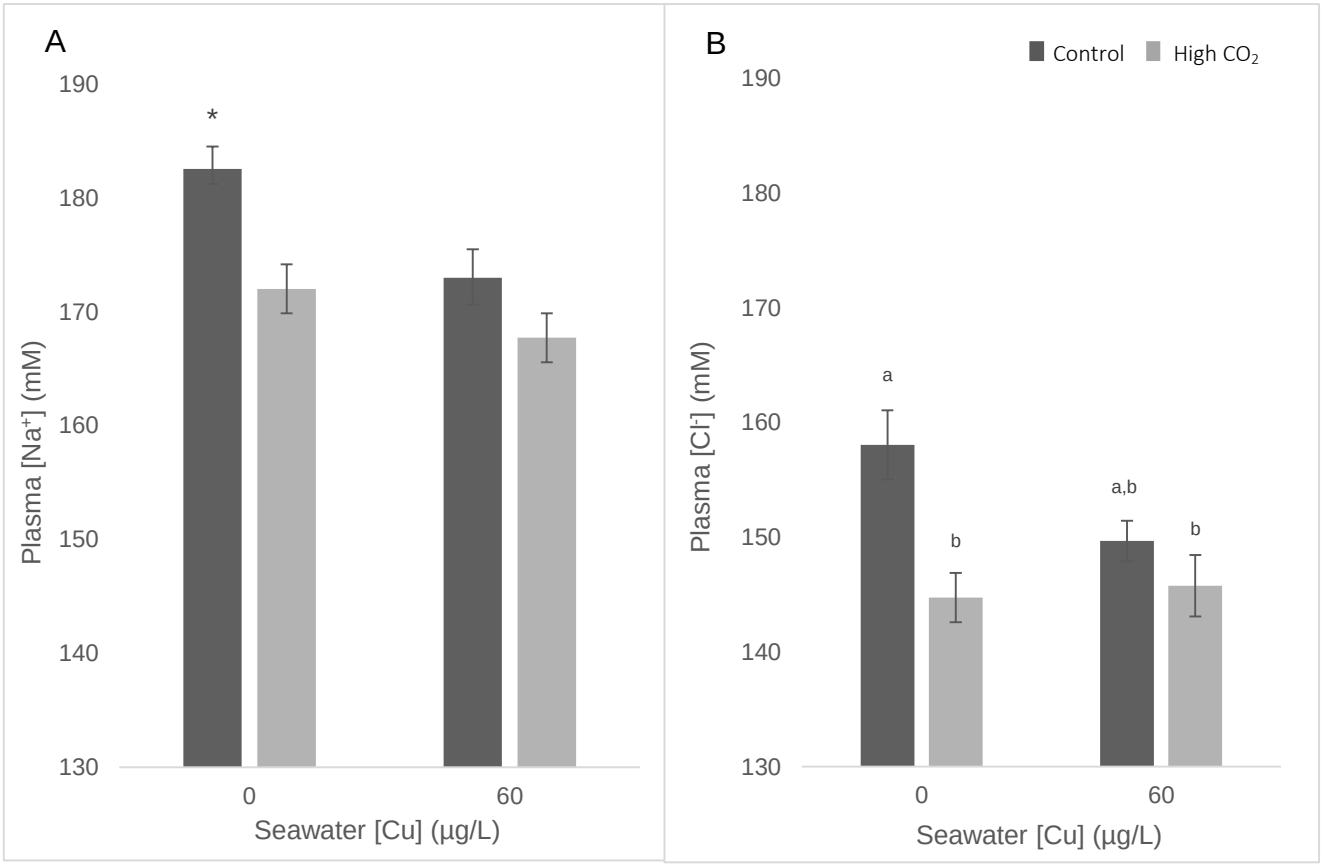


Chapter 3 Figure 12: Total Cu concentrations measured in hepatic tissue of *D. labrax* after 14 d exposure. Value represent Mean ± SEM. N=6,6,7,7

7.2.3 Plasma Ion Status



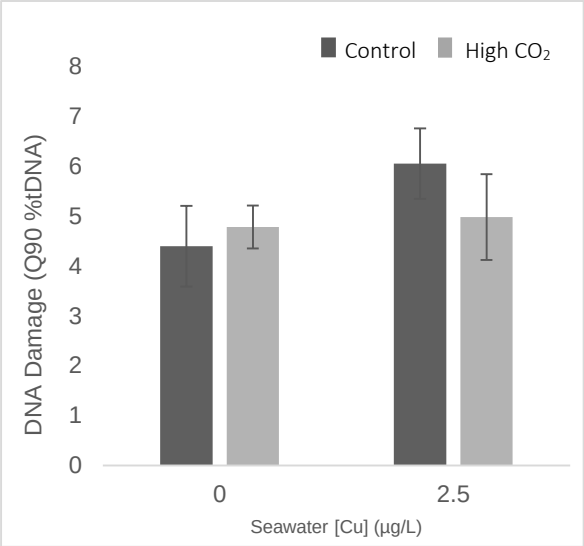
Chapter 3 Figure 13: Strong ion difference as calculated from ionic analysis of *D. labrax* plasma following 24 h exposure. Value represent Mean ± SEM. N=7,7,8,8.



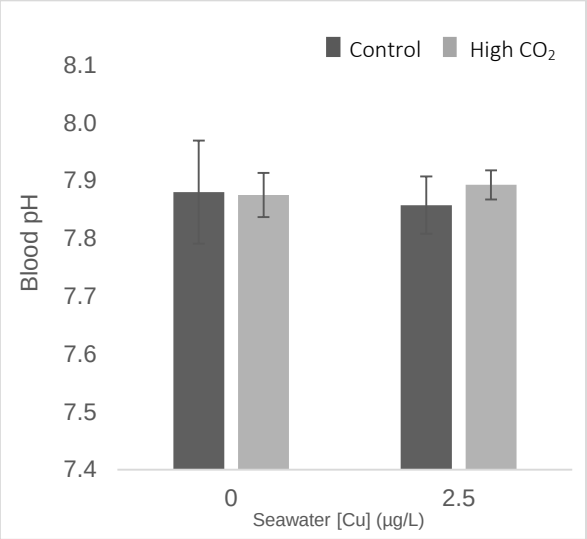
Chapter 3 Figure 14: Sodium (A) and chloride (B) concentrations of *D. labrax* plasma following 24 h exposure. Values represent mean ± SEM. N=7,7,8,8.

7.3 Preliminary Study:

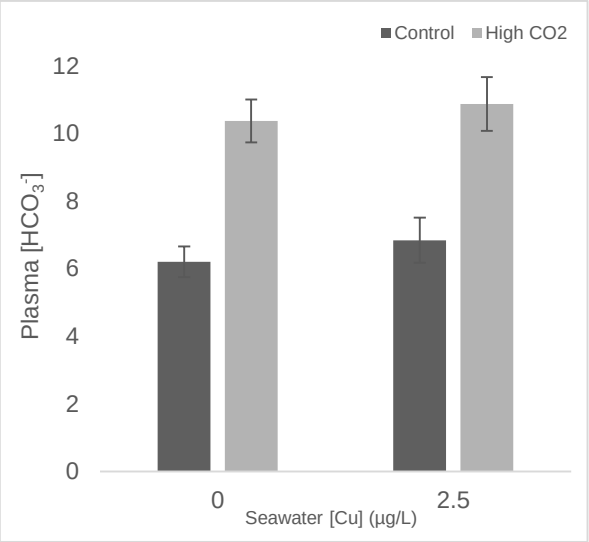
Sea bass exposed to Cu (2.5 ± 0.2 μg/L) under control and high CO_2 (nominal 400 and 1200 μatm).



Chapter 3 Figure 15: DNA damage in red blood cells of sea bass following 14 day *in vivo* exposure, as measured by comet assay. Values represent treatment means for Q90 of log transformed percentage tail intensity (log%tDNA) ± SEM. N=4.



Chapter 3 Figure 17: Blood pH of sea bass following 14 day *in vivo* exposure to varying concentrations of Cu and CO_2. Values represent Mean ± SEM. N=4.



8. Appendices

Chapter 3 Table 1: Ionic composition of control and high bicarbonate salines used for *in vitro* exposure of sea bass erythrocytes.

	Control (mM)	High Bicarbonate (mM)
$[Na^+]$	175	175
$[Ca^{2+}]$	8.8	8.8
$[K^+]$	3.6	3.6
$[Mg^{2+}]$	0.7	0.7
$[NH_4^+]$	0.6	0.6
$[Cl^-]$	130	124
$[HCO_3^-]$	6	12
$[SO_4^{2-}]$	4.4	4.4
TOTAL	**329.1**	**329.1**

Chapter 3 Table 2: Nominal carbonate chemistry of artificial salines used for *in vitro* exposure of sea bass erythrocytes.

Treatment	$[HCO_3^-]$ (mM)	Copper (µg/L)	Temperature (°C)	Osmolarity (Osm)	Plasma pH	CO_2 (mM)	TCO_2 (mM)	PCO_2 (µatm)
Control	6	0	15.0	362	7.88	0.09	6.09	2321.7
					7.60	0.19	6.19	4689.1
High Bicarbonate	12	0	15.0	362	8.16	0.09	12.09	2301.5
					7.88	0.19	12.19	4647.2
Cu	6	45	15.0	362	7.88	0.09	6.09	2321.7
					7.60	0.19	6.19	4689.1
High Bicarbonate + Cu	12	45	15.0	362	8.16	0.09	12.09	2301.5
					7.88	0.19	12.19	4647.2

Chapter 3 Table 4: Seawater carbonate chemistry and copper concentrations in four experimental treaments under which European sea bass were held during the 24 h exposure. Values shown are means (±SEM). † indicates values calculated using CO2sys (Pierrot, 2006).

Treatment	Temperature (°C)	pH_{NBS}	Salinity	Cu (µg/L)	TA (µmol/kg)†	TCO_2 (µmol/kg)	pCO_2 (µatm)†	HCO_3^- (µmol/kg)†	CO_3^{2-} (µmol/kg)†	ΩCa†	ΩAr†
Control	15.4 (±0.0)	8.1 (±0.01)	34.5 (±0.0)	0.8 (±0.3)	1955.6 (±10.2)	1796.8 (±7.6)	434.5 (±7.5)	1668.5 (±6.2)	112.2 (±2.3)	2.7 (±0.1)	1.7 (±0.0)
High CO_2	15.4 (±0.0)	7.7 (±0.00)	34.5 (±0.0)	0.8 (±0.5)	1960.1 (±11.4)	1920.3 (±10.3)	1121.4 (±10.3)	1826.6 (±9.8)	52.2 (±0.8)	1.2 (±0.0)	0.8 (±0.0)
Cu	15.4 (±0.1)	8.1 (±0.01)	34.5 (±0.1)	62.2 (±0.8)	2129.1 (±12.4)	1944.0 (±9.8)	426.5 (±8.2)	1795.5 (±8.6)	132.8 (±2.9)	3.2 (±0.1)	2.0 (±0.0)
High CO_2 + Cu	15.4 (±0.1)	7.7 (±0.01)	34.6 (±0.1)	65.2 (±1.4)	2244.8 (±9.9)	2194.6 (±10.6)	1202.2 (±20.7)	2086.5 (±10.2)	63.7 (±0.8)	1.5 (±0.0)	1.0 (±0.0)

Chapter 3 Table 5: Seawater carbonate chemistry and copper concentrations in four experimental treaments under which European sea bass were held during the 14 day exposure. Values shown are means (±SEM). † indicates values calculated using CO2sys (Pierrot, 2006).

Treatment	Temperature (°C)	pH_{NBS}	Salinity	Cu (µg/L)	TA (µmol/kg)†	TCO_2 (µmol/kg)	pCO_2 (µatm)†	HCO_3^- (µmol/kg)†	CO_3^{2-} (µmol/kg)†	ΩCa†	ΩAr†
Control	15.1 (±0.06)	8.10 (±0.02)	34.8 (±0.06)	0.3 (±0.4)	2269.3 (±85.2)	2081.9 (±76.6)	469.5 (±19.5)	1926.3 (±68.9)	138.2 (±8.8)	3.3 (±0.2)	2.1 (±0.1)
High CO_2	15.2 (±0.1)	7.72 (±0.02)	34.9 (±0.1)	0.5 (±0.5)	2169.1 (±76.1)	2122.0 (±72.0)	1179.3 (±40.1)	2017.8 (±68.0)	60.4 (±4.2)	1.4 (±0.1)	0.9 (±0.1)
Cu	15.2 (±0.1)	8.11 (±0.02)	34.9 (±0.1)	82.6 (±5.0)	2288.9 (±99.3)	2095.8 (±85.8)	462.5 (±13.8)	1936.4 (±74.8)	142.3 (±11.7)	3.4 (±0.3)	2.2 (±0.2)
High CO_2 + Cu	15.2 (±0.1)	7.71 (±0.02)	34.9 (±0.1)	82.4 (±1.0)	2168.4 (±51.3)	2123.1 (±48.4)	1196.9 (±41.4)	2019.0 (±45.8)	59.7 (±3.1)	1.4 (±0.1)	0.9 (±0.0)

9. References

Adeyemi, J. A., Deaton, L. E., Pesacreta, T. C., & Klerks, P. L. (2012). Effects of copper on osmoregulation in sheepshead minnow, *Cyprinodon variegatus* acclimated to different salinities. *Aquatic Toxicology, 109*(0), 111-117.

Arellano, J. M., Storch, V., & Sarasquete, C. (1999). Histological changes and copper accumulation in liver and gills of the Senegales sole, *Solea senegalensis*. *Ecotoxicology and Environmental Safety, 44*(1), 62-72.

Baker, J. T. (1969). Histological and electron microscopical observations on copper poisoning in the winter flounder (*Pseudopleuronectes americanus*). *Journal of the Fisheries Board of Canada, 26*(11), 2785-2793.

Bao, Y., Qiao, F., & Song, Z. (2012). Historical simulation and twenty-first century prediction of oceanic CO_2 sink and pH change. *Acta Oceanologica Sinica, 31*(5), 87-97.

Barth, A., Bauer, R., Gedrange, T., Walter, B., Klinger, W., & Zwiener, U. (1998). Influence of hypoxia and hypoxia/hypercapnia upon brain and blood peroxidative and glutathione status in normal weight and growth-restricted newborn piglets. *Experimental and Toxicologic Pathology, 50*(4), 402-410.

Berge, J. A., Bjerkeng, B., Pettersen, O., Schaanning, M. T., & Øxnevad, S. (2006). Effects of increased sea water concentrations of CO_2 on growth of the bivalve *Mytilus edulis* L. *Chemosphere, 62*(4), 681-687.

Bibby, R., Widdicombe, S., Parry, H., Spicer, J., & Pipe, R. (2008). Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. *Aquatic Biology, 2*, 67–74.

Bielmyer, G. K., Gatlin, D., Isely, J. J., Tomasso, J., & Klaine, S. J. (2005). Responses of hybrid striped bass to waterborne and dietary copper in freshwater and saltwater. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 140*(1), 131-137.

Bogdanova, A. Y., Gassmann, M., & Nikinmaa, M. (2002). Copper ion redox state is critical for its effects on ion transport pathways and methaemoglobin formation in trout erythrocytes. *Chemico-Biological Interactions, 139*(1), 43-59.

Brewer, G. J. (2007). A brand new mechanism for copper toxicity. *Journal of Hepatology, 47*(4), 621-622.

Buckley, J. T., Roch, M., McCarter, J. A., Rendell, C. A., & Matheson, A. T. (1982). Chronic exposure of coho salmon to sublethal concentrations of copper-I. Effect on growth, on accumulation and distribution of copper, and on copper tolerance. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 72*(1), 15-19.

Burlinson, B., Tice, R. R., Speit, G., Agurell, E., Brendler-Schwaab, S. Y., Collins, A. R., Nakajima, M. (2007). Fourth international workgroup on genotoxicity testing: results of the *in vivo* Comet assay workgroup. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 627*(1), 31-35.

Chakoumakos, C., Russo, R. C., & Thurston, R. V. (1979). Toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH, and hardness. *Environmental Science & Technology, 13*(2), 213-219.

Christensen, G., & Tucker, J. (1976). Effects of selected water toxicants on the *in vitro* activity of fish carbonic anhydrase. *Chemico-Biological Interactions, 13*(2), 181-192.

Claiborne, J. B., Perry, E., Bellows, S., & Campbell, J. (1997). Mechanisms of acid-base excretion across the gills of a marine fish. *Journal of Experimental Zoology, 279*, 509-520.

Dickson, A., Sabine, C., & Christian, J. (2007). Guide to Best Practices for Ocean CO_2 Measurements.

Dickson, A. G. (1990). Standard potential of the reaction: and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K. *The Journal of Chemical Thermodynamics, 22*, 113-127.

Dickson, A. G., & Millero, F. J. (1987). A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Research, 34*, 1733-1743.

Dixson, D. L., Munday, P. L., & Jones, G. P. (2010). Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecology Letters, 13*(1), 68-75.

Doney, S. C., Fabry, V. J., Feely, R. A., & Kleypas, J. A. (2009). Ocean acidification: the other CO_2 problem. *Annual Review of Marine Science, 1*(1), 169-192.

Edeker, B. L., Rasmussen, G. T., & Britigan, B. E. (1995). Bicarbonate and phosphate ions protect transferrin from myeloperoxidase-mediated damage. *Journal of leukocyte biology, 58*(1), 59-64.

Esbaugh, A., Heuer, R., & Grosell, M. (2012). Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. *Journal of Comparative Physiology B, 182*(7), 921-934.

Farrell, A., MacLeod, K., & Scott, C. (1988). Cardiac performance of the trout (*Salmo gairdneri*) heart during acidosis: effects of low bicarbonate, lactate and cortisol. *Comparative Biochemistry and Physiology Part A: Physiology, 91*(2), 271-277.

Figueiredo-Fernandes, A., Ferreira-Cardoso, J. V., Garcia-Santos, S., Monteiro, S. M., Carrola, J., Matos, P., & Fontaínhas-Fernandes, A. (2007). Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. *Pesquisa Veterinária Brasileira, 27*(3), 103-109.

Franke, A., Clemmesen, C. (2011). Effect of ocean acidification on early life stages of Atlantic herring (*Clupea harengus L.*). *Biogeosciences Discuss, 8*, 7097–7126.

Friederich, G. E., Walz, P. M., Burczynski, M. G., & Chavez, F. P. (2002). Inorganic carbon in the central California upwelling system during the 1997-1999 El Nino-La Nina event. *Progress in Oceanography, 54*(1-4), 185-203.

Gabbianelli, R., Lupidi, G., Villarini, M., & Falcioni, G. (2003). DNA damage induced by copper on erythrocytes of gilthead sea bream (*Sparus aurata*) and mollusk (*Scapharca inaequivalvis*). *Archives of Environmental Contamination and Toxicology, 45*(3), 350-356.

Georgalis, T., Perry, S. F., & Gilmour, K. M. (2006). The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology, 209*(3), 518-530.

Gilmour, K. M., & Perry, S. F. (2009). Carbonic anhydrase and acid-base regulation in fish. *Journal of Experimental Biology, 212*(11), 1647-1661.

Götze, S., Matoo, O. B., Beniash, E., Saborowski, R., & Sokolova, I. M. (2014). Interactive effects of CO_2 and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. *Aquatic Toxicology, 149*, 65-82.

Grosell, M., McDonald, M. D., Wood, C. M., & Walsh, P. J. (2004). Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*): I. Hydromineral balance and plasma nitrogenous waste products. *Aquatic Toxicology, 68*(3), 249-262.

Grosell, M., McGeer, J. C., & Wood, C. M. (2001). Plasma copper clearance and biliary copper excretion are stimulated in copper-acclimated trout. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 280*(3), 796-806.

Grosell, M., & Wood, C. M. (2002). Copper uptake across rainbow trout gills. *Journal of Experimental Biology, 205*(8), 1179-1188.

Grosell, M., Wood, C. M., & Walsh, P. J. (2003). Copper homeostasis and toxicity in the elasmobranch *Raja erinacea* and the teleost *Myoxocephalus octodecemspinosus* during exposure to elevated water-borne copper. *Comparative Biochemistry and Physiology Toxicology & Pharmacology, 135*(2), 179-190.

Hall, J. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of experimental botany, 53*(366), 1-11.

Hamza-Chaffai, A., Cosson, R., Amiard-Triquet, C., & El Abed, A. (1995). Physico-chemical forms of storage of metals (Cd, Cu and Zn) and metallothionein-like proteins in gills and liver of marine fish from the Tunisian coast: ecotoxicological consequences. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 111*(2), 329-341.

Hans, S., Fehsenfeld, S., Treberg, J. R., & Weihrauch, D. (2014). Acid–base regulation in the Dungeness crab (*Metacarcinus magister*). *Marine Biology, 161*(5), 1179-1193.

Hayashi, M., Fuse, S., Endoh, D., Horiguchi, N., Nakayama, K., Kon, Y., & Okui, T. (2006). Accumulation of copper induces DNA strand breaks in brain cells of Long-Evans Cinnamon (LEC) rats, an animal model for human Wilson Disease. *Exp. Anim., 55*(5), 419-426.

Hayashi, M., Kita, J., & Ishimatsu, A. (2004). Comparison of the acid-base responses to CO_2 and acidification in Japanese flounder (*Paralichthys olivaceus*). *Marine Pollution Bulletin, 49*(11-12), 1062-1065.

Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Hatziolos, M. E. (2007). Coral reefs under rapid climate change and ocean acidification. *Science, 318*(5857), 1737-1742.

Ivanina, A. V., Beniash, E., Etzkorn, M., Meyers, T. B., Ringwood, A. H., & Sokolova, I. M. (2013). Short-term acute hypercapnia affects cellular responses to trace metals in the hard clams *Mercenaria mercenaria*. *Aquatic Toxicology, 140*, 123-133.

Kamunde, C., Grosell, M., Higgs, D., & Wood, C. M. (2002). Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. *Journal of Experimental Biology, 205*(2), 279-290.

Kim, S.G., & Kang, J.C. (2004). Effect of dietary copper exposure on accumulation, growth and haematological parameters of the juvenile rockfish, *Sebastes schlegeli*. *Marine Environmental Research, 58*(1), 65-82.

Langenbuch, M., & Pörtner, H.-O. (2003). Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempi*) as a function of ambient CO2: pH-dependent limitations of cellular protein biosynthesis? *Journal of Experimental Biology, 206*(22), 3895-3903.

Larsen, B. K., Pörtner, H. -O., & Jensen, F. B. (1997). Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology, 128*(2), 337-346.

Lovell, D. P., & Omori, T. (2008). Statistical issues in the use of the comet assay. *Mutagenesis, 23*(3), 171-182.

McGeer, J. C., Szebedinszky, C., Gordon McDonald, D., & Wood, C. M. (2000). Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout 2: Tissue specific metal accumulation. *Aquatic Toxicology, 50*(3), 245-256.

Mehrbach, C., Culberson, C. H., Hawley, J. E., & Pytkowicz, R. M. (1973). Measurements of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnology and Oceanography, 18*(6), 897-906.

Michaelidis, B., Spring, A., & Pörtner, H. -O. (2007). Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish *Sparus aurata*. *Marine Biology, 150*(6), 1417-1429.

Millero, F., Santana-Casiano, J., & González-Dávila, M. (2010). The formation of Cu^{2+} complexes with carbonate and bicarbonate ions in $NaClO_4$ solutions. *Journal of Solution Chemistry, 39*(4), 543-558.

Millero, F. J. (2009). Effects of ocean acidification on the speciation of metals in seawater. *Oceanography, 22*(4), 72.

Munday, P. L., Dixson, D. L., Donelson, J. M., Jones, G. P., Pratchett, M. S., Devitsina, G. V., & Døving , K. B. (2009). Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proceedings of the National Academy of Sciences, 106*(6), 1848-1852.

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sorensen, C., Watson, S.-A., & Munday, P. L. (2012). Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change, 2*(3), 201-204.

NOAA. (2015). Trends in Carbon Dioxide. Retrieved 15.08.15, from http://www.esrl.noaa.gov/gmd/ccgg/trends/weekly.html

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Yool, A. (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature, 437*(7059), 681-686.

Parveen, A., & Javed, M. (2010). Effect of water-borne copper on the growth performance of fish *Catla catla*. *International Journal of Agriculture & Biology, 12*.

Pavlidis, M., Berry, M., Divanach, P., & Kentouri, M. (1997). Diel pattern of haematocrit, serum metabolites, osmotic pressure, electrolytes and thyroid hormones in sea bass and sea bream. *Aquaculture International, 5*(3), 237-247.

Pelgrom, S., Lock, R., Balm, P., & Bonga, S. W. (1995). Integrated physiological response of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure. *Aquatic Toxicology, 32*(4), 303-320.

Pierrot, D. E., Wallace, D.W.R. (2006). MS Excel Program Developed for CO_2 System Calculations. *ORNL/CDIAC-105a*.

Pilgaard, L., Malte, H., & Jensen, F. B. (1994). Physiological effects and tissue accumulation of copper in freshwater rainbow trout (*Oncorhynchus mykiss*) under normoxic and hypoxic conditions. *Aquatic Toxicology, 29*(3–4), 197-212.

Playle, R. C., Dixon, D. G., & Burnison, K. (1993). Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modelling of metal accumulation. *Canadian Journal of Fisheries and Aquatic Sciences, 50*(12), 2678-2687.

Pörtner, H., Langenbuch, M., & Reipschläger, A. (2004). Biological impact of elevated ocean CO_2; concentrations: Lessons from animal physiology and earth history. *Journal of Oceanography, 60*(4), 705-718.

Pörtner, H.-O., Langenbuch, M., & Reipschlager, A. (2004). Biological impact of elevated ocean CO_2 concentrations: Lessons from animal physiology and earth history. *Journal of Oceanography, 60*(4), 705-718.

Pörtner, H. O., Reipschläger, A., & Heisler, N. (1998). Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level.

Rehncrona, S., Hauge, H. N., & Siesjô, B. K. (1989). Enhancement of iron-catalyzed free radical formation by acidosis in brain homogenates: Difference in effect bylactic acid and CO_2. *Journal of Cerebral Blood Flow & Metabolism, 9*(1), 65-70.

Riahi, K., Grübler, A., & Nakicenovic, N. (2007). Scenarios of long-term socio-economic and environmental development under climate stabilization. *Technological Forecasting and Social Change, 74*(7), 887-935.

Riggs, A. F. (1988). The Bohr Effect. *Annual Review of Physiology, 50*(1), 181-204.

Roberts, D. A., Birchenough, S. N., Lewis, C., Sanders, M. B., Bolam, T., & Sheahan, D. (2013). Ocean acidification increases the toxicity of contaminated sediments. *Global change biology, 19*(2), 340-351.

Roesijadi, G., & Robinson, W. (1994). Metal regulation in aquatic animals: mechanisms of uptake, accumulation and release. *Aquatic Toxicology, 102*, 125-133.

Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., Rios, A. F. (2004). The oceanic sink for anthropogenic CO_2. *Science, 305*(5682), 367-371.

Segner, H. (1987). Response of fed and starved roach, *Rutilus rutilus*, to sublethal copper contamination. *Journal of Fish Biology, 30*(4), 423-437.

Segner, H., & Braunbeck, T. (1990). Qualitative and quantitative assessment of the response of milkfish, *Chanos chanos*, fry to low-level copper exposure. *Pathol. Mar. Sci.*, 347-368.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low-levels of DNA damage in individual cells. *Experimental Cell Research, 175*(1), 184-191.

Small, D., Calosi, P., White, D., Spicer, J., & Widdicombe, S. (2010). Impact of medium-term exposure to CO_2 enriched seawater on the physiological functions of the velvet swimming crab *Necora puber*. *Aquatic Biology, 10*(1), 11-21.

Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K., Miller, H. (2007). The physical science basis. *Contribution of working group i) to the fourth assessment report of the intergovernmental panel on climate change*, 235-337.

Somero, G. (1986). Protons, osmolytes, and fitness of internal milieu for protein function. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 251*(2), 197-213.

Stagg, R. M., & Shuttleworth, T. J. (1982). The effects of copper on ionic regulation by the gills of the seawater-adapted flounder (*Platichthys flesus* L.). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 149*(1), 83-90.

Stewart, P. A. (1983). Modern quantitative acid–base chemistry. *Canadian Journal of Physiology and Pharmacology, 61*(12), 1444-1461.

Taha, T. A., Mullen, T. D., & Obeid, L. M. (2006). A house divided: Ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1758*(12), 2027-2036.

Toews, D. P., Holeton, G. F., & Heisler, N. (1983). Regulation of the acid-base status during environmental hypercapnia in the marine teleost fish *Conger conger*. *Journal of Experimental Biology, 107*(1), 9-20.

Ueda, J.-I., Takai, M., Shimazu, Y., & Ozawa, T. (1998). Reactive oxygen species generated from the reaction of copper (II) complexes with biological reductants cause DNA strand scission. *Archives of biochemistry and biophysics, 357*(2), 231-239.

Valko, M., Rhodes, C., Moncol, J., Izakovic, M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*(1), 1-40.

Vannucci, R. C., Brucklacher, R. M., & Vannucci, S. J. (1997). Effect of carbon dioxide on cerebral metabolism during hypoxia-ischemia in the immature rat. *Pediatric research, 42*(1), 24-29.

Vesela, A., & Wilhelm, J. (2002). The role of carbon dioxide in free radical reactions in organism. *Physiological research, 51*(4), 335-340.

Vitale, A., Monserrat, J., Castilho, P., & Rodriguez, E. (1999). Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 122*(1), 121-129.

Wang, T., Knudsen, P., Brauner, C., Busk, M., Vijayan, M., & Jensen, F. (1998). Copper exposure impairs intra-and extracellular acid-base regulation during hypercapnia in the fresh water rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B, 168*(8), 591-599.

Whiteside, J. R., Box, C. L., McMillan, T. J., & Allinson, S. L. (2010). Cadmium and copper inhibit both DNA repair activities of polynucleotide kinase. *DNA Repair, 9*(1), 83-89.

Woodcock, J. (2006). Sphingosine and ceramide signalling in apoptosis. *IUBMB life, 58*(8), 462-466.

Yamamoto, K., & Kawanishi, S. (1989). Hydroxyl free radical is not the main active species in site-specific DNA damage induced by copper (II) ion and hydrogen peroxide. *Journal of Biological Chemistry, 264*(26), 15435-15440.

Zimmer, A. M., Barcarolli, I. F., Wood, C. M., & Bianchini, A. (2012). Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity

in euryhaline guppies acclimated to both fresh water and sea water. *Aquatic Toxicology, 122–123*(0), 172-180.

CHAPTER 4

Acid-base regulation may protect against copper-induced DNA damage in shore crabs (*Carcinus maenas*) but is inhibited at higher copper concentrations

CHAPTER 4

ACID-BASE REGULATION MAY PROTECT AGAINST COPPER-INDUCED DNA DAMAGE IN SHORE CRABS (*CARCINUS MAENAS*) BUT IS INHIBITED AT HIGHER COPPER CONCENTRATIONS

1. Abstract

Marine animals acutely subjected to seawater with elevated CO_2 partial pressure experience a corresponding extracellular acidosis. Many fish and crustaceans are able to regulate these acid-base perturbations by the elevation of extracellular bicarbonate ions (HCO_3^-). These animals can use this mechanism to completely restore normal blood pH well within 24 hours of exposure to elevated CO_2. We provide evidence that the acid-base regulatory responses to high CO_2 may provide a protective effect against copper-induced DNA damage at low concentrations (10 µg/L). Additionally we provide the first evidence of the Cu (40 µg/L) induced inhibition of acid-base regulation in crabs under high CO_2 exposure.

Shore crabs (*Carcinus maenas*) were exposed to various waterborne Cu concentrations over 24 hours (20 µg/L Cu) and 14 days (10, 15 and 40 µg/L Cu) under both normocapnic (450 µatm) and high CO_2 (1200 µatm) seawater conditions. Analysis of haemolymph acid-base variables (pH, TCO_2) revealed complete pH regulation in all animals exposed to high CO_2 and/or copper with the exception of the high CO_2 + 40 µg/l Cu treatment, where copper inhibited this regulation. Regulation of haemolymph pH when challenged with elevated external pCO_2 was achieved by ~4 mM elevations in haemolymph [HCO_3^-]. Exposure of crabs to 40 µg/L waterborne Cu resulted in the inability to accumulate extracellular HCO_3^- leading to an uncompensated respiratory acidosis.

Assessment of haemocyte DNA damage using comet assay showed a dose-dependent increase in DNA damage with increasing seawater Cu concentration between 0 and 15 µg/L under normocapnia, however this trend did not continue between 15 and 40 µg/L with no further damage apparent at the highest Cu concentration (40 µg/L). Crabs exposed to Cu under high CO_2 showed a trend towards lower levels of DNA damage compared to the same Cu level under normocapnia: with complete protection observed in the high CO_2 + 10 µg/L Cu exposure. We suggest

that elevated haemolymph [HCO_3^-], resulting from the acid-base regulatory response to high CO_2, has the capacity to reduce Cu-induced DNA damage in haemocytes and may even lower background levels of such damage in animals not exposed to Cu. Protection appeared progressively limited in its effect with increasing Cu concentration and was absent at the highest Cu levels, most likely due to the inhibition of HCO_3^- accumulation.

There were no differences in measurable copper concentrations in any tissues following 24 h exposure, however stark changes in these concentrations were apparent after 14 d. Following 14 d the gills were the primary site of copper accumulation, with concentrations 23 to 28-fold higher than animals not exposed to copper. Haemolymph copper concentrations showed little change due to exogenous copper but were lower after 14 d when compared with 24 h. This was attributed to the breakdown of haemocyanin under starved conditions, which coincided with an observed increase in hepatopancreatic copper levels which has previously been reported in shrimp (*Crangon crangon*) and lobsters (*Nephrops norvegicus*).

2. Introduction

As we have discussed in previous chapters (1 and 3) copper toxicity and elevated environmental pCO_2 are naturally linked due to the well described influence that water pH is known to have on the speciation of Cu, and as a consequence its bioavailability to aquatic organisms.

Lowering the pH of a copper solution results in an exponential increase in toxic and bioavailable Cu^{2+} ions (Chakoumakos et al., 1979). Millero et al. (2010) explained how this change in speciation relates to carbonate chemistry in the context of ocean acidification and may lead to significant increases in more harmful copper species, including a doubling of toxic Cu^{2+} ions by early next century. The few studies to consider the effects of high CO_2 and copper on marine biota suggest that the physiological impacts are by no means predictable and may not be attributable to simple changes in environmental Cu speciation. Lewis (2015, submitted) found that DNA damage induced by copper exposure was significantly exacerbated by simultaneous exposure to high pCO_2 (1481 µatm). Elevated seawater pCO_2 reduced the uptake of Cd^{2+} in hard clams (*Mercenaria mercenaria*) but increased that of Cu^{2+} (Ivanina et al., 2013). Tomanek et al. (2011) reported the reversal of detrimental

tumour suppressor inhibition by Cu^{2+} and Cd^{2+} under elevated pCO_2 in the same clam species, and Larsen et al. (1997) found a complete removal of acute Cu lethality in Atlantic cod (*Gadus morhua*). Summarily, the effects of copper under high CO_2 appear both metal and species specific. However, given the clear potential for the additive, interactive or synergistic effects of these two stressors, Cu presents a mechanistically interesting and environmentally relevant stressor for research aimed at better understanding the effects of such contaminants in the context of ocean acidification.

In chapter 3 we explored the potential indirect ameliorating effect that acid-base regulatory mechanism may have on European sea bass (*Dicentrarchus labrax*) associated with the accumulation of bicarbonate ions (HCO_3^-). Conversely with respect to potential benefit to the animal, we also found evidence suggestive of inhibition of this acid-base regulatory strategy at higher copper concentrations (80 µg/L). Both findings have considerable implications for species success and survivorship as well as implications for teleosts as a whole. In the present study we assess the same toxicological and physiological endpoints in an unrelated species that employs largely the same acid-base regulatory strategy. In this way we aim to draw mechanistic and physiological comparisons between the two species. To do so we consider the effects that exposure to high CO_2 and copper have, both separately and in combination, on shore crabs (*Carcinus maenas*). Shore crabs represent a hardy species convenient for laboratory work. They inhabit coastal waters where there exists clear potential for combined exposure to terrestrially introduced Cu under future climate change scenarios. Not only this but the intertidal environment they inhabit often leads to frequent exposure of these animals to hypoxic/hypercapnic seawater in rock pools at low tide, as well as coastal contaminants such as copper, making such work of equal relevance to their current life history.

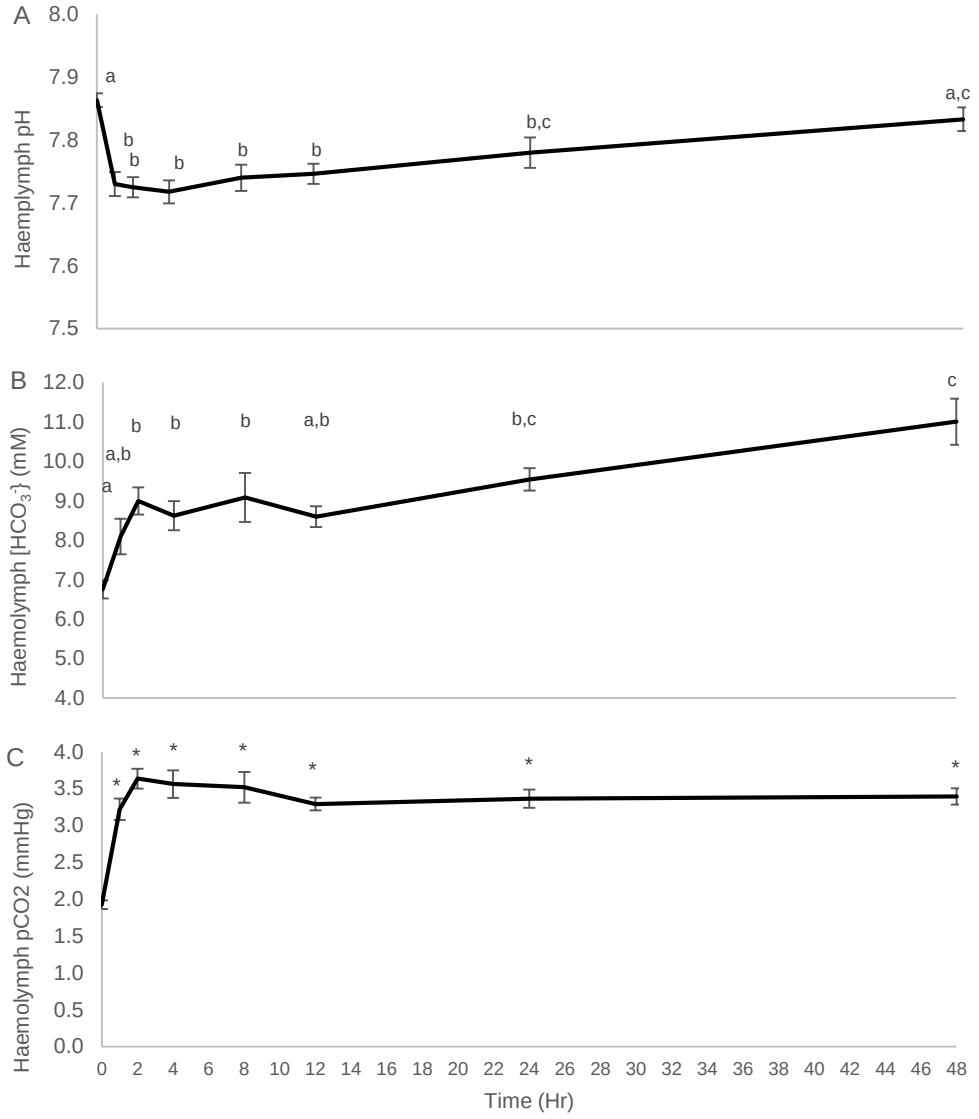
In the present study, we aimed to assess whether changes to the carbonate chemistry of crab haemolymph, resulting from the acid-base regulatory response to high CO_2, have the capacity to modulate the toxicity of internalised Cu, perhaps in a similar (but opposite) way that changes to seawater chemistry modulate the bioavailability of Cu to organisms.

1.1 Acid-base Regulation

One of the primary impacts that increased environmental CO_2 concentrations has on crabs is the short term disruption of their internal acid-base regulation. As discussed in Chapter 1 and 2 acid-base regulation strives towards maintaining physiologically optimum internal pH, even when subjected to internal or environmental changes (Claiborne, 1998). This is achieved primarily through the active accumulation of bicarbonate ions (HCO_3^-; Fehsenfeld & Weihrauch, 2013).

1.2 Chapter 2 Excerpt: Acid-base Regulation in Shore crabs (*C. maenas*)

Exposure to elevated seawater CO_2 (nominal 1200 µatm) caused a rapid and sustained increase in haemolymph pCO_2. This resulted in a transient extracellular acidosis during the first 24 hours. A 1.6-fold increase in extracellular [HCO_3^-] from 6.8 to 11.0 mM enabled recovery of extracellular pH to control values within 48 hours following the onset of exposure.



Chapter 2 Figure 3: Haemolymph pH, bicarbonate and pCO_2 concentrations of Shore crabs (*C. maenas*) as a function of time following onset of environmental hypercapnia at 1200 µatm. Values represent mean ± SEM. N = 20, 8, 8, 8, 8, 8, 7 and 10 for 0, 1, 2, 4, 8, 12, 24 and 48 hours respectively. Haemolymph pH and bicarbonate: Subsets showing significant differences are labelled with letters (ANOVA, $p<0.05$). PCO_2: A significant difference when compared to control (0 hours) is denoted by an asterisk (ANOVA, $p<0.05$).

Based on the findings from work on sea bass (Chapter 3) which suggested cells may be protected from Cu-induced DNA damage under high CO_2, we test the hypothesis that extracellular HCO_3^- accumulated as an acid-base regulatory measure may serve to reduce toxicity to haemocytes within that fluid. Haemocytes serve as a proxy for other internal tissues. DNA damage within haemocytes was employed as a sensitive and widely used measure of intracellular toxicity under Cu exposure.

3. Methods

3.1 Experimental Approach

In order to assess the potential effect of simultaneous exposure to copper and high CO_2 on DNA damage in haemocytes, crabs were exposed to copper under both normocapnic (450 µatm CO_2) and hypercapnic (1200 µatm CO_2) conditions, in addition to controls without copper under both CO_2 partial pressures. With the aim of assessing temporal and dose-dependency of effects in crabs we ran two consecutive experiments, 24 h and 14 d, and employed multiple copper concentrations.

In the 24 h experiment crabs were randomly assigned to 1 of 4 treatments with respect to copper and CO_2: 1) Control (pCO_2: 450 µatm), 2) High CO_2 (pCO_2: 1200 µatm), Copper only (pCO_2: 450 µatm + Cu: 20 µg/L), or 4) High CO_2 + Cu (pCO_2: 1200 µatm + Cu: 20 µg/L).

The 14 d treatment differed only in duration and the use of 3 copper concentrations of increasing severity (10, 15 and 40 µg/L) and the associated combined treatment with high CO_2: totalling 8 treatments.

Haemolymph was then sampled to characterise acid-base chemistry and haemocytes were then extracted for analysis of DNA damage using the comet assay. Seawater pCO_2 was elevated 48 hours prior to commencing the 24 h experiment (prior to addition of any copper) in order to allow elevation of haemolymph bicarbonate, as per their acid-base regulation strategy (Chapter 2).

3.2 Animals

A mixture of male and female shore crabs (58.2±1.4 g) were collected from the Exe estuary in Devon, UK. After capture they were transported in aerated tanks to the

Aquatic Resources Centre within the department of Biosciences at the University of Exeter where they were kept in aerated 45 litre holding tanks for 7 days prior to start of each exposure. The holding tanks were fed with artificial seawater produced using Tropic Marin® salts dissolved in deionised water (Nominal: 15° C, pH: 8.15, salinity: 35, pCO_2: 400 µatm). Each holding aquarium contained 10 animals. Crabs were fed with mussels daily prior to the start of the exposure bar the 3 days prior to commencing the experiment.

3.3 Experimental Set-up

Each treatment set-up consisted of 10 x 1.2 litre aquaria (each containing 1 animal) all fed by a 115 litre reservoir tank. Water was fed to each aquarium at a rate of 65±2 ml per minute via lengths of Tygon tubing through which water was pumped using a submersible pump (Eheim Universal 1250) positioned in the bottom of the reservoir tank. The total water volume for each of the four treatments was 130 litres.

Seawater was maintained at a temperature of 15 °C and salinity of 35. Water chemistry (see Appendix Tables 1 and 2) was controlled via the reservoir tanks. Control and copper treatment header tanks were bubbled vigorously with compressed air (pCO_2 =400 µatm). The high pCO_2 conditions of the remaining two treatments was achieved through use of two pH stat systems (Aqua Medic) connected to a compressed CO_2 cylinder (BOC Canada Ltd.). Electronically controlled solenoids were able to open or close the flow of CO_2 to the air stones within each of the two reservoir tanks according to real-time pH measurements from pH probes within the tanks. Each was set to release CO_2 if the pH deviated 0.01 pH units above the target values for the two treatments. Aeration, also from the centralised air supply, ensured both sufficient oxygen levels were maintained and that pH would rise quickly rise back up to the target value after the addition of CO_2 by the pH stat system. All aeration and CO_2 input was achieved via gas impermeable Tygon tubing attached to weighted diffusers (limewood air stones).

Within each of the two experiments (24 h and 14 d) copper treatments were dosed with 1 mM $CuSO_4$ in order to achieve a target seawater concentrations of total copper (24 h = 20 µg/l, 14 d = 10, 15 and 40 µg/l) (see Tables 1 and 2). Due to the closed system design partial water changes (50 %) were carried out every 48 hours using

temperature equilibrated water of the correct pH and CO_2 level. Copper treatments were re-dosed accordingly.

3.4 Determination of Seawater Chemistry

Throughout the exposure pH (Hannah Instruments HI 8314), temperature (Metrohm 826 pH Mobile), and salinity (YSI Inc. Salinity, Conductivity, Temperature Meter Model. 30/10) were measured on a daily basis. Over the 14 d experiment water samples were taken every third day of the exposure for dissolved inorganic carbon (DIC) analysis. These were drawn from the aquaria into glass vials and preserved using 4 % (w/v) mercuric chloride (added to achieve a final concentration of 0.04 %) to prevent microbial influences on the water DIC. DIC samples were analysed using custom-built apparatus centred upon an infra-red CO_2 analyser (Qubit Systems) using methods adapted from Friederich et al. (2002). Additionally 50 ml of seawater was taken at random from treatment tanks on the first, seventh and fourteenth days of the exposure for analysis of total copper. These samples were stored in HCl acid-washed 50 ml tubes and acidified using 50 µl of conc. HCl before being stored in the dark prior to analysis. Copper samples were sent to Plymouth Marine Laboratories for total copper analysis using ICP-MS. The CO_2sys program for Microsoft Excel (Pierrot et al. 2006) was used to calculate a suite of seawater chemistry variables, including pCO_2, HCO_3^- and total alkalinity, from the measured parameters (salinity, temperature, pH, DIC).

3.5 Crab Sampling

A volume of between approximately 800 µl of prebranchial haemolymph was taken from the base of a rear walking leg immediately upon removal from the experimental tanks. This was done rapidly to minimise the potential for stress-induced changes to haemolymph acid-base chemistry or those imposed by emersion (Truchot, 1986). The bled crab was then weighed and sexed and the body mass recorded after adding the known weight of removed haemolymph. Finally the whole crab was terminated by flash-freezing in liquid nitrogen and then stored at -80 $^{\circ}$C. Crabs were later dissected for analysis of gill and hepatopancreas Cu content by ICP-MS, conducted at the Camborne School of Mines (University of Exeter).

3.6 Measurement of Haemolymph Acid-base Variables

Haemolymph pH was measured immediately upon removal from the animals. This was done using a pH meter (Hannah Instruments HI 8314) and microprobe (Cole Parmer, Accumet) in controlled temperature water bath (15 °C). The probe was calibrated prior to usage using pH 7.00 and 10.00 specific buffers (calibrated to 7.04 and 10.11 at 15 °C), rinsed and checked against the pH 7 standard between each measurement. Submersion of the probe in isotonic saline prior to each measurement prevented introduction of deionised water and therefore unnecessary osmotic stress to the cells.

An aliquot of 300 µl was then added to another 1.5 ml microcentrifuge tube already containing 100 µl of anti-coagulant (see Söderhäll & Smith, 1983) (pH balanced to 7.78, kept at 4 °C) for analysis of DNA damage. These samples were then kept in the dark at 0 °C until the Comet assay (see Methods 3.7) could be run (within 2 hours). A final aliquot of 200 µl was then decanted into a further tube and flash frozen for ICP-MS analysis of Cu content.

The remaining haemolymph was stored on ice in sealed glass haematocrit tubes for analysis of TCO_2. Haemolymph TCO_2 was determined using a total CO_2 analyser (Corning 965), calibrated using blank runs and a 10 mM $NaHCO_3$ solution as the standard. Blanks and standards were run between samples to assess any drift in calibration over the period of analysis which was later accounted for. A volume of 50 µl was used for both samples and standards, delivered using a gas-tight syringe (Hamilton) rinsed thoroughly with de-ionised water between each use. Given the pH and TCO_2, haemolymph variables not directly measured could be calculated from pK_1 and CO_2 solubility constants for shore crabs derived from Truchot (1976) (see Chapter 2).

3.7 Comet Assay

Unfrosted microscope slides (Thermo Scientific) were coated on one side with 1 % high melting point (HMP) agarose and allowed to set at room temperature overnight prior to commencing the assay.

A 150 µl subsample of the 400 µl haemolymph/anti-coagulant mix was pipetted into a fresh 1.5 ml micro-centrifuge tube and centrifuged at 1000 rpm for 4 minutes at 4 °C

(Fresco Biofuge, Heraeus). The visibly less dense supernatant (accounting for ~130 µl) was then removed to leave ~20 µl of cell fraction. These volumes were optimized to produce sufficient per-frame cell density for later microscopic analysis.
The comet assay was then carried out and slides visualized to quantify DNA damage as per Chapter 3 and according to the protocol set out by Singh et al. (1988).
Comets with denser tails are formed when more DNA strand breaks have occurred, so tail intensity (%) was used as a measure of assessing DNA damage. Cells are manually captured within an on-screen score area which was automatically analysed by Comet Assay IV software, calculating the intensity of the tail area as a percentage of the entire comet.

3.8 Statistics

Comet assay data was measured as percentage tail intensity (%tDNA) and as such was transformed using a logarithmic function prior to analysis. A varying proportion of the data consisted of low values or zeros such that it was necessary to add one to each percentage prior to transformation: $\log_{10}$(%tDNA+1). The mean is greatly influenced by extreme observations in tail intensity rendering it largely uninformative (Gabbianelli et al., 2003). As such the 90th percentile (Q90) for each animal was used as a summary statistic. The median or Q75 would have been equally suitable (Lovell & Omori, 2008). Differences between treatments were determined by comparing the Q90 values using one-way ANOVA, followed by Tukey's post-hoc test.
All other analyses of treatment effects were conducted using one-way ANOVA, also employing Tukey's post-hoc test if the initial ANOVA revealed significant differences. In all cases the results were accepted as significant if $p < 0.05$. Statistical analyses were carried out using SPSS version 20.0 and histograms were produced using Microsoft Excel 2013.
Chauvenet's criterion was applied to ICP-MS tissue copper concentration data sets in order to lessen the effect of a few extreme outliers on the mean tissue concentrations. Only 7 data points were removed from a total of 181 across the four tissues analysed.

4. Results

4.1 Twenty-four Hour Exposure

4.1.1 DNA Damage

Following 24 h exposure DNA damage was not significantly affected by treatment, with no significant deviation from control values [ANOVA: $F_{(3,34)}$=2.71, p=0.06, Fig. 1]. Although not significantly so, mean DNA damage did appear highest in the combined high CO_2 + Cu treatment, around 50 % higher than controls and twice as high as the copper only treatment.

4.1.2 Acid-base Status

Crabs in the 24 h treatment showed clear evidence of acid-base regulation in both the high CO_2 and high CO_2 + Cu treatments. Elevations in ambient pCO_2 resulted in significant concomitant increases in haemolymph pCO_2, from an average of 1.7 to 2.8 mmHg [ANOVA: $F_{(3,33)}$=16.0, p<0.001, Fig. 2a]. Haemolymph pH was unaffected by the elevations in pCO_2 and not significantly different between treatments [ANOVA: $F_{(3,33)}$=1.06, p=0.378, Fig. 2b]. Any extracellular acidosis resulting from the elevation in seawater pCO_2 was compensated by this time point, through the accumulation of extracellular HCO_3^- which rose approximately 4 mM in both high CO_2 treatments [ANOVA: $F_{(3,33)}$=18.16, p<0.001, Fig. 2c]. The simultaneous exposure to Cu (20 µg/L) and high CO_2 appeared to have no measurable influence on acid-base regulation.

4.1.3 Tissue Accumulation

Exposure to copper for 24 h caused no elevation in haemolymph copper concentrations, evidenced by a lack difference from control: all treatments were within the range of 38-46 µg/g [ANOVA: $F_{(3,36)}$=0.54, p=0.658, , Fig. 3]. Gill [Cu] also revealed no significant treatment effect [ANOVA: $F_{(3,35)}$=2.00, p=0.131, Fig. 4] . However the mean concentrations in the copper only treatment were 54 % higher than gills from crabs not exposed to copper. Unsurprisingly given the duration of exposure there was no evidence of copper accumulation in the hepatopancreatic tissue of the crabs following 24 h exposure. None of the treatments were significantly different from control [ANOVA: $F_{(3,33)}$=0.19, p=0.901, Fig. 5]. Hepatopancreatic data from this short

exposure did however serve as an effective baseline against which to compare the same tissue following 14 d exposure.

4.2 Fourteen Day Exposure

4.2.1 DNA Damage

After the 14 d trial, levels of haemocytic DNA damage in control crabs were directly comparable to those from the control animals in 24 h experiment (~20 %tDNA). Copper exposure appeared to induce DNA damage in a dose-dependent manner between 0-15 µg/L, although this trend did not continue when copper was further increased to 40 µg/L. DNA damage was significantly reduced under high CO_2 in the 10 µg/L Cu treatment [ANOVA: $F_{(7,56)}=7.19$, $p<0.001$, Fig. 6]. In this treatment, DNA damage was comparable with control animals suggesting the high ambient CO_2 may afford full protection at this Cu concentration. Cells from animals in all the combined high CO_2 + copper treatments (including 0 µg/L) showed reduced levels of DNA when compared to the same concentration under normocapnic conditions, albeit only significantly so at the lowest copper concentration (10 µg/L).

4.2.2 Acid-Base Regulation

After 14 d haemolymph pCO_2 remained elevated in all crabs exposed to high ambient CO_2 conditions [ANOVA: $F_{(7,59)}=13.79$, $p<0.001$, Fig. 7a]. Crabs appeared to acid-base regulate effectively under high CO_2 in non-copper treatments. Acid-base regulation was however differentially effected by copper dose with effective acid-base regulation observed in the 10 and 15 µg/L Cu exposures, but not in the 40 µg/L [ANOVA: $F_{(7,59)}=7.09$, $p<0.001$, Fig. 7b]. In the two lowest copper treatments, as in all non-copper exposed crabs, acid-base regulation was achieved by marked elevation in extracellular [HCO_3^-] (increase of 3-4 mM) [ANOVA: $F_{(7,59)}=11.60$, $p<0.001$, Fig. 7c]. Clear inhibition of acid-base regulation was apparent in the 40 µg/L Cu exposure, characterised by an uncompensated acidosis (0.2 pH units lower), likely owing to the complete lack of elevation in haemolymph [HCO_3^-]. Extracellular [HCO_3^-] appeared marginally elevated in the normocapnic 40 µg/L Cu treatment: 7.0 mM compared with the no copper control of 5.5 mM. In combination with a slightly elevated mean pH (0.03

units) this may be indicative of a mild alkalosis induced by the 40 µg/L copper exposure.

4.2.3 Tissue Accumulation

Copper exposure did not result in elevated haemolymph [Cu] following 14 d exposure: copper-exposed animals showed no significant difference from control [ANOVA: $F_{(3,29)}$=0.72, p=0.546, Fig. 8]. There was however a clear accumulation in the gills of crabs at this time point with copper being markedly elevated in both copper conditions (3200-4000 µg/g) compared with both control and high CO_2 conditions (130-140 µg/g) [ANOVA: $F_{(3,30)}$=5.48, p<0.01, Fig. 9]. Unlike the gills, copper did not appear to accumulate in hepatopancreatic tissue as a result of waterborne exposure to copper, with no differences apparent between any of the treatments [ANOVA: $F_{(3,30)}$=0.082, p=0.970, Fig. 10]. However, accumulation of exogenous Cu in this tissue may have been masked by large elevations in hepatopancreatic Cu concentrations seemingly not due to waterborne Cu. All treatments, including those not exposed to Cu, showed much higher (5-fold) Cu concentrations when compared with the 24 h exposure (discussed below).

5. Discussion

Copper-induced haemocytic DNA damage after 14 d waterborne exposure but not significantly so after 24 h. This induction of DNA damage by copper appeared dose-dependent at lower concentrations (0-15 µg/L) but no further increases in DNA damage were apparent from 15-40 µg/L, possibly owing to the absence of apoptotic cells from the comet assay at this highest concentrations. Crabs were able to acid-base regulate under exposure to 1200 µatm CO_2 in all treatments with the exception of 40 µg/L where copper appeared to inhibit normal acid-base regulatory function. Accumulation of exogenous copper was not apparent in any of the tissues measured following the 24 h exposure but after 14 d gills appeared to be the primary tissue for accumulation. Increases in hepatopancreatic copper concentrations after 14 days coincided with reduction in haemolymph levels, and rather than treatment effect, were attributed to the breakdown of haemocyanin as a result of starvation. This changes in hepatopancreatic concentrations after 14 d, as well as high background haemolymph copper concentrations after both durations caused by the ubiquity of haemocyanin

limited the ability to assess the effects of exogenous copper in both hepatopancreas and haemolymph.

5.1 Twenty-four Hour Exposure

Exposure to copper for 24 h appeared to have no significant influence on the level of DNA damage induced in crab haemocytes. Interpretation of the data was made more difficult by high variance, potentially masking a trend towards higher DNA damage in those animals exposed to Cu under high CO_2 conditions. Such a trend would provide support for Cu bioavailability and toxicity being primarily determined by seawater chemistry (particularly pH). However without statistical support we cannot ascertain whether such a relationship is in effect or not. Crabs showed clear evidence of acid-base regulation following this 24 h exposure by the accumulation of extracellular HCO_3^- as a means of buffering their internal pH. This is in support of previous work by the authors (*Chapter 2*) on the same species at the same pCO_2, as well as work on other species by Pane and Barry (2007) at much higher pCO_2 (~10,000 µatm), suggesting that shore crabs are consistent in time-requirement and physiological strategy with respect to their acid-base regulatory strategy. Copper (20 µg/L) appeared to have no influence on the ability of the shore crabs to acid-base regulate under high CO_2 following 24 h exposure.

Haemolymph copper concentrations appeared unaffected by exposure to waterborne copper after 24 hours. Rtal and Truchot (1996) demonstrated that, at least under more severe copper exposure (2 mg/L), the haemolymph of shore crabs is subject to elevation as a result of exogenous copper. They suggest that haemolymph concentrations are determined by the rates of uptake offset against tissue sequestration and excretion. The lack of apparent increase in haemolymph copper under exposure in the present study may be a result of homeostatic regulation and/or our inability to detect small changes brought about by waterborne exposure against the relatively high background haemolymph [Cu] due to the abundance of haemocyanin, a Cu-based metalloprotein.

Gill accumulation was also not significantly affected after 24 hours. Although mean copper accumulation was markedly higher in the normocapnic copper treatment, variance was high in all treatments limiting our ability to draw strong conclusions from this observation. Given that neither haemolymph nor hepatopancreatic copper

concentrations appeared elevated in the normocapnic copper treatment, this may suggest that the basolateral membrane be differentially permeable to copper under normocapnic conditions compared with high CO_2: possibly due to altered copper speciation. The result would be a greater accumulation of copper in the gill under normocapnia. This is speculation and cannot be determined without further assessment, for example the use of radiolabelled ^{67}Cu (McCarter & Roch, 1984).

Unsurprisingly, there was no evidence of hepatopancreatic accumulation after 24 h. These data did however provide us with an important assessment of the effects of the experimental procedure itself on heptopancreatic concentrations, known for example to be affected by starvation (Djangmah, 1970; Watts et al., 2014).

5.2 Fourteen Day Exposure

DNA damage following 14 d exposure at varying Cu concentrations showed a markedly more complex pattern. Firstly, DNA damage appeared to increase with increasing Cu concentration from 0-15 µg/L Cu. This dose dependent response is somewhat unsurprisingly and is likely due to the induction of DNA strand breaks caused by ionic copper on entry into the haemocytes. Damage to biological molecules such as DNA occurs through the catalysed production of reactive oxygen species and free radicals by Cu^{2+} or Cu^{+} within the cell (Valko et al., 2005). Under 15 µg/L Cu DNA damage was greatly elevated when compared with the similar exposure concentration of 20 µg/L for just 24 h. This is likely a result of the longer exposure duration, and possibly a result of lowered antioxidant status associated with 14 d starvation (Dissanayake et al., 2008). It may also be a cumulative effect of cellular imbalances between DNA damage and repair over time.

Although DNA damage was still marginally higher than in controls, the dose-dependent trend towards increasing damage under increasing Cu severity was not apparent at 40 µg/L Cu. The reason for the discontinuity of this trend is as yet not understood. One explanation may be the induction of haemocyte apoptosis under the more severe Cu exposure regime. Given that 15 µg/L Cu induced very high levels of DNA damage (>40 %tDNA) it is reasonable to suggest that Cu exposure as high as 40 µg/L might be sufficient to induce apoptosis in a proportion of the haemocytes. Cells removed from the population as a result of apoptosis will reduce the effectiveness of comet assay as a sensitive marker for Cu toxicity. Apoptosis occurs

when cells are sufficiently damaged or stressed such that repair and defence mechanism are overwhelmed and the cell initiates a programmed cell death. Cells initiate apoptosis in order to safely remove themselves from the population where they might otherwise lead to pathogenic consequences. In the context of Cu exposure this is often associated with loss of cell membrane integrity brought about by lipid peroxidation (Gabbianelli et al., 2003) and the subsequent elevation of cytosolic Ca^{2+} (Marchi et al., 2004). For example, exposure of black tiger shrimp (*Penaeus monodon*) to 500 µg/L Cu for just 48 hours resulted in a significant elevation in haemocyte apoptosis (Xian et al., 2010). It is reasonable to suggest that longer term exposure to lower levels of copper may have a similar effect. In this instance the unknown proportion of the cells under the greatest stress are effectively being removed from the population, and any remaining apoptotic cells not subsequently destroyed during the lysis step of the comet assay appear as a distinctly separated head and tail and such cannot be scored. The result may be a biased appraisal of intracellular stress in the highest Cu concentration whereby only 'surviving' cells are subject to assessment of DNA damage.

Exposure to high CO_2 appears to have a marked ameliorating effect on DNA damage with a clear trend towards reduced damage in all high CO_2 treatments independent of Cu concentration. This relationship was statistically significant under 10 µg/L Cu, suggesting that the simultaneous exposure to high CO_2 may afford the haemocytes some protection from copper induced toxicity. Previous work by the authors (*Chapter 3*) provides clear *in vitro* evidence for the protective influence of HCO_3^- against Cu-induced DNA damage in sea bass erythrocytes, pointing to the likelihood of a similar role for HCO_3^- in the similar observed effect in crabs. Cells exposed to Cu in artificial media containing 12 mM HCO_3^- were completely protected from Cu-induced DNA damage when compared with significantly elevated damage in those exposed under 6 mM HCO_3^-. This suggests that acid-base regulating organisms in high CO_2 environments may have some passive protection against intracellular stress as a consequence of the HCO_3^- accumulated through their acid-base regulatory strategy. This might serve to explain the apparent protective effect observed under the combined 10 µg/L Cu and high CO_2 exposure as well as the reduced intracellular stress suggested by the trend towards lower DNA damage measured in all high CO_2

treatments. The mechanisms by which HCO_3^- might impart this protective influence on cells is discussed in detail in Chapter 3.

If such a protective effect were in action it is less starkly apparent in the higher copper concentrations (15 and 40 µg/L). In the highest Cu concentration this may be somewhat explained by discrepancies in the Cu exposure regime such that the Cu only treatment received an average of 33 µg/L and the high CO_2 + Cu treatment 47 µg/L. Treatments were maintained in the same manner and as such we are unable explain the disparity in seawater copper concentrations. Changes in speciation under high CO_2 could conceivably differentially alter adsorption to the polyurethane and Tygon surfaces within the experimental set-up and/or sedimentation out of solution in complexation with dissolved organic carbon.

However, changes to the animals acid-base status may provide a more obvious explanation for the apparent lack of protective effect under 40 µg/L Cu (i.e. bicarbonate accumulation was prevented at this high level of copper), but cannot account for the lack of a clear protective effect under 15 µg/L Cu.

Haemolymph pH and [HCO_3^-] showed a clear pattern of acid-base regulation in all treatments up to 20 µg/L (24 h) and 15 µg/L Cu (14 d). This is characterized by a maintenance of control pH in all treatments including those with elevated seawater pCO_2 whereby a compensatory elevation in [HCO_3^-] was in effect. In the high CO_2 + 40 µg/L Cu exposure however, the respiratory acidosis was not compensated, suggesting an inhibition of acid-base regulation by Cu. This has been reported in previous studies in fish and is generally attributed to the inhibition by Cu of carbonic anhydrase (CA; Zimmer et al., 2012): a vital enzyme for the hydration of CO_2 thought to be involved in producing the HCO_3^- for pH_e regulation (Claiborne et al., 1997). In this treatment the lack of significantly elevated HCO_3^- may explain the absence of an observable protective effect against DNA damage, and even if it were present it may only partially protect against the induction of apoptosis (Chapter 3) proposed to be occurring at this Cu concentration (discussed above). Similar inhibition of acid-base regulation has been reported previously in both fresh and seawater teleost fishes that employ largely the same acid-base regulatory strategy, albeit at higher Cu concentrations: Atlantic cod (*Gadus morhua*) at 400 µg/L Cu (Larsen et al., 1997), freshwater rainbow trout (*Oncorhynchus mykiss*) at 600 µg/L Cu (Wang et al., 1998),

European sea bass (*D. labrax*) at 60 µg/L Cu (Chapter 3). Under normocapnia, Cu did not appear to induce any perturbations in the acid-base status of the animals. Copper exposure is often associated with metabolic acidosis (Boitel & Truchot, 1990; Weeks et al., 1993) although typically at considerably higher Cu concentrations (>500 µg/L).

In the present study the gills of copper exposed crabs were greatly elevated when compared with those in control and high CO_2 treatments (23 to 28-fold higher). These data promote gills as the primary site of copper accumulation in crabs under waterborne exposure. This is supported by previous literature to consider the copper accumulation in this and related crab species (Martins et al., 2011; Weeks et al., 1993). Accumulation of copper in the gills can have profound effect on ion transport with consequent physiological detriment to osmotic and acid-base regulation in crabs (Thurberg et al., 1973; Weeks et al., 1993). Respiratory impairment has also been reported (Spicer & Weber, 1992). Osmoregulatory impairment during waterborne copper exposure is also frequently reported in fishes (Adeyemi et al., 2012; De Boeck et al., 2007; Hansen et al., 1993).

The hepatopancreatic tissue did not appear to be a major site of copper accumulation following either 24 h or 14 d exposure as has been reported in previous studies in the same species (Rtal & Truchot, 1996). Copper concentrations in the hepatopancreatic tissues of all crabs from the 14 d exposure were much higher than those post 24 h exposure. Higher concentrations in all treatments including those not exposed to Cu suggest these differences may be unrelated to duration or severity of Cu exposure. These results are more easily explained by the lack of food availability and the influence this has on both hepatosomatic index (HSI) and the copper content of the hepatopancreatic tissue. Under conditions of starvation crabs will increasingly utilize lipid stores from within the hepatopancreatic tissue. Although water content in this tissue increases during this process the resulting reduction in mass, and even more so in dry mass, of the hepatic tissue is in itself enough to amplify the relative concentration of Cu. Arguably of greater affect is the breakdown of haemocyanin when food is not available. This results in transfer of Cu from the haemolymph to the hepatopancreas (Djangmah, 1970; Watts et al., 2014). As such resulting elevations in hepatopancreatic copper concentrations due to starvation over the two week period appear to have significantly outweighed any measurable accumulation resulting from waterborne exposure to Cu. This idea is further supported by the average 30 %

reduction in haemolymph copper levels measured at 14 days when compared with animals assessed at 24 h.

5.3 Interpretation of Findings

The interpretation of the results of the present study is complicated by interactions between the two stressors and the mechanisms influencing their physiological impact. Specifically, copper (at high concentrations) appears to impair the ability of crabs to accumulate extracellular bicarbonate for acid-base regulation in response to high CO_2. This HCO_3^- accumulation in turn appears to have an important influence on the toxicity of internalised copper. While there is good evidence of a protective effect seemingly afforded to the cells during high CO_2 exposure to 10 µg/L Cu, evidence of such an effect is not visible at higher Cu concentrations. This is further complicated by the apparent inhibition of acid-base regulation at the highest Cu concentration. That said, all treatments including the high CO_2 control showed a trend towards reduced DNA damage under high CO_2. The similar protective effect found *in vitro* (Chapter 3), showing that a doubling of [HCO_3^-] protected sea bass erythrocytes from Cu-induced DNA damage. In the present study extracellular HCO_3^- was not elevated to such a degree (65 % in control) and less so with increasing Cu exposure concentration (50 % in high CO_2 + 15 µg/L Cu), possibly reducing the capacity for a protective influence. Other physiological considerations complicate interpretation of the data. One of these relates to nutritional state. If crabs had been fed, the acid-base status of their extracellular fluid would have been considerably effected by fluxes of acid-base variables necessary for digestion. Food and excreted faeces would also add dissolved organic matter (DOM) to seawater that would influence and complicate the copper-speciation and therefore exposure conditions. Secondarily, non-uniform consumption would result in variation of nutritional status with consequent bearing on a number of physiological parameters: not of least which might include oxidative stress and DNA damage, possibly through variations in anti-oxidant status (Dissanayake et al., 2008). As such starvation was a necessary part of the current experimental design.

However, starvation in itself would also likely effected anti-oxidant status although at least to a similar degree in all treatments. One side effect of this starvation was the considerable influence it had on Cu storage in the hepatopancreatic tissue, undermining attempts to quantify accumulation of the metal in this tissue under

waterborne Cu exposure. Furthermore, the non-haemocyanin Cu concentration of crab haemolymph proved difficult to measure without radiolabelling exogenous Cu, as per Martins et al. (2011). This was not possible within the remit of the present study and traditional methods resulted in a high copper background such that accurate determination of any small changes extracellular free Cu was not possible. Such information would provide context for interpretation of the DNA damage results. For example if Cu uptake across the gills was significantly higher under high CO_2 conditions, the findings of equal or marginally lower DNA damage in these treatments would be compelling evidence for some reduction of Cu toxicity after internalisation. This would lend further support to a protective mechanism afforded to the cells by HCO_3^-, as described in crabs in the present study and European sea bass *in vitro* (Chapter 3). Furthermore, rates of copper uptake could not be determined using only end-of-exposure snapshots of tissue accumulation. As such any differential bioavailability of waterborne Cu to the animals under varying pCO_2 and pH, as is predicted by models of speciation (Millero, 2009) and bioavailability (Chakoumakos et al., 1979) could not be elucidated. The finding that 40 µg/L Cu appears insufficient to induce a metabolic acidosis under normocapnia, as reported at higher concentrations (Boitel & Truchot, 1989), but was sufficient to inhibit the normal acid-base regulatory strategy of crabs is of considerable interest. This presents the first evidence of such an inhibitory effect in crabs under high CO_2. Moreover it is apparent at Cu concentrations an order of magnitude below similar effects reported in fish (Larsen et al., 1997; Wang et al., 1998). Inhibition of effective acid-base regulatory ability in these animals could have profound consequence for correct functioning of a number of physiologically important enzymes and reduced affinity of haemocyanin for O_2 as per the Bohr effect (Riggs, 1988).

In fact the precise mechanisms by which crabs accumulate HCO_3^- in order to buffer their extracellular pH against respiratory acidosis are not fully known (discussed in Chapter 2) and as such we can only hypothesise the way in which Cu may inhibit this process. Accumulation of HCO_3^- may be achieved via electroneutral exchange of HCO_3^- with Cl^- across the gill or may result from the CA catalysed hydration of endogenous CO_2 coupled with excretion of the resulting H^+ ions: as is proposed in models of acid-base regulation in fishes (Claiborne et al., 1997). Both may be affected by a Cu-induced structural damage to the gills (Nonnotte et al., 1993): the latter by inhibition of CA. Inhibition of CA mediated hydration of CO_2 has been reported in fish

(Christensen & Tucker, 1976; Vitale et al., 1999; Zimmer et al., 2012) suggesting potential for the same to occur in the crabs, thereby preventing accumulation of extracellular HCO_3. Regardless of the mechanism of inhibition, the inability of these Cu exposed animals to accumulate HCO_3^- presents a major physiological perturbation with detrimental consequences for the animal.

Evidence suggests that terrestrial crabs exposed to hypercapnia without the ion rich seawater with which to exchange acid-base relevant ions draw HCO_3^- from their exoskeleton in order to buffer their extracellular pH. It is conceivable that faced with chronic high CO_2 and a reduced ability (under Cu exposure) to accumulate compensatory HCO_3^- by other means, shore crabs may employ a similar mechanism: possibly at the expense of exoskeletal integrity, growth and normal molting cycles, (Boitel & Truchot, 1989; Weeks et al., 1993)

The implications of the present study are focussed around two seemingly contradictory and dose-dependent effects. Under high CO_2, such as is predicted under near-future climate change scenarios, crabs appear protected from the lowest Cu concentrations with respect to haemocyte DNA damage. This may be a consequence of a protective effect by the HCO_3^- they accumulate in order to buffer their extracellular fluid. The mechanism for such protection is currently unknown, but may include: reduction of capacity for cell entry through complexation, the protection of superoxide dismutase from inactivation, and the stabilization of iron-transferrin complexes that prevent intracellular oxidative damage (discussed in Chapter 3). Such a protective mechanism would not only serve to benefit acid-base regulating organisms under ocean acidification but those who currently experience hypercapnic conditions on a regular basis, such as in intertidal rock pools. This often occurs in combination with hypoxia which is also known to modulate the effects of toxic metals (Johnson, 1988). Coastal animals such as these are also the most likely marine species to encounter elevated levels of waterborne Cu as a result of human activity (industry, mining, domestic and marine applications). These findings have potentially far reaching implications for any aquatic organisms that are able to regulate their internal pH through the accumulation of HCO_3^- ions. The implication being that under future climate scenarios in which the oceans have an elevated pCO_2, these acid-base regulating organisms may be better protected against copper toxicity. The knock-on effect of this CO_2-derived protection

may be conformational shifts in the species distribution in any copper contaminated areas such as rivers, estuaries, harbours and coastal ecosystems.

With respect to energy allocation, the 'passive' cellular protection may at first appear to reduce energy requirement under Cu exposure, therefore resulting in an energy budget more conducive to investment in growth and reproduction (discussed in Chapters 3 and 5). However this is overlooking the energetic cost of chronic acid-base regulation which may present a significant energetic cost in itself. Short-term extracellular pH regulation in crabs may be important for the effective transport of oxygen via haemoglobin (Truchot, 1989), however the allocation of energy to acid-base regulatory mechanisms may have detrimental effects to other physiological processes or behaviours. For example, Appelhans et al. (2012) describes a 41 % reduction in the feeding behaviour of *C. maenas* under prolonged conditions (10 wks) of more extreme elevations in CO_2 (3500 µatm), however these effects were not present at concentrations comparable to the present study. Hammer et al. (2012) found changes in the metabolic profile of *C. maenas* following 4 weeks of exposure to varying degrees of elevated CO_2. These changes were largely attributed to reductions in important intracellular osmolytes such as amino acids, similar to recorded changes under hypo-osmotic stress. Acid-base status and ion regulation are inextricably linked through the exchange of acid-base equivalents (H^+/HCO_3^-) for osmolytes (Na^+/Cl^-) across the gill surface (Pörtner et al., 2004), giving rise to the potential for detrimental changes in the ionic composition of body fluids (Cameron, 1989).

Putting aside these potential costs and homeostatic disruptions, there may be more significant developmental limitations on the ability of acid-base regulatory mechanisms to provide increased copper tolerance. Even in more tolerant taxa, gametes, zygotes and early embryonic stages lack the specialized gill function that provides later developmental stages with the ion-transport mechanisms required to effectively acid-base regulate (Melzner et al., 2009). This would leave early developmental stages in future high CO_2 ocean scenarios just as vulnerable to copper toxicity as their modern day conspecifics, limiting the benefit to proliferation of this advantageous copper tolerance.

Despite these caveats, the result of such protective effect may be to create a clear differentiation in their ability to handle Cu exposure between acid-base regulators and those organisms not able to regulate their internal pH: 'acid-base conformers'. While acid-base regulators may become more tolerant to low level Cu exposure, acid-base

conformers such as mussels (*Mytilus edulis*) may be under greater stress due to the predicted increases in bioavailability (Millero, 2009). This assertion is supported by both increases in the uptake of cellular Cu uptake in hard clams (*Mercenaria mercenaria*; Ivanina et al., 2013) and mussels (*M. edulis*; Hans et al., 2014), as well as increases Cu-induced DNA damage in mussels (*M. edulis*; *Lewis, 2015, submitted*) under high CO_2.

That said, the present study also demonstrates the detrimental effect of Cu on the normal homeostatic strategy of acid-base regulators. Current findings suggest Cu exposure as low as 40 µg/L may be sufficient to disrupt acid-base regulation with a suite of potential downstream effects on normal physiological functioning. As such Cu at and above this concentration presents a contaminant of serious concern for shore crabs in the context of hypercapnic environments, both in their current ecological niche and under future ocean acidification scenarios.

The acclamatory responses of these animals should also be assessed. Long-term pre-exposure of shore crabs to sublethal concentrations of Cu has been shown to reduce the severity of toxic effects upon re-exposure (Truchot & Rtal, 1998). This response may be linked to reductions in the uptake of waterborne Cu across the gill and changes to the efficiency of Cu transport from the haemolymph to other tissues. Equally, evidence suggests that the commonly reported metabolic acidosis induced in shore crabs by Cu is eventually reversed under prolonged exposure (>20 d) (Boitel & Truchot, 1989). This literature suggests that the duration of the present study is insufficient to provide such acclamatory benefits. Assessment of how such chronic exposure mediates Cu uptake and toxicity, particularly in combination with stressors such as high CO_2, is vital in understanding longer term tolerance of this animal to Cu.

5.5 Future Work

Some of the key directions for this area of research were discussed previously in Chapter 3. In summary, elucidation of the potential protective mechanism described in the present study might focus around characterising the role of HCO_3^- in preventing DNA damage or other intracellular cellular stress. Such work would look to employ alternative biomarkers for intracellular stress and might consider whether similar protective mechanism are in effect in tissues with higher a copper burden (such as the gills or hepatopancreas). This work might consider employing more

severe hypercapnic conditions, or direct injection into the circulatory system, to further elevate extracellular HCO_3^- concentrations, thereby exaggerating the potential for a protective effect.

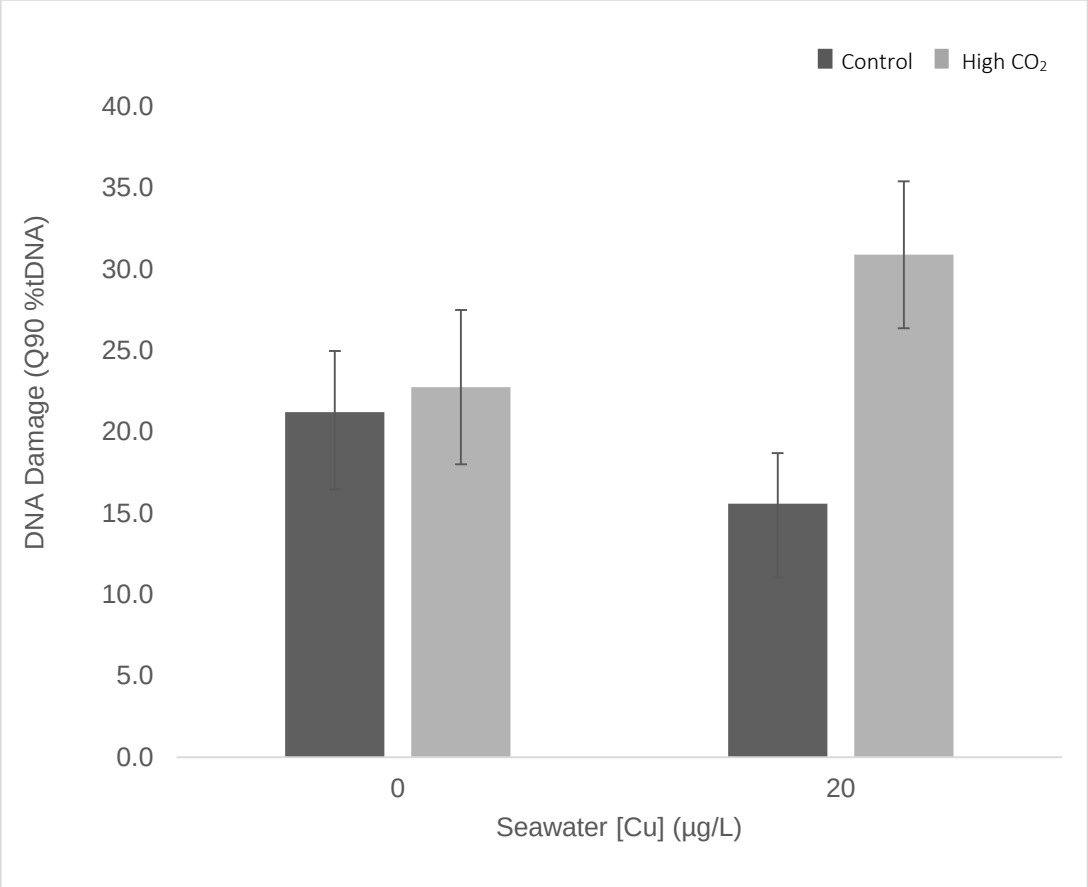
With respect to the inhibition of acid-base regulation, further research should aim to assess the mechanism by which Cu inhibits acid-base regulation in these animals. This would be made easier with a more precise understanding of the how crabs accumulate extracellular HCO_3^-: endogenously, exogenously or both? Better understanding of how acid-base regulatory mechanisms are effected by Cu and vice versa may enable broader application of these findings, and similar findings in fishes, to a wider range of marine biota. For example where acid-base regulatory strategies are similar or conserved, the potential may exist for similar Cu-induced dysfunction (or protection). As a result better predictions may be possible as to the susceptibility of certain animals to high CO_2 in the context of background Cu contamination, and equally Cu tolerance in the context of a high CO_2 environment. Such information is vital for the modelling of future population effects of combined stressors as well as informing policy on the influx of chemical stressors via human activity. Although shore crabs are not of economic values they serve as model species from which we can make inferences about the responses of physiologically similar decapod crustaceans of considerable value to European and global fisheries: most notably lobsters, prawns and edible crabs.

More generally, with respect to the future impacts of ocean acidification on marine organisms, less research has been conducted on those aquatic organisms thought to be more tolerant to elevations in environmental pCO_2, such as active metazoans with effective acid-base regulatory systems: teleosts, crustaceans and many brachyurans. Melzner et al. (2009) rightly suggests that more comparative work between vulnerable and tolerant species will provide a better understanding of the physiological traits that will determine the winners and losers of future high CO_2 oceans.

6. Figures

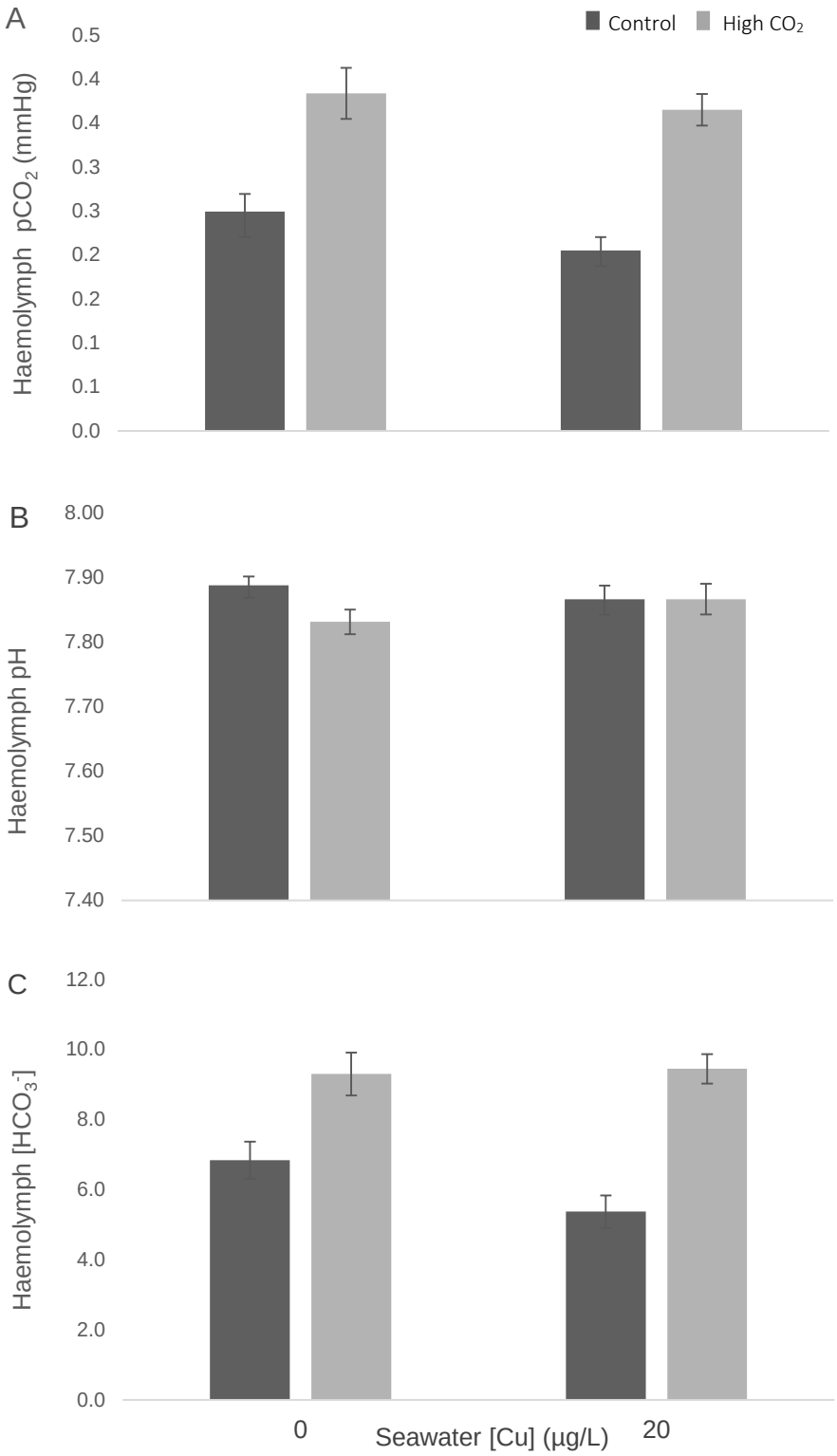
6.1 Twenty-four Hour Experiment

6.1.2 DNA Damage



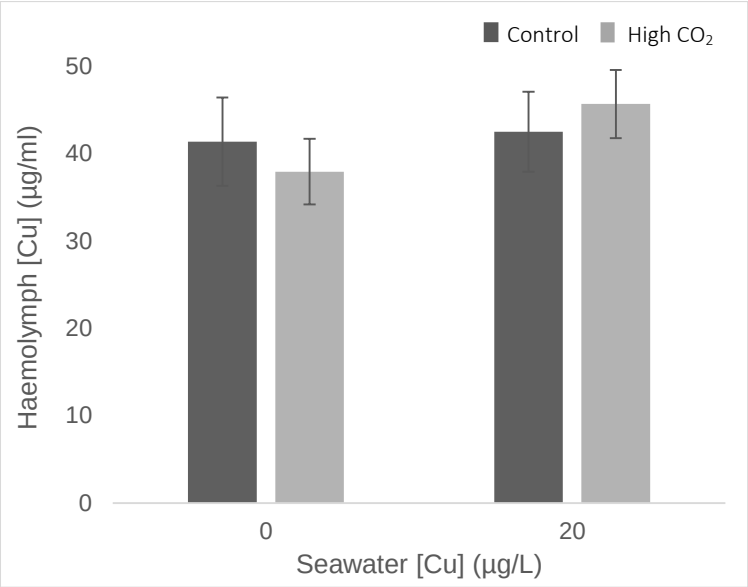
Chapter 4 Figure 19: DNA damage in haemocytes of shore crabs (*C. maenas*) following 24 hour *in vivo* exposure, as measured by comet assay. Values represent treatment means for Q90 of log transformed percentage tail intensity (log%tDNA) ± SEM. N=10.

6.1.3 Acid-base Status

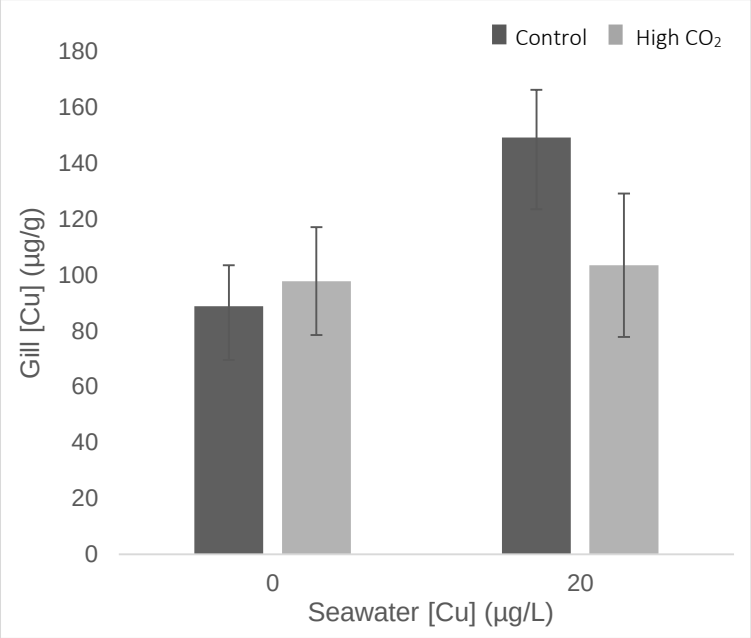


Chapter 4 Figure 20: Acid-base parameters (pCO_2 (A), pH (B) and HCO_3^- (C)) of haemolymph extracted from shore crabs (*C. maenas*) following 24 hour *in vivo* exposure. Values represent Mean ± SEM. N=9, 8,10,10. Significant difference is represented by an *asterisk*.

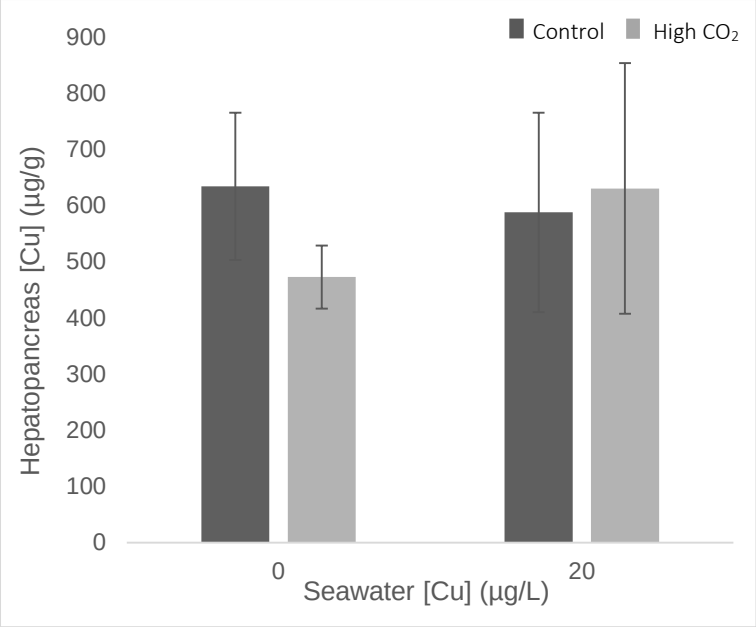
6.1.4 Tissue Concentrations



Chapter 4 Figure 21: Total Cu concentrations measured in haemolymph of shore crabs (*C. maenas*) following 24 h exposure. Values represent Mean ± SEM. N=10.



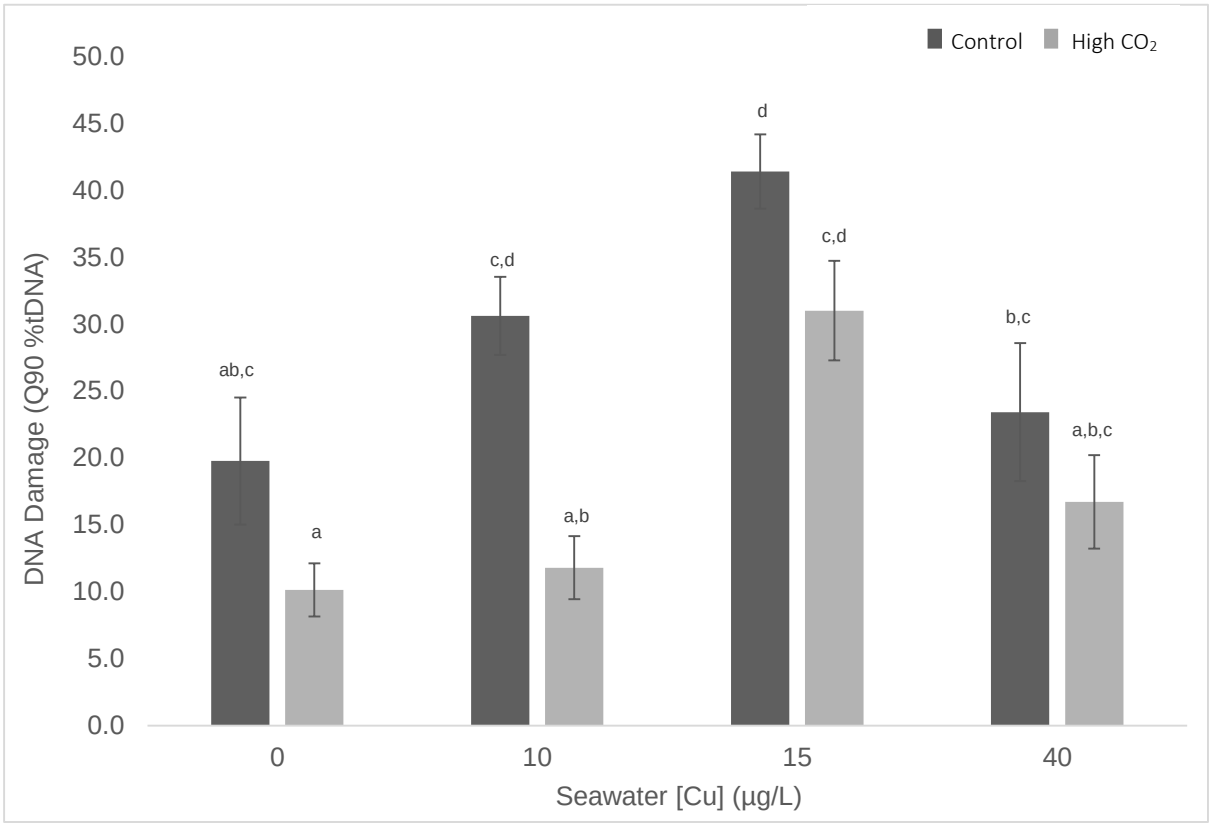
Chapter 4 Figure 22: Total Cu concentrations measured in gill tissue of shore crabs (*C. maenas*) following 24 h exposure. Values represent Mean ± SEM. N=10.



Chapter 4 Figure 23: Total Cu concentrations measured in hepatopancreatic tissue of shore crabs (*C. maenas*) following 24 h exposure. Values represent Mean ± SEM. N=9,9,9,10.

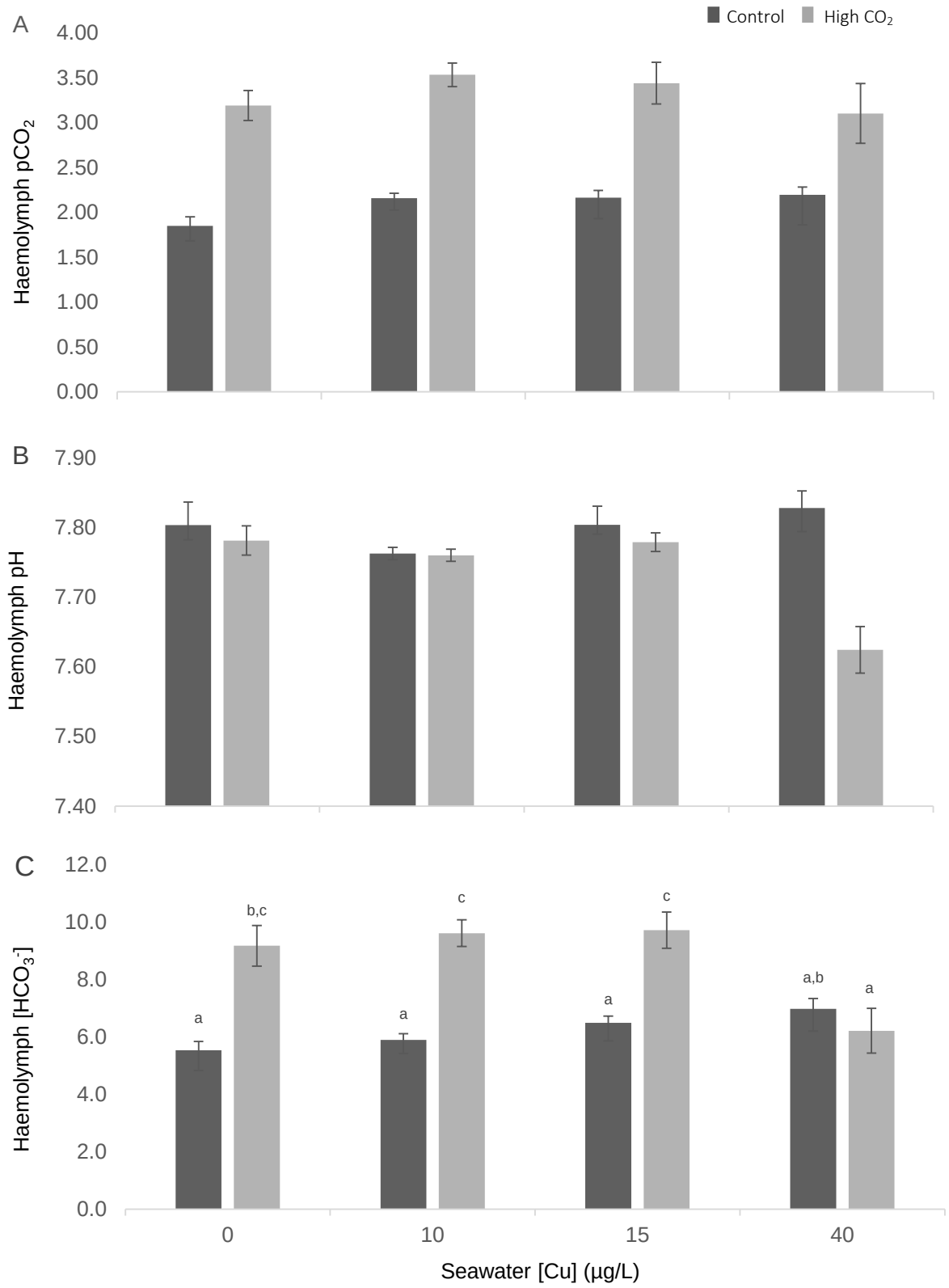
6.2 Fourteen Day Exposure

6.2.1 DNA Damage



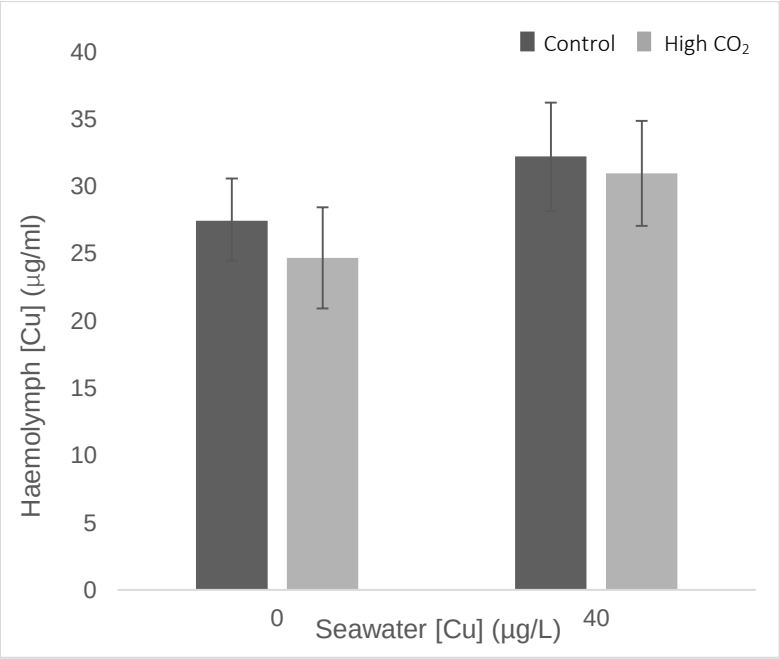
Chapter 4 Figure 24: DNA damage in haemocytes of shore crabs (*C. maenas*) following 14 d *in vivo* exposure, as measured by comet assay. Values represent treatment means for Q90 of log transformed percentage tail intensity (log%tDNA) ± SEM. N=8,7,9,7,7,9,8,9.

6.2.2 Acid-base Status

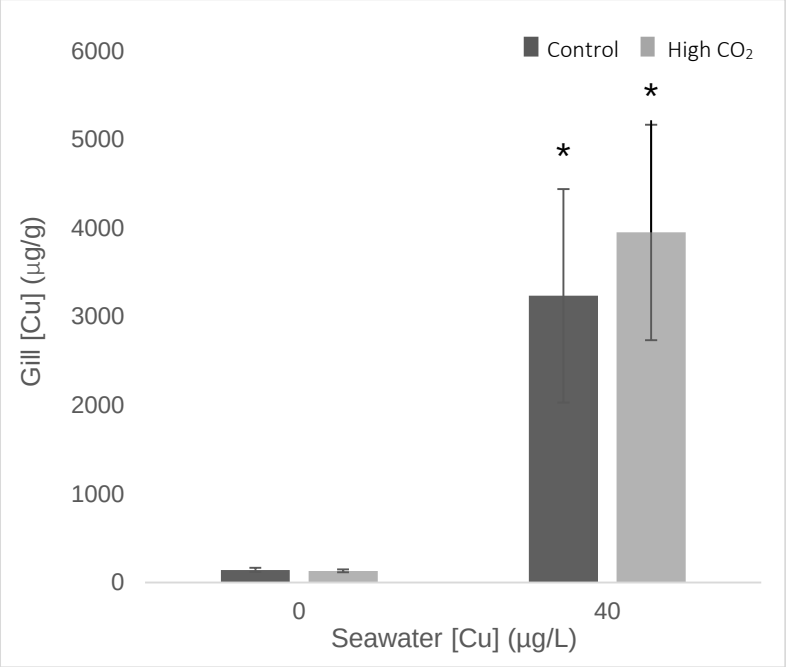


Chapter 4 Figure 25: Acid-base parameters (pCO_2 (A), pH (B) and HCO_3^-(C)) of haemolymph extracted from shore crabs (*C. maenas*) following 14 day *in vivo* exposure. Values represent Mean ± SEM. N=8, 7,9,7,7,9,8,9.

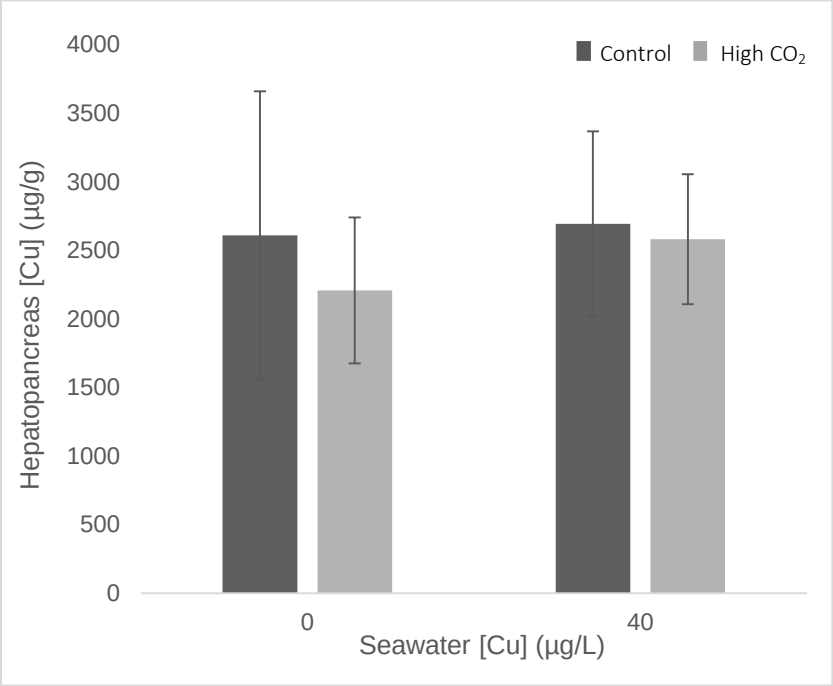
6.2.3 Tissue Concentrations



Chapter 4 Figure 26: Total Cu concentrations measured in haemolymph of shore crabs (*C. maenas*) following 14 d exposure. Values represent Mean ± SEM. N=8,8,9,9.



Chapter 4 Figure 27: Total Cu concentrations measured in gill tissue of shore crabs (*C. maenas*) following 14 d exposure. Values represent Mean ± SEM. N=8,8,9,8. Significant difference is denoted by an *asterisk* (ANOVA $p<0.05$)



Chapter 4 Figure 28: Total Cu concentrations measured in hepatopancreatic tissue of shore crabs (*C. maenas*) following 14 d exposure. Values represent Mean ± SEM. N=9,9,9,10.

7. Appendices

Chapter 4 Table 1: Seawater carbonate chemistry and copper concentrations in four experimental treatments under which shore crabs (*C. maenas*) were held during the 24 h exposure. Values represent Mean ± SEM. † calculated using CO2sys (Pierrot, 2006)

Treatment	pH_{NBS}	Copper (µg/L)	Temperature (°C)	Salinity	TA (µmol/kg)†	TCO_2 (µmol/kg)	pCO_2 (µatm)†	HCO_3^- (µmol/kg)†	CO_3^{2-} (µmol/kg)†	ΩCa†	ΩAr†
Control	8.10 (±0.01)	0.37 (±0.29)	15.4 (±0.05)	34.4 (±0.04)	1959.0 (±4.3)	1789.6 (±5.5	405.2 (±9.0)	1656.1 (±6.6)	118.5 (±2.1)	2.8 (±0.05)	1.8 (±0.03)
High CO_2	7.66 (±0.02)	0.12 (±0.09)	15.3 (±0.03)	34.4 (±0.02)	1952.3 (±4.2)	1923.2 (±8.1)	1231.2 (±74.8)	1829.7 (±7.8)	48.0 (±2.5)	1.1 (±0.06)	0.7 (±0.04)
Copper	8.13 (±0.01)	19.71 (±0.28)	15.4 (±0.02)	34.4 (±0.02)	2142.1 (±4.8)	1948.5 (±4.2)	407.4 (±7.60)	1794.9 (±4.8	138.5 (±2.0)	3.3 (±0.05)	2.1 (±0.03)
High CO_2 + Cu	7.71 (±0.01)	19.03 (±0.42)	15.4 (±0.02)	34.4 (±0.04)	2245.0 (±5.00	2200.3 (±4.2)	1249.9 (±30.4)	2092.7 (±4.0)	61.4 (±1.4)	1.5 (±0.03)	0.9 (±0.02)

Chapter 4 Table 2: Seawater carbonate chemistry and copper concentrations in eight experimental treatments under which shore crabs (*C. maenas*) were held during the 14 day exposure. Values represent Mean ± SEM. † calculated using CO2sys (Pierrot, 2006).

Treatment	pH_{NBS}	Copper (µg/L)	Temp.(°C)	Salinity	TA (µmol/kg)†	TCO_2 (µmol/kg)	pCO_2 (µatm)†	HCO_3^- (µmol/kg)†	CO_3^{2-} (µmol/kg)†	ΩCa†	ΩAr†
Control	8.09 (±0.07)	0.47 (±0.18)	15.2 (±037)	34.8 (±0.23)	2253.6 (±427.4	2067.7 (±395.0)	468.8 (±101.3)	1913.5 (±360.9)	136.8 (±37.2	3.2 (±0.89)	2.1 (±0.57)
High CO_2	7.72 (±0.07)	0.92 (±0.19)	15.2 (±0.40)	34.9 (±0.20)	2243.4 (±472.2)	2195.2 (±458.6)	1216.9 (±250.4)	2087.2 (±434.8)	62.8 (±19.7)	1.5 (±0.47)	1.0 (±0.30)
10 µg/L Cu	8.13 (±0.11)	10.69 (±1.56)	15.0 (±0.56)	35.0 (±0.10)	2353.1 (±223.1)	2146.7 (±168.1)	449.6 (±90.5)	1977.7 (±132.1)	152.2 (±43.4)	3.6 (±1.04)	2.3 (±0.66)
High CO_2 + 10 µg/L Cu	7.68 (±0.07)	10.90 (±1.10)	15.0 (±0.38)	34.9 (±0.20)	2071.4 (±291.5)	2034.9 (±274.4)	1217.6 (±167.1)	1935.8 (±259.5)	53.6 (±15.6)	1.3 (±0.37)	0.8 (±0.24)
15 µg/L Cu	8.14 (±0.07)	14.59 (±1.55)	15.0 (±0.30)	34.9 (±0.39)	2393.9 (±220.7)	2178.5 (±211.9	440.5 (±91.9)	2003.1 (±199.8)	159.0 (±22.9)	3.89 (±0.55)	2.4 (±0.35)
High CO_2 + 15 µg/L Cu	7.68 (±0.07)	14.22 (±2.23)	15.2 (±0.26)	35.1 (±0.20)	2123.5 (±264.7)	2086.5 (±258.7)	1257.6 (±233.3)	1984.6 (±245.7)	55.2 (±12.3)	1.3 (±0.29)	0.8 (±0.19)
40 µg/L Cu	8.11 (±0.08)	33.8 (±2.93)	15.2 (±0.33)	34.9 (±0.24)	2279.3 (±506.6)	2086.1 (±458.2)	459.1 (±90.9)	1927.0 (±412.2)	142.1 (±48.6)	3.4 (±1.16)	2.2 (±0.75)
High CO_2 + 40 µg/L Cu	7.72 (±0.07)	47.1 (±2.34)	15.2 (±0.44)	34.9 (±0.23)	2259.7 (±456.3)	2211.0 (±444.0)	1223.8 (±240.9)	2102.2 (±421.1)	63.4 (±18.7)	1.5 (±0.45)	1.0 (±0.29)

8. References

Adeyemi, J. A., Deaton, L. E., Pesacreta, T. C., & Klerks, P. L. (2012). Effects of copper on osmoregulation in sheepshead minnow (*Cyprinodon variegatus*) acclimated to different salinities. *Aquatic Toxicology, 109*(0), 111-117.

Appelhans, Y., Thomsen, J., Pansch, C., Melzner, F., & Wahl, M. (2012). Sour times: Seawater acidification effects on growth, feeding behaviour and acid–base status of *Asterias rubens* and *Carcinus maenas*. *Marine Ecology Progress Series, 459*, 85-98.

Boitel, F., & Truchot, J. (1989). Effects of sublethal and lethal copper levels on haemolymph acid-base balance and ion concentrations in the shore crab (*Carcinus maenas*) kept in undiluted sea water. *Marine Biology, 103*(4), 495-501.

Boitel, F., & Truchot, J.-P. (1990). Comparative study of the effects of copper on haemolymph ion concentrations and acid-base balance in shore crabs (*Carcinus maenas*) acclimated to full-strength or dilute seawater. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 95*(2), 307-312.

Cameron, J. N., & Iwama, G. K. (1989). Compromises between ionic regulation and acid-base regulation in aquatic animals. *Canadian Journal of Zoology, 67*(12), 3078-3084.

Chakoumakos, C., Russo, R. C., & Thurston, R. V. (1979). Toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH, and hardness. *Environmental Science & Technology, 13*(2), 213-219.

Christensen, G., & Tucker, J. (1976). Effects of selected water toxicants on the *in vitro* activity of fish carbonic anhydrase. *Chemico-Biological Interactions, 13*(2), 181-192.

Claiborne, J. B. (1998). *The Physiology of Fishes* (D. H. Evans Ed.): CRC press.

Claiborne, J. B., Perry, E., Bellows, S., & Campbell, J. (1997). Mechanisms of acid-base excretion across the gills of a marine fish. *Journal of Experimental Zoology, 279*, 509-520.

De Boeck, G., Hattink, J., Franklin, N. M., Bucking, C. P., Wood, S., Walsh, P. J., & Wood, C. M. (2007). Copper toxicity in the spiny dogfish (*Squalus acanthias*): Urea

loss contributes to the osmoregulatory disturbance. *Aquatic Toxicology, 84*(2), 133-141.

Dissanayake, A., Galloway, T. S., & Jones, M. B. (2008). Nutritional status of *Carcinus maenas* (Crustacea: Decapoda) influences susceptibility to contaminant exposure. *Aquatic Toxicology, 89*(1), 40-46.

Djangmah, J. S. (1970). The effects of feeding and starvation on copper in the blood and hepatopancreas, and on blood proteins of *Crangon vulgaris* (*fabricius*). *Comparative Biochemistry and Physiology, 32*(4), 709-IN708.

Fehsenfeld, S., & Weihrauch, D. (2013). Differential acid–base regulation in various gills of the green crab (*Carcinus maenas*): Effects of elevated environmental pCO_2. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 164*(1), 54-65.

Friederich, G., Walz, P., Burczynski, M., & Chavez, F. (2002). Inorganic carbon in the central California upwelling system during the 1997–1999 El Nino–La Nina event. *Progress in Oceanography, 54*(1), 185-203.

Gabbianelli, R., Lupidi, G., Villarini, M., & Falcioni, G. (2003). DNA damage induced by copper on erythrocytes of gilthead sea bream (*Sparus aurata*) and mollusk (*Scapharca inaequivalvis*). *Archives of Environmental Contamination and Toxicology, 45*(3), 350-356.

Hammer, K. M., Pedersen, S. A., & Størseth, T. R. (2012). Elevated seawater levels of CO_2 change the metabolic fingerprint of tissues and hemolymph from the green shore crab (*Carcinus maenas*). *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*.

Hans, S., Fehsenfeld, S., Treberg, J. R., & Weihrauch, D. (2014). Acid–base regulation in the Dungeness crab (*Metacarcinus magister*). *Marine Biology, 161*(5), 1179-1193.

Hansen, H. J. M., Olsen, A. G., & Rosenkilde, P. (1993). The effect of Cu^{2+} on osmoregulation in rainbow trout (*Oncorhynchus mykiss*) assayed by changes in plasma salinity and gill lipid metabolism. *Science of The Total Environment, 134, Supplement 2*(0), 899-905.

Ivanina, A. V., Beniash, E., Etzkorn, M., Meyers, T. B., Ringwood, A. H., & Sokolova, I. M. (2013). Short-term acute hypercapnia affects cellular responses to trace metals in the hard clams (*Mercenaria mercenaria*). *Aquatic Toxicology, 140*, 123-133.

Johnson, I. (1988). The effects of combinations of heavy metals, hypoxia and salinity on ion regulation in *Crangon crangon* (L.) and *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 91*(2), 459-463.

Lallier, F., & Truchot, J.-P. (1989). Haemolymph oxygen transport during environmental hypoxia in the shore crab, *Carcinus maenas*. *Respiration Physiology, 77*(3), 323-336.

Larsen, B. K., Pörtner, H. O., & Jensen, F. B. (1997). Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology, 128*(2), 337-346.

Lovell, D. P., & Omori, T. (2008). Statistical issues in the use of the comet assay. *Mutagenesis, 23*(3), 171-182.

Marchi, B., Burlando, B., Moore, M. N., & Viarengo, A. (2004). Mercury- and copper-induced lysosomal membrane destabilisation depends on $[Ca^{2+}]_i$ dependent phospholipase A2 activation. *Aquatic Toxicology, 66*(2), 197-204.

Martins, C. D. M. G., Barcarolli, I. F., de Menezes, E. J., Giacomin, M. M., Wood, C. M., & Bianchini, A. (2011). Acute toxicity, accumulation and tissue distribution of copper in the blue crab (*Callinectes sapidus*) acclimated to different salinities: *In vivo* and *in vitro* studies. *Aquatic Toxicology, 101*(1), 88-99.

McCarter, J. A., & Roch, M. (1984). Chronic exposure of coho salmon to sublethal concentrations of copper—III. Kinetics of metabolism of metallothionein. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 77*(1), 83-87.

Melzner, F., Gutowska, M., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M. C., Pörtner, H.-O. (2009). Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences, 6*, 2313-2331.

Millero, F., Santana-Casiano, J., & González-Dávila, M. (2010). The formation of Cu^{2+} complexes with carbonate and bicarbonate ions in $NaClO_4$ solutions. *Journal of Solution Chemistry, 39*(4), 543-558.

Millero, F. J. (2009). Effect of ocean acidification on the speciation of metals in seawater. *Oceanography, 22*(4), 72.

Nonnotte, L., Boitel, F., & Truchot, J.-P. (1993). Waterborne copper causes gill damage and hemolymph hypoxia in the shore crab (*Carcinus maenas*). *Canadian Journal of Zoology, 71*(8), 1569-1576.

Pane, E. F., & Barry, J. P. (2007). Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. *Marine Ecology Progress Series, 334*, 1-9.

Pierrot, D. E., Wallace, D.W.R. (2006). MS Excel program developed for CO_2 system calculations. *ORNL/CDIAC-105a*.

Pörtner, H., Langenbuch, M., & Reipschläger, A. (2004). Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. *Journal of Oceanography, 60*(4), 705-718.

Riggs, A. F. (1988). The Bohr effect. *Annual review of physiology, 50*(1), 181-204.

Rtal, A., & Truchot, J.-P. (1996). Haemolymph transport and tissue accumulation of exogenous copper in the shore crab, *Carcinus maenas*. *Marine Pollution Bulletin, 32*(11), 802-811.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low-levels of DNA damage in individual cells. *Experimental Cell Research, 175*(1), 184-191.

Söderhäll, K., & Smith, V. J. (1983). Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution. *Developmental & Comparative Immunology, 7*(2), 229-239.

Spicer, J. I., & Weber, R. E. (1992). Respiratory impairment by water-borne copper and zinc in the edible crab (*Cancer pagurus*) (L.) (Crustacea: Decapoda) during hypoxic exposure. *Marine Biology, 112*(3), 429-435.

Thurberg, F., Dawson, M., & Collier, R. (1973). Effects of copper and cadmium on osmoregulation and oxygen consumption in two species of estuarine crabs. *Marine Biology, 23*(3), 171-175.

Tomanek, L., Zuzow, M. J., Ivanina, A. V., Beniash, E., & Sokolova, I. M. (2011). Proteomic response to elevated pCO_2 level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. *The Journal of Experimental Biology, 214*(11), 1836-1844.

Truchot, J.-P. (1986). Changes in the haemolymph acid-base state of the shore crab, *Carcinus maenas*, exposed to simulated tidepool conditions. *The Biological Bulletin, 170*(3), 506-518.

Truchot, J.-P. (1976). Carbon dioxide combining properties of the blood of the shore crab (*Carcinus maenas*) (L): carbon dioxide solubility coefficient and carbonic acid dissociation constants. *The Journal of Experimental Biology, 64*(1), 45-57.

Truchot, J.-P., & Rtal, A. (1998). Effects of long-term sublethal exposure to copper on subsequent uptake and distribution of metal in the shore crab (*Carcinus maenas*). *Journal of Crustacean Biology*, 224-231.

Valko, M., Morris, H., & Cronin, M. (2005). Metals, toxicity and oxidative stress. *Current medicinal chemistry, 12*(10), 1161-1208.

Vitale, A., Monserrat, J., Castilho, P., & Rodriguez, E. (1999). Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 122*(1), 121-129.

Wang, T., Knudsen, P., Brauner, C., Busk, M., Vijayan, M., & Jensen, F. (1998). Copper exposure impairs intra-and extracellular acid-base regulation during hypercapnia in the fresh water rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B, 168*(8), 591-599.

Watts, A. J., McGill, R. A., Albalat, A., & Neil, D. M. (2014). Biophysical and biochemical changes occur in *Nephrops norvegicus* during starvation. *Journal of Experimental Marine Biology and Ecology, 457*, 81-89.

Weeks, J. M., Jensen, F. B., & Depledge, M. H. (1993). Acid-base status, haemolymph composition and tissue copper accumulation in the shore crab (*Carcinus maenas*) exposed to combined copper and salinity stress. *Marine ecology progress series. Oldendorf, 97*(1), 91-98.

Xian, J.A., Wang, A.L., Ye, C.X., Chen, X.D., & Wang, W.N. (2010). Phagocytic activity, respiratory burst, cytoplasmic free-Ca^{2+} concentration and apoptotic cell ratio of haemocytes from the black tiger shrimp, *Penaeus monodon* under acute copper stress. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 152*(2), 182-188.

Zimmer, A. M., Barcarolli, I. F., Wood, C. M., & Bianchini, A. (2012). Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. *Aquatic Toxicology, 122–123*(0), 172-180.

CHAPTER 5

The combined effects of high CO_2 and copper on juvenile sea bass (*Dicentrarchus labrax*): feeding, growth, tissue metal accumulation, and calcium carbonate production

CHAPTER 5

THE COMBINED EFFECTS OF HIGH CO_2 AND COPPER ON JUVENILE SEA BASS (*DICENTRARCHUS LABRAX*): FEEDING, GROWTH, TISSUE METAL ACCUMULATION, AND CALCIUM CARBONATE PRODUCTION

1. Abstract

Anthropogenically released carbon dioxide from the atmosphere is dissolving in the oceans elevating partial pressure of CO_2 (pCO_2), resulting in a concomitant reduction in pH, termed ocean acidification. The homeostatic mechanism employed to regulate internal chemistry when challenged with these external changes may incur an energetic cost to teleost fishes. Ocean acidification therefore not only has the potential to impact growth in fishes directly but may alter the behaviour of chemical stressors in seawater with negative effects predicted due to increased bioavailability at lower pH. Copper is a ubiquitous marine toxicant that enters water systems both directly through its use as an aquatic biocide, and indirectly as a consequence of its vast and proliferating number of industrial and technological applications. Even at sublethal exposure copper has profound effects on fish physiology and behaviour. Of major concern to both wild and farmed populations is the impact such a stressor may have on growth and food conversion efficiency (FCE), particularly in the face of near-future climate-induced changes to water chemistry.

Here the extent to which copper and high CO_2 exposure affects growth and FCE in juvenile sea bass (*Dicentrarchus labrax*) are considered. Furthermore the effects of these combined stressors on calcium carbonate production are considered and provide some of the first evidence linking food consumption and carbonate production rates.

We exposed juvenile sea bass to copper (50 µg/L) under control (540 µatm) and/or high CO_2 (1150 µatm) for 28 days during which food consumption and carbonate excretion were monitored. Specific growth rate (SGR) and food conversion efficiency were unaffected by both copper and high CO_2 treatments, both separately and in combination. Regression analysis suggested that food consumption was a strong

determinant in carbonate production rate, presumably owing to the extra supply of calcium to the gut via the diet.

Consistent with many previous studies copper was found to accumulate in the livers of copper exposed animals and was associated with higher hepatosomatic indices and total liver copper burden as a percentage of body mass. Plasma copper concentrations appeared unaffected by exposure to waterborne copper and muscle did not appear to be a major site of copper accumulation with no treatments exceeding those concentrations found in control animals. Irrespective of copper exposure, fish held under elevated pCO_2 conditions averaged 17 % higher plasma [Cu] and 45 % lower muscle [Cu], compared to control fish. This may arise from CO_2-induced changes to copper homeostasis mechanisms with respect to relative tissue burden.

2. Introduction

2.1 Ocean acidification

Research has shown that ocean acidification, characterized by elevated water pCO_2 and lowered pH, can affect a range of physiological processes in marine organisms including protein biosynthesis (Franke, 2011), growth (Michaelidis 2005, Langdon & Atkinson 2005), feeding (Fernandez-Reiriz et al., 2011), reproduction (Fitzer et al., 2012), immune function (Bibby et al., 2008) and olfactory discrimination (Munday et al., 2009). However, defining the scale of effect is difficult as sensitivity to OA varies with species (Kroeker et al., 2010, Hendriks et al., 2010, Ries et al., 2009). Chronic hypercapnia can reduce growth rates in early life stage marine teleosts (Baumann, et al., 2011). These effects are as yet without clear physiological explanation but may be a result of increased metabolic load: possibly associated with the energetic cost of acid-base and osmo- regulation. Current research, including previous work conducted by the authors (see Chapter 2), indicates that many marine organisms including European sea bass are capable of rapidly compensating for the effects of elevated water on blood acid-base status (Chapter 2). This primarily involves acid-base regulation with subsequent effects on ion regulation. Compensatory responses may come at increased energetic cost with potential downstream effects on processes like growth and reproduction. Adverse effects on such parameters would transfer directly

to the population level, influencing both fishery models and stock management (Le Quesne, 2012).

2.2 Copper

Both the nutritive and toxicological properties of copper with respect to marine animals have been discussed at length in previous chapters. The focus of this chapter is the potential for copper and high CO_2 to impact growth rates in a commercially important marine fish species, European sea bass. Exposure to copper has been found previously to retard growth in teleost fish (Buckley et al., 1982; Parveen & Javed, 2010), even at concentrations as low as 20 % of the 96 h LC_{50} (Hansen et al., 2002). Given the previously discussed potential for future changes to seawater carbonate chemistry to increase the proportion of harmful copper in the environment, there exists clear scope for ocean acidification to exacerbate the effects of copper on fish growth. Copper is therefore an environmentally relevant model contaminant for assessing the combined impacts of ocean acidification and chemical stressors on fish growth.

As the primary influxes of copper to the marine environment are from terrestrial sources, it is prudent to study marine species that inhabit coastal or estuarine habitats during their life cycle. European sea bass (*Dicentrarchus labrax*) inhabit coastal and estuarine waters during their juvenile stage. They also represent and a species of considerable commercial value. Commercial landings are in excess of £4.5 million and recreational fishing of this species contributes around £100 million per annum to the UK economy alone. However, these values pale in comparison to global revenue from sea bass farms (particularly in the Mediterranean) where production exceeds 160,000 tonnes per year and equates to £500 million, without recreational contribution (FAO, 2015). However wild sea bass populations are currently under considerable threat due to over-fishing and populations are in decline. Over the last few years the International Council for the Exploration of the Sea (ICES) have recommended increasingly stringent changes to fishing practices and the UK government has formally requested the European Commission take emergency measures to protect stocks. In response a ban on pelagic trawling during the spawning season of was introduced this year in addition to a daily catch limit on sports fishermen, and a maximum monthly catch for gear type trawls (Hirst, 2015).

When considering the combined effects of copper and hypercapnia it must be recognised that high CO_2/ low pH exposure is not limited to natural water systems any more than it is limited to future climate change scenarios. Recirculating aquaculture facilities (RAS) represent an often overlooked environment in which fish are exposed to hypercapnic conditions. The increasing popularity of sea bass over the last three decades has seen a considerable proliferation in the number of aquaculture facilities (particularly in Europe). Wherever there exists high stocking density and low water turnover, the accumulation of metabolic products such as CO_2 (Ishimatsu et al., 2005), can lead to hypercapnic conditions of varying severity (Crocker, 1996): 10-40 times higher than typical marine environments (Moran, 2011). Even open water cage systems vary greatly with respect to water turnover. This may be particularly low in sheltered bays or areas where currents and tides have only a small appreciable effect. Potential for such high pCO_2 in combination with industrial run-off and the wide and proliferating use of copper based chemicals and materials as biocides, present realistic scenarios for these two stressors to be exerting negative effects on farmed animals now, as well as the aforementioned effects on wild populations under ocean acidification in the future.

2.3 Study Focus

In this study physiological endpoints are assessed that are pertinent to informing climate change models and considering the potential for high CO_2 and copper to impact commercially-relevant indices. These endpoints include feeding, growth and feed conversion efficiency (FCE).), as well as the uptake of metal into tissues relevant to human consumption (i.e. muscle). In addition, the production/excretion of calcium carbonate via the gut was measured, which has implications to understanding surface ocean carbonate chemistry and its response to change models. Feed conversion efficiency is a measure of how efficiently animals convert ingested food into changes in body mass: expressed in this paper as body mass gain (g) per feed consumed (g). Any effects on growth and FCE may have considerable impacts on individual survivorship, population viability and fishery/aquaculture profitability. In addition to growth and food conversion indices the impact of these two stressors on the gut production of calcium carbonate ($CaCO_3$) was measured and an assessment of how food consumption affects this. Marine teleosts produce $CaCO_3$ in their intestine as an

excretory by-product of their osmoregulatory strategy. Faced with the constant threat of dehydration in a hyperosmotic saline environment, marine fish must constantly drink Ca^{2+}-rich seawater (Smith, 1930). Alkalinization of the intestine (pH 8.5 - 9.2) via the secretion of bicarbonate (HCO_3^-) across the intestinal lumen, causes precipitation of Ca^{2+} to form $CaCO_3$, which is then excreted within the faeces (Wilson et al., 2009). This avoids excess rates of calcium absorption, and the potential for renal stone formation, whilst aiding water absorption. Ocean acidification is predicted to increase the rate of carbonate production due to increases in intestinal HCO_3^- secretion resulting from acid-base regulation under high pCO_2 conditions (Grosell et al., 2005; Heuer et al., 2012). Copper also has the potential to affect carbonate production as it causes osmotic dysregulation (Cardeilhac et al., 1979; Stagg & Shuttleworth, 1982; Wilson & Taylor, 1993) with potential consequences for drinking rate. Furthermore copper inhibits the enzyme carbonic anhydrase (Zimmer et al., 2012), a key enzyme for HCO_3^- secretion from the intestinal epithelium (Grosell et al., 2005). Inhibition of this enzyme could therefore inhibit intestinal $CaCO_3$ formation. As such it is difficult to predict the effect that combined copper and high CO_2 exposure may have on carbonate.

The physiological consequences of ocean acidification and copper on growth and FCE, both independently and in combination are ultimately of concern as they may have downstream effects on stock density, leading to reduced fishery and aquaculture yields (Fabry et al., 2008, Cooley & Doney, 2009, Le Quesne & Pinnegar 2012). For this reason working towards a better understanding of how fish respond to the challenge of ocean acidification in the context of environmental co-stressors such as copper is of great importance, both for conservation and fishery management. In the present study the impacts of elevated CO_2 (1150 µatm) and copper (50 µg/L) on feeding, growth, FCE, tissue copper accumulation and gut carbonate production were assessed in the juvenile European sea bass using a 2x2 factorial experimental approach.

3. Methods

3.1 Animals

Juvenile sea bass were sourced from Ecloserie Marine de Gravelines in July 2014 at a mean weight of approximately 5 g and transferred to the aquaculture research facility at Cefas (Centre for Environment, Fisheries and Aquaculture Science), Weymouth, Devon. They were allowed to acclimatize for 14 days (nominal: temp.: 20 °C, salinity: 35 and CO_2: 390 µatm) prior to the study. Fish were fed ~50 mg of feed (Skretting Diamond Gamma 1.2-1.5 mm) twice daily throughout the acclimation period bar the 12 hours prior to the light anaesthesia required for initial weighing. Weighing was only conducted at the start and finish of the study in order to minimize the physiological and behavioural disturbance caused by anaesthesia and handling. Prior to the experiment, fish were lightly anaesthetised using aerated seawater containing sodium bicarbonate buffered 135 mg/L Tricaine Methanesulfonate (MS-222) before full recovery in clean seawater. At the end of the 28 d study, individual weights were taken following humane termination by terminal anaesthesia (400 mg/L MS-222); confirmed by destruction of the brain. Specific growth rate (SGR) was calculated according to the equation: $SGR = (\ln W_f - W_i)/ \Delta t \times 100$. Where W_f = final weight of fish, W_i = initial weight of fish, and t = time (in days). Fulton's Condition Factor (FCF) was calculated using the fish's weight (W) in grams and fork length (L) in cm, according to: $FCF = W \times L^3 \times 100$.

Liver Copper Burden as a Percentage of Total Body Weight was calculated using the measured concentration of copper in liver tissue (µg/g) and the mass of the liver (g) to find the copper burden in each liver (µg). This could then be considered as a percentage of the known final mass of each fish (g). Hepatosomatic index (HSI) was calculated according to the equation: HSI = LW/BW x 100, where LW and BW are liver and body weight, respectively.

Immediately after terminal sampling ~30 µl of blood was acquired via tail ablation. The liver and a subsample of muscle tissue were then dissected out and stored at -20 °C. Liver and muscle samples were later freeze dried at -50 °C and homogenised at the University of Exeter before acid-digestion in hydrogen peroxide and nitric acid at Cefas, Lowestoft. Digested samples were analysed for Cu concentration by ICP-MS at the Camborne School of Mines, Penryn, Cornwall, UK. Blood samples were acid-

digested in high purity hydrogen peroxide and nitric acid (SpA Grade, Romil Ltd.) at the University of Exeter before analysis by ICP-MS at Plymouth University, Plymouth, UK.

3.2 Experimental setup and CO_2 control

Natural seawater was used for experiments (salinity = 34.9 ± 0.02) pumped from the coastal inlet pipe just offshore from Cefas Laboratories, Weymouth, UK before being filtered to 0.45 µm and UV sterilized. The pCO_2 of the water was adjusted by passing sterilised seawater through 4 interlinked columns (0.2 m Ø x 2.2 m) against a counter current gas flow totalling 2.4 l/min output per treatment. Gas was pumped through the base of each column via a ceramic fine bubble diffuser (FBS-775; Diffused Gas Technologies, USA) with an AF30 head (0.19 m diameter, 22 µm pore size) regulated by a Q-Flow 140 flow meter (Vogtlin Instruments, Switzerland). Experimental pCO_2 values were achieved by mixing compressed air dried by a MMP140 dew point drying and filtration system (Millenium Medical Products UK) with pure CO_2 (certified 99.5 % food grade, BOC).The gas mixtures of 390 and 1150 µatm CO_2 (Table 2) were, independently measured using a CO_2/H_2O gas analyser (LI-840, LI-COR, Nebraska, USA) calibrated against certified CO_2 gas mixtures (0 and 2000 µatm), BOC, UK). Equilibrated water for each treatment was delivered to experimental tanks (10 L) at a rate of 119 ± 1 ml/min. Copper was added directly into seawater delivery tubing after gas equilibrium at a rate of 600 µl/min from a 50.8 mg/L stock using a peristaltic pump in order to achieve delivery of seawater containing 50 µg/L Cu in to each experimental unit.

3.3 Feeding Regime

Fish were fed twice daily on commercially available Skretting Diamond Gamma 1.2-1.5 mm pellets, totalling 2.5 %BW/day. Uneaten food and faeces (including carbonate precipitates) were collected separately at the end of each day, stored at -20 °C, and later dried to constant weight at 80 °C in order to ascertain net ingested food (FI).

3.4 Carbonate Analysis

Faeces, and any loose carbonates collected from the experimental tanks were bleached with 5 % sodium hypochlorite to remove organic material and rinsed

thoroughly with deionised water to remove traces of bleach before being homogenised in 10 ml of deionised water (Nanopure) by sonication. Samples were pooled by week for individual fish and double titrated (Metrohm Titrando 907) as per (Wilson, 2002). In this procedure samples are acidified to below pH 4.0 using hydrochloric acid and then titrated back to the starting pH with sodium hydroxide. Bubbling the samples with nitrogen during this double titration removes HCO_3^- and CO_3^{2-} as CO_2 gas. The molar difference between the acid and base required is therefore equal to the number of moles of HCO_3^- + CO_3^{2-} equivalents (Wilson, 2002).

Contribution of dietary calcium to carbonate production was calculated from measured weekly food consumption and estimated body mass for each week (calculated from SGR using initial and final weights). Weekly consumption by body weight was then converted to an hourly food intake rate. Given the measured calcium content of the feed (2.06 %; *titration as above*) and the molecular weight of calcium (40.078) the rate of dietary calcium intake was calculated in µequiv/kg/h. Estimates of calcium intake were derived from the typical calcium content of seawater (10 mM at salinity 35; Millero et al., 2008) and estimates of drinking rate for small fish with higher metabolic rates (5 ml/kg/hr; Wilson et al., 2009).

3.5 Statistics

All comparisons of means between the four treatments were done using Analysis of Variance (ANOVA) and were subject to Tukey's post-hoc analysis if the ANOVA revealed significant differences. Comparison between data sets grouped according to copper or high CO_2 were done using an independent samples T-test. In all analyses the results were accepted as significant if $p < 0.05$. All statistical analyses were carried out using SPSS version 20.0 and histograms were produced using Microsoft Excel 2013.

4. Results

4.1 Growth

Regardless of treatment, fish body mass increased over the 4 weeks of exposure, increasing from a mean of 6.77 (± 0.15) to 10.71 (± 0.42), representing a 60-70 % increase in body mass. Comparison of mean SGR between the four treatments revealed no significant differences [ANOVA: $F_{(3,27)}=0.94$, $p=0.43$, Table 1]. However,

both copper treatments showed slightly lower specific growth rates when compared to control and high CO_2 treatments (4-6 % lower than control, 9-11 % lower than high CO_2).

Changes in Fulton's Condition Factor (FCF) between start and finish of the experiment (Δ FCF) revealed no difference between treatments [ANOVA: $F_{(3,27)}$=0.915, p=0.447, Table 1]. Equally, at the end of the exposure (final FCF), there were no differences across treatments [ANOVA: $F_{(3,27)}$=0.154, p=0.926, Table 1]. Food conversion efficiency showed a weak trend towards lower efficiency in the two copper treatments, particularly the combined high CO_2 + Cu treatment, however these differences were not statistically significant, [ANOVA: $F_{(3,27)}$=1.38, p=0.27, Table 1].

There was no difference between treatments in mass relative food consumption expressed as percentage body weight consumed per day [ANOVA: $F_{(3,27)}$=0.574, p=0.64, Table 1]. However in all four treatments appetite showed a downward trend throughout the course of the trial and was significantly lower in the final week when compared with the three previous weeks, Control: [ANOVA: $F_{(3,20)}$=4.47, p=0.03], High CO_2: [ANOVA: $F_{(3,32)}$=13.86, p<0.001], Copper: [ANOVA: $F_{(3,24)}$=7.22, p=0.001], High CO_2 + Cu: [ANOVA: $F_{(3,32)}$=14.21, p<0.01],

4.2 Carbonate Production

Linear regression analysis (Fig. 1) revealed that carbonate production rate was positively correlated with food consumption rate in all treatments Control: [$F_{(1,22)}$= 31.8, p<0.001], High CO_2: [$F_{(1,34)}$= 11.77, p<0.01], Copper: [$F_{(1,26)}$= 12.66, p<0.01], High CO_2 + Cu: [$F_{(1,33)}$= 35.34, p<0.001]. As such analysis of between-treatments carbonate production rates was calculated after accounting for food consumption. Carbonate production rates were marginally higher (7-10 %) in the high CO_2 and copper treatments, although comparison of means revealed no significant differences [ANOVA: $F_{(3,27)}$=0.75, p=0.533] (Table 1). This was indicative of production being unaffected by copper exposure or elevated ambient pCO_2, either separately or simultaneously. The calculated contribution of dietary Ca^{2+} to carbonate production (67 %) was 7.5 times higher than the estimated Ca^{2+} contribution from drinking seawater (9 %). The relative contribution of dietary Ca^{2+} to carbonate production was not significantly different between treatments ANOVA [$F_{(3,24)}$= 2.203, p=0.114, Fig. 2],

although this contribution did appear ~9 % higher in the Control treatment when compared to all others.

4.3 Tissue Accumulation of Copper

In all treatments the liver contained high copper concentrations between 250 to 450 µg/g (Fig. 4). These values were an order of magnitude greater than those measured in other tissues (plasma: 260-315 ng/ml, Fig. 5, muscle: 7- 15 µg/g, Fig. 6). Comparison of the four distinct treatments revealed no significant differences in liver copper burden. However when grouped according to 'copper' or 'no-copper' exposure, the livers of copper-exposed fish contained significantly higher concentrations of copper (>26 %) than those maintained in copper free water (T-test: $t(29)=-2.33$, $p=0.03$, Fig. 4 inset). The same grouped analysis revealed that copper exposed animals had an increased total liver burden of copper (>30 %) as a percentage of their body mass (T-test: $t(29)=-4.35$, $p<0.001$, Fig. 3 inset). They also had larger livers proportional to their body mass as indicated by their hepatosomatic index (T-test: $t(24.94)=-2.16$, $p=0.04$, Table 1).

Copper concentrations in the plasma were far lower than in the muscle or liver (260-315 ng/ml). While there were no significant differences in the copper plasma concentrations between treatments [ANOVA: $F_{(3,29)}=0.63$, $p=0.599$, Fig. 5], copper concentrations were 17 % higher in both high CO_2 treatments.

Muscle tissue was not a major site of copper accumulation and control animals showed no difference in tissue copper concentration compared to the copper-treated groups. However, copper concentrations in the muscle of fish exposed to high CO_2 were significantly lower (~45 %) than animals kept under control pCO_2, independent of copper exposure [ANOVA: $F_{(3,27)=}11.71$, $p<0.001$, Fig. 6]. Tukey's post-hoc tests showed that High CO_2 and High CO_2 + Cu treatments were significantly different from Control and Copper treatment ($p<0.001$).

5. Discussion

Over the 28 d trial FCE, growth and condition factor of juvenile sea bass were unaffected by exposure to high CO_2 and copper, independently and in combination. Food intake was also unaffected by treatment suggesting that in the short term this

was not a strategy to compensate for any increased energy demands resulting from the treatments. Food intake did decrease in the fourth week in all treatments. This may simply be explained by the normal reduction in appetite (as a % of their body mass) as fish grow. The excretion rates of calcium carbonate were similarly unaffected by treatment, however there was a strong correlation between food intake and carbonate production. This was attributed to dietary calcium intake. Liver tissue was a site of copper accumulation in copper-exposed animals and these animals also showed increased liver mass relative to their body weight. There was a trend towards lower muscle [Cu] and higher plasma [Cu] under high CO_2, possibly suggestive of some differential impact of blood chemistry on transport/storage under these conditions. This observation was statistically supported with respect to muscle [Cu] where concentrations under normocapnic conditions were more than double those measured in the elevated pCO_2 treatments. The converse observation measured in plasma [Cu] was not supported statistically.

Specific growth rate was unaffected by any of the treatments. This seems to suggest that the mechanisms employed by fish to maintain homeostasis under high CO_2 and copper exposure (primarily acid-base, osmo- regulation and copper detoxification/excretion processes) were not sufficient to have a measurable effect on growth over the time-frame studied, at least when food was available to excess. To date, few studies have considered growth rates of fish under realistic near-future CO_2 levels making comparisons difficult. Moreover, the available studies present contradictory findings. Munday et al. (2009) reported increased growth rates in larval stages of clownfish (*Amphiprion percula*) under 550, 750 and 1030 µatm CO_2, while Baumann et al., (2011) reported reduced growth rates in early life stages of the estuarine inland silverside fish (*Menidia beryllina*) under 1000 µatm. Copper has well-documented effects on growth at a range of concentrations, with susceptibility differing between species and under differing water chemistry. Such effects often coincide with decreased appetite and altered behaviours relating to feeding or activity in both freshwater and seawater fish species (Buckley et al., 1982; De Boeck et al., 1997; Liu et al., 2010; Parveen & Javed, 2010; Wong et al., 1999). The present study revealed no impact of copper exposure on growth in sea bass under control or high CO_2 conditions. This suggests that the concentrations used in the present study were insufficient to elicit the negative physiological changes typically attributable to

decreases in growth, for example increased metabolic load (McGeer et al., 2000). Equally, it may be that bass are among the more tolerant of the species measured to-date. Furthermore it could be argued that results pertaining to growth rates under low copper concentrations that showed no effect are less often published than detrimental effects under higher concentrations. Concentrations used in the present study are relatively low compared to those used in much of the previous literature (typically above 150 µg/L into the mg/l range). This is particularly pertinent when considering a seawater exposure where copper toxicity is considered to be reduced by increased hardness and alkalinity (Chakoumakos et al., 1979), in addition to reduced bioavailability due to natural chelating agents in seawater such as dissolved organic carbon (DOC; Di Toro et al., 2001).

Lower growth rates under copper exposure are often attributed to suppression of appetite (70 and 140 µg/L; Buckley et al., 1982), reduced feeding behaviour (6-15 µg/L; Drummond et al., 1973) or the increased metabolic cost associated with mechanisms related to copper-induced disruption of physiological processes, for example counteracting oxidative stress or osmoregulatory/ acid-base regulatory dysfunction. Although detailed assessment of energy allocation was not possible in the present study there was no loss of appetite and feeding behaviour appeared unaffected by treatment throughout the trial in all animals. Although there is some evidence of decreased growth rates under copper exposure independent of changes to food consumption (Collvin, 1985), the fact that feeding behaviour and appetite were seemingly unaffected by copper exposure was no doubt a major factor in the maintenance of growth rate for these fish. Given the fundamental importance of appetite, food location, and feeding behaviour in determining energy availability, one might be expect these factors to outweigh the energetic costs associated with exposure to copper (or high CO_2). Detrimental effects on both the physiology and behaviour associated with food intake have been documented under both high CO_2 (Munday, 2015) and copper (Atchison et al., 1987) separately, and changes to feeding behaviours occurred at concentrations lower than that which produced any measured physiological impacts. The conservative copper exposure concentration in the present study may be sufficiently low so as not to inhibit feeding. The same may be true for the level of CO_2 exposure. As a consequence food intake was not decreased and

therefore neither was energy availability to the animals, allowing maintenance of growth rates consistent with controls.

This idea is further supported by a lack of difference in food conversion efficiency between treatments, suggesting that for fish exposed to copper and/or high CO_2 any costs post-ingestion (i.e. metabolic costs) were insufficient to effect overall growth. We might expect any increased metabolic costs to affect other processes such as reproduction. There is evidence to support such effects at more extreme levels of CO_2 (10,000 µatm; Bencic et al., 2000; Ingermann et al., 2002) and as well as under copper exposure (32.5 and 162 µg/L Cu; Benoit, 1975; McKim & Benoit, 1971 respectively). At copper concentrations (37 µg/L), similar to those of the present study, egg production was inhibited (Pickering et al. 1977). High pCO_2 can have detrimental effects on sperm motility (Ingermann et al., 2002) and therefore fertilizing ability (Bencic et al., 2000), possibly as a consequence of disruption in of sperm intracellular pH (Ingermann et al., 2002). Unfortunately, we are unable to comment on the combined effects of copper and high pCO_2 on the reproductive capability of animals in the present study as they were not sexually mature.

Despite potential for both copper and high CO_2 to affect rates of calcium carbonate excretion through changes to drinking rates and intestinal HCO_3^- availability, respectively, carbonate production rates were unaffected in all treatments. This study provides some of the first compelling evidence that food consumption rate is a strong determinant of carbonate production rate. Carnivorous fish such as sea bass will have a naturally calcium-rich diet due to the skeletal composition of their vertebrate and invertebrate prey: typically calcium phosphate and calcium carbonate, respectively. Feed pellets in the present study consisted of 2.06 % calcium and were therefore representative of a natural (fish-based) diet that contain around 3.2 % calcium (wet weight): derived from Cameron (1985) . As such, feeding is a considerable source of calcium to these animals making it unsurprising that the rate of its excretion as calcium carbonate would be directly influenced by the rate of its uptake through the diet. Given the lack of available information on carbonate production rates under fed conditions, the data from this study will assist in improving the accuracy of current global fish carbonate production models (Wilson et al., 2009). The greatly increased production rate of carbonate compared with the existing literature on unfed animals (Heuer et al., 2012; Perry et al., 2011; Wilson et al., 2009) enables more accurate modelling of the

contribution of teleosts to the marine carbon cycle. To-date such models have used an estimated constant derived from metabolic differences between active (fed) and sedentary (unfed) animals (Kerr, 1982) to scale up carbonate production from unfed fish data (Wilson et al., 2009). The carbonate excretion rate data in the present study provides the first quantitative evidence on animals fed naturally high-calcium diets that can be compared with unfed carbonate excretion data from a similar size class in the same species to more accurately inform this scaling factor. For example, Reardon *et al.* (in prep.) used a scaling factor to predict carbonate excretion rates in 10 g sea bass (as per the present study) based on measurements from 100 g bass. The predicted unfed excretion rate for 10 g bass (at 18 °C) is 117 µequiv./kg/hr, equating to 39 % of the mean excretion rates measured in the fed animals in the present study.

NB: Data from the present study will be contained within Reardon et al. (in prep) for comparative analysis of the influence of feeding on carbonate production rate.

Furthermore assessment was made of the relative contribution of dietary Ca^{2+} to estimates of Ca^{2+} ingested as a result of drinking seawater. Consumption of ~2 % BW food per day resulted in a dietary contribution of Ca^{2+} that was 7.5-fold higher than the estimated contribution from drinking (Fig. 2) This is potentially even higher in natural diets due to the higher Ca^{2+} content of fish (~3.2 %; Cameron, 1985) compared with the feed pellets used in the present study (2.06 %). The cation contribution to the excreted carbonate not accounted for varies between 17-28 % across the four treatments. This is likely comprised of magnesium ions (Mg^{2+}), both from the diet and from the seawater, as this proportion is typical of the Mg mole% (4-40 %) found in gut carbonates excreted by marine fish (Wilson et al., 2009; Salter et al, 2012; 2014).

The total burden of copper, when expressed as a percentage of body mass appeared higher in the livers of copper-exposed animals. This is evidence that copper is being internalised from the seawater medium and is differentially accumulating in certain tissues. Higher liver concentrations in copper-exposed bass are consistent with a wealth of literature highlighting hepatic tissue as the primary site for copper storage and detoxification (Arellano et al., 1999; Buckley et al., 1982; Jezierska & Witeska, 2006) . These same animals were also found to have a higher hepatosomatic indices

when compared with those not exposed to copper. Dose-dependent increases in HSI have been reported previously as a result of waterborne copper exposure (150-300 µg/L; Liu et al. (2010) and were speculatively attributed to increased lipid content (fatty liver syndrome). Other histological changes in liver tissue are frequently reported under exposure to copper (>100 µg/L) including disorganization of hepatic parenchyma (Oliva et al., 2009) and vacuolization (Arellano et al., 1999; Liu et al., 2010; Oliva et al., 2009), but do not consistently correlate with increases in HSI and so cannot explain this observation in the present study.

High CO_2/low pH did not appear to impact the accumulation of copper in the liver. Previous work using the same species and comparable copper concentrations (80 µg/L for 14 days) found copper accumulation in the gill as well as the liver under similar conditions to this study (Chapter 4). Although the lack of differential accumulation might at first suggest similar bioavailability between high CO_2 and normocapnic treatments in the present study, the rate of copper uptake cannot be established from the tissue accumulation data alone as these are affected by rates of excretion which may be different under differing treatments. As such these data are not necessarily at odds with predictive models of copper speciation and bioavailability under future ocean acidification conditions (Millero, 2009) which suggests there will be increased bioavailability and a greater potential for toxicity under high CO_2 conditions. As plasma copper concentrations were far lower than the other tissues measured and did not vary significantly between treatments. This could indicate that plasma [Cu] is under tight homeostatic control and as such was not subject to a high copper load under exposure to environmental copper. Although not significantly so, plasma copper concentrations were 17 % higher in both high CO_2, irrespective of copper exposure. This may be indicative of a CO_2-induced shift in tissue transport/storage mechanisms under these conditions. The opposite trend is apparent in the muscle.

Muscle tissue was not a major site of copper accumulation with no concentrations exceeding those of the control animals and no differences apparent between those exposed to copper and not. However, high CO_2 exposure resulted in a considerable decrease (~45 %) in muscle copper concentration, independent of copper exposure. This may suggest that high CO_2 conditions promote depuration from muscle tissue, possibly resulting from the binding of intramuscular copper with the elevated bicarbonate ions present in the muscle during acid-base regulation (Larsen et al.,

1997). Bogdanova et al. (2002) observed the cellular entry of copper-chloride complexes via Cl^-/HCO_3^- exchangers in rainbow trout erythrocytes. It possible that inward movement of HCO_3^- ions and/or outward flow of Cl^- ions during intramuscular acid-base regulation provide a depuration route for elevated intracellular copper. This further supports the idea that high CO_2 may shift the balance of tissue copper burden marginally from the muscle to the plasma: as could be interpreted from trends in plasma [Cu] in the present study. However this is speculative as plasma [Cu] elevations under high CO_2 (17 %) were not statistically significant.

These findings have positive implications for both bass fisheries and aquaculture in that copper does not appear to bioaccumulate to a measurable degree in tissues sold for human consumption (muscle). Furthermore, these data suggest that environments of elevated CO_2 do not increase bioaccumulation, and may even act to reduce the already low copper concentrations of these tissues.

5.1 Summary

Sea bass showed tolerance to both high CO_2 (1150 µatm) and copper (50 µg/L) exposure both separately and simultaneously. While the liver remained a site of copper accumulation under waterborne exposure the low pH of the high CO_2 seawater appeared to have no measurable effect on the accumulation of the copper within this tissue. We are unable to comment on the bioavailability and rate of uptake of copper from the environment. Plasma and muscle copper concentrations suggested that neither tissue was a site of copper accumulation but that high CO_2 may affect the copper burden of these tissues: lowering that of the muscles and increasing that of the plasma. In the context of human health protection, concentrations of copper in the muscle tissue remained very low, particularly under high CO_2 conditions.

Neither copper nor CO_2 had any perceivable impact on the overall health of the animals as measured by growth, food conversion efficiency and condition factor. Overall neither stressor, at the concentrations examined, appear to have any effect on growth or food conversion suggesting sea bass aquaculture and fisheries are unlikely to be greatly affected by predicted end-of-century CO_2 partial pressures, even in the context of background copper contamination (>50 µg/L). Food consumption was found to be strongly determinant in carbonate production rates with considerable implications for informing global teleost inputs into the marine carbon cycle.

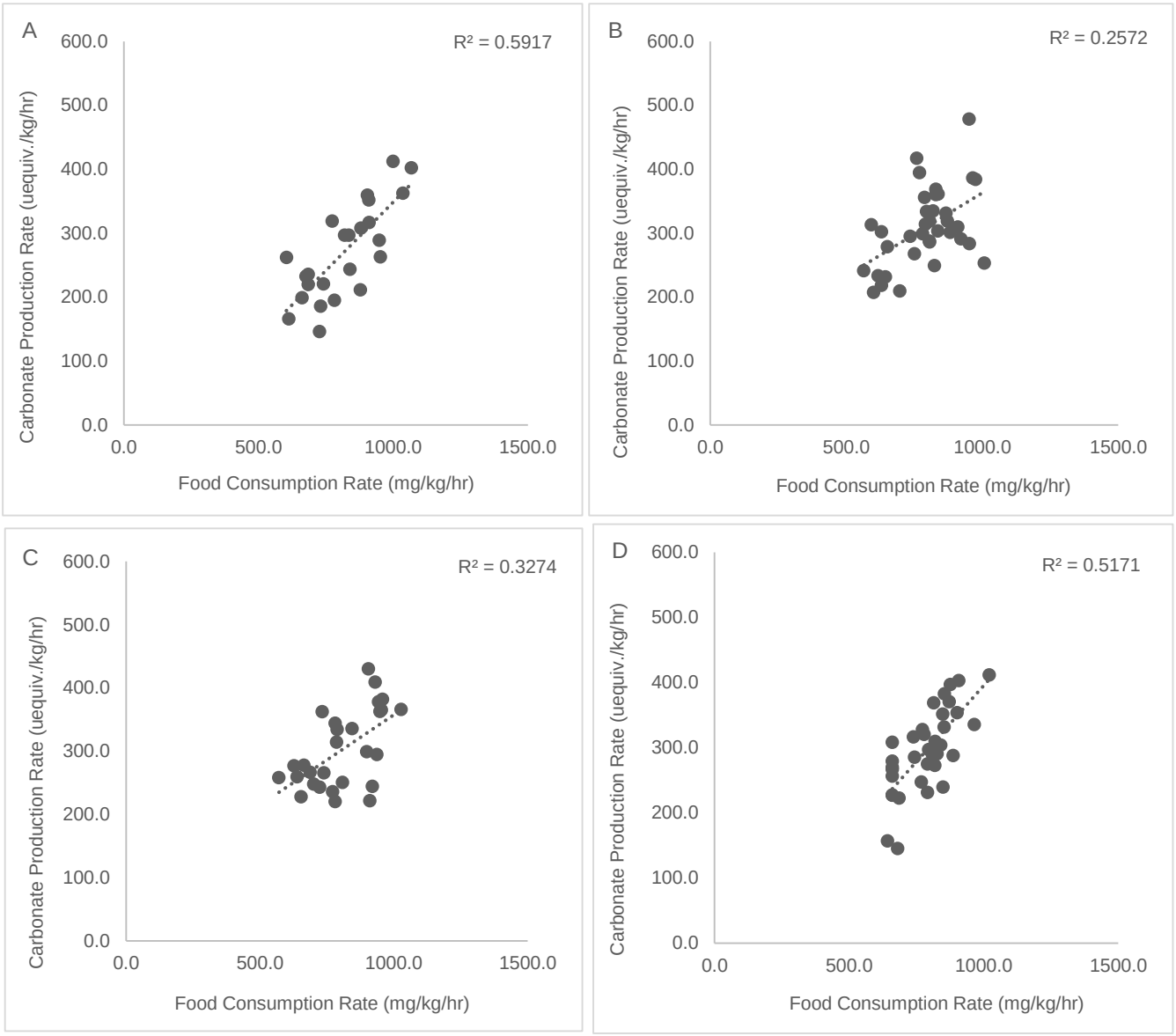
In addition, acclimation and/or adaptation are realistic possibilities in the face of chronic exposure to stressors such as these and have been reported for both copper and high CO_2. Recovery of appetite and growth rate during chronic exposure to copper have previously been documented (Buckley et al., 1982; Lett et al., 1976), although acclimation may be non-heritable (McKim & Benoit, 1971). Another recent study showed that detriment to growth and survival in juvenile fish exposed to 1000 µatm was not apparent in their offspring (Miller et al., 2012), demonstrating that non-genetic parental effects positively alter the way in which future generations respond to the same conditions. Conversely, detrimental effects attributed to elevated HCO_3^- during acid-base regulation (Nilsson et al., 2012), such as behavioural alteration (Domenici et al., 2011; Munday, 2015; Munday et al., 2014) and changes to sensory preference/ability (Dixson et al., 2010; Munday et al., 2009; Simpson et al., 2011), have been found to persist across generations in wild species under chronic high CO_2 exposure (Munday et al., 2014).

5.2 Future Work

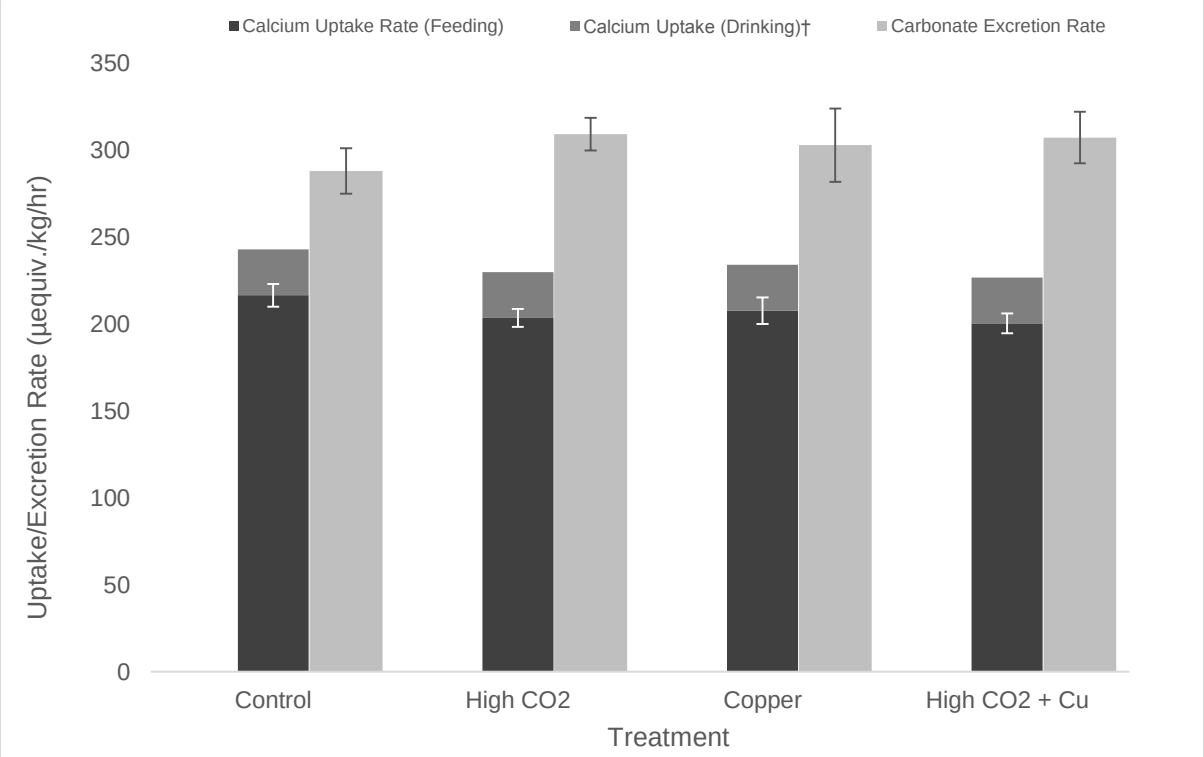
Historically fish studies on copper have focussed largely on freshwater species where copper contamination is more common and severe due to the proximity to terrestrial influxes. For this reason experiments have typically employed copper concentrations exceeding those likely to be experienced by marine animals. Coastal species and marine species with life stages in estuaries or freshwater, like sea bass, are perhaps the exceptions and may encounter copper concentrations greater than those ever likely to be experienced by their open ocean counterparts. However, even with this in mind a good deal of the literature is only relevant to areas of severe contamination. With a wealth of background information on severe and acute copper toxicity, future work should aim to better understand the physiological effects associated with lower, more environmentally realistic (<5 µg/L), exposure concentrations over longer time frames (>30 d). Of equal importance, as per the present study and previous work (*Chapters 3 & 4*), we must aim to better understand how copper effects animals in the context of changing co-stressors, particularly those associated with future changes to ocean chemistry: temperature, CO_2, salinity. Not only this, but how copper affects the animals ability to deal with such environmental perturbations. Each stressor will likely influence the physiological response to any one or all of the others. Temperature for example is likely effect on the response of fishes to high CO_2 (Munday et al., 2009).

As such the ultimate aim for this type of research may be a chronic, multi-stressor assessment of copper under modelled future water chemistry with respect to the aforementioned physicochemical parameters. Furthermore we should aim to elucidate the effects that changing ocean chemistry will have on the bioavailability of copper to a range of marine phyla, not least fishes. Finally, research should consider to what extent copper is a representative model for other metals and the degree to which the findings in the present study may be generalizable to these contaminants.

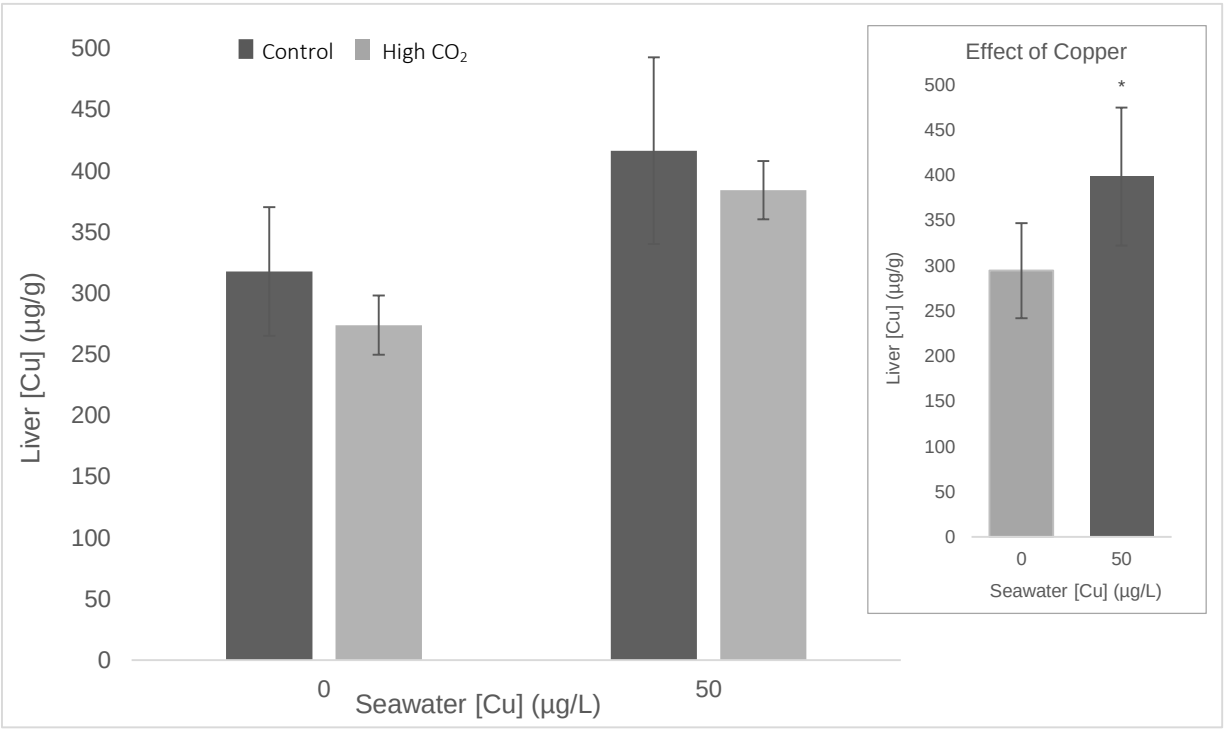
6. Figures



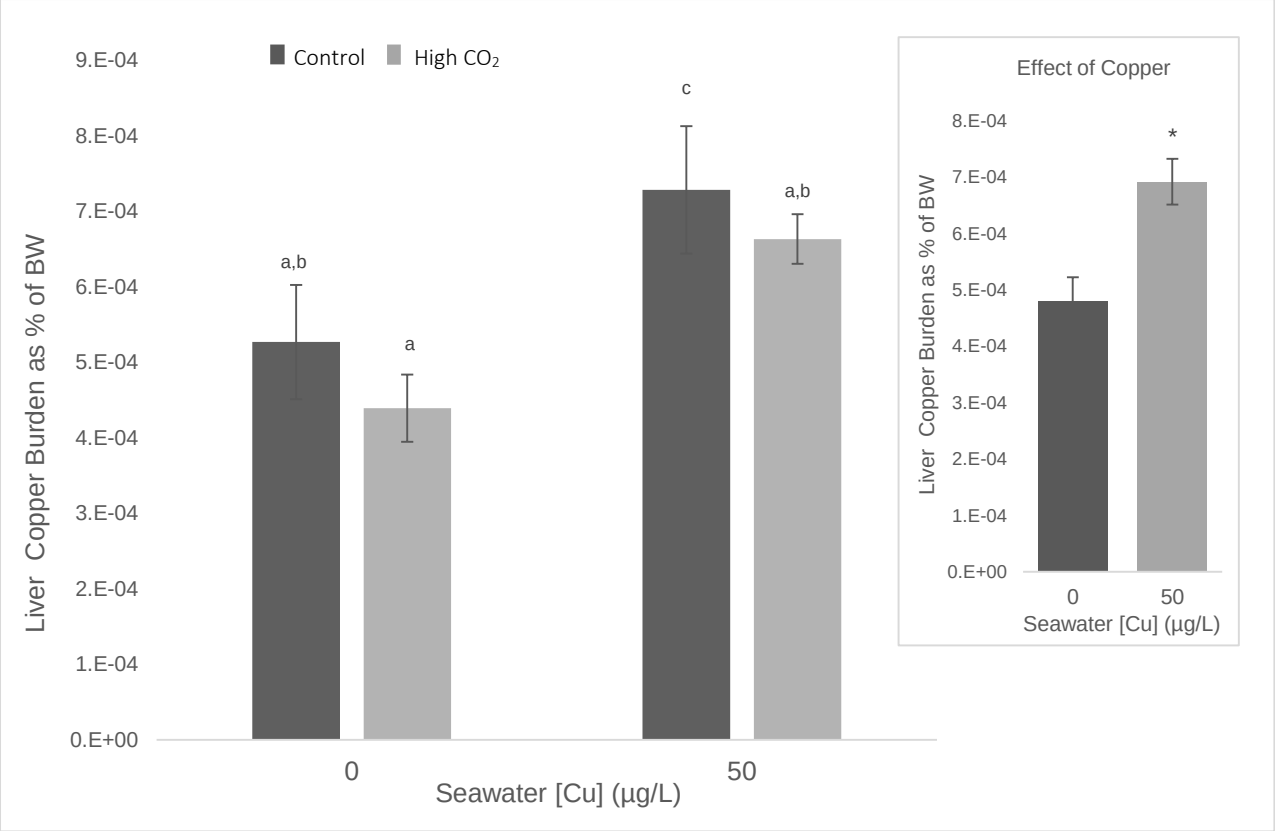
Chapter 5 Figure 1: : Linear regression plots of Carbonate Production Rate as determined by Food Consumption Rate for all treatments: A) Control, B) High CO_2, C) Copper and D) High CO_2 + Cu. Values represent weekly averages for each animal. All treatment showed a significant correlation (ANOVA $p<0.01$).



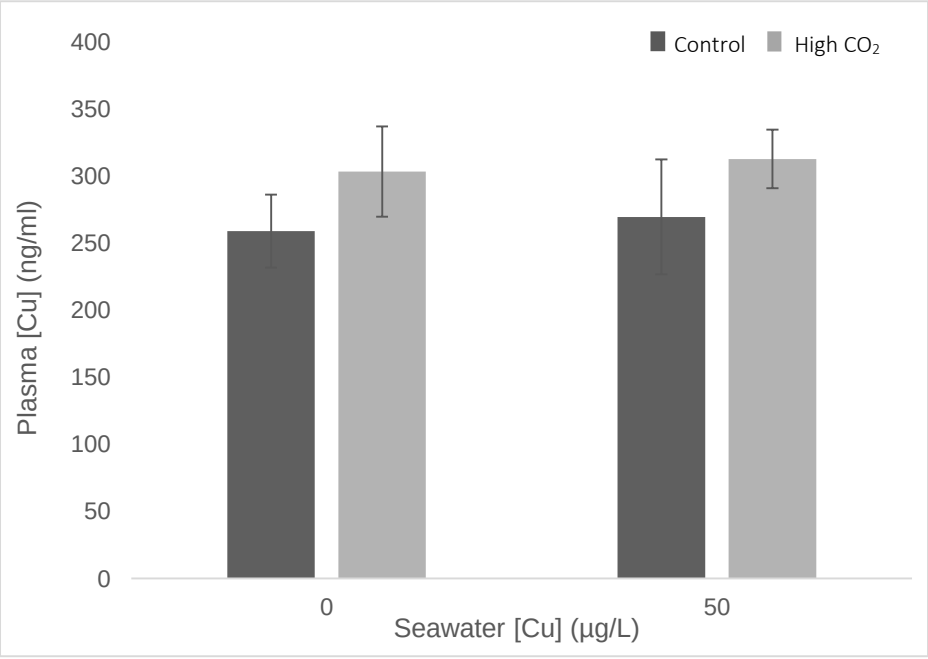
Chapter 5 Figure 2: Relative contribution of Ca^{2+} from feeding and drinking to total carbonate production (primarily $CaCO_3$). Values represent Means (±SEM for Feeding Uptake and Carbonate Excretion). †Calculated using estimated drinking rates of 5 ml/kg/hr (Grosell et al., 2004) and calcium content of seawater at a salinity of 35 (Millero et al., 2008)



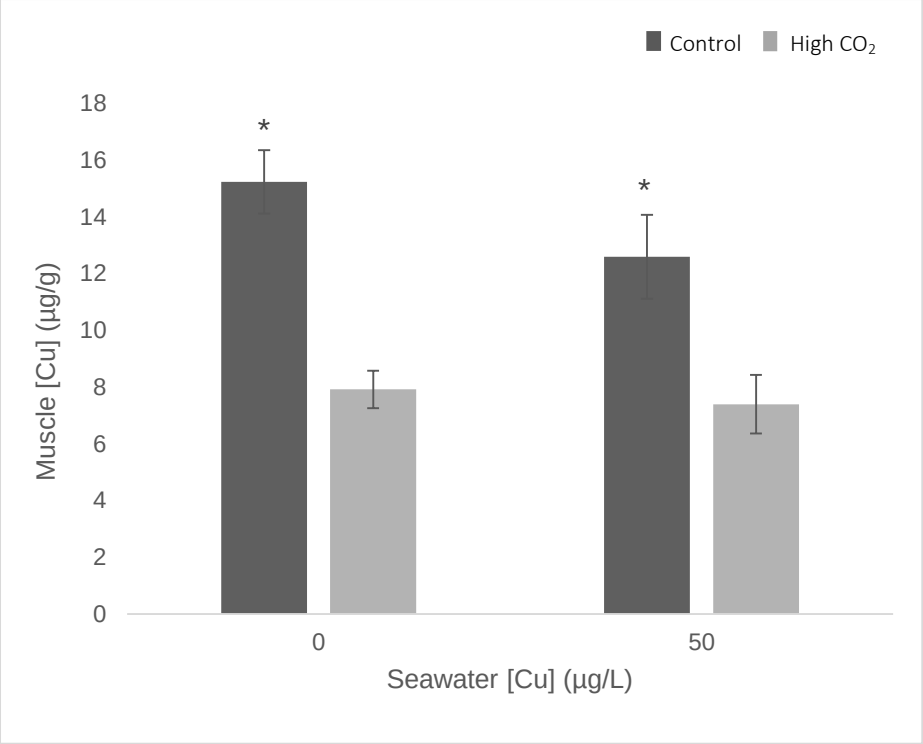
Chapter 5 Figure 3: Copper concentrations measured (ICP-MS) in the liver tissues of sea bass kept under two copper exposures at control (540 µatm) and high CO_2 (1150 µatm). Main graph displays all treatments. N=6,9,7,9 . Inset displays grouped data of fish exposed to copper or no copper. N=15,16. Asterisk denotes significant difference (t-test $p<0.05$). Values represent Mean ±SEM.



Chapter 5 Figure 2: Liver copper burden as a percentage of fish body mass in sea bass kept under two copper exposures at control (540 µatm) and high CO_2 (1150 µatm). Main graph displays all treatments. N=6,8,7,9 . Inset displays grouped data of fish exposed to copper or no copper. N=14,16. Letter denote letter denote homogenous subsets (ANOVA $p<0.05$, with Tukey's post-hoc test). Asterisk denotes significant difference (t-test $p<0.05$). Values represent Mean ±SEM.



Chapter 5 Figure 3: Copper concentrations measured (ICP-MS) in the plasma of sea bass kept under different seawater copper and CO_2 conditions. Values represent Mean ±SEM. N=7,10,7,9.



Chapter 5 Figure 4: Copper concentrations measured (ICP-MS) in the muscle tissues of sea bass kept under different seawater copper and CO_2 conditions. Asterisk denotes significant (ANOVA $p<0.05$). Values represent Mean ±SEM. N=6,9,7,9.

7. Appendices

Chapter 5 Table 1: Summary of parameters measured in sea bass over the 4 week experiment. Fulton's Condition Index is displayed as Final Condition Index (Change in Condition Index Δ). Value represent Mean ± SEM. N = Control (6), High CO_2 (9), Copper (7), High CO_2 + Cu (9).

Treatment	**Specific Growth Rate** (%BW/day)	**Food Conversion Efficiency** (ΔgBW/g)	**Hepatosomatic Index** (%BW)	**Food Consumption** (%BW/d)	**Carbonate Production Rate per Food Intake** (μequiv./kg/hr/mgFI)	**Fulton's Condition Index:** (Final (Δ))
Control	1.78 (± 0.12)	0.92 (± 0.05)	1.66 (± 0.11)	1.96 (± 0.07)	41.9 (± 3.7)	1.25 (0.06) (± 0.02)
High CO_2	1.88 (± 0.08)	1.01 (± 0.03)	1.56 (± 0.06)	1.90 (± 0.07)	46.3 (± 2.7)	1.24 (0.04) (± 0.02)
Copper	1.71 (± 0.11)	0.89 (± 0.03)	1.90 (± 0.16)	1.93 (± 0.05)	44.6 (± 1.8)	1.26 (0.04) (± 0.03)
High CO_2 + Copper	1.67 (± 0.11)	0.81 (± 0.06)	1.76 (± 0.10)	1.86 (± 0.05)	41.6 (± 2.5)	1.24 (0.01) (± 0.02)

Chapter 5 Table 2: Seawater carbonate chemistry and copper concentrations in four experimental treaments to which sea bass were exposed. Values represent Mean ± SEM. † calculated using CO2sys (Pierrot, 2006).

Treatment	pH_{NBS}	Copper (µg/L)	Temp. (°C)	Salinity	TA (µmol/kg)†	TCO_2 (µmol/kg)	pCO_2 (µatm)†	HCO_3^- (µmol/kg)†	CO_3^{2-} (µmol/kg)†	ΩCa†	ΩAr†
Control	8.07 (±0.00)	0.3 (±0.2)	20.2 (±0.01)	34.9 (±0.03)	2296.1 (±2.4)	2090.0 (±2.3)	531.7 (±1.6)	1920.5 (±2.2)	152.4 (±0.4)	3.6 (±0.01)	2.4 (±0.01)
High CO_2	7.75 (±0.01)	1.6 (±0.7)	20.1 (±0.02)	35.0 (±0.04)	2236.8 (±5.7)	2157.9 (±3.1)	1173.1 (±44.9)	2041.0 (±3.0)	79.1 (±1.6)	1.9 (±0.04)	1.2 (±0.03)
Copper	8.06 (±0.00)	52.1 (±1.5)	20.3 (±0.04)	34.9 (±0.03)	2289.9 (±2.4)	2089.7 (±2.3)	547.2 (±1.9)	1923.5 (±2.2)	148.6 (±0.4)	3.6 (±0.01)	2.3 (±0.01)
High CO_2 + Cu	7.76 (±0.00)	51.3 (±1.0)	20.2 (±0.01)	35.1 (±0.02)	2237.4 (±4.9)	2154.8 (±4.9)	1132.2 (±3.9)	2037.9 (±4.6)	80.6 (±0.2)	1.9 (±0.00)	1.3 (±0.00)

8. References

Arellano, J. M., Storch, V., & Sarasquete, C. (1999). Histological changes and copper accumulation in liver and gills of the Senegales sole, *Solea senegalensis*. *Ecotoxicology and Environmental Safety, 44*(1), 62-72.

Atchison, G. J., Henry, M. G., & Sandheinrich, M. B. (1987). Effects of metals on fish behaviour: a review. *Environmental Biology of fishes, 18*(1), 11-25.

Bao, Y., Qiao, F., & Song, Z. (2012). Historical simulation and twenty-first century prediction of oceanic CO_2 sink and pH change. *Acta Oceanologica Sinica, 31*(5), 87-97.

Baumann, H., Talmage, S., Gobler, C.J. (2011). Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. *Nature Climate Change, 2*(1), 38-41.

Bencic, D., Cloud, J., & Ingermann, R. (2000). Carbon dioxide reversibly inhibits sperm motility and fertilizing ability in steelhead (*Oncorhynchus mykiss*). *Fish Physiology and Biochemistry, 23*(4), 275-281.

Benoit, D. (1975). Chronic effects of copper on survival, growth, and reproduction of the bluegill (*Lepomis macrochirus*). *Transactions of the American Fisheries Society, 104*(2), 353-358.

Bogdanova, A. Y., Gassmann, M., & Nikinmaa, M. (2002). Copper ion redox state is critical for its effects on ion transport pathways and methaemoglobin formation in trout erythrocytes. *Chemico-Biological Interactions, 139*(1), 43-59.

Buckley, J. T., Roch, M., McCarter, J. A., Rendell, C. A., & Matheson, A. T. (1982). Chronic exposure of coho salmon to sublethal concentrations of copper—I. Effect on growth, on accumulation and distribution of copper, and on copper tolerance. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 72*(1), 15-19.

Cameron, J. (1985). The bone compartment in a teleost fish, *Ictalurus punctatus*: size, composition and acid-base response to hypercapnia. *Journal of Experimental Biology, 117*(1), 307-318.

Cardeilhac, P., Simpson, C., Lovelock, R., Yosha, S., Calderwood, H., & Gudat, J. (1979). Failure of osmoregulation with apparent potassium intoxication in marine teleosts: a primary toxic effect of copper. *Aquaculture, 17*(3), 231-239.

Chakoumakos, C., Russo, R. C., & Thurston, R. V. (1979). Toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH, and hardness. *Environmental Science & Technology, 13*(2), 213-219. 3

Collvin, L. (1985). The effect of copper on growth, food consumption and food conversion of perch (*Perca fluviatili*s L.) offered maximal food rations. *Aquatic Toxicology, 6*(2), 105-113.

Crocker, C. E., & Cech, J. J. (1996). The effects of hypercapnia on the growth of juvenile white sturgeon, *Acipenser transmontanus*. *Aquaculture, 147*(3), 293-299.

De Boeck, G., Vlaeminck, A., & Blust, R. (1997). Effects of sublethal copper exposure on copper accumulation, food consumption, growth, energy stores, and nucleic acid content in common carp. *Archives of Environmental Contamination and Toxicology, 33*(4), 415-422.

Di Toro, D. M., Allen, H. E., Bergman, H. L., Meyer, J. S., Paquin, P. R., & Santore, R. C. (2001). Biotic ligand model of the acute toxicity of metals: 1) Technical Basis. *Environmental Toxicology and Chemistry, 20*(10), 2383-2396.

Drummond, R., Spoor, W., & Olson, G. (1973). Some short-term indicators of sublethal effects of copper on brook trout, *Salvelinus fontinalis*. *Journal of the Fisheries Board of Canada, 30*(5), 698-701.

FAO. (2015). Cultured Aquatic Species Information Programme *Dicentrarchus labrax* (Linnaeus, 1758). Retrieved 01.08.15

Fitzer, S. C., Caldwell, G. S., Close, A. J., Clare, A. S., Upstill-Goddard, R. C., & Bentley, M. G. (2012). Ocean acidification induces multi-generational decline in copepod naupliar production with possible conflict for reproductive resource allocation. *Journal of Experimental Marine Biology and Ecology, 418–419*, 30-36.

Fivelstad, S., Haavik, H., Løvik, G., & Olsen, A. B. (1998). Sublethal effects and safe levels of carbon dioxide in seawater for Atlantic salmon postsmolts (*Salmo salar* L.): ion regulation and growth. *Aquaculture, 160*(3-4), 305-316.

Foss, A., Røsnes, B., & Øiestad, V. (2003). Graded environmental hypercapnia in juvenile spotted wolffish (*Anarhichas minor Olafsen*): Effects on growth, food conversion efficiency and nephrocalcinosis. *Aquaculture, 220*(1), 607-617.

Franke, A., Clemmesen, C., (2011). Effect of ocean acidification on early life stages of Atlantic herring (*Clupea harengus* L.). *Biogeosciences Discussion 8*, 7097–7126.

Grosell, M., McDonald, M. D., Walsh, P. J., & Wood, C. M. (2004). Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) II: copper accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues. *Aquatic Toxicology, 68*(3), 263-275.

Grosell, M., Wood, C. M., Wilson, R. W., Bury, N. R., Hogstrand, C., Rankin, C., & Jensen, F. B. (2005). Bicarbonate secretion plays a role in chloride and water absorption of the European flounder intestine. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 288*(4), 936-946.

Hansen, J., Lipton, J., Welsh, P., Morris, J., Cacela, D., & Suedkamp, M. (2002). Relationship between exposure duration, tissue residues, growth, and mortality in rainbow trout (*Oncorhynchus mykiss*) juveniles sub-chronically exposed to copper. *Aquatic Toxicology, 58*(3), 175-188.

Heuer, R. M., Esbaugh, A. J., & Grosell, M. (2012). Ocean acidification leads to counterproductive intestinal base loss in the gulf toadfish (*Opsanus beta*). *Physiological and biochemical zoology, 85*(5), 450-459.

Hirst, D. (2015). *UK and European sea bass conservation measures*. House of Commons Library: House of Commons.

Ingermann, R., Holcomb, M., Robinson, M., & Cloud, J. (2002). Carbon dioxide and pH affect sperm motility of white sturgeon (*Acipenser transmontanus*). *Journal of Experimental Biology, 205*(18), 2885-2890.

Ishimatsu, A., Hayashi, M., & Kikkawa, T. (2008). Fishes in high-CO_2, acidified oceans. *Marine Ecology-Progress Series, 373*, 295-302.

Ishimatsu, A., Hayashi, M., Lee, K. S., Kikkawa, T., & Kita, J. (2005). Physiological effects on fishes in a high - CO_2 world. *Journal of Geophysical Research: Oceans (1978–2012), 110*(C9).

Jezierska, B., & Witeska, M. (2006). The metal uptake and accumulation in fish living in polluted waters. *Soil and Water Pollution Monitoring, Protection and Remediation* (pp. 107-114): Springer.

Kerr, S. (1982). Estimating the energy budgets of actively predatory fishes. *Canadian Journal of Fisheries and Aquatic Sciences, 39*(3), 371-379.

Larsen, B. K., Pörtner, H. -O., & Jensen, F. B. (1997). Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology, 128*(2), 337-346.

Lett, P. F., Farmer, G., & Beamish, F. (1976). Effect of copper on some aspects of the bioenergetics of rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Board of Canada, 33*(6), 1335-1342.

Liu, X., Luo, Z., Xiong, B., Liu, X., Zhao, Y., Hu, G., & Lv, G. (2010). Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*. *Ecotoxicology and Environmental Safety, 73*(6), 1286-1291.

McKim, J., & Benoit, D. A. (1971). Effects of long-term exposures to copper on survival, growth, and reproduction of brook trout (*Salvelinus fontinalis*). *Journal of the Fisheries Board of Canada, 28*(5), 655-662.

Miller, G. M., Watson, S.-A., Donelson, J. M., McCormick, M. I., & Munday, P. L. (2012). Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. *Nature Climate Change, 2*(12), 858-861.

Millero, F. J. (2009). Effect of ocean acidification on the speciation of metals in seawater. *Oceanography, 22*(4), 72.

Millero, F. J., Feistel, R., Wright, D. G., & McDougall, T. J. (2008). The composition of standard seawater and the definition of the reference-composition salinity scale. *Deep Sea Research Part I: Oceanographic Research Papers, 55*(1), 50-72.

Moran, D., & Støttrup, J. G. (2011). The effect of carbon dioxide on growth of juvenile Atlantic cod (*Gadus morhua* L.) *Aquatic Toxicology, 102*(1-2), 24-30.

Munday, P. (2015). *Effects of ocean acidification on the life history and behaviour of tropical marine fishes.* Paper presented at the 145th Annual Meeting of the American Fisheries Society.

Munday, P. L., Crawley, N. E., & Nilsson, G. E. (2009). Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes. *Marine Ecology Progress Series, 388*, 235-242.

Munday, P. L., Donelson, J. M., Dixson, D. L., & Endo, G. G. K. (2009). Effects of ocean acidification on the early life history of a tropical marine fish. *Proceedings of the Royal Society B: Biological Sciences, 276*(1671), 3275-3283.

Oliva, M., Garrido, M., Márquez, D. S., & de Canales, M. G. (2009). Sublethal and lethal toxicity in juvenile Senegal sole (*Solea senegalensis*) exposed to copper: a preliminary toxicity range-finding test. *Experimental and Toxicologic Pathology, 61*(2), 113-121.

Parveen, A., & Javed, M. (2010). Effect of water-borne copper on the growth performance of fish *Catla catla*. *International Journal of Agriculture & Biology, 12*.

Perry, C. T., Salter, M. A., Harborne, A. R., Crowley, S. F., Jelks, H. L., & Wilson, R. W. (2011). Fish as major carbonate mud producers and missing components of the tropical carbonate factory. *Proceedings of the National Academy of Sciences*.

Pickering, Q., Brungs, W., & Gast, M. (1977). Effect of exposure time and copper concentration on reproduction of the fathead minnow (*Pimephales promelas*). *Water Research, 11*(12), 1079-1083.

Pierrot, D. E., Wallace, D.W.R. (2006). MS Excel program developed for CO_2 system calculations. *ORNL/CDIAC-105a*.

Roberts, D. A., Birchenough, S. N., Lewis, C., Sanders, M. B., Bolam, T., & Sheahan, D. (2013). Ocean acidification increases the toxicity of contaminated sediments. *Global change biology, 19*(2), 340-351.

Smith, H. W. (1930). *The absorption and excretion of water and salts by marine teleosts* (Vol. 93).

Stagg, R. M., & Shuttleworth, T. J. (1982). The effects of copper on ionic regulation by the gills of the seawater-adapted flounder (*Platichthys flesus* L.). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 149*(1), 83-90.

Wilson, R., Millero, F., Taylor, J., Walsh, P., Christensen, V., Jennings, S., & Grosell, M. (2009). Contribution of fish to the marine inorganic carbon cycle. *Science, 323*(5912), 359-362.

Wilson, R.W, Wilson, J. M., & Grosell, M., (2002). Intestinal bicarbonate secretion by marine teleost fish - how and why? *Biochemica et Biophysica Acta, 1566*, 182-193.

Wilson, R. W., & Taylor, E. W. (1993). Differential responses to copper in rainbow trout (*Oncorhynchus mykiss*) acclimated to sea water and brackish water. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 163*(3), 239-246.

Wong, P., Chu, L., & Wong, C. (1999). Study of toxicity and bioaccumulation of copper in the silver sea bream (*Sparus sarba*). *Environment International, 25*(4), 417-422.

Zimmer, A. M., Barcarolli, I. F., Wood, C. M., & Bianchini, A. (2012). Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. *Aquatic Toxicology, 122–123*(0), 172-180.

CHAPTER 6

General Discussion and Conclusions

Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

Global fisheries produce $80-85 billion in revenue every year and account for as much as $235 billion when considering associated ancillary services (Dyck & Sumaila, 2010). Additionally, fish provide 15% of the animal protein requirements of 3 billion people globally. Worldwide fisheries are already underperforming due to habitat degradation, pollution and overfishing. The effects of climate change represent an additional threat with predicted changes in primary productivity, distribution and potential yield (Sumaila, 2012). Needless to say, it is vital that we better understand the potential impacts that ocean acidification may have on fish physiology and the potential for downstream costs to success at both an individual and population level. Furthermore is the need for consideration of such impacts in the context of ubiquitous environmental stressors, such as copper, an environmentally and mechanistically relevant co-stressor for work on coastal marine species.

6.1 Acid-base Regulation

For two essentially unrelated marine species of vertebrate (European sea bass, *Dicentrarchus labrax*) and invertebrate (shore crabs, *Carcinus maenas*), this thesis provides clear evidence for the ability of both to regulate the pH of their extracellular fluids (pH_e) when challenged with elevated ambient carbon dioxide (CO_2) predicted for the next century (~1200 µatm; IPCC, 2014). Sea bass were particularly effective in the speed with which they regulated their pH_e (<30 mins), whilst crabs took as long as 48 hours. Both species appeared to buffer their pH_e in the same manner: by the accumulation of bicarbonate ions (HCO_3^-). Although HCO_3^- uptake has been considered by all previous studies to be the primary means of regulation, there may be some role for H^+ extrusion, which was not measured in the present studies. One explanation for the considerable temporal difference in the ability of these two animals to acid-base regulate, may be explained by their ecological niche. Most environments in which shore crabs are exposed to hypercapnic seawater, for example tide pools, will also be hypoxic. Given that hypoxia typically induces respiratory alkalosis in crabs

(Burnett & Johansen, 1981), largely due to hyperventilation, it is thought that combined hypoxia and hypercapnia minimise perturbations to internal pH/pCO_2, having a net regulatory effect (Truchot, 1986). The slow accumulation of these changes in water chemistry in these instances has possibly negated the need for crabs to develop rapid and efficient acid-base regulatory mechanisms to deal with hypercapnia alone. Sea bass on the other hand also experience combined hypoxia/hypercapnia, but are far more likely to experience acute changes in these variables as they swim into or out of such areas. They may therefore have adapted to rapidly compensate their acid-base status in order to must maintain performance under such circumstances.

6.1.1 Copper Affects Acid-base Regulation

Acid-base regulation was clearly affected by the presence of waterborne copper. Copper (40 µg/L) was found to inhibit the ability of crabs to accumulate HCO_3^- as part of their acid-base regulatory strategy, resulting in an uncompensated respiratory acidosis under exposure to high CO_2 (1200 µatm). The same inhibition was not present at lower concentrations suggesting an effect threshold between 15 and 40 µg/L.

There was indirect evidence (based on strong ion difference) for a similar effect in sea bass under 80 µg/L copper although direct measurement of pH and HCO_3^- was not possible. These findings suggest that copper-induced inhibition of acid-base regulation may occur at concentrations well below those few papers to describe this effect to-date: ~400 µg/L (Larsen et al., 1997), ~600 µg/L (Wang et al., 1998). Although copper concentrations around the UK coast are typically ≤5 µg/L, these finding have considerable implications for fish and crustaceans inhabiting even moderately contaminated water. Freshwater fish, for example, are generally considered to be more at risk to the detrimental effects of copper as they are exposed to far higher concentrations of than marine species and as a result of different copper speciation: typically more bioavailable Cu^{2+} (Paquin et al., 2002). Yet they are equally reliant on the same acid-base regulatory strategies. As such the potential for inhibited acid-base regulation may be far higher in these species as well as any marine species in coastal or estuarine areas of greater copper contamination. Very little is yet known about the implications for these animals if they were faced with chronically inhibited acid-base regulatory capability, but it's likely that disrupted enzyme function and decreased

oxygen carrying capacity would occur with knock-on effects for a whole suite of physiological functions.

Future research on the mechanism of copper-induced inhibition of acid-base regulation would likely be closely linked with better understanding the precise way in which animals accumulate HCO_3^-: the source of these ions is yet unknown. There are currently two models for acid-base regulation in fish and it's likely that the same mechanism is conserved in crustaceans (*see Chapter 1 for detail*). One suggests an exogenous source of these HCO_3^- ions, transported inward across the gill (Esbaugh et al., 2012; Truchot, 1979), and the other suggests an endogenous source through carbonic anhydrase (CA) catalysed hydration of CO_2, coupled with excretion of the simultaneously generated H^+ ions (Claiborne et al., 1997). Better understanding this accumulation will help identify targets for assessment of likely means by which copper exposure results in inhibition. For example, Zimmer et al. (2012) found inhibition of branchial CA in both fresh and seawater acclimated fish exposed to 20 μg/L Cu exposure. Continued research in this area might also seek to assess the relative contribution of H^+ excretion to acid-base regulation when compared with HCO_3^- accumulation. The two models and the relative contribution of H^+ excretion may have implications for energy cost, for example through differential reliance on ATP-dependent transporters.

6.1.2 Potential Costs of Acid-base Regulation

One of the major repercussions that chronically elevated HCO_3^- may have on fishes has come to light in the last 6 years. Phil Munday and colleagues have catalogued numerous behavioural and olfactory effects in ecologically and economically important coral reef fish species under high CO_2 and have attributed these changes to altered neurotransmission as a result of elevated extracellular HCO_3^- and reduced Cl^-. Fish tested under both laboratory conditions (Munday et al., 2013) and in the environment at naturally high CO_2 seeps (Munday et al., 2014) showed behavioural changes and olfactory impairments characterized by attraction to predator cues, increased boldness and unsuitable habitat selection. Nilsson et al. (2012) describe how changes to the intracellular ion status of one of the main inhibitory receptors within the vertebrate brain, GABA-A, during acid-base regulation (increase in $[HCO_3^-]$ and decrease in $[Cl^-]$), may induce excitation of nerve transmission rather than its inhibition during stimulation

of the receptor with GABA. The same disruption may also be responsible for changes in auditory preference found in clownfish (*Amphriprion percula*) under ocean acidification, in the absence of changes to otolith growth (Simpson et al., 2011). As such, one of the primary impacts of chronic acid-base regulation under ocean acidification may be the induction of involuntary excitation of nerves that have GABA-A receptors. The resulting shifts in sensory preference and altered behaviours have clear potential for deleterious ecological consequences.

Furthermore, the energetic cost of acid-base regulation (and osmoregulation) is a significant unknown in consideration of the physiological impact of elevated seawater pCO_2. Both of these homeostatic mechanisms rely on ATP-dependent ion transporters and are under increased demand in the face on environmental perturbations in pCO_2. Additionally, the acclamatory changes in regulation of acid-base and osmo- regulation relevant genes under chronic exposure may also affect the energetic cost of these processes to an unknown degree (Deigweiher et al., 2008). What is likely is that chronic acid-base regulation will incur an increased energy cost to the organism. What is not known is the scale of this cost and to what extent it will be of detriment to other physiological processes: particularly those with direct bearing on individual and population success such as growth and reproduction.

In Chapter 5 one of the potential downstream effects of increased energetic cost was considered by measurement of growth rates and food conversion efficiency in sea bass under high CO_2 (1140 µatm). Neither endpoint appeared significantly affected by hypercapnic conditions when compared with control and the conclusion is that any increased energetic cost of regulatory processes was either insufficient to elicit a measurable response to whole organism growth, or was compensated by reduced costs elsewhere (e.g. reduced activity as found for some metals in freshwater fish; Allin & Wilson, 1999, 2000; Sloman & Wilson, 2005). Also, the fact that food consumption was not significantly elevated in any of the treatments suggests that animals were not compensating for increased energy demand through increased consumption and that food availability was not a limiting factor in determining growth rates under any of the treatments. This data is among only a handful of studies to consider the impacts of high CO_2 at realistic near-future partial pressures on growth (Baumann et al., 2012; Munday et al., 2009; Munday et al., 2011) and possibly the only study to consider growth on post-hatch life stages <2000 µatm pCO_2. The implication growth data from the present study is that sea bass aquaculture (where

food is supplied at optimum rates) yields are unlikely to be affected by elevations in pCO_2 in the next century. Furthermore, data from the present study suggests that the influence of environmental contamination from copper (<50 µg/L) is unlikely to have a bearing on maintenance of normal growth rates under normo- or hypercapnic conditions. The implications of these findings are more difficult to interpret for fisheries however. Any, as yet unquantified, reductions in activity employed to offset energetic costs, or detrimental behavioural changes linked to the brain processing of sensory information under chronic acid-base regulation (discussed above) are likely to have considerable effects on species success.

Further research into the potential energetic consequences of chronic increased pressure on acid-base regulatory mechanisms might consider how any altered energy budget may influence energy availability to other processes like reproduction, activity, social behaviour etc. and the extent to which animals may prioritise energy allocation under any imposed energy deficit.

A large portion of the work within this thesis aimed at drawing comparisons between two largely unrelated species that employ very similar acid-base regulatory strategies. Future work might be directed towards assessing the differential impact of high CO_2 on these acid-base regulators when compared with animals that do not (or only partially) regulate their internal pH: termed here 'acid-base conformers'. Many bivalves, such as mussels (*Mytilus galloprovincialis*) do not regulate their coelomic fluid pH and as such are subject to reductions in metabolic rate which are likely responsible for observed reduction in growth rate (Michaelidis et al., 2005). What this type of work highlights is a need to better understand how responses to high CO_2 in acid-base regulators and conformers will differ. This type of information will help make broader inferences on the responses/tolerances of groups of species and better inform various models including commercial projections, local population dynamics and global distributions under ocean acidification.

6.2 Protective Mechanisms

As well as some of the more purely acid-base regulation focussed work. We considered some of the physiological implications of exposure to copper, separately and in combination with high CO_2. The environmental and mechanistic relevance of choosing copper as a co-stressor under decreased water pH and elevated pCO_2 are

discussed in previous chapters. In short this choice revolved around the environmentally relevant effect of decreased water pH on copper speciation (Millero, 2009) and therefore bioavailability/toxicity (Chakoumakos et al., 1979) as well as potential for increased dissolution of legacy copper from sediments (Roberts et al., 2013). This was in addition to a number of similar physiological perturbations induced by copper exposure when compared with high CO_2 (acid-base/osmo-regulation, growth and sensory physiology, which were not considered in this thesis).

Assessment of the susceptibility of both sea bass erythrocytes and crab haemocytes to DNA damage under copper exposure suggested that HCO_3^- accumulated for acid-base regulation may afford some cellular protection. These results were most discernible *in vitro* for sea bass erythrocytes and under low copper concentrations *in vivo* for shore crab haemocytes. The mechanism by which cells are protected is as yet unknown (see Chapter 4 for discussion). Various protective effects under hypercapnia have been reported previously in fish (decreased mortality; Larsen et al. 1997), clams (reduced production of ROS; Ivanina et al. 2013), oysters (reversed suppression of tumour suppressor mRNA production; Götze et al. 2014), as well as rats and piglets (reduced free radical damage; Barth et al., 1998; Vannucci et al., 1997). While these protective effects remain unexplained this study is not the only ones to suggest such a role for HCO_3^-, which was proposed by another author (Vesela & Wilhelm, 2002) as the source of the aforementioned protection in rats and piglets under combined hypoxia/hypercapnia.

The implications of such a protective effect at a cellular level make it difficult to infer what the whole organism repercussions might be. Again, a good starting point for such consideration is the influence it may have on energy expenditure. When challenged with elevated waterborne copper exposure, fish and crustacean copper homeostasis employs a suite of mechanisms to minimise the detrimental effects associated with increased internalised copper. Production of metallothioneins (Roesijadi, 1992), changes in regulation (Casanova et al., 2013) and production of anti-oxidants (Lenartova et al., 1997), as well as increased detoxification and excretion rates (Ahearn et al., 2004; Grosell et al., 2001) may each incur an energetic cost. Putting aside the unavoidable energetic cost for these animals of accumulating HCO_3^- (as discussed), acid-base regulating animals may be afforded some ‘passive’ protection against DNA damage, production of free radical and ROS, and inhibition of anti-oxidants (see Chapter 4). The result may be a less energetically costly response to

copper when compared with the same exposure under normocapnia. Whether there are actual energetic benefits under copper and high CO_2 exposure will be determined by the net balance between energy saved as a result of the protective influence of HCO_3^- versus the energy used in accumulating that HCO_3^-. Either way this again highlights potential differences between acid-base regulators and acid-base conformers. Where acid-base regulating animals may be afforded protection against waterborne copper, those that do not appear to be more susceptible to impacts such as DNA damage (Lewis, 2015 submitted). This is likely a consequence of the low pH of both their coelomic fluid and the surrounding seawater influencing copper bioavailability, accumulation and binding both internally and externally (Ivanina et al., 2013). The result may be to create winners and losers with respect to copper tolerance under future high CO_2 water environments, with one of the primary drivers of the differentiation being acid-base regulating ability. This differentiation is complicated by animals that partially regulate their internal pH. Animals like these, such as some bivalves (Michaelidis et al., 2005; Lewis, 2015 submitted), are not true acid-base regulators like teleosts and most crustaceans, but do accumulate some HCO_3^- through the dissolution of their shells which can lessen the impact of the largely uncompensated acidosis (Michaelidis et al., 2005).

Furthermore, fish and crustaceans like shore crabs may be considerably limited in their ability to acid-base regulate during early life stages: although no research has considered this to-date for obvious technical reasons of the difficulty in sampling and/or measuring extracellular fluid in such small animals. Equally, these early life stages may be differentially vulnerable to copper toxicity during certain developmental stages (Chapman, 1978; McKim et al., 1978). These factors have the potential to create bottlenecks in the tolerance of these ‘protected’ species under copper exposure, possibly affording them far less benefit in terms population success than may at first seem.

Going forward, one of the primary areas of focus for work on the combined impacts of high CO_2 and chemical stressors like metals should be determining whether elevated CO_2 will lessen or exacerbate some of the key physiological impacts. Sensitive biomarkers such as DNA damage that reflect cellular toxicity, and whole organism endpoints such as growth that provide information about energetic cost and condition are both good targets for this kind of research.

Conversely, as has been shown, copper can also influence the ability of aquatic animals to regulate their acid-base status when challenged with high CO_2. Given the considerable implications for the health of the organism resulting from limited or ineffective pH regulation, knowing the concentration of copper at which inhibition occurs is vital for assessing the ability of a given organism to cope with a high CO_2 environment. The copper concentrations required to inhibit this regulation may vary greatly between species, and possibly under differing water conditions.

Finally, one of the important extensions to this direction of research is the assessment of how representative these findings are of other harmful metal contaminants. Speciation and the resulting changes in potential for toxicity under low pH will be metal-specific. For example, there is evidence to suggest that metals like Cd^{2+}, respond in the opposite way to copper under high CO_2 with respect to bioavailability, uptake and toxicity (Ivanina et al., 2013). As such, information on toxicity, protective effects, dose-responses, uptake and accumulation on different metals should ultimately work towards informing the development of a marine-orientated model, similar in nature to the biotic ligand model used widely for freshwater ecotoxicology, for predicting the physiological effects of metals under the physicochemical water parameters predicted under ocean acidification.

6.3 Carbonate Production

The assessment of carbonate production in juvenile sea bass throughout the duration of the growth study (28 days) was somewhat of an aside from the main focus of the project. The data it yielded however proved very interesting. Although high CO_2 (and/or copper) showed no measurable impact on carbonate production, as might be predicted (Grosell et al., 2005; Wilson et al., 2009), the precise quantification of food consumption enabled us to assess the correlation between food intake and carbonate production. These data yielded clear evidence for a correlation between food intake and calcium production (Chapter 5). Furthermore, given estimates of drinking rates from juveniles of the same species (Varsamos et al., 2004) and the measured composition of the food pellets, the relative contribution of food and drinking to calcium carbonate production in fed fish could be calculated. This is the first quantitative evidence of this relationship for fish feeding on diets that are naturally high in calcium (due to the bonemeal content) and has considerable application to global models of

carbonate production (Wilson et al., 2009). These models previously used estimates derived from metabolic differences between active (fed) and sedentary (unfed) animals (Kerr, 1982) to upscale carbonate production from unfed fish data by a fixed factor (α): 2.5 in Wilson et al. (2009). Reardon et al (unpub.) has subsequently refined that the value of α to within the range 2.77-2.99 (mean: 2.93) using unpublished data collected on fed and unfed fishes. However, in those studies fish were deliberately fed diets with unnaturally low calcium content (e.g. squid muscle, prawns with shells removed). This was to exclude the potential for dietary sources of carbonate to pass through the gut unaltered and become mixed in with the endogenously-produced carbonates excreted by the gut. However, carnivorous fish in the wild, like sea bass, are likely to consume high calcium diets (whole prey containing calcium phosphate endoskeletons or calcium carbonate exoskeletons). Data in the present study on carbonate production from sea bass fed on a commercial pelleted diet that includes bonemeal with a similar calcium content to that of natural fish diets (Chapter 5) is therefore likely to be very relevant to estimating gut carbonate production rates in wild fish populations. The fact that the carbonate production rates in sea bass here were substantially (3.2-fold) higher than unfed sea bass of a comparable size (mean 13.6 g) and temperature (18 °C) will mean that previous models of global fish carbonate production may have grossly underestimated this output from fish. The model previously used (Wilson et al., 2009) is soon to be re-run (Prof. Simon Jennings, UEA and Cefas) using unpublished updated data from a study by Reardon and Wilson, and this will now include the data from Chapter 5 on sea bass fed a high calcium diet. It will be exciting to see the outcome of this revised model and how this may affect the global carbonate budget and its impact on surface ocean chemistry (Wilson et al., 2009).

6.4 References

Ahearn, G., Mandal, P., & Mandal, A. (2004). Mechanisms of heavy-metal sequestration and detoxification in crustaceans: a review. *Journal of Comparative Physiology B, 174*(6), 439-452.

Allin, C. J., & Wilson, R. W. (1999). Behavioural and metabolic effects of chronic exposure to sublethal aluminum in acidic soft water in juvenile rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Fisheries and Aquatic Sciences, 56*(4), 670-678.

Allin, C. J., & Wilson, R. W. (2000). Effects of pre-acclimation to aluminium on the physiology and swimming behaviour of juvenile rainbow trout (*Oncorhynchus mykiss*) during a pulsed exposure. *Aquatic Toxicology, 51*(2), 213-224.

Barth, A., Bauer, R., Gedrange, T., Walter, B., Klinger, W., & Zwiener, U. (1998). Influence of hypoxia and hypoxia/hypercapnia upon brain and blood peroxidative and glutathione status in normal weight and growth-restricted newborn piglets. *Experimental and Toxicologic Pathology, 50*(4), 402-410.

Baumann, H., Talmage, S. C., & Gobler, C. J. (2012). Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. *Nature Climate Change, 2*(1), 38-41.

Burnett, L., & Johansen, K. (1981). The role of branchial ventilation in hemolymph acid-base changes in the shore crab (*Carcinus maenas*) during hypoxia. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 141*(4), 489-494.

Casanova, F. M., Honda, R. T., Ferreira-Nozawa, M. S., Aride, P. H. R., & Nozawa, S. R. (2013). Effects of copper and cadmium exposure on mRNA expression of catalase, glutamine synthetase, cytochrome p450 and Hheat shock protein 70 in Tambaqui Fish (*Colossoma Macropomum*). *Gene Expression to Genetical Genomics, 6*, 1.

Chakoumakos, C., Russo, R. C., & Thurston, R. V. (1979). Toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH, and hardness. *Environmental Science & Technology, 13*(2), 213-219.

Chapman, G. A. (1978). Toxicities of cadmium, copper, and zinc to four juvenile stages of chinook salmon and steelhead. *Transactions of the American Fisheries Society, 107*(6), 841-847.

Claiborne, J. B., Perry, E., Bellows, S., & Campbell, J. (1997). Mechanisms of acid-base excretion across the gills of a marine fish. *Journal of Experimental Zoology, 279*, 509-520.

Deigweiher, K., Koschnick, N., Pörtner, H. -O., & Lucassen, M. (2008). Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. *AJP - Regulatory, Integrative and Comparative Physiology, 295*(5), 1660-1670.

Dyck, A., & Sumaila, U. (2010). Economic impact of ocean fish populations in the global fishery. *Journal of Bioeconomics, 12*(3), 227-243.

Esbaugh, A., Heuer, R., & Grosell, M. (2012). Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. *Journal of Comparative Physiology B, 182*(7), 921-934.

Götze, S., Matoo, O. B., Beniash, E., Saborowski, R., & Sokolova, I. M. (2014). Interactive effects of CO_2 and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. *Aquatic Toxicology, 149*, 65-82.

Grosell, M., McGeer, J. C., & Wood, C. M. (2001). Plasma copper clearance and biliary copper excretion are stimulated in copper-acclimated trout. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 280*(3), R796-R806.

Grosell, M., Wood, C. M., Wilson, R. W., Bury, N. R., Hogstrand, C., Rankin, C., & Jensen, F. B. (2005). Bicarbonate secretion plays a role in chloride and water absorption of the European flounder intestine. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 288*(4), 936-946.

IPCC. (2014). The Physical Science Basis: Working group I contribution to the IPCC 5th assessment report – changes to the underlying scientific/technical assessment.

Ivanina, A. V., Beniash, E., Etzkorn, M., Meyers, T. B., Ringwood, A. H., & Sokolova, I. M. (2013). Short-term acute hypercapnia affects cellular responses to trace metals in the hard clams (*Mercenaria mercenaria*). *Aquatic Toxicology, 140*, 123-133.

Kerr, S. (1982). Estimating the energy budgets of actively predatory fishes. *Canadian Journal of Fisheries and Aquatic Sciences, 39*(3), 371-379.

Larsen, B. K., Pörtner, H. -O., & Jensen, F. B. (1997). Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology, 128*(2), 337-346.

Lenartova, V., Holovska, K., Pedrajas, J. R., Lara, E. M., Peinado, J., Barea, J. L., Kosuth, P. (1997). Antioxidant and detoxifying fish enzymes as biomarkers of river pollution. *Biomarkers, 2*(4), 247-252.

McKim, J., Eaton, J., & Holcombe, G. W. (1978). Metal toxicity to embryos and larvae of eight species of freshwater fish—II: copper. *Bulletin of Environmental Contamination and Toxicology, 19*(1), 608-616.

Michaelidis, B., Ouzounis, C., Paleras, A., & Pörtner, H. -O. (2005). Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels (*Mytilus galloprovincialis*). *Marine Ecology Progress Series, 293*(2), 109-118.

Millero, F. J. (2009). Effect of ocean acidification on the speciation of metals in seawater. *Oceanography, 22*(4), 72.

Munday, P. L., Cheal, A. J., Dixson, D. L., Rummer, J. L., & Fabricius, K. E. (2014). Behavioural impairment in reef fishes caused by ocean acidification at CO_2 seeps. *Nature Climate Change, 4*(6), 487-492.

Munday, P. L., Donelson, J. M., Dixson, D. L., & Endo, G. G. K. (2009). Effects of ocean acidification on the early life history of a tropical marine fish. *Proceedings of the Royal Society B: Biological Sciences, 276*(1671), 3275-3283.

Munday, P. L., Gagliano, M., Donelson, J. M., Dixson, D. L., & Thorrold, S. R. (2011). Ocean acidification does not affect the early life history development of a tropical marine fish. *Marine Ecology Progress Series, 423*, 211-221.

Munday, P. L., Pratchett, M. S., Dixson, D. L., Donelson, J. M., Endo, G. G., Reynolds, A. D., & Knuckey, R. (2013). Elevated CO_2 affects the behaviour of an ecologically and economically important coral reef fish. *Marine Biology, 160*(8), 2137-2144.

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sorensen, C., Watson, S.A., & Munday, P. L. (2012). Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change, 2*(3), 201-204.

Paquin, P. R., Gorsuch, J. W., Apte, S., Batley, G. E., Bowles, K. C., Campbell, P. G. C., Wu, K. B. (2002). The biotic ligand model: a historical overview. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, 133*(1-2), 3-35.

Roberts, D. A., Birchenough, S. N., Lewis, C., Sanders, M. B., Bolam, T., & Sheahan, D. (2013). Ocean acidification increases the toxicity of contaminated sediments. *Global Change Biology, 19*(2), 340-351.

Roesijadi, G. (1992). Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology, 22*(2), 81-113.

Sloman, K. A., & Wilson, R. W. (2005). Anthropogenic impacts upon behaviour and physiology. *Fish physiology, 24*, 413-468.

Sumaila, U. R., Cisneros-Montemayor, A. M., Dyck, A., Huang, L., Cheung, W., Jacquet, J., & Pauly, D. (2012). Impact of the Deepwater Horizon well blowout on the economics of US Gulf fisheries. *Canadian Journal of Fisheries and Aquatic Sciences*, *69*(3), 499-510.

Truchot, J.-P. (1986). Changes in the haemolymph acid-base state of the shore crab, (*Carcinus maenas*), exposed to simulated tidepool conditions. *The Biological Bulletin, 170*(3), 506-518.

Truchot, J.-P. (1979). Mechanisms of the compensation of blood respiratory acid - base disturbances in the shore crab, *Carcinus maenas* (L.). *Journal of Experimental Zoology, 210*(3), 407-416.

Vannucci, R. C., Brucklacher, R. M., & Vannucci, S. J. (1997). Effect of carbon dioxide on cerebral metabolism during hypoxia-ischemia in the immature rat. *Pediatric Research, 42*(1), 24-29.

Varsamos, S., Bonga, S. W., Charmantier, G., & Flik, G. (2004). Drinking and Na^+/K^+ -ATPase activity during early development of European sea bass, *Dicentrarchus labrax*: ontogeny and short-term regulation following acute salinity changes. *Journal of Experimental Marine Biology and Ecology, 311*(2), 189-200.

Vesela, A., & Wilhelm, J. (2002). The role of carbon dioxide in free radical reactions in organism. *Physiological research, 51*(4), 335-340.

Wang, T., Knudsen, P., Brauner, C., Busk, M., Vijayan, M., & Jensen, F. (1998). Copper exposure impairs intra-and extracellular acid-base regulation during hypercapnia in the fresh water rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B, 168*(8), 591-599.

Wilson, R., Millero, F., Taylor, J., Walsh, P., Christensen, V., Jennings, S., & Grosell, M. (2009). Contribution of fish to the marine inorganic carbon cycle. *Science, 323*(5912), 359-362.

Zimmer, A. M., Barcarolli, I. F., Wood, C. M., & Bianchini, A. (2012). Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. *Aquatic Toxicology, 122–123*(0), 172-180.

APPENDICES

Appendix 1: Manuscript “Ocean acidification increases copper toxicity differentially in two key marine invertebrates with distinct acid-base responses” – written by Dr. Ceri Lewis.

Enclosed is a copy of a manuscript currently in submission. This study considers a number of endpoints similar to the work conducted in chapters 3 and 4 of this thesis. It provides support for the assertions/predictions made in the thesis for widely differing effects under combined high CO_2 and copper exposure between acid-base regulators and acid-base ‘conformers’, as discussed in Chapters 3, 4 and General Discussion.

Contribution to the following paper involved assistance in the running of the experiments and collection of data.

The study was led by Dr. Ceri Lewis (University of Exeter), who also wrote the following manuscript. Details of others contributors to the study are contained within the paper itself.

Ocean acidification increases copper toxicity differentially in two key marine invertebrates with distinct acid-base responses.

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Abstract

Ocean acidification (OA) is expected to indirectly impact biota living in contaminated coastal environments by altering the bioavailability and potentially toxicity of many pH-sensitive metals. Here, we show that OA (pH 7.71; pCO_2 1480 µatm) significantly increases the toxicity responses to a global coastal contaminant (copper ~0.1 µM) in two keystone benthic species; mussels (*Mytilus edulis*) and purple sea urchins (*Paracentrotus lividus*). Mussels showed an extracellular acidosis in response to OA and copper individually which was enhanced during combined exposure. In contrast, urchins maintained extracellular fluid pH under OA by accumulating bicarbonate but exhibited a slight alkalosis in response to copper either alone or with OA. Importantly, copper-induced damage to DNA and lipids was significantly greater under OA compared to control conditions (pH 8.14; pCO_2 470 µatm) for both species. However, this increase in DNA-damage was four times lower in urchins than mussels, suggesting that internal acid-base regulation in urchins may substantially moderate the magnitude of this OA-induced copper toxicity effect. Thus, changes in metal toxicity under OA may not purely be driven by metal speciation in seawater and may be far more diverse than either single-stressor or single-species studies indicate. This has important implications for future environmental management strategies.

Introduction:

Ocean acidification (OA), the drop in ocean pH associated with increasing levels of carbon dioxide in the atmosphere and hence the oceans, is now widely considered to be one of the most pervasive human impacts on global marine biodiversity (Gattuso et al., 2015; Halpern et al., 2008). Recent projections published as part of the Representative Concentration Pathways (RCP) database suggest that atmospheric pCO_2 levels may exceed 1000 µatm early in the next century (RCP 8.5)(Bopp et al., 2013) causing the average pH of the world's surface waters to drop by as much as 0.43 units to around 7.73 (Bopp et al., 2013; IPCC, 2013). There is now a wealth of evidence that this change in ocean carbonate chemistry has the potential to impact upon the health and physiology of a wide range of marine invertebrate species (Fabricius et al., 2014; Fabry et al., 2008b; Kroeker et al., 2013; Orr et al., 2005; Pörtner, 2008) with 63 % of echinoderms and 51.6 % of molluscs tested so far showing negative impacts of near-future OA, making them amongst the most sensitive phyla (Wittmann & Pörtner, 2013).

Whilst the physiological impacts of OA for many marine biota have been widely studied, the potential for OA to interact with additional environmental stressors remains poorly understood. Changes in ocean carbonate chemistry are happening against a background of additional anthropogenically driven changes such as warming, sea level rise, increasing hypoxic and anoxic zones and chronic coastal pollution. The urgent need for 'multi-stressor' studies is now widely acknowledged by the OA community (Crain et al., 2008) but to date such studies have tended to focus on combining OA with either temperature, salinity or hypoxia (Byrne & Przeslawski, 2013; Przeslawski et al., 2015; Schram et al., 2014). Of particular concern for

environmental monitoring purposes is the potential for the predicted changes in ocean pH to alter the behaviour and bioavailability of historical and chronic coastal contaminants, such as metals (Hoffmann et al., 2012; Ivanina et al., 2013; Millero et al., 2009).

Metals are one of the most common types of coastal contaminant globally and are found in high concentrations in the waters and sediments of many coastal and estuarine systems (Bryan & Langston, 1992; Schiff et al., 2007). For example, concentrations of total dissolved copper in U.K. coastal and estuarine waters can range from chronic low levels of 0.004 µM (Jones & Bolam, 2007) to as high as 1.61 µM in highly contaminated habitats (Bryan & Gibbs, 1983). OA is expected to alter the bioavailability of waterborne metals (Millero et al., 2009) as a result of changes in their speciation in seawater, driven by the declining pH. The toxic free-ion concentration of copper (Cu^{2+}) is predicted to increase by 115 % in coastal waters in the next 100 years due to reduced pH (Pascal et al., 2010; Richards et al., 2011), while the free-ion concentration of other metals including cadmium (Cd) may decrease or be unaffected (Labarthe et al., 2012; Labarthe et al., 2011; Pascal et al., 2010). Increased metal accumulation under near-future OA conditions has been demonstrated for two bivalves species so far (Goetze et al., 2014) and for marine organisms exposed to the same nominal concentrations of any metal, greater metal toxicity effects would be predicted under near-future OA where reduced seawater pH increases free ion availability.

Whilst many transition metals including copper are essential for biological functions, elevated levels can overwhelm an organism's antioxidant defences and induce

oxidative damage of cellular components such as lipids, proteins and DNA via the production of reactive oxygen species (Stohs & Bagchi, 1995). In addition copper is known to exert a number of physiological impacts that are similar to those observed in response to exposures to OA conditions, such as growth reduction, disturbance of acid-base and osmotic regulation, and enzyme inhibition (Parveen & Javed, 2010; Wang et al., 1998). Marine animals acutely subjected to seawater with elevated pCO_2 experience a corresponding extracellular acidosis (Pörtner, 2008; Widdicombe & Spicer, 2008). Many fish and crustaceans are able to regulate these acid-base perturbations by the elevation of extracellular bicarbonate ions (HCO_3^-) whilst other invertebrates, such as mussels and some urchin species, are generally considered to be less able to acid-base regulate (Collard et al., 2013; Gazeau et al., 2013). Furthermore, copper has been demonstrated to inhibit carbonic anhydrase, a vital enzyme for acid-base regulation and identified as an enzyme of interest for OA physiological studies (Hofmann et al., 2008). These overlapping physiological and toxicity effects of OA and copper pollution suggest the potential for additional interactions in the responses of an organism to both stressors when exposed in combination on top of those driven purely by the metal speciation changes. Understanding these potential interactions is vital for understanding the impact of OA on coastal metal contamination and its impacts on both commercially and ecologically important biota.

We examined a suite of physiological and toxicity responses to combined OA and copper (0.1 µM) exposures in two ecologically important marine invertebrates with known sensitivities to OA as a single stressor. The common mussel *Mytilus edulis* is both an economically important shellfish species and provides a key ecosystem

service, forming important substratum for many epibionts and influencing ecosystem functioning via their role in nutrient and mineral cycling (Gazeau et al., 2013). The sea urchin *Paracentrotus lividus* is an ecologically important herbivore in coastal benthic habitats with additional economic importance as a food source. We used a simple factorial design to test the hypothesis that OA increases the toxicity response to copper of these two key benthic invertebrates.

Results and Discussion:

Acid-base responses differed between mussels and urchins:

The ability to compensate for OA-induced changes in extracellular pH is believed to be a key determinant of an organisms' ability to tolerate near-future OA (Melzner et al., 2009; Wittmann & Pörtner, 2013). Interestingly, we found very different acid-base responses between mussels and urchins to both the OA conditions and the copper exposures. In mussels, haemolymph pCO_2 levels increased slightly but non-significantly with exposure to OA alone, increased further with exposure to copper alone and showed the greatest increase in pCO_2 in the combined OA-copper exposures (Fig. 1a; two-way GLM model for OA $F_{1,39} = 6.60$, $P = 0.014$; for copper $F_{1,39} = 26.73$, $P < 0.001$; interaction term $F_{1,39} = 1.32$, $P = 0.258$). Haemolymph bicarbonate levels (HCO_3^-) in mussels showed a similar pattern of change in response to this elevated pCO_2 (Fig. 1b), with a small but significant increase of ~0.2 mM under OA conditions and about double this increase when exposed to copper alone (Fig. 1b; two-way GLM model for OA $F_{1,39} = 9.75$, $P = 0.004$; for copper $F_{1,39} = 26.57$, $P < 0.001$; interaction term $F_{1,39} = 1.95$, $P = 0.171$). Combined exposure to OA and copper caused a further increase in haemolymph bicarbonate level of ~ 1 mM. These pCO_2 changes

combined with limited bicarbonate responses drove a slight but non-significant acidosis of the haemolymph in mussels exposed to OA conditions, whilst copper induced a stronger and significant acidosis of haemolymph from 7.56 to 7.43 (Fig. 1c). The combined OA and copper exposure caused an even greater acidosis reducing haemolymph pH to 7.33, however statistical analysis revealed this was not an interactive effect (Fig. 1c; two-way GLM model for OA $F_{1,39} = 3.74$, $P = 0.061$; for copper $F_{1,39} = 15.58$, $P < 0.001$; interaction term $F_{1,39} = 0.34$, $P = 0.562$).

The increased haemolymph pCO_2 in the combined OA and copper treatment compared to the OA (no copper) treatment might be explained by changes in mussel ventilation rate as a behavioural mechanism to reduce acidosis, since they appear unable to accumulate substantial bicarbonate as a buffering mechanism. Whilst ventilation rate was not measured here, increased mussel gaping (i.e. greater amount of time ventilating the gills) under reduced seawater pH conditions has been reported elsewhere (Bamber, 1990). Hyperventilation in response to copper may be less likely as it could be counter-productive by increasing exposure of gills to the waterborne metal. Mussels have been shown to reduce the amount of time spent with their shells open in response to exposures to metals such as copper (Fdil et al., 2006). These results support the general consensus that mussels are not good acid-base regulators with a limited ability to buffer their haemolymph using bicarbonate (Gazeau et al., 2013), instead responding to periods of hypercapnia with metabolic suppression or changes in ventilation rate.

Conversely, we found that urchins were able to employ physiological mechanisms to regulate coelomic fluid pH against the CO_2-induced drop in external seawater pH. In

sea urchins gas exchange, i.e. uptake of O_2 and elimination of CO_2, relies solely on a favourable diffusion gradient due to the lack of any active ventilatory mechanism, so is generally considered to be diffusion-limited (Farmanfarmaian, 1966). Despite this inability to regulate internal pCO_2 levels, urchins appear to have varying abilities to acid-base regulate depending on species (Collard et al., 2013; Spicer, 1995; Spicer et al., 2011). In the present experiments with *P. lividus*, we observed a rise in coelomic fluid pCO_2 in both the OA (alone) and OA with copper treatments, but a slight yet significant reduction in pCO_2 caused by copper alone (Fig. 1d; two-way GLM model for OA $F_{1,32} = 35.14$, $P < 0.001$; for copper $F_{1,32} = 6.33$, $P = 0.018$; interaction term $F_{1,32} = 0.78$, $P = 0.385$). Coelomic fluid bicarbonate levels were significantly elevated by > 2 mM in response to this elevated pCO_2 in both OA treatments, whilst exposure to copper alone did not affect bicarbonate levels (Fig. 1e; two-way GLM model for OA $F_{1,32} = 29.25$, $P < 0.001$; for copper $F_{1,32} = 0.07$, $P = 0.793$; interaction term $F_{1,32} = 0.06$, $P = 0.809$).

All *P. lividus* in the present study maintained a coelomic fluid pH between 7.60 and 7.76, independent of exposure to OA conditions or elevated copper (Fig. 1f; two-way GLM model for OA $F_{1,32} = 0.56$, $P = 0.461$; for copper $F_{1,32} = 9.31$, $P = 0.005$; interaction term $F_{1,32} = 1.83$, $P = 0.186$), most likely attributed to the 48 % and 55 % increase, respectively, in the coelomic fluid bicarbonate levels in the two OA treatments buffering against the effect of the increased pCO_2. Increased protein concentrations of coelomic fluid have also been suggested to play a role in this buffering capacity(Bookbinder & Shick, 1986) however this was not evident in our data (see below). This agrees with previous studies on *P. lividus* which have shown full compensation of coelomic fluid pH at seawater pCO_2 of 1293 µatm (pH of 7.7) and a partial ability to buffer against an

external pCO_2 rise over a wider seawater pCO_2 range (pCO_2 583 - 2364 µatm; pH 8.0 – 7.4) (Catarino et al., 2012; Collard et al., 2013). In contrast, exposing urchins to copper combined with OA caused a slight but significant alkalosis of their coelomic fluid. This corresponded to slightly lower coelomic fluid pCO_2 in the two copper treatments (alone and combined with OA) compared to their corresponding treatments without copper (control and OA).

These differences in acid-base responses between mussels and urchins to OA and copper exposures can be illustrated using Davenport diagrams (Fig. 2a and 2b). In mussels the decrease in haemolymph pH due to OA exposure is increased by the additional presence of copper with very little compensation from the elevation of bicarbonate ions. The pattern observed reflects a primarily respiratory acidosis of varying severity caused by the treatments. In urchins under OA conditions (with or without copper) the coelomic fluid pH is buffered by additional bicarbonate (i.e. a fully compensated respiratory acidosis) whilst copper caused a slight mixed respiratory/metabolic alkalosis. Our contrasting findings for mussels and urchins, and the contrasting findings of other studies across a number of aquatic species to similar copper concentrations (Bielmyer et al., 2005; Boitel & Truchot, 1990; Spicer &. Weber, 1992), suggest highly species-specific and concentration-dependent acid-base responses to copper.

Oxidative stress responses also differed between mussels and urchins:

Superoxide dismutase (SOD) is an important cytosolic anti-oxidant enzyme. SOD activity has been shown to be induced in a number of marine invertebrate species in response to a range of environmental metals including copper (Geracitano et al., 2002;

Rhee et al., 2011), with inhibition being reported for exposures using higher concentrations (Jing et al., 2006; Maria & Bebianno, 2011). After 14 days exposure to our four treatments, SOD activity in mussel haemolymph showed no significant changes in response to any of these treatments, (Fig. 3a, two-way GLM model for copper: $F_{1,39} = 2.57$, $P = 0.118$; for OA: $F_{1,39} = 3.15$, $P = 0.084$; interaction term $F_{1,39} = 1.73$, $P = 0.198$). There was an average ~ 2 fold increase in SOD activity in response to copper alone compared to the treatments with no copper, however this response varied between individuals such that there was no significant difference overall. In urchins, however, SOD activity was significantly increased following exposure to copper under both ambient and OA conditions (Fig. 3b; two-way GLM model for copper $F_{1,32} = 7.07$, $P = 0.013$). OA conditions had no effect on extracellular fluid SOD activity in urchins (Fig. 3a; two-way GLM model for OA $F_{1,32} = 0.05$, $P = 0.484$; interaction term $F_{1,32} = 0.08$, $P = 0.777$).

Lipid peroxidation was significantly induced by exposure to copper in mussels under both ambient and OA conditions (Fig. 3c, two-way GLM model, for copper: $F_{1,39} = 17.77$, $P < 0.001$). There was no additional increase in lipid peroxidation when OA and copper were combined (two-way GLM model, for OA: $F_{1,39} = 0.22$, $P = 0.640$; interaction term $F_{1,39} = 0.05$, $P = 0.486$). Urchins again showed a different response, with no increase in lipid peroxidation induced by exposure to copper alone, most likely due to the protective effect of the increased SOD activity. A significant increase in lipid peroxidation was, however, induced by copper when exposed under OA conditions, despite the higher SOD levels (Fig. 3d; two-way GLM model for copper $F_{1,32} = 0.54$, $P = 0.467$; for OA $F_{1,32} = 11.19$, $P = 0.002$). No significant interaction term was present (interaction OA x copper $F_{1,32} = 3.59$, $P = 0.068$). This suggests a much greater toxicity

effect of copper on lipids under the combined treatments that overwhelms the anti-oxidant defence capabilities in urchins in the combined OA and copper treatment.

Protein levels, measured as part of both the SOD and TBARS assays using the Bradford assay, were found to be ~20% lower in haemolymph/ coelomic fluid in response to copper exposure in both mussels and urchins independent of OA treatment. Mussels in the ambient pH and OA treatments were found to have haemolymph protein levels of 3.17 and 3.44 mg protein ml^{-1}. Protein levels were significantly lower at 2.41 and 2.73 mg protein ml^{-1}, respectively, in the treatments with the addition of copper (two-way GLM model for copper $F_{1,30} = 8.26$, $P = 0.007$). In urchins protein levels were generally slightly lower with 1.84 and 1.97 mg protein ml^{-1} in the ambient pH and OA treatments. The additional presence of copper lowered these levels to 1.46 and 1.56 mg protein ml^{-1}, respectively, but this was not a significant effect (two-way GLM model for copper: $F_{1,30} = 3.45$, $P = 0.073$).

DNA Damage was increased under OA in both species

Elevated copper induces DNA damage in the form of single strand breaks by the production of reactive oxygen species via the Fenton reaction and by base modifications such as 8-OHdG (a major product of DNA oxidation) (Stohs & Bagchi, 1995). In mussels copper-induced DNA damage was only observed in the combined OA and copper exposures, with no increase in DNA damage caused by exposure to copper alone (Figure 3e). Combined exposure of adult mussels to both OA and copper in combination, however, resulted in a 1.9-fold increase in DNA damage from 14 % DNA damage in the ambient (pH 8.1, no copper) treatment to 27 % in the combined exposures. Statistical analysis reveals a significant interaction term between OA

conditions and the presence of copper on DNA fragmentation (Two-way GLM model, for OA $F_{1,39}$ = 12.54, P < 0.001; for copper $F_{1,39}$ = 8.25, P = 0.007; interaction term $F_{1,39}$ = 23.17, P < 0.001).

This indicates that mussels are able to cope with this low concentration of copper under the ambient pH/pCO_2 conditions, either via their antioxidant defences preventing damage occurring in the first place or via efficient DNA repair activities. This does not hold true under OA conditions. The strong increase in DNA damage when exposed to copper under OA conditions has two likely explanations; a) the lack of anti-oxidant response (SOD) to the copper under OA conditions, and/or b) the increased availability and presumably uptake of the toxic free Cu^{2+} ion under the reduced seawater pH of OA conditions.

In urchins both OA and copper exposure were found to significantly affect DNA damage in coelomocytes. Exposure to copper induced a significant increase in DNA damage in both the pH 8.1 and pH 7.7 treatments, but as with mussels, DNA damage was significantly higher again in the OA treatment (Fig. 3f two-way GLM model for copper $F_{1,32}$ = 138.12, P < 0.001; for OA $F_{1,32}$ = 23.81, P = 0.038). No significant interaction term between copper and OA was found ($F_{1,32}$ = 4.71, P = 0.104). This suggests that whilst increased SOD levels appear to provide some protection against lipid peroxidation it has not prevented copper-induced DNA damage from occurring in the urchin coelomocytes. This toxicity effect of copper on DNA is then significantly greater under OA conditions.

The urchins' acid-base regulation strategy of increasing extracellular bicarbonate levels may actually act 'protectively' against copper toxicity and explain the overall reduced toxicity driven by the difference in the DNA damage response to combined copper and OA that we observed between mussels and urchins. It has been widely shown that copper is less toxic to freshwater species in hard water (i.e. higher alkalinity) than in soft water (Flemming & Trevors, 1989). A protective effect of hypercapnia on copper toxicity has also been suggested by the work of Larsen et al.(1997) in the cod *Gadus morhua*. Free copper (II) ions (Cu^{2+}) will form a range of complexes with bicarbonate ions such that the amount of free Cu^{2+} is only a small fraction of the total copper present in a high bicarbonate solution, with the relative concentrations of copper species varying with pH (Stiff, 1971). Elevated bicarbonate levels should therefore reduce the proportion of the toxic free copper ions and so reduce the amount that is bioavailable to cause damage. Whilst the damage to urchin's lipids did not follow the same pattern as for DNA, this might be explained by their different positions within the cell, with the TBARS assay measuring mostly damage to lipids on the outside of cell membranes whilst DNA is within the cell nucleus, perhaps making lipids more susceptible to damage by the remaining free Cu^{2+} present. More detailed biochemical studies of the precise mechanisms of this damage would be required to fully explain these differences. A hypothesis resulting from this work requiring further testing could be that species which regulate their extracellular pH in response to OA by the elevation of internal bicarbonate levels will show reduced overall copper toxicity responses compared to those which are unable to acid-base regulate using bicarbonate.

This increased copper toxicity as a result of OA conditions in both test species is consistent with two other studies looking at metal-induced DNA damage under near-future OA. Roberts et al. (2013) found that DNA damage in the sediment dwelling amphipod *Corophium volutator* exposed to naturally contaminated sediments, which contained a range of metals and polycyclic aromatic hydrocarbons (PAHs), was higher under OA conditions than extant pCO_2 conditions (Roberts et al., 2013). More recently an increase in copper-induced DNA damage in the sperm of the polychaete *Arenicola marina* was reported when worms were exposed to copper under elevated pCO_2 (Campbell et al., 2014). Synergistic toxicities have been reported between OA and copper in adults of the copepod *Amphiascoides atopus* (Pascal et al., 2010), where the LC_{50} for copper was reduced from 0.65 mg l^{-1} at pH 8.1 to 0.32 mg l^{-1} at a CO_2-driven reduction of pH of 6.5. Increased copper toxicity under OA has also been reported for larvae of the polychaete worms *Pomatoceros lamarckii* (Lewis et al., 2013) and *Arenicola marina* (Campbell et al., 2014). Larvae are often considered to be the most sensitive life history stages to environmental stressors, particularly in free spawning marine invertebrates with bi-phasic life histories. We have now demonstrated that this increased toxicity under experimental OA can also be found in adult marine invertebrates at environmentally relevant concentrations.

Conclusions:

Our data has clearly shown that near-future OA significantly increases the sub-lethal toxicity responses of two key coastal marine invertebrates to coastally relevant concentrations of the common metal pollutant copper. We found that for both mussels (*Mytilus edulis*) and urchins (*Paracentrotus lividus*) copper-induced damage to DNA was significantly greater when animals were exposed to nominal 0.1 µM copper under OA (high pCO_2/ low pH) conditions compared with animals exposed under extant pCO_2 levels. This relative increase in copper-induced DNA damage under OA was four-times greater in mussels than in urchins (despite the measured seawater copper concentrations actually being lower in the mussel exposures than for the urchins). In mussels OA seemed to suppress the response of the anti-oxidant enzyme SOD to copper exposures, whilst in urchins lipid peroxidation was also increased when exposed to copper under OA. So whilst copper-induced toxicity was clearly increased in both species under near-future OA, there were slight differences in the biochemical details of how the two species responded.

Demonstrating an increase in the toxicity of copper in two ecologically and economically important coastal invertebrates under near future OA is a significant cause for concern given the widespread nature of coastal metal contamination. This is particularly the case given the relatively low concentrations of this coastal contaminant used in the present study, which are relevant to measurements of copper contamination for UK coastal waters generally and which are often exceeded in contaminated locations globally (Bryan & Gibbs, 1983; Jones & Bolam, 2007). Furthermore, the changes in seawater copper speciation expected with a reduction in seawater pH will be compounded by an increase in the total copper released from

sediments under OA conditions (Millero et al., 2009). Subsequently the OA-induced increase in bioavailable copper is expected to be two-fold. We cannot fully determine whether these interactions between OA and copper are additive (the sum of the individual stressor effects) or synergistic interactions (greater than the sum of the individual stressor effects) due to the limited number of copper concentrations or seawater pH levels used here. However, the strong interaction terms in the analysis for some of the end points measured here are suggestive that synergies are likely to be present. Recent meta-analysis studies have shown that synergistic interactions between multiple abiotic stressors in the marine environment are more common than additive interactions (Crain et al., 2008; Przeslawski et al., 2015). This highlights a real need for the potential for interactions between climate driven stressors and coastal pollution to be studied in much greater detail rather than relying on predictive modelling approaches for environmental risk assessments.

Our results have clear implications for ecotoxicological assessments, highlighting a need to better understand how OA will alter the behaviour and toxicity of not just copper or metals generally but a wider range of environmental contaminants. Such knowledge is vital for those charged with protecting our marine habitats in order to provide justifiable predictions of OA impacts in coastal regions. In demonstrating a contrasting toxicity response in two ecologically important marine invertebrate species, related to acid-base regulation, we show that organismal responses may be far more diverse than either single stressor or single species studies have previously indicated. Furthermore, our results imply that not only may we be under-estimating OA impacts for coastal invertebrates exposed to chronic metal pollution, but also that OA may impact a much wider range of species, not traditionally considered as OA

sensitive, due to this indirect effect on metal toxicity. This suggests that further investigations on OA-pollutant interactions in a wider range of organisms will be important to better understand the near future impact of toxic coastal contaminants for marine organisms globally.

Methods:

Adult *Paracentrotus lividus* specimens (4 – 6 cm diameter) were purchased from Dunmannus Sea Farm Ltd. in Cork, Ireland. Adult *Mytilus edulis* specimens were collected by hand from the intertidal range of the River Exe estuary, Exmouth, Devon, UK. Individuals were left for 7 days in 30 litre holding tanks at 15 °C in ambient artificial seawater (pH 8.1, 470 µatm pCO_2, salinity = 35) to acclimatise prior to the exposures. Ten individuals per treatment were exposed to one of the following four treatments for 14 days at 15 °C; (1) ambient conditions (pH 8.1) with no added copper; (2) ambient conditions (pH 8.1) with 0.1 µM copper sulphate added; (3) OA conditions (pH 7.7) with no added copper; (4) OA conditions (pH 7.7) with 0.1 µM copper sulphate added.

Seawater pH values of 7.7 were targeted to represent near-future OA treatments as projected according to scenario RCP 8.5 and the 2013 IPCC WGI AR5 (IPCC, 2013; Meinshausen et al., 2011); full seawater chemistry is provided in Tables 1 and 2. Seawater pH in the OA conditions was nominally maintained at pH 7.7 (to a resolution of 0.05 units) using pH computers (Aqua Medic, Bissendorf, Germany) which controlled the release of CO_2 gas directly into the header tanks. Partial water changes (50 %) were carried out every 48 hours using temperature equilibrated water of the correct pH and CO_2 level and copper concentrations were re-dosed appropriately. Water samples were taken every third day for measurements of dissolved inorganic

carbon (DIC) and metals. Seawater DIC analysis was carried out as described in detail in Lewis et al. (2013) using a custom built system and using Dickson seawater standards (Dickson 1990). Copper concentrations in seawater were determined using ICP-MS.

Following the 14 day exposure samples of extracellular fluid (haemolymph for mussels and coelomic fluid for urchins) were collected from each individual. Extracellular fluid for TCO_2 analysis was stored in 100 µl hematocrit (micro capillary) tubes sealed with paraffin oil and the Critoseal^{TM} capillary tube sealant (Fisher) then analysed using a Corning 965 CO_2 analyser (Corning Ltd., UK). Extracellular fluid for oxidative stress assays was snap frozen in liquid nitrogen and stored at -80 °C until analysis. Immediately following extraction extracellular fluid pH was measured at 15 °C using a pH meter (Hannah Instruments HI 8314) and microprobe (Cole Parmer, Accumet) calibrated prior to usage using pH_{NBS} 7.00 and 10.00 specific buffers (calibrated to 7.04 and 10.11 at 15 °C). 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).

Superoxide dismutase (SOD) activity was measured using the nitroblue tetrazolium (NBT) microplate assay (Parry & Pipe, 2004). Lipid peroxidation was determined using the thiobarbituric acid reactive substances (TBARS) assay (Camejo et al., 1999) which quantifies malondialdehyde (MDA), a secondary product of lipid peroxidation, via its reaction with thiobarbituric acid. DNA damage in haemocytes/ coelomocytes was measured as single strand DNA breaks using the comet assay. Two hundred

microlitres of haemolymph/ coelomic fluid from each individual was used immediately after sampling for the comet assay according to the methods described by Lewis & Galloway (2008), using alkaline conditions at 5 °C.

Data were analysed using 2-way analysis of variance (ANOVA) general linear models with the fixed factors; ‘pH’ and ‘copper concentration’.

References:

1 Halpern, B. S. *et al.* A global map of human impact on marine ecosystems. *Science* **319**, 948-952.

2 Gattuso, J. P. *et al.* Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. *Science* **349**, 45-55 (2015).

3 Bopp, L. *et al.* Multiple stressors of ocean ecosytems in the 21st century: projections with CMIP5 models. *Biogeosciences* **10**, 6225-6245 (2013).

4 IPCC. Climate Change 2013: The Physical Science Basis. Working Group I Contribution to the IPCC 5th Assessment Report – Changes to the Underlying Scientific/Technical Assessment.

5 Fabry, V. J., Seibel, B. A., Feely, R. A. & Orr, J. C. Impacts of ocean acidification on marine fauna and ecosystem processes. *Ices Journal of Marine Science* **65**, 414-432.

6 Kroeker, K. J. *et al.* Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. *Global Change Biology* **19**, 1884-1896.

7 Orr, J. C. *et al.* Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681-686.

8 Fabricius, K. E., De'ath, G., Noonan, S. & Uthicke, S. Ecological effects of ocean acidification and habitat complexity on reef-associated macroinvertebrate communities. *Proc. R. Soc. B-Biol. Sci.* **281**.

9 Pörtner, H.O. Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Marine Ecology Progress Series* **373**, 203-217.

10 Wittmann, A. C. & Pörtner, H.-O. Sensitivities of extant animal taxa to ocean acidification. *Nature Climate Change* **3**, 995-1001.

11 Crain, C. M., Kroeker, K. & Halpern, B. S. Interactive and cumulative effects of multiple human stressors in marine systems. *Ecology Letters* **11**, 1304-1315.

12 Byrne, M. & Przeslawski, R. Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories. *Integrative and Comparative Biology* **53**, 582-596.

13 Schram, J. B., Schoenrock, K. M., McClintock, J. B., Amsler, C. D. & Angus, R. A. Multiple stressor effects of near-future elevated seawater temperature and decreased pH on righting and escape behaviours of two common Antarctic gastropods. *Journal of Experimental Marine Biology and Ecology* **457**, 90-96.

14 Przeslawski, R., Byrne, M. & Mellin, C. A review and meta-analysis of the effects of multiple abiotic stressors on marine embryos and larvae. *Global Change Biology* **21**, 2122-2140.

15 Millero, F. J., Woosley, R., Ditrolio, B. & Waters, J. Effect of ocean acidification on the speciation of metals in seawater. *Oceanography* **22**, 72-85 (2009).

16 Hoffmann, L. J., Breitbarth, E., Boyd, P. W. & Hunter, K. A. Influence of ocean warming and acidification on trace metal biogeochemistry. *Marine Ecology Progress Series* **470**, 191-205.

17 Ivanina, A. V. *et al.* Short-term acute hypercapnia affects cellular responses to trace metals in the hard clams (*Mercenaria mercenaria*). *Aquatic Toxicology* **140**, 123-133.

18 Bryan, G. W. & Langston, W. J. Bioavailability, accumulation and effects of heavy-metals in sediments with special reference to United-Kingdom estuaries - A review. *Environmental Pollution* **76**, 89-131.

19 Schiff, K., Brown, J., Diehl, D. & Greenstein, D. Extent and magnitude of copper contamination in marinas of the San Diego region, California, USA. *Marine Pollution Bulletin* **54**, 322-328.

20 Jones, B. & Bolam, T. Copper speciation survey from UK marinas, harbours and estuaries. *Marine Pollution Bulletin* **54**, 1127-1138.

21 Bryan, G. W. & Gibbs, P. E. Heavy metals in the Fal estuary, Cornwall: A study of long term contamination by mining waste and its effects on estuarine organisms. *Occasional Publications of the Marine Biological Association* **2**, 112.

22 Pascal, P.-Y., Fleeger, J. W., Galvez, F. & Carman, K. R. The toxicological interaction between ocean acidity and metals in coastal meiobenthic copepods. *Marine Pollution Bulletin* **60**, 2201-2208.

23 Richards, R., Chaloupka, M., Sano, M. & Tomlinson, R. Modelling the effects of 'coastal' acidification on copper speciation. *Ecological Modelling* **222**, 3559-3567.

24 Lacoue-Labarthe, T. *et al.* Temperature and pCO_2 effect on the bioaccumulation of radionuclides and trace elements in the eggs of the common cuttlefish, *Sepia officinalis*. *Journal of Experimental Marine Biology and Ecology* **413**, 45-49.

25 Lacoue-Labarthe, T. *et al.* Effects of increased pCO_2 and temperature on trace element (Ag, Cd and Zn) bioaccumulation in the eggs of the common cuttlefish, *Sepia officinalis*. *Biogeosciences* **6**, 2561-2573 (2009).

26 Lacoue-Labarthe, T. *et al.* Effects of ocean acidification on trace element accumulation in the early-life stages of squid (*Loligo vulgaris*). *Aquatic Toxicology* **105**, 166-176.

27 Goetze, S., Matoo, O. B., Beniash, E., Saborowski, R. & Sokolova, I. M. Interactive effects of CO_2 and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. *Aquatic Toxicology* **149**, 65-82.

28 Stohs, S. J. & Bagchi, D. Oxidative mechanisms in the toxicity of metal-ions. *Free Radical Biology and Medicine* **18**, 321-336.

29 Parveen, A. & Javed, M. Effect of water-borne copper on the growth performance of fish *Catla catla*. *International Journal of Agriculture and Biology* **12**, 950-952.

30 Wang, T. *et al.* Copper exposure impairs intra- and extracellular acid-base regulation during hypercapnia in the fresh water rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* **168**, 591-599.

31 Widdicombe, S. & Spicer, J. I. Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? *Journal of Experimental Marine Biology and Ecology* **366**, 187-197.

32 Collard, M. *et al.* Buffer capacity of the coelomic fluid in echinoderms. *Comparative biochemistry and physiology. Part A, Molecular & integrative physiology* **166**, 199-206.

33 Gazeau, F. *et al.* Impacts of ocean acidification on marine shelled molluscs. *Marine Biology* **160**, 2207-2245.

34 Hofmann, G. E., O'Donnell, M. J. & Todgham, A. E. Using functional genomics to explore the effects of ocean acidification on calcifying marine organisms. *Marine Ecology-Progress Series* **373**, 219-225.

35 Melzner, F. *et al.* Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **6**, 2313-2331.

36 Bamber, R. N. The effects of acidic seawater on three species of lamellibranch mollusc. *Journal of Experimental Marine Biology and Ecology* **143**, 181-191.

37 Fdil, M. A. *et al.* Valve movement response of the mussel *Mytilus galloprovincialis* to metals (Cu, Hg, Cd and Zn) and phosphate industry effluents from Moroccan Atlantic coast. *Ecotoxicology* **15**, 477-486.

38 Farmanfarmaian, A. (1966) *Physiology of Echinodermata* (ed R.A. Boolootian) Interscience Publishers.

39 Spicer, J. I., Widdicombe, S., Needham, H. R. & Berge, J. A. Impact of CO_2-acidified seawater on the extracellular acid-base balance of the northern sea urchin *Strongylocentrotus droebachiensis*. *Journal of Experimental Marine Biology and Ecology* **407**, 19-25.

40 Spicer, J. I. Oxygen and acid-base status of the sea urchin *Psammechinus miliaris* during environmental hypoxia. *Marine Biology* **124**, 71-76.

41 Bookbinder, L. H. & Shick, J. M. Anaerobic and aerobic energy-metabolism in ovaries of the sea-urchin *Strongylocentrotus droebachiensis*. *Marine Biology* **93**, 103-110.

42 Catarino, A. I., Bauwens, M. & Dubois, P. Acid-base balance and metabolic response of the sea urchin *Paracentrotus lividus* to different seawater pH and temperatures. *Environmental Science and Pollution Research* **19**, 2344-2353..

43 Bielmyer, G. K., Brix, K. V., Capo, T. R. & Grosell, M. The effects of metals on embryo-larval and adult life stages of the sea urchin, *Diadema antillarum*. *Aquatic Toxicology* **74**, 254-263.

44 Boitel, F. & Truchot, J.-P. Comparative-study of the effects of copper on hemolymph ion concentrations and acid-base-balance in shore crabs *Carcinus maenas* acclimated to full-strength or dilute seawater. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **95**, 307-312.

45 Spicer, J. I. & Weber, R. E. Respiratory impairment by water-borne copper and zinc in the edible crab *Cancer-pagurus* (l) (Crustacea, Decapoda) during hypoxic exposure. *Marine Biology* **112**, 429-435.

46 Rhee, J.-S. *et al.* Expression of superoxide dismutase (SOD) genes from the copper-exposed polychaete, *Neanthes succinea*. *Marine Pollution Bulletin* **63**, 277-286.

47 Geracitano, L., Monserrat, J. M. & Bianchini, A. Physiological and antioxidant enzyme responses to acute and chronic exposure of *Laeonereis acuta* (Polychaeta, Nereididae) to copper. *Journal of Experimental Marine Biology and Ecology* **277**, 145-156.

48 Jing, G., Li, Y., Xie, L. P. & Zhang, R. Q. Metal accumulation and enzyme activities in gills and digestive gland of pearl oyster (*Pinctada fucata*) exposed to copper. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* **144**, 184-190.

49 Maria, V. L. & Bebianno, M. J. Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* **154**, 56-63.

50 Flemming, C. A. & Trevors, J. T. Copper toxicity and chemistry in the environment - a review. *Water Air and Soil Pollution* **44**, 143-158.

51 Larsen, B. K., Pörtner, H. -O. & Jensen, F. B. Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology* **128**, 337-346.

52 Stiff, M. J. Copper/bicarbonate equilibria in solutions of bicarbonate ion at concentrations similar to those found in natural water. *Water Research* **5**, 171.

53 Roberts, D. A. *et al.* Ocean acidification increases the toxicity of contaminated sediments. *Global Change Biology* **19**, 340-351.

54 Campbell, A. L., Mangan, S., Ellis, R. P. & Lewis, C. Ocean acidification increases copper toxicity to the early life history stages of the polychaete *Arenicola marina* in artificial seawater. *Environmental Science and Technology* (2014).

55 Lewis, C., Clemow, K. & Holt, W. V. Metal contamination increases the sensitivity of larvae but not gametes to ocean acidification in the polychaete *Pomatoceros lamarckii* (Quatrefages). *Marine Biology* **160**, 2089-2101.

56 Meinshausen, M. *et al.* The RCP greenhouse gas concentrations and their extensions from 1765 to 2300. *Climate Change* **109**, 213-241.

57 Truchot, J.-P. Carbon dioxide combining properties of blood of shore crab *Carcinus maenas* - carbon dioxide solubility coefficient and carbonic acid dissociation constants. *Journal of Experimental Biology* **64**, 45-57.

58 Parry, H. E. & Pipe, R. K. Interactive effects of temperature and copper on immunocompetence and disease susceptibility in mussels (*Mytilus edulis*). *Aquatic Toxicology* **69**, 311-325.

59 Camejo, G., Wallin, B. & Enojärvi, M. *Free radical and antioxidant protocols. Methods in molecular biology* Vol. 108 (ed Amstrong D) 377–387 (Humana).

60 Lewis, C. & Galloway, T. Genotoxic damage in polychaetes: A study of species and cell-type sensitivities. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis* **654**, 69-75.

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Author Contributions:

CL designed and led the study, CL, RE, KE, EV and SN all contributed to the running of the experiments and the collection and analysis of the data; CL wrote the manuscript; RWW advised on all aspects of design and interpretation and contributed to the manuscript. All authors read and approved the final version of the manuscript.

Additional Information:

Competing financial interests

The authors declare no competing financial interests.

Figure Legends:

Figure 1: Acid-base parameters in the haemolymph of *Mytilus edulis* (a, c, e) and coelomic fluid of *Paracentrotus lividus* (b, d, f) following 14 day exposures to elevated pCO_2 with and without the presence of 0.1 µM copper; (a, b) haemolymph/coelomic fluid pH, (c, d) haemolymph/coelomic fluid bicarbonate concentrations, and (e, f) haemolymph/coelomic fluid pCO_2. [N.B. * represent significant differences]

Figure 2: Davenport diagram illustrating the relationship between pH, bicarbonate and pCO_2 in the haemolymph and coelomic fluid of (a) *Mytilus edulis* and (b) *Paracentrotus lividus* respectively. Lines represent isopleths of equal pCO_2 (mmHg). Position calculated from means ± SEM for haemolymph/coelomic fluid pH and $[HCO_3^-]$ according to pK_1 values calculated from (J. P. Truchot, 1976).

Figure 3: Oxidative stress indicators in the mussel *Mytilus edulis* (a, c, e) and the adult purple urchin *Paracentrotus lividus* (b, d, f) following 14 day exposures to elevated pCO_2 with and without the presence of 0.1 µM copper; (a, b) Activity of the anti-oxidant enzyme superoxide dismutase (SOD) activity; (c, d) lipid peroxidation measured as malondialdehyde (MDA) levels; (e, f) DNA damage, measured as percentage of single strand breaks in haemocytes/coelomocytes.

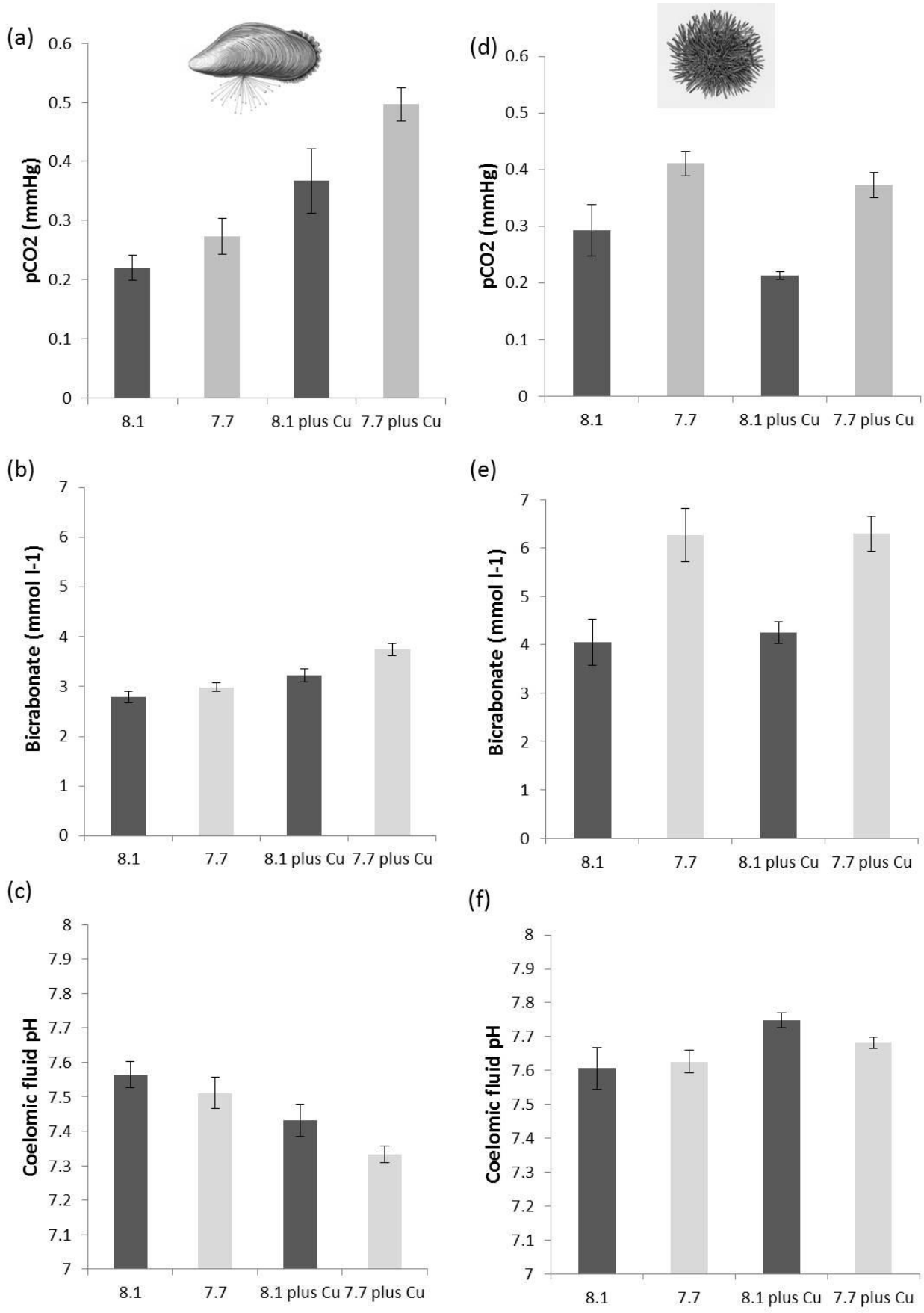
Tables:

Table 1: Seawater carbonate chemistry and copper levels for the four experimental treatment solutions for the *Mytilus edulis* exposures.

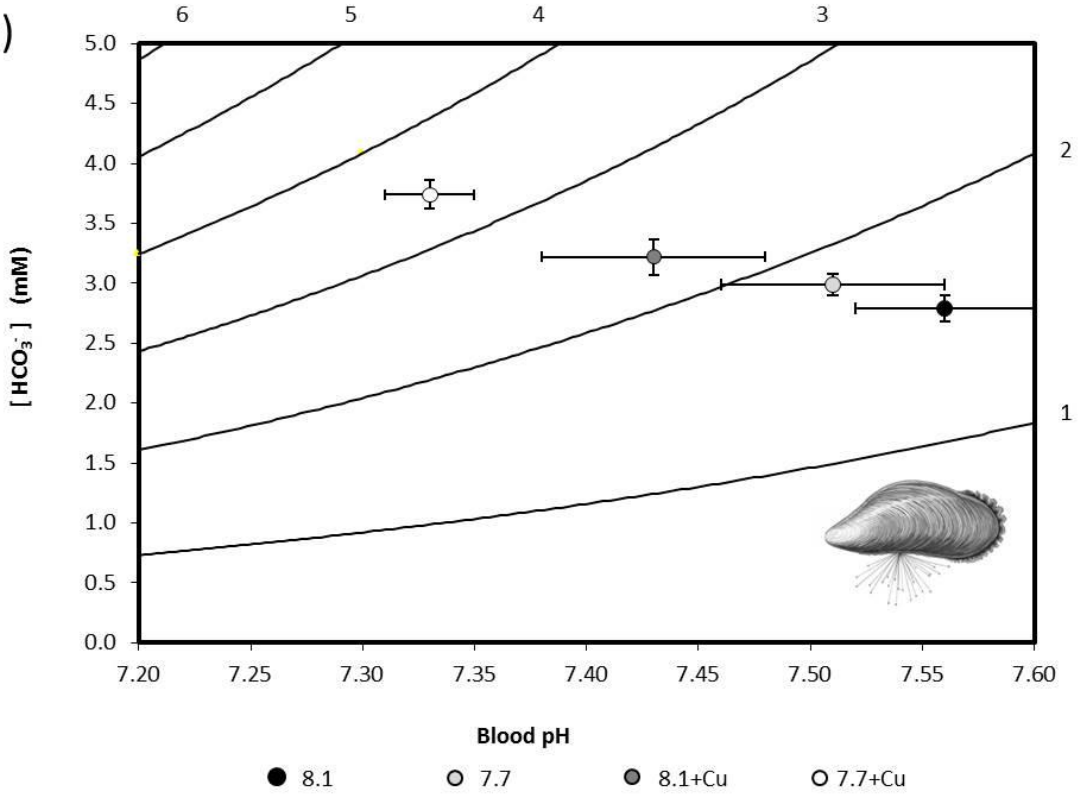
Treatment	Temperature (°C)	pH_{NBS}	Salinity	Copper (µM)	TA (µmol/kg)†	TCO_2 (µmol/kg)	pCO_2 (µatm)†	HCO_3^- (µmol/kg)†	CO_3^{2-} (µmol/kg)†	ΩCa†	ΩAr†
8.1	15.0 (± 0.1)	8.14 (± 0.01)	35.68 (± 0.04)	0.016 (± 0.006)	2345.8 (± 68.6)	2132.4 (± 60.4)	436.4 (± 10.9)	1960.2 (± 53.3)	156.0 (± 7.7)	3.7 (± 0.2)	2.4 (± 0.1)
7.7	15.0 (± 0.1)	7.68 (± 0.01)	35.96 (± 0.06)	0.011 (± 0.002)	2303.1 (± 63.6)	2264.3 (± 63.3)	1373.0 (± 69.2)	2152.5 (± 60.2)	60.6 (± 2.7)	1.4 (± 0.1)	0.9 (± 0.4)
8.1 + Cu	14.6 (± 0.1)	8.09 (± 0.01)	35.60 (± 0.04)	0.047 (± 0.007)	2635.3 (± 55.0)	2423.1 (± 38.4)	536.2 (± 7.6)	2238.5 (± 39.7)	164.1 (± 7.1)	3.9 (± 0.2)	2.5 (± 0.1)
7.7 + Cu	14.5 (± 0.1)	7.68 (± 0.01)	35.74 (± 0.07)	0.052 (± 0.007)	2662.8 (± 56.4)	2568.7 (± 41.7)	1481.4 (± 16.1)	2486.6 (± 49.7)	73.0 (± 3.1)	1.7 (± 0.1)	1.1 (± 0.1)

Table 2: Seawater carbonate chemistry and copper levels for the four experimental treatment solutions for the *Paracentrotus lividus* exposure.

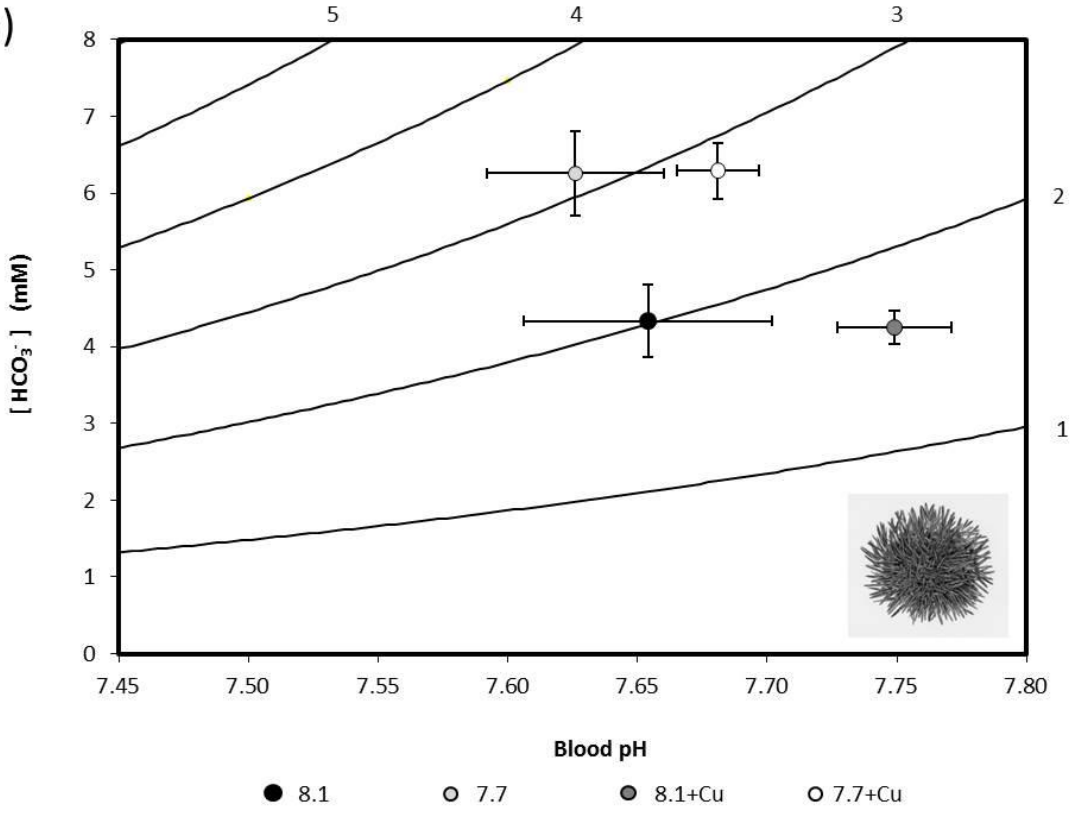
Treatment	Temperature (°C)	pH_{NBS}	Salinity	Copper (µM)	TA (µmol/kg)†	TCO_2 (µmol/kg)	pCO_2 (µatm)†	HCO_3^- (µmol/kg)†	CO_3^{2-} (µmol/kg)†	ΩCa†	ΩAr†
8.1	15.6 (± 0.1)	8.14 (± 0.01)	34.06 (± 0.02)	0.031 (± 0.006)	2599.7 (± 35.2)	2364.9 (± 25.5)	470.7 (± 15.8)	2168.9 (± 19.2)	178.7 (± 8.0)	4.3 (± 0.2)	2.8 (± 0.1)
7.7	15.6 (± 0.1)	7.72 (± 0.01)	34.11 (± 0.02)	0.028 (± 0.004)	2538.5 (± 36.4)	2467.9 (± 28.6)	1297.5 (± 85.0)	2337.0 (± 28.3)	83.3 (± 12.4)	2.0 (± 0.3)	1.3 (± 0.2)
8.1 + Cu	15.3 (± 0.1)	8.13 (± 0.01)	34.25 (± 0.03)	0.084 (± 0.002)	2673.0 (± 30.6)	2452.6 (± 21.4)	528.4 (± 17.9)	2262.0 (± 15.8)	170.9 (± 7.1)	4.1 (± 0.2)	2.6 (± 0.1)
7.7 + Cu	15.23 (± 0.1)	7.71 (± 0.01)	34.50 (± 0.05)	0.088 (± 0.005)	2655.1 (± 22.9)	2608.8 (± 21.4)	1483.1 (± 31.9)	2481.3 (± 20.2)	72.2 (± 1.8)	1.7 (± 0.1)	1.1 (± 0.1)

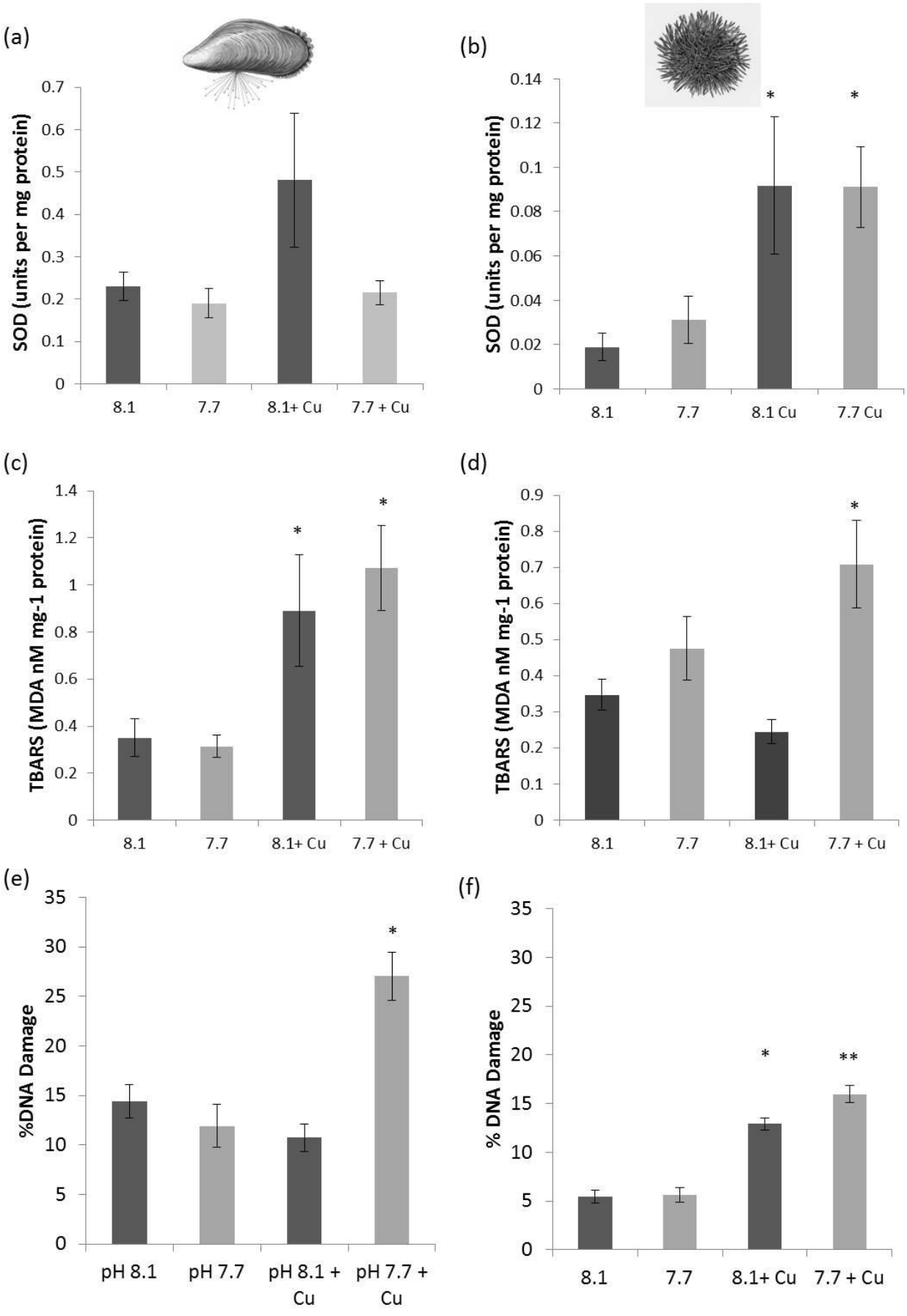

(a)
pCO2 (mmHg)
0
0.1
0.2
0.3
0.4
0.5
0.6
8.1
7.7
8.1 plus Cu
7.7 plus Cu
(d)
pCO2 (mmHg)
0
0.1
0.2
0.3
0.4
0.5
0.6
8.1
7.7
8.1 plus Cu
7.7 plus Cu
(b)
Bicrabonate (mmol l-1)
0
1
2
3
4
5
6
7
8.1
7.7
8.1 plus Cu
7.7 plus Cu
(e)
Bicrabonate (mmol l-1)
0
1
2
3
4
5
6
7
8.1
7.7
8.1 plus Cu
7.7 plus Cu
(c)
Coelomic fluid pH
7
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8
8.1
7.7
8.1 plus Cu
7.7 plus Cu
(f)
Coelomic fluid pH
7
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8
8.1
7.7
8.1 plus Cu
7.7 plus Cu

(a)


6
5
4
3
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
1
[HCO3-] (mM)
7.20
7.25
7.30
7.35
7.40
7.45
7.50
7.55
7.60
Blood pH
8.1
7.7
8.1+Cu
7.7+Cu

(b)


5
4
3
8
7
6
5
4
3
2
1
0
2
1
[HCO3-] (mM)
7.45
7.50
7.55
7.60
7.65
7.70
7.75
7.80
Blood pH
8.1
7.7
8.1+Cu
7.7+Cu


(a)
SOD (units per mg protein)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
8.1
7.7
8.1+ Cu
7.7 + Cu
(b)
SOD (units per mg protein)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
*
8.1
7.7
8.1 Cu
7.7 Cu
(c)
TBARS (MDA nM mg-1 protein)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
8.1
7.7
8.1+ Cu
7.7 + Cu
(d)
TBARS (MDA nM mg-1 protein)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
8.1
7.7
8.1+ Cu
7.7 + Cu
(e)
%DNA Damage
35
30
25
20
15
10
5
0
*
pH 8.1
pH 7.7
pH 8.1 + Cu
pH 7.7 + Cu
(f)
% DNA Damage
35
30
25
20
15
10
5
0
*
**
8.1
7.7
8.1+ Cu
7.7 + Cu