Physiological and biochemical responses of the intertidal crabs *Hemigrapsus crenulatus* and *H. sexdentatus* to hypoxia and salinity

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by Thomas Falconer

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Abstract

Many marine habitats, intertidal zones in particular, are changing due to the global temperature shifts of climate change. These areas are characterized by daily, seasonal and irregular changes in dissolved oxygen and salinity. This forces species to adapt and often regulate in order to survive. Nutrient enrichment is becoming one of the foremost environmental issues impacting species distribution and diversity worldwide. Hypoxia is a decline in water oxygen levels that is increasing globally through anthropogenic aquatic nutrient loading. The New Zealand crabs *H. crenulatus* and *H. sexdentatus* are commonly found in intertidal zones, such as estuaries and rocky shores, which are subject to daily variations in dissolved oxygen and salinity. Consequently, these species are faced with homeostatic challenges involving their physiological and biochemical regulatory systems. The current study investigated the physiological and biochemical responses of the crabs to decreasing dissolved oxygens (including hypoxia) and salinity variations.

The crabs were tolerant of hypoxia maintaining metabolic rates (MO_2) down to a critical oxygen tension (P_{CRIT}) of 52.5 mmHg for *H. crenulatus* and 47.8 mmHg for *H. sexdentatus*. These critical oxygen tensions were unaffected by salinity for both species. At higher oxygen levels (above each species P_{CRIT}) salinity effected the metabolic rate of both species. A bradycardic heart rate response (decrease in heart rate) was also seen for both species in low dissolved oxygen levels (10 mmHg). Salinity had no effect on the heart rates of *H. crenulatus*, whereas *H. sexdentatus* had an increased heart rate when exposed to hypo- and hyper-salinities. The biochemical studies investigated various haemolymph parameters (osmolarity, chloride, potassium, sodium, glucose, lactate, and haemocyanin). The dissolved oxygen treatments had little effect on any of the haemolymph parameters tested for either species which is indicative of a lack of stress in these animals. Salinity, however, affected the haematological composition of both crabs. For *H. crenulatus* osmolarity, chloride, and potassium were all hyper-regulated at low salinities and hypo-regulated at high salinities. In contrast, *H. sexdentatus* conformed haemolymph osmolarity and chloride in the high salinities. Haemolymph glucose showed no apparent trends in

relation to dissolved oxygen or salinity for either species which is again suggestive of a lack in stress response.

The physiological and biochemical abilities of both New Zealand crab species are indicative of a strong regulatory response in both varying dissolved oxygens and salinities. These findings may have important environmental implications in the future as global warming intensifies. These species could be used as biological indicators of ecological stress and could therefore be implemented in risk assessment analyses.

Chapter One

1. General introduction

1.1. Context

Intertidal environments are subject to change over short time periods (hours) as well as extended periods of time, such as seasonal change. Organisms in such environments have evolved to tolerate a range of environmental perturbations in their natural habitats. Marine invertebrates can be subjected to extended periods of increased hypoxia (reduced oxygen levels) changes in salinity and even aerial exposure on a daily basis. When environmental fluctuations exceed that which an organism can tolerate homeostatic processes, growth and survival of animals can become compromised (Díaz and Rosenberg, 1995). Human-induced environmental changes are becoming more prominent as urbanisation and industrialisation increases and nutrients, waste and toxic by-products are input into the environment. These lead to new research areas for animal physiology and biochemistry and will increase our understanding of species responses to environmental disruptions. In the face of habitat and global changes it is paramount to understand the tolerance limits of organisms in these environments if we are to assess ecological risk and to estimate protective limits.

Aquatic environments such as estuaries and tidal coastal zones can be challenging to animals due to fluctuations in salinity and dissolved oxygen (DO). Increased concentrations of trace metals, like copper, zinc, cadmium, silver, and mercury, in waterbodies may also make them less habitable for invertebrates and vertebrates due to increased toxicity. Metals and other toxicants in high concentrations can have an additive effect with DO and/or salinity further increasing the likelihood of species mortality. Pollutants have become enhanced in many coastal regions worldwide due to the proximity of humans (Matthiessen and Law, 2002; Amando et al., 2006) and could affect survival of ecologically important species and thus ecosystem functioning (Vaalgamaa and Conley, 2008). To survive osmotic stressors within these habitats aquatic animals can employ one of two physiological mechanisms to balance their internal environment: they can either osmoregulate (maintaining water and salt balance through internal processes) or osmoconform

(maintaining body fluids at the same salt concentration as the external medium) (Lee et al., 2010).

Intertidal crabs in environments with constant change, such as estuaries, encounter various physiological and homeostatic challenges including environmental salinity and DO changes and increased levels of toxic metals. Naturally occurring and anthropogenic xenobiotics (foreign compounds) are constantly brought into aquatic ecosystems directly or indirectly through land runoff (which can involve toxic pharmaceuticals, such as oestrogen, or metals) (Livingstone, 1998). Many estuaries are susceptible to contaminant runoff due to proximity and vulnerability to human-sourced pollution. The effects of contaminants and pollution such as sewage and metals leeching from piping can encompass entire ecosystems causing deleterious effects to organismal internal processes (Monserrat et al., 2007).

1.2. Environmental change

Conservation efforts are now facing a challenge on a scale never seen before over the past century, global changes in the form of global warming. This is due to the alarming increases in global temperature and the resultant environmental and ecological impacts that come with such drastic change. In the context of marine environments we are seeing serious issues arising with increasing sea level, oxygen and salt content changes. Over the past century we have seen historically uncharacteristic rises in sea levels. Globally, levels have risen anywhere between 10 and 25 cm which is attributed to the melting of polar and glacial ice (Ledley et al., 1999). Tectonic and isostatic changes have also influenced sea level change which is partially attributed to land subsidence or emergence, and/or ocean basin deformation (Kennish, 2002). These levels are only going to rise over the coming decades with a predicted 2.6 – 15.3 cm increase by 2020 (Kennish, 2002). What this means for coastal and estuarine regions is that shorelines will retreat with increases in erosion and losses of wetlands through flooding (Wolanski and Chappell, 1996). In these areas salinity, and DO, are expected to be substantially altered with salinity regime changes predicted to significantly alter the composition of estuarine communities (Kennish, 2002).

1.2.1. Hypoxia

In many of the world's coastal zones hypoxia is being accelerated because of human activities and increased nutrient loading. As the demand for food and human populations increase resource intensifications are likely to rise. Humans' dependency on crops and fossil-fuels, as well as waste water runoff and urbanization, are driving global warming and therefore hypoxia and eutrophication (Rabalais et al., 2010). As the global climate changes, temperatures will rise potentially increasing the likelihood of both natural and human caused-hypoxia (Vaquer-Sunyer and Duarte, 2008). Hypoxia in nature is most commonly attributed to the photosynthetic release of O_2 through microbial respiration (Rabalais et al., 2010).

Areas of hypoxia and anoxia (no oxygen) have been on the rise historically since the 19th century (Diaz and Rosenberg, 1998; Vaquer-Sunyer and Duarte, 2008; Gilbert et al., 2010; Rabalais et al., 2010). From the 1960s to 2008 the number of hypoxic sites around the world had increased an order of magnitude (40 to 400 sites) (Díaz and Rosenberg, 2008; Gilbert et al., 2010). This rate is predicted to increase with the exacerbated effects of global warming. This indicates that hypoxic and anoxic waters are becoming more common, severe and prevalent on a global scale. Therefore, many regions may become uninhabitable for organisms resulting in larger scale and more numerous biological "dead zones".

Environmental hypoxia is a common natural occurrence in many marine environments such as shallow coastal and benthic zones (Paschke et al., 2010). Environmental fluctuations of this nature tend to follow seasonal patterns but may also be a result of anthropogenic eutrophication and the influx of freshwater (Paschke et al., 2010). Eutrophication is a result of an increased rate in the supply of organic material to an ecosystem (Nixon, 1995). This phenomenon is most commonly associated with the growth of algae and higher forms of plant life as a direct response to nutrient enrichment especially nitrogen and/or phosphorus (Anderson et al., 2006; Díaz and Rosenburg, 2008).

Hypoxia as a result of anthropogenic eutrophication is becoming one of the foremost impacts on marine species distribution worldwide (Nixon, 1995; Anderson et al., 2006; Turner et al., 2008; Vaquer-Sunyer and Duarte, 2008). The duration and severity of hypoxia can disrupt ecosystem communities resulting in mass mortality of aquatic life (Díaz and Rosenburg, 1995; Grantham et al., 2004; Paschke et al., 2010). Certain marine environments

are more susceptible to anthropogenic nutrient loading due to their proximity to anthropogenic urbanization and industrialisation. Marine environments such as estuaries and semi-enclosed seas in proximity to humans are highly susceptible to increased levels of hypoxia (Grantham et al., 2004).

Few examples of large scale permanent areas affected by hypoxia/anoxia have been documented (two examples are seen in the Baltic Sea (Conley et al., 2002) and the Black Sea (Díaz, 2001)). Seasonal hypoxic events have, however, been investigated more in depth with numerous study sites across the globe. Study sites range from the Gulf of Mexico (Rabalais et al., 2002), Denmark estuarine and surrounding waterways (Conley et al., 2007), and Chesapeake Bay (Hagy et al., 2004). Prolonged seasonal hypoxia in Denmark waterways changed benthic faunal diversity with reductions seen in as little as one week (Conley et al., 2007). Within three to five weeks of hypoxia a collapse in the community structure had occurred. In these waterways changes in species diversity can be seen on an unprecedented scale where, in 2002, Conley et al. (2007) observed benthic faunal diversity and composition changes over several thousands of square kilometres.

High temperatures can intensify the negative effects of hypoxia in marine environments. High temperatures coupled with hypoxia negatively affect growth, survival, behaviour, food consumption, reproduction and distribution of estuarine fish species such as the Atlantic sturgeon and striped bass (Secor and Gunderson, 1998; Brandt et al., 2009). Species will tend to select water strata which correspond to their optimal thermal temperature range (Brandt et al., 2009). When species inhabit environments that exceed their tolerance range in either dissolved oxygen and/or temperature they can undergo regulatory and other homeostatic challenges. Although most crustacea and fish species can actively avoid areas with low DO and high temperatures, through individual or population migrations, seasonal changes in temperature can decrease O_2 levels to beyond their tolerance ranges (Pihl et al., 1991). This occurs when surface temperatures exceed that of the species tolerance. When DO levels in the lower water column are reduced this can result in a temperature-oxygen “squeeze”. In this scenario the optimal environment for growth and consumption is somewhere in the middle of the water column (which is hard for many crustacea to inhabit) or disappears entirely (Hagy et al., 2004; Brandt et al., 2009).

Brandt et al. (2009) found environmental water changes that reflected a temperature-oxygen squeeze in various summertime waterways. These include Chesapeake

Bay, the Hudson River, the Connecticut River and other water reservoirs in Tennessee, Texas, and Sacramento. These changes have led to various consequences to striped bass distribution and mortality, which ranged from individual reduction in consumption and/or growth, by being forced into suboptimal habitats, to direct mortality from respiratory and thermal stress (Brandt et al., 2009).

1.2.2. Salinity

Increased prevalence of storms and flooding can lead to changes in DO but can also result in reductions in salinity (Elliott and Quintino, 2007). River diversions can reduce freshwater input in estuaries, such as in San Francisco Bay (Nichols et al., 1986). Salinity, hydrological and sediment regimes of estuarine and coastal marine habitats can be changed because of freshwater outflows. Surface water diversions can occur anthropogenically to satisfy agricultural, industrial and municipal needs of man. This leads to the degradation of environments, the organisms that reside within them, and the trophodynamics of the affected system (Kennish, 2002).

Salinity as a stressor can affect the homeostatic functioning of aquatic organisms. In crustacea the salinity surrounding developing embryos drives growth, survival, duration of development, initial mass, and larval salinity tolerance (Giménez and Anger, 2001; Giménez, 2003). Salinity can also influence the distribution and abundance of decapod species. In *Neohelice granulata* differences between populations of juveniles and adults of the Patos Lagoon estuary in Southern Brazil are a result of differences in salinity. This phenomenon is likely associated with the species recruitment and reproductive strategies (Bianchini et al., 2008). The reproductive physiology of crustacea is influenced by salinity, with direct effects on the number and quality of larvae produced reflecting the salinity of the water where they were cultured (Bianchini et al., 2008).

Lipids are a major energy store in crustaceans and are key for maintaining physiological and structural integrity of sub-cellular and cellular membranes (Luvizotto-Santos et al., 2003). Salinity can have deleterious effects on lipid mobilization and regulation in crabs. An example is seen where juvenile and adult *N. granulata* acclimatized to low salinities induced lipid mobilization in the muscles and gills (Luvizotto-Santos et al., 2003; Bianchini et al., 2008). Lipid concentrations can also decrease after hypo-osmotic stress and

increase after hyper-osmotic stress (Luvizotoo-Santos et al., 2003). Haemolymph glucose levels of invertebrates are also affected by salinity where alterations commonly occur under hyper- or hypo-salinity stress (Santos and Nery, 1987; Bianchini et al., 2008).

1.3. Physiological and biochemical responses of decapod crabs to environmental stressors

Environmental and toxicological work often uses biomarkers and bioindicators within an ecosystem, or a particular species, in order to identify the risks associated with abiotic stressors. A biomarker has been broadly defined as "a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also, of toxic effect" (Depledge and Fossi, 1994). Biomarkers are behavioural, biochemical, cellular, or physiological responses that can be characterized as a functional measure of exposure to a stressor (Adams et al., 2001). Oxygen consumption is commonly used as a physiological biomarker particularly in decapod crustaceans and crayfish (Ansell, 1973; Taylor, 1977; Bradford and Taylor, 1982; McMahon, 2001; Depledge and Galloway, 2005; Kuklina et al., 2013). This is because an imbalance in the physiological responses of the respiratory systems reflects the environmental status (Depledge and Galloway, 2005; Kuklina et al., 2013).

Decapod crabs have various physiological responses to cope with environmental stressors such as hypoxia or salinity stress. At low ambient oxygen levels crustaceans are often able to tolerate hypoxic exposure and regulate O_2 (McMahon, 2001). In situations of acute hypoxia some crustacea are able to increase cardiac output, shunting blood and water flow to vital areas of the organism (McMahon, 2001). The majority of crustaceans, including decapods, display increased scaphognathite frequency upon initial introduction to hypoxia (Taylor, 1982; McMahon and Wilkens, 1983). The rate and depth of scaphognathite pumping increases in O_2 depleted water with both actions resulting in an increased ventilation volume (Burggren and McMahon, 1983; McMahon, 2001). Below normal

environmental levels, along with an increased gill ventilation rate, a reduced heart-beat frequency (bradycardia) is also found as PO_2 declines (Airriess and McMahon, 1994).

Hyperventilation is another means of resisting hypoxia where increased hyperventilation is associated with alkalosis and CO_2 washout (McMahon, 2001). Hypoxia-induced hyperventilation is seen in many crustacean species such as the crab *Cancer magister*, which can maintain hyperventilation even at extremely low O_2 levels (2.96kPa) (Airriess and McMahon, 1994). Bradycardia is also a common response to hypoxia in decapods such as in homarid lobsters which responded to a long term environmentally reduced PO_2 exposure with bradycardic and hyperventilation responses (Butler et al., 1978).

In order to cope with external salinity stress, crabs can either osmoregulate or osmoconform. Osmoregulation is crucial for the survival of many estuarine organisms where disruptions to osmoregulatory processes can be fatal (Monserrat et al., 2007). Osmoregulation and the transportation of ions can happen diffusively or through carriers across many crustacean membranes. Permeability exists across the gills of decapod crabs where fluids and ions, such as Na^+ and Cl^-, are transferred at varying rates depending on their environments (McNamara and Faria, 2012). In crabs the gills are the primary organ for salt and osmotic transport (Morris, 2001). The gills of marine crabs have an extensive surface area allowing for maximal ion and salt transport especially in cases of environmental extremes (hypo- and hyper-salinity waters). A correlation was also found between the environmental salinity and the cuticular permeability of decapods, however cuticular surface plays a minor role in osmoregulation in contrast to the gills (Péqueux, 1995).

Decapod crustaceans display a variety of biochemical and endocrine responses to hypoxia including ion pumps, ion exchangers and ion transporters in the gills and other osmoregulatory tissues (Wheatly and Gao, 2004; Jayasundara et al., 2007; Towle et al., 2011; McNamara and Faria, 2012). The effectiveness of O_2 transport can be modified at these tissues where changes to the O_2-binding affinity and oxygen carrying capacity of haemocyanin is a common response (McMahon, 2001). Neural control of pericardial organs can release many neuroendocrine substances which can influence the rate and stroke amplitude of crustacean heartbeats (stroke amplitude can also vary independent of heart rate) (Wilkens et al., 1996). These substances are released directly into the haemolymph before it enters the heart (McMahon, 2001). This can lead to potent *in vivo* effects on the cardiovascular system of these animals which is a profound adaptive response to hypoxia.

In crustacea changes to enzyme gene expression activity of the osmoregulatory tissues occurs at a molecular level in response to fluctuating salinities. Marine euryhaline species that inhabit brackish niches can employ branchial pumping mechanisms to maintain ion balance (Lucu and Towle, 2003). The success of these animals, in part, comes from gene expression and/or activity of gill carbonic anhydrase, arginine kinase, alkaline phosphatase, Na^+/K^+ -ATPase, and H^+ -ATPase molecular transporters invoked by salinity (Bianchini et al., 2008). The commonly accepted model for ion transport in gill epithelial cells involves Na^+/H^+ and Cl^-/HCO_3^- exchanges (Morris, 2001). These exchangers can directly move Na^+ and Cl^- into the gill cells through the apical membrane and can increase in activity rates in relation to its stress levels (Morris, 2001). Na^+/K^+ -ATPase is an important basal membrane pump for many regulating crustacea species. These exchanging pumps can increase in activity when animals are exposed to hypo- or hyper-saline solutions. For example in the intertidal crab species *Hemigrapsus nudus* ATPase enzyme-specific activity approximately doubled between crabs acclimated to 50% seawater in contrast to crabs acclimated to 100% seawater (Corotto and Holliday, 1996).

1.3. Background and previous physiological research on *H. crenulatus and H. sexdentatus*

The purple rock crab *Hemigrapsus sexdentatus* (formerly *H. edwardsii* and *Hemiplax edwardsii*) is an endemic species distributed throughout intertidal habitats of New Zealand. It is commonly considered a rocky mid- to high-shore crab and is regularly found on mud, gravel and rocky substrata (Hicks, 1973). *H. sexdentatus* also inhabits varying brackish environments such as estuaries (Hicks, 1973). The hairy-handed crab *H. crenulatus* on the other hand can be found in various habitats ranging from rock pools and reefs with little sand or mud sediment, to more muddy and sandy substrata (Hicks, 1973). This species is also endemic to New Zealand and is considered more of an estuarine/mud-flat species commonly burrowing within sand, mud and beneath stones (Taylor and Seneviratna, 2005). In contrast *H. sexdentatus* is prevalent within non-silted rocky and boulder beach environments (Taylor and Seneviratna, 2005). Both crabs are euryhaline (able to tolerate a

wide range of salinities) and the adults of these species are hyper-osmotic regulators, constantly regulating to the changing external environment (such as changes to rainfall, tidal and salinity changes and the occurrence of freshwater streams) (Seneviratna and Taylor, 2006).

To date there has been little research on either *H. crenulatus* or *H. sexdentatus* in the context of multiple and/or acute salinity or DO stressors. Lee et al. (2010) has, however, investigated the effects of prolonged copper and salinity exposure on the haemolymph parameters of *H. crenulatus* with exposures running for 96 hours. In that study four salinities were used (50, 75, 100, 125%) made from a stock of 35.7 ppt (parts per thousand) sea water with each variant tested with one of three copper concentrations; 0 μgL^{-1} (control) 125 μgL^{-1} and 250 μgL^{-1} Cu(II). Here *H. crenulatus* regulated its haemolymph osmolarity in the dilute salinities, displaying a hyper-osmotic regulatory response, however in salinities in excess of 100% seawater regulation broke down and *H. crenulatus* osmoconformed (Lee et al., 2010). Results from this study were also indicative of an additive effect of salinity when exposed to higher levels of copper. This suggests that additional stressors will further challenge homeostatic regulation in species within constantly changing habitats. Taylor and Seneviratna (2005) showed that embryos of both *H. sexdentatus* and *H. crenulatus* are remarkably tolerant to acute hyposaline exposure where post-gastrula stages survived 10% seawater (produced from a 35.7 ppt stock of seawater) for trials equivalent to environmental tidal changes. The embryos were even able to survive 6 hour exposures to 1% seawater with minor mortalities, indicating that normal development of these species could be possible in environments with fluctuations in salinity over hours or even days (Taylor and Seneviratna, 2005).

Other than these studies, the physiological and biochemical responses to hyper- and hypo-salinity stressors has yet to be thoroughly investigated for either species. To the best of our knowledge there are also no studies on the tolerances of hypoxia and the effects of hypoxia and salinity as a combined stressor for either species.

1.4. Aims and hypotheses

The effects of hypoxia and salinity stress have yet to be thoroughly investigated in the two New Zealand crustacean species *Hemigrapsus crenulatus* and *H. sexdentatus*. Little is known about the responses of these animals when put under acute short term (6 hour) stress at environmental extremes, such as those that might occur from eutrophication and global change through global warming and industrialisation. I will be investigating four levels of hypoxia (6, 25, 50, 100% DO) and five salinity levels (2, 25, 50, 100, 150%).

Chapter two investigates the oxygen consumption responses of both species at the various hypoxia and salinity levels. It is hypothesised that oxyregulation will occur where O_2 is highest (100% DO) and will drop as O_2 decreases until a hypoxia-induced bradycardic response is found. It is hypothesised that at the extremes of salinity (2% and 150%) there will be increased effects of hypoxia especially at the lower limits, i.e. at 6% DO. These effects may be greater for *H. sexdentatus* which use behavioural mechanisms to avoid stressors, unlike *H. crenulatus* which do not employ avoidance mechanisms. *H. sexdentatus* also has larger gills and therefore respiratory surface area, than *H. crenulatus* which may intensify the salinity effects.

Chapter three investigates the cardiac response of both species to the various hypoxia and salinity levels. It is hypothesised that the heart rates of both species will progressively decrease in declining hypoxia with a bradycardic response at the lowest 6% DO. Similarly for salinity we expect to see contrasting results for both species where increases in the environmentally extreme salinities are predicted to show an exacerbated heart rate response. I predict that the effects are likely to be greater for *H. sexdentatus* than for *H. crenulatus* due to the differences in environmental niches of the species. In this case a more defined change in heart rate is predicted between the environmentally equivalent and extreme treatments.

Chapter four examines the biochemical responses of both species to hypoxia and salinity stress. Here investigations will describe the osmolarity, Cl^-, K^+, Na^+ concentrations as well as the glucose, lactate and haemocyanin concentrations of the haemolymph. The osmolality, Cl^-, K^+, and Na^+ will be investigating the crabs' osmoregulatory abilities. Glucose,

lactate and haemocyanin concentration analyses explore the impacts of acid-base balance, and the degree of anaerobic metabolism/whether or not it is activated. I predict that both species' osmolarity, Cl^-, K^+, and Na^+ will be unaffected by hypoxia. However, in hyper-salinities both species are predicted to hypo-regulate and in hypo-salinities these species are predicted to hyper-regulate these haemolymph constituents. For both species haemolymph glucose, lactate, and haemocyanin concentrations are all predicted to increase in the more stressful treatments (i.e. hypoxic 6% DO, and 150% hyper-salinity and 2% hypo-salinity) in contrast to the other treatments. Again, these affects are predicted to be greater for *H. sexdentatus* compared to *H. crenulatus* due to the differences in their natural habitats.

Chapter five uses the findings from the current research and discusses the results and implications for each species. Here discussions will compare and contrast the differences between *H. crenulatus* and *H. sexdentatus* and how these species relate to one another and similar decapod species. Chapter five will also consider implications for these animals in their future habitats as well as where future work may be required to further understand the effects stressors have on New Zealand decapod species.

Chapter Two

2. Effects of hypoxia and salinity on oxygen uptake in the crabs *Hemigrapsus crenulatus* and *H. sexdentatus*

2.1. Introduction and aims

Estuaries and intertidal zones can be some of the most challenging environments for decapods to inhabit. Fluctuations in salinity and dissolved oxygen can occur over short time periods (hours) with common appearances of hypoxic waters due to contaminants and freshwater outlets. In order to survive these variable conditions organisms must not only be able to cope with extremes of single stressors but must also be able to withstand the effects of multiple stressors. Oxygen consumption in crustaceans has been extensively studied for many species of crabs, such as *Chasmagnathus granulata* which exhibit daily variation in oxygen and aerobic metabolism (Maciel et al., 2004), and the intertidal crabs *Hemigrapsus oregonensis* and *H. nudus* which display seasonal and temperature differences in oxygen consumption rates in Spanish Bank (Vancouver, Canada) (Dehnel, 1960). There is, however, sparse literature on the physiological effects of stressors on the two endemic New Zealand crab species with, to the best of our knowledge, no research on the effects of multiple stressors on the oxygen consumption of these species.

Determining stressor tolerance of animals is a common practice of ecologists and animal physiologists. The concept and definition of biological 'stress' has been debated, however, it can be defined as a condition in which the homeostatic functioning of an animal is disturbed by intrinsic or extrinsic stimuli (Bonga, 1997). When environmental stressors (outside of the organism's normal environmental range) disrupt homeostasis, animals often coordinate behavioural and physiological responses if they are to overcome the stress. Intertidal decapod crabs are well adapted to moderate environmental fluctuations displaying a suite of physiological mechanisms to minimise acute (1-6 hours) hypoxic and/or salinity stress (Pillai and Diwan, 2002). Measurement of the animals' responses to short-term stressors can therefore represent their tolerance to certain environmental changes and conditions.

Metabolic rate (traditionally measured as the rate of oxygen consumption (MO_2)) is a good indicator of the overall metabolic status of an animal and is often used to determine their tolerance to stressors (Dehnel, 1960; Ketpadung and Tangkrock-olan, 2006). It is a measure of the energy utilized by an organism and results from changes in tissue oxygen demand (Clarke and Fraser, 2004). This is because oxygen is used in the production of ATP through aerobic metabolism. The rate at which oxygen is consumed is a measure of metabolic rate and is a useful marker of stress in crabs (Gillooly et al., 2001; Clarke and Fraser, 2004; Ketpadung and Tangkrock-olan, 2006).

Crustaceans can maintain MO_2 in hypoxia using several physiological responses, including modified O_2 transport, binding affinity and carrying capacity of haemocyanin as well as modulation of ventilatory and cardiovascular systems (McMahon, 2001). This regulation of oxygen (oxyregulation), however, occurs until MO_2 can no longer be maintained at which point the physiological systems of the animal have reached their limits. Beyond this point the animal's oxygen uptake depends on oxygen availability and reduces over time (Yeager and Ultsch, 1989). A progressive drop in oxygen consumption is found during extreme hypoxic events where the partial pressure of O_2 reaches a critical level. This is seen in both oxyregulators and oxyconformers and is a result of oxygen supply being insufficient to cover the aerobic metabolic demands of the organism (Ekau et al., 2010). The point at which an oxyregulator can no longer maintain MO_2 and transitions to an oxyconformer is termed the P_{CRIT} (critical oxygen tension) (Burnett, 1997; Reiber and McMahon, 2008). After this point anaerobic metabolism will be activated and resultant increases in ATP production, through glycolysis, with the concomitant accumulation of L-lactate and the degradation of phosphagen occurs in crustaceans (Pörtner and Grieshaber, 1993). It is generally accepted that the lower the P_{CRIT} value of an animal the greater it can tolerate hypoxia and regulate oxygen consumption.

Salinity is another one of the main environmental factors affecting the metabolic rates of aquatic organisms (Findley et al., 1978). It is a limiting factor influencing the abundance and distribution of crab species, particularly in estuarine environments (Barnes, 1967). Metabolic rates of crabs often stabilize when the animals are allowed to acclimate to salinity gradually over long periods of time. However, this response may be very different when animals are exposed to temporary fluctuations in salinity with most crustaceans seen

to increase their oxygen consumption rates in response to reduced salinity (Taylor, 1977; Stickle and Sabourin, 1979). Invertebrates exhibit intraspecific variations in their tolerances and osmoregulatory abilities due to the environments they inhabit (Stancyk and Shaffer, 1977; Hicks, 1980). For example, *Callinectes sapidus* sampled from low salinities are better able to osmoregulate in lower salinities than animals sampled from high salinities (Guerin and Stickle, 1992). *C. sapidus* from the Laguna Madre (hypersaline lagoon along the western coast of the Gulf of Mexico, Texas) and from a brackish waterway near a mouth of a river (San Antonio Bay, Texas) also displayed different salinity tolerances (Guerin and Stickle, 1992).

Closed box respirometry is useful in the measure of progressive changes in hypoxic conditions and the subsequent cardiovascular and respiratory responses in various aquatic invertebrate species (Wheatly and Taylor, 1981; Kedwards et al., 1996). This method involves placing animals within a sealed container where oxygen is consumed over time. The speed at which oxygen is consumed depends on the size of the container and the size and metabolic rate of the animal. The combination of these factors therefore dictate the rate at which hypoxia is generated be it acute or gradually over time.

To determine the effects of multiple stressors the current study aimed to assess the physiological tolerances of *H. crenulatus* and *H. sexdentatus* to hypoxia and salinity. This was achieved by implementing four hypoxia levels (6, 25, 50 and 100%) and measuring the physiological responses of the animals in the form of oxygen consumption (MO_2). Investigations also looked at the rate at which the animals consumed oxygen at specific levels of salinity (2, 25, 50, 100 and 150% made from a 35 ppt seawater stock). At each salinity treatment animals were tested at each of the four dissolved oxygen levels (6, 25, 50 and 100%). Animals were sealed in a respirometer with fully saturated water (this is considered to be the 100% DO treatment), they are left to consume the oxygen down to the lowest 6% DO. These data were used to identify the critical oxygen tensions at which either species switches from an oxyregulatory to an oxyconforming response and will be useful in assessing the environmental tolerances of both endemic crab species to their respective hypoxia-prone environments.

2.2. Collection and maintenance of *H. crenulatus* and *H. sexdentatus*

Both endemic crab species *Hemigrapsus crenulatus* (hairy-handed crab) and *H. sexdentatus* (rocky-shore crab) were collected from the Avon-Heathcote Estuary/Ihutai and coastal areas near Christchurch, New Zealand. Male hairy-handed crabs were collected from the Avon-Heathcote estuary whereas male rocky-shore crabs were collected from Waipara beach (52°89'96.7"N 25°01'11.5"E). The hairy-handed crab was obtained using a baited dropnet (similar to methods employed by Brown et al., 2004), whereas the rocky-shore crab was collected by hand. The average size of *H. crenulatus* was 28.6 ± 2.8 mm and the average size of *H. sexdentatus* was 38.8 ± 3.5 mm. Crabs were transported and held in the aquarium system in the School of Biological Sciences building, University of Canterbury. This system consists of recirculating seawater (~30 ppt) kept at 15 ± 2 °C and is subjected to a 12-hour light 12-hour dark photoperiod, daylight commencing at 8.00 am. The method of a 12:12 light/dark photoperiod cycle is one regularly employed in animal research of this nature (Brown et al., 2004; Taylor and Seneviratna, 2005; Sabatini et al., 2009; Lee et al., 2010; Lauer et al., 2012).

Animals were provided with various sized rocks, stones and foliage to hide under as well as a rocky substrata. Parts of the rocks within the aquaria were exposed to air allowing the crustacea to perch out of the water simulating an intertidal zone. Water exchange rates were kept low (~2 - 5 L min^{-1}) and all animals were fed half a large green lipped mussel (*Perna canalicula*) twice weekly. Due to the aquaria sizes a maximum of 15 *H. crenulatus* and 8 *H. sexdentatus* were housed per tank. Crabs were acclimated to the aquaria seawater conditions at 15°C for a minimum of 1 week and fasted for a minimum of 24 hours prior to experimentation.

Decapods, such as *H. crenulatus* can show gender and seasonal-specific physiological and biochemical responses to stressors (Díaz-Jaramillo et al., 2013). Male *H. crenulatus'* stress responses and hepatopancreas samples were better indicators of estuarine sediment pollution than females when put under oxidative stress (Díaz-Jaramillo et al., 2013). Therefore, only males of either species were used for experimentation. Animals were selected randomly from the tanks and after experimentation used animals were kept

separate from unused animals. All animal procedures were approved by the University of Canterbury Animal Ethics Committee (No: 2015/23R).

Figure 2.1: Photographs of H. crenulatus *(left) and* H. sexdentatus *(right).*

Figure 2.2: Photograph of the Waipara beach collection site for H. sexdentatus *showing a freshwater stream outlet onto the beach and the collection area for the crabs*.

2.3. Methods and Materials

Oxygen consumption was measured using closed-boxed respirometry. Crabs were acclimated to the University of Canterbury's aquarium facility settings (15 ± 2 ^{o}C ~30 ppt salinity) for at least 7 days before experimentation commenced. Prior to experimentation, crabs were fasted for a minimum of 24 hours and weighed before being placed into a respirometer. The respirometer consisted of a 400 ml plastic container (10.5 x 6.2 x 6.2 cm) with a screw-on plastic lid. At the centre of the lid a 3 cm hole was made in which a bung containing an oxygen electrode was fitted (Fig. 2.3). The respirometer was filled with seawater of the desired salinity and aerated with an Aqua One Stellar 380D (air output: 190L/hr, max pressure: 3kpa) air pump for 10 min before the crabs were inserted and experiments began. All experiments were run at 15 ± 1 ^{o}C by placing each respirometer in a water bath with either a Grant Instruments GD100 series stirred water circulator or a TECHNE TE 10D thermoregulator set to 15 ^{o}C.

Each electrode was connected to a model 781 Strathkelvin oxygen meter (Strathkelvin Instruments) and calibrated before the trials began. This involved calculating the PO_2 for air-saturated water at the trial salinity (correcting for atmospheric pressure) and the zero value (using a solution of sodium sulphite dissolved in the trial water) before each experiment. Upon calibration, all air bubbles were removed from the respirometer. The crab was then placed in the respirometer and the lid was immediately screwed firmly on, with the bung and electrode fitted. An initial reading was recorded at the commencement of the trial which was used as the 100% oxygenated value. Recordings continued until the crab had depleted the amount of dissolved oxygen down to 6% of the initial 100% value (for example if the initial reading was 150 mmHg the trial would be stopped at 9 mmHg or 6%).

Oxygen consumption was recorded throughout the entirety of the experiment where each oxygen meter was connected to an ADInstruments PowerLab 26T which was then connected to a computer. The output from this was recorded through LabChart 7 computer software. Sample readings for change in oxygen partial pressure (PO_2) in mmHg were

calculated at the commencement of the experiment and every hour thereafter (five recordings taken 1 minute apart at each hour) using this equation:

Equation 2.1:

$$\Delta PO_2 = \frac{PO_2 i - PO_2 f}{t}$$

Where $PO_2 i$ is the initial reading at each of the oxygen consumption intervals, $PO_2 f$ is the final reading after 5 mmHg of change, and t is how long it took for the change to occur in minutes.

Using sample readings at specific oxygen consumption intervals (at the commencement of the experiment and at each hour thereafter), the following equation was used to calculate MO_2:

Equation 2.2:

$$MO_2 = \frac{(a \times \Delta PO_2 \times V)}{(w \times t)}$$

Here a is the oxygen capacitance of water in µmol L^{-1} $mmHg^{-1}$ at 15 °C (i.e. 1.6218), ΔPO_2 is the change in oxygen partial pressure in mmHg, V is the volume of the respirometer (accounting for the crabs volume), w is the weight of the crab in g, and t is the time in hours.

Figure 2.3: Photograph showing H. crenulatus *within the 400 mL respirometer.*

The oxygen consumption experiments were conducted on a total of 30 *H. crenulatus* and 30 *H. sexdentatus*, with 6 crabs used for each trial (see Table 2.1.). A maximum of three respirometers containing animals were experimented at a time owing to the size of the water baths and the number of Strathkelvin oxygen meters available. Experimental measurements commenced once each oxygen electrode was calibrated and, when more than one animal was being tested, once all animals were placed within their respective respirometers with the bungs and electrodes fitted. Oxygen consumption was recorded until each animal had consumed oxygen down to 6% of the initial dissolved oxygen (~10 mmHg) or after 6 hours, which ever occurred first. After the experimental procedure, the bung was removed from the respirometer and the water was flushed with oxygenated water allowing the crabs to recover for a minimum of half an hour before being returned to the aquarium.

Table 2.1: The average wet weight (± standard deviation) of H. crenulatus *and* H. sexdentatus *used in the closed-box respirometry experiments measuring oxygen consumption rate (MO_2). n = 6 for all experiments.*

Salinity treatment	*H. crenulatus* mean mass ± Standard deviation (g)	*H. sexdentatus* mean mass ± Standard deviation (g)
150%	10.7 ± 2.5	41.3 ± 16.2
100%	13.2 ± 1.5	30.5 ± 10.4
50%	10.9 ± 2.8	38.8 ± 8.6
25%	10.4 ± 2.1	27.1 ± 9.8
2%	9.1 ± 1.3	28.1 ± 10.9

Statistical analysis was carried out using the R statistical programme using a significance level of $P < 0.05$. Normality of data was tested using a quantile-quantile normality plot as well as a Shapiro-Wilk normality test. Homogeneity of variance was tested by plotting the fitted against the residual values as well as a Levene's test for homogeneity of variance. Where treatment effects were shown to be significant, such as for calculating the P_{CRIT} of each species at each salinity condition, post-hoc Tukey's HSD analyses were performed. Oxygen consumption rates of the animals at each treatment (tested excluding values below the P_{CRIT} value) were conducted using an analysis of covariance (ANCOVA). This was used to test if one linear regression line, fitted to the raw data, was significantly different to another in slope and/or elevation.

In order to determine the P_{CRIT} of each treatment data were initially interpreted graphically followed by a piecewise regression function applied to the data (this is also referred to as segmented regression or 'brokenstick' analysis). One value was given here when analysing the accumulated raw data. In order to validify this value each individual crab's data were tested using the same statistical method and the average of this value was then compared to the accumulated piecewise data analysis.

2.4. Results

Critical oxygen tensions (P_{CRIT})

Analysing the raw data a clear effect of hypoxia on oxygen consumption was found for both species. During progressive hypoxia oxygen consumption decreased for both species with decreasing external PO_2 until a critical oxygen tension was reached (Fig. 2.4 and Fig. 2.5). For *H. crenulatus* there were no significant differences between the P_{CRIT}s of the different salinity treatments (post-hoc Tukey's HSD tests all P >0.05). The assumptions of normality (Shapiro-Wilk test P = 0.598) and homogeneity (Levene's test P = 0.339) were both met. For *H. sexdentatus* a similar pattern emerged where there were no significant differences between the P_{CRIT}s of each salinity treatment (P >0.05) except between the 2% and 100% treatments (P = 0.035). For *H. sexdentatus* the assumptions of normality (Shapiro-Wilk test P = 0.163) and homogeneity (Levene's test P = 0.098) were also met.

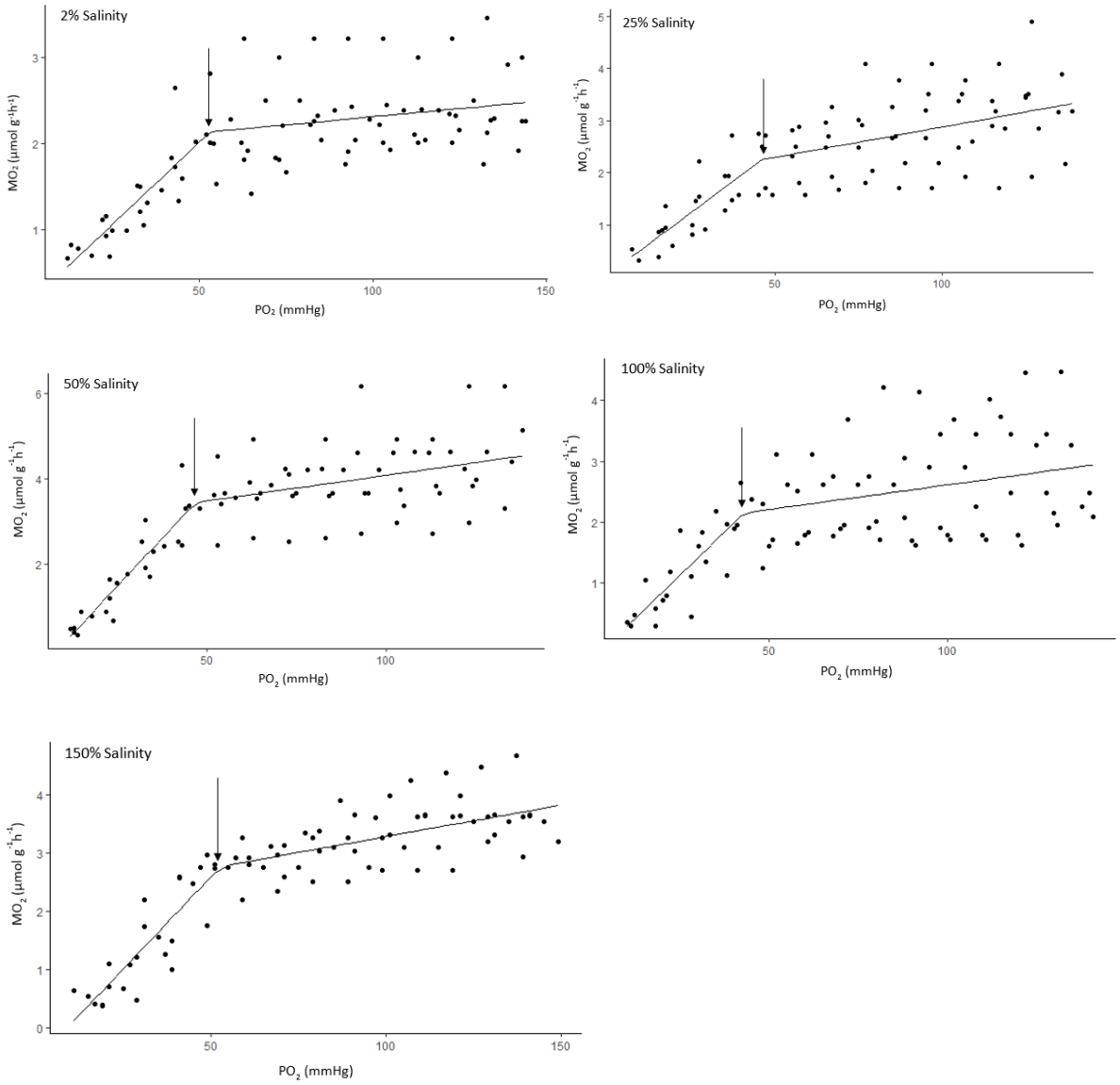

Figure 2.4: H. crenulatus *oxygen consumption (MO_2) under progressive hypoxia (PO_2) to the five treatment salinities (2, 25, 50, 100 & 150%). The solid lines show the regression line and the arrows indicate the calculated P_{CRIT} value.*

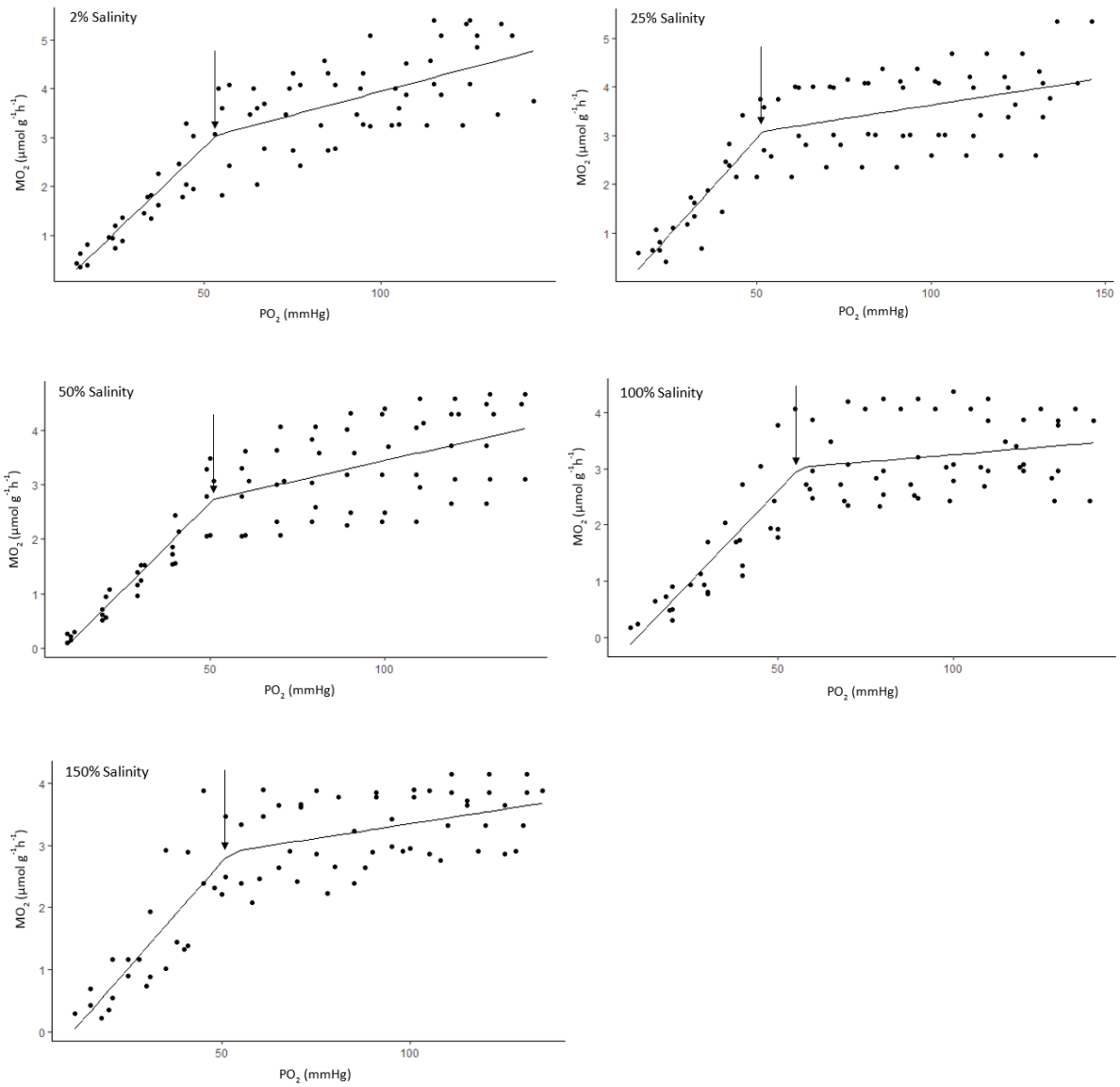

Figure 2.5: H. sexdentatus *oxygen consumption (MO_2) under progressive hypoxia (PO_2) for the five treatment salinities (2, 25, 50, 100 & 150%). The solid lines show the regression line and the arrows indicate the calculated P_{CRIT} value.*

The piecewise regression analysis was conducted on both individual oxygen consumption rates and the accumulated oxygen consumption rates, from the raw data, of all six individuals in each salinity treatment. From this we get an average P_{CRIT} based on the total data (represented by the arrows in figures 2.4 and 2.5) as well as an average based on the means of each individuals P_{CRIT} value (Table 2.2). This double check is used to attain the most accurate P_{CRIT} value for each species.

Table 2.2: The P_{CRIT} for the total raw data averages and the mean for the individual P_{CRIT} averages (mmHg) for H. crenulatus *and* H. sexdentatus *as well as the total average ± standard error of the mean (S.E.M). For each trial n = 6.*

H. crenulatus	Raw data total P_{CRIT} average (mmHg)	Individual P_{CRIT} average (mmHg)	Total average + S.E.M.
2%	53.70	48.61	50.15 ± 0.07
25%	51.82	52.20	52.01 ± 0.11
50%	51.01	51.05	51.03 ± 0.16
100%	56.53	57.31	56.92 ± 0.11
150%	52.36	50.80	51.58 ± 0.12
Average	53.08	51.99	52.54 ± 0.11
H. sexdentatus			
2%	53.13	54.51	53.82 ± 0.16
25%	45.99	41.04	43.52 ± 0.14
50%	46.71	47.74	47.23 ± 0.14
100%	42.83	44.03	43.43 ± 0.13
150%	52.86	48.79	50.83 ± 0.13
Average	48.30	47.22	47.76 ± 0.14

The above data and statistical analyses suggest that there was no significant difference between the P_{CRIT} values of either crab species to differing salinities with one exception seen between 2% and 100% salinities for *H. sexdentatus*. The average P_{CRIT} across all salinities for *H. crenulatus* was 52.54 ± 0.11 mmHg and for *H sexdentatus* was 47.76 ± 0.14. This data suggests that below ~53 mmHg *H. crenulatus* will switch from an oxyregulating to an oxyconforming regulatory response. *H. sexdentatus* are predicted to change their regulatory response type at a lower ~48 mmHg.

Effect of salinity on oxygen consumption rates

Physiological responses to hypoxia of both species varied under closed box respirometry. *H. sexdentatus* had an average MO_2 of 3.48 µmol g^{-1} h^{-1} consumption rate compared to 2.99 µmol g^{-1} h^{-1} for *H. crenulatus,* averaged across all salinity treatments in high oxygen tensions (above 47 mmHg). An analysis of covariance (ANCOVA) was performed comparing regression lines to determine whether a salinity treatment significantly differed from another before the critical oxygen tension (i.e. values above 53 mmHg for *H. crenulatus* and values above 48 mmHg for *H. sexdentatus*). This was used to assess whether metabolic rates of the animals changed whilst oxyregulating at different salinities.

If a regression line has a similar slope but crosses the y-axis at different values the lines are parallel. This means that the change in O_2 consumption rate is similar for both treatments but one group is consuming more. Where a difference in slope is found and a different intercept this can be interpreted as a difference in the rate of change of O_2 consumption and that the treatments result in different consumption rates, meaning a significant change in O_2 consumption between treatments. Where a line has no slope this suggests O_2 consumption has not changed over the change in DO.

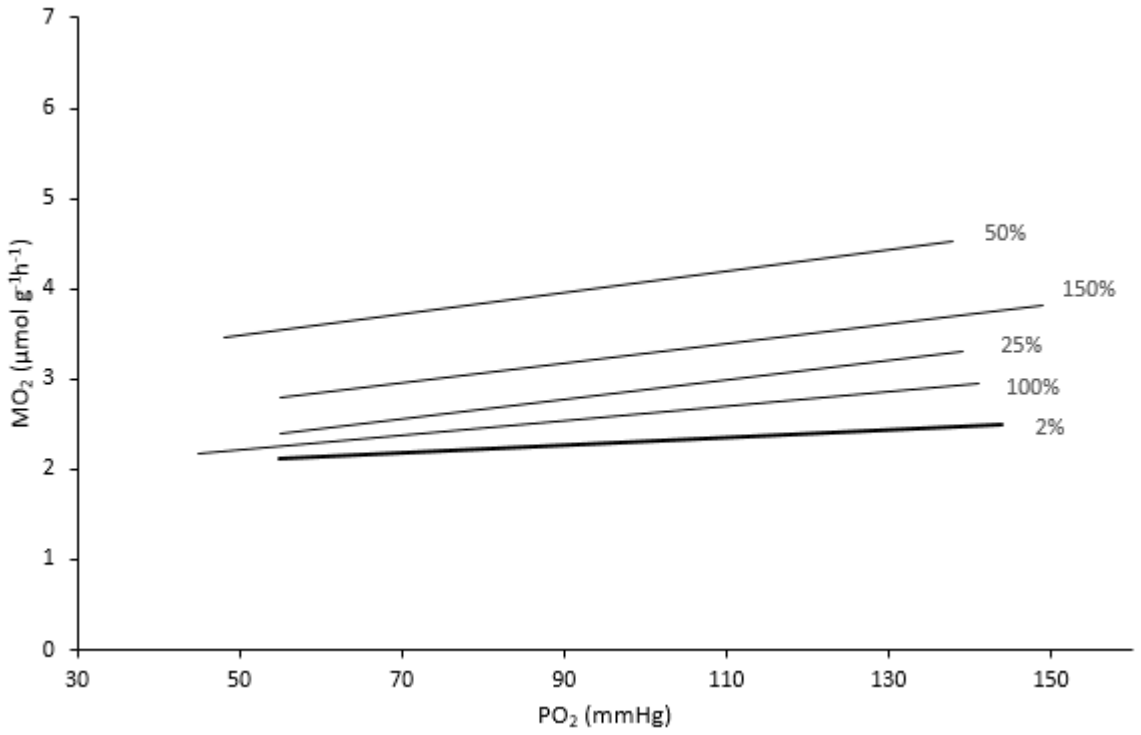

Figure 2.6: Oxygen consumption (MO_2) values of H. crenulatus *under progressively declining oxygen levels (PO_2) represented using regression lines. Each regression line represents a different salinity and bold lines indicate that a regression line does not significantly fit the data.*

Table 2.3: Regression equations, correlation coefficients and degrees of freedom for the H. crenulatus *salinity regression lines.*

Salinity	Regression equation	Correlation coefficient (r)	Degrees of freedom (df)
2%	y = 0.0041x + 1.893	0.230	53
25%	y = 0.0107x + 1.814	0.358	51
50%	y = 0.0119x + 2.888	0.357	52
100%	y = 0.008x + 1.806	0.273	57
150%	y = 0.0109x + 2.198	0.558	55

Table 2.4: Consumption F and P-values, intercept F and P-values and degrees of freedom for each salinity treatment regression line comparison of H. crenulatus*.*

Salinity treatment comparisons (%)	Consumption rate change F-value	Consumption rate change P-value	Intercept (consumption rate) F-value	Intercept (consumption rate) P-value	Degrees of freedom (df)
2 vs. 25	2.052	0.155	22.812	<0.05*	102
2 vs. 50	2.443	0.121	178.569	<0.05*	103
2 vs. 100	0.692	0.408	5.213	0.024*	108
2 vs. 150	4.191	0.043*	127.844	<0.05*	106
25 vs. 50	0.041	0.84	62.035	<0.05*	101
25 vs. 100	0.233	0.631	3.13	0.08	106
25 vs. 150	0.001	0.975	12.079	<0.05*	104
50 vs. 100	0.45	0.504	86.82	<0.05*	107
50 vs. 150	0.049	0.825	38.98	<0.05*	105
100 vs. 150	6.582	0.012*	0.293	0.589	110

For *H. crenulatus* there is no significant difference in the change of O_2 consumption rates (at dissolved oxygens above the P_{CRIT}) over time between treatments. However, exceptions were found when comparing the 2% and 150% treatments and the 100% and 150% salinity treatments (Table 2.4). There were, however, differences in the oxygen consumption rate between most treatments (Fig 2.6). Exceptions were found for the 25% vs. 100% and 100% vs. 150% treatments which have similar consumption rates.

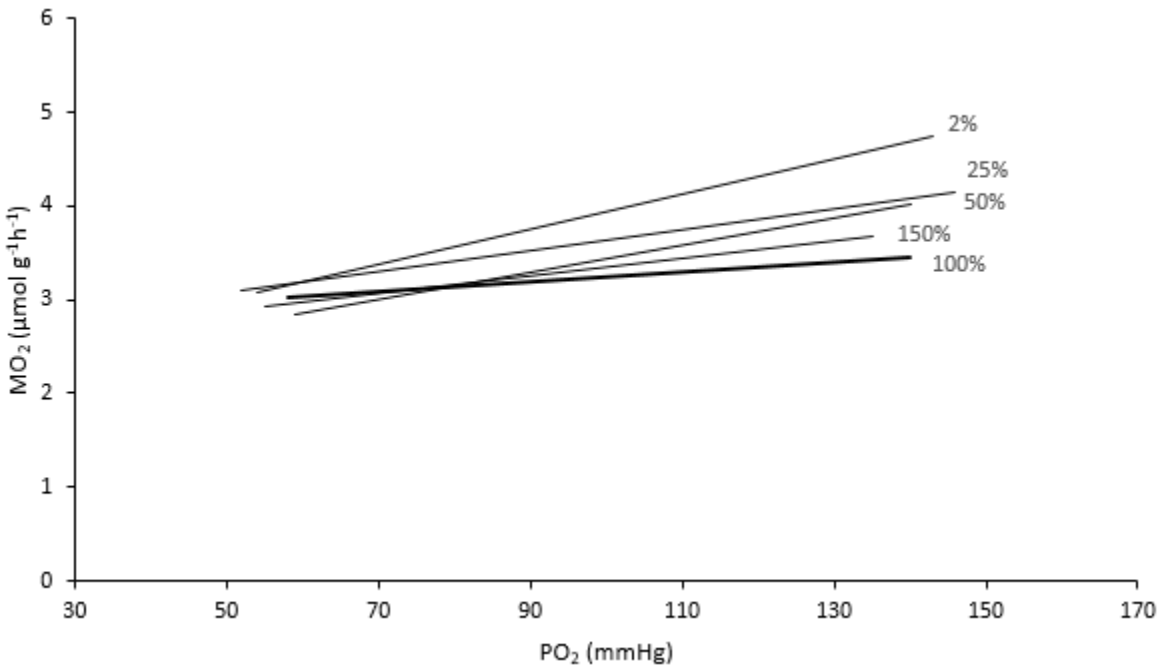

Figure 2.7: Oxygen consumption (MO_2) values of H. sexdentatus *under progressively declining oxygen levels (PO_2) represented using regression lines. Each regression line represents a different salinity and bold lines indicate that a regression line does not significantly fit the data.*

Table 2.5: Regression equations, correlation coefficients and degrees of freedom for the H. sexdentatus *salinity regression lines.*

Salinity	Regression equation	Correlation coefficient (r)	Degrees of freedom (df)
2%	y = 0.0188x + 2.072	0.539	50
25%	y = 0.0112x + 2.509	0.379	53
50%	y = 0.0145x + 1.989	0.449	50
100%	y = 0.0052x + 2.728	0.197	49
150%	y = 0.0094x + 2.405	0.391	48

Table 2.6: Consumption F and P-values, intercept F and P-values and degrees of freedom for each salinity treatment regression line comparison of H. sexdentatus*.*

Salinity treatment comparisons (%)	Consumption rate change F-value	Consumption rate change P-value	Intercept (consumption rate) F-value	Intercept (consumption rate) P-value	Degrees of freedom (df)
2 vs. 25	1.815	0.181	3.919	0.051	101
2 vs. 50	0.519	0.473	11.245	0.001*	98
2 vs. 100	5.713	0.019*	21.222	<0.05*	97
2 vs. 150	3.017	0.086	17.7	<0.05*	96
25 vs. 50	0.359	0.55	1.892	0.172	101
25 vs. 100	1.206	0.275	6.808	0.01*	100
25 vs. 150	0.115	0.735	4.481	0.037*	99
50 vs. 100	2.742	0.101	1.427	0.235	97
50 vs. 150	0.921	0.34	0.312	0.578	96
100 vs. 150	0.701	0.405	0.417	0.52	95

A similar pattern emerged for *H. sexdentatus* with no significant differences between oxygen consumption rate changes over time for the majority of the salinity treatments (Fig. 2.7). However, one exception was found when comparing 2% vs. 100% salinity (Table 2.6). For *H.* sexdentatus differences were found between O_2 consumption rates between some of the salinity treatments. The 2% salinity was found to have a significantly higher consumption rate than the 50%, 100% and 150% treatments (Fig. 2.7). The 25% salinity treatment was also found to have a significantly higher O_2 consumption rate than the 100% and 150% treatments (Fig. 2.7). Many of the treatments displayed no difference in consumption rates or consumption rate changes over time (2% vs. 25%, 25% vs. 50%, 50%

vs. 100%, 50% vs. 150%, and 100% vs. 150%) (Table 2.6). This suggests that for *H. sexdentatus* O_2 consumption rates in response to salinities above 25% are maintained at a similar level.

Between species oxygen consumption rate comparisons.

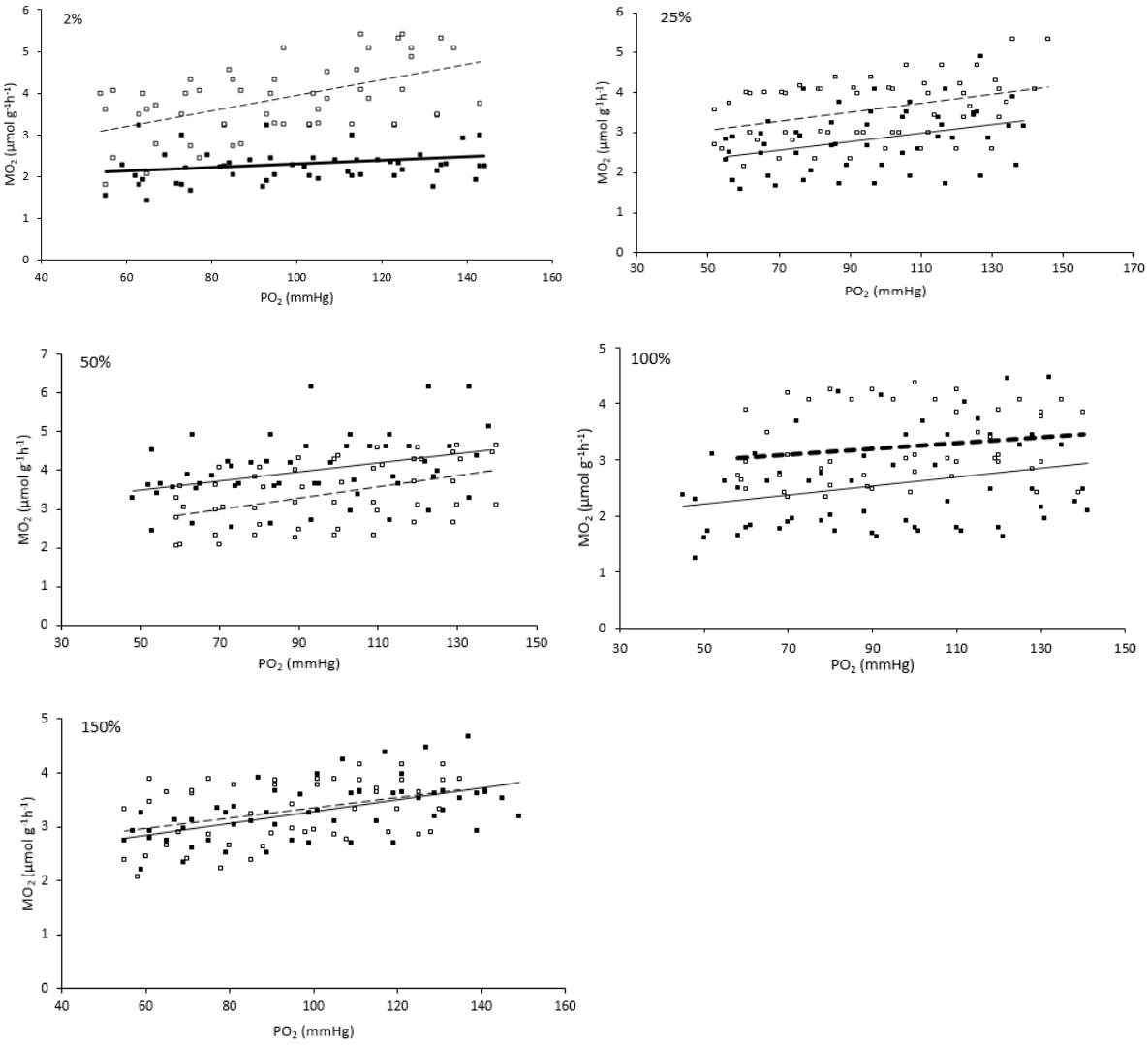

Figure 2.8: Oxygen consumption rate (MO_2) comparison of H. crenulatus *(■) and* H. sexdentatus *(□) under progressively declining oxygen levels (PO_2). Each graph represents a different salinity. Solid lines represent the regression lines for* H. crenulatus *and dashed lines represent the regression lines for* H. sexdentatus*. Bold lines indicate that a regression line does not significantly fit the data.*

Table 2.7: Consumption F and P-values, intercept F and P-values and degrees of freedom for the species regression line comparisons at each salinity.

Salinity	Consumption rate F-value	Consumption rate P-value	Intercept F-value	Intercept P-value	Degrees of freedom (df)
2%	9.507	0.00264*	174.346	<0.05*	101
25%	0.007	0.934	27.529	<0.05*	102
50%	0.187	0.666	18.207	<0.05*	100
100%	0.255	0.615	21.102	<0.05*	104
150%	0.14	0.709	0.511	0.476	101

Differences in O_2 consumption rates between species were dependent upon the salinity treatment. For the 2% treatment the two species differed in their consumption rates and consumption rate changes over time. This result contrasts the 25%, 50%, and 100% treatments in which there were no significant differences between the O_2 consumption rate changes rather a difference was found for the oxygen consumption rate (Fig 2.8). Furthermore, the 150% treatment showed a unique result in which both crab species appeared to consume oxygen at the same rate and had the same change in rate over time as one another (Table 2.7).

2.5. Discussion

Crustaceans are remarkable in their abilities to exploit a wide range of niches with fluctuations of environmental parameters. Such changes are seen to vary from short term (hours) to long time sequences i.e., days and seasonal changes. Because of their physiological, biochemical and behavioural abilities these aquatic animals can tolerate and maintain homeostatic functioning in fluctuating temperatures, salinities and low oxygen levels. Crabs inhabiting intertidal zones are exposed to variations in salinity where species found higher up shore can be subject to high salinities. In hypoxic waters crustacea can utilise various physiological tools to survive, these include a bradycardia response, hyperventilation, hypometabolism and the modulation of haemocyanin oxygen affinity (Bonvillain et al., 2012). The metabolic responses were investigated for the two New Zealand species. Although mechanisms underlying salinity and hypoxia tolerance have been extensively studied for decapod crabs in the past, this is the first study to investigate these two stressors, and the combination of these stressors, on the New Zealand crabs *H. crenulatus* and *H. sexdentatus*.

Metabolic rates and critical oxygen tensions (P_{CRIT}) of the crabs

The metabolic rates of decapods in response to hypoxia are believed to be primarily dependent on the physiological state of the animal and the environment in which they are found (Herreid, 1980). Among other factors, pollutants, pH, temperature, activity, size and salinity also play a role in the determination of the metabolism of aquatic animals when exposed to hypoxic conditions (Rosas et al., 1999). Decapods will typically attempt to maintain MO_2 as the water oxygen is reduced, however, for most species this is only achieved down to a critical oxygen tension (P_{CRIT}) (McMahon, 2001). At this tension most oxyregulating species can no longer maintain their oxygen consumption rate which drastically declines. At the critical level the animal switches from an oxyregulatory to an oxyconforming response.

The MO_2 of the New Zealand species was maintained down to a PO_2 of 52.54 ± 0.11 mmHg for *H. crenulatus* and 47.76 ± 0.14 mmHg for *H. sexdentatus* at which point MO_2 was no longer maintained and declined with the declining PO_2. This value represents the P_{CRIT} of the animal and is considered to be the point at which an oxyregulator switches to an oxyconforming response. The two methods used to calculate the critical oxygen tension of these species (combined data P_{CRIT} (n = 6) and individual P_{CRIT}s) were in accord with one another (53.08, 51.99 for *H. crenulatus* and 48.30, 47.22 for *H. sexdentatus*) validating the findings. The critical oxygen tensions of these species are within the range found for other decapods, such as the freshwater crayfishes *Austropotamobius pallipes* (40 mmHg) and *Procambarus clarkii* (44.8 ± 2.4 mmHg) and are also in agreement with previous findings for decapod crabs such as *Lithodes santolla* (67.5 mmHg) and *Carcinus maenas* (~60-80 mmHg) (Taylor, 1976; Wheatly and Taylor, 1981; Reiber and McMahon, 1998; Paschke et al., 2010). Other crab species have weaker regulatory abilities, such as *Callinectes danue*, which becomes dependent on water oxygen availability below 97 mmHg (Rantin et al., 1996). Other species completely oxyconform and are entirely dependent on the water oxygen availability no matter the PO_2, such as the crab *Paralithodes kamtschatica* (Cameron, 1989). The P_{CRIT} values for *H. crenulatus* and *H. sexdentatus* were not significantly different across the various salinities. This suggests that dissolved oxygen is the key driver in determining the critical oxygen tension of these species when they are exposed to multiple DO and salinity stressors.

Although salinity was not a factor for either species' P_{CRIT} level it did play a role in their MO_2 at dissolved oxygens above the P_{CRIT}. Most estuarine fauna are likely to alter their metabolic rates in varying salinities (Stickle and Sabourin, 1979). For *H. crenulatus* there were few differences in O_2 consumption rate changes over time between the different treatments, there was, however, a difference in the oxygen consumption rates between almost all treatments (Fig. 2.6). Differences in O_2 consumption rate changes over time were found between the 2% and 150% and the 100% and 150% treatments. This suggests that at salinity extremes (hyposaline 2% and hypersaline 150%) MO_2 is significantly altered over time in contrast to environmentally normal salinities in *H. crenulatus*. O_2 consumption rate differences were found between all treatments except for between the 25% vs. 100% and 100% vs. 150% treatments. For the 100% vs. 150% treatment this could be attributed to the

difference in the change of oxygen consumption over time (at DO's above the P_{CRIT}). However, the results for the 25% vs. 100% treatments (where there is no difference in change of O_2 consumption rates over time) suggest the O_2 consumption rates, and change in rates over time, are similar between these treatments.

A similar result was found for *H. sexdentatus* where O_2 consumption rate changes over time were not significantly different from one another with a single exception found between the 2% and 100% treatments. For *H. sexdentatus*, in contrast to *H. crenulatus*, oxygen consumption rates were similar between most salinities (Fig. 2.7). Exceptions were found between the 2% salinity, which had a significantly higher consumption rate than the 50%, 100% and 150% treatments, and the 25% salinity treatment, which also had a significantly higher O_2 consumption rate than the 100% and 150% treatments. These findings indicate that *H. sexdentatus* is able to maintain its MO_2 in high salinities. This might be due to *H. sexdentatus* residing in a rocky shore environment, in comparison to *H. crenulatus'* intertidal estuarine environment, which are likely to encounter higher salinities (due to being higher up the shoreline and more likely to have water evaporate). In this particular environment (Waipara beach) *H. sexdentatus* may also be more likely to encounter flooding from excessive rain and encounter more freshwater, therefore lower salinities as well, due to the freshwater stream outlet directly onto the beach (Fig. 2.2).

Differences in O_2 consumption rates between species were dependent upon the salinity treatment. For the 2% treatment the two species differed in their consumption rates and consumption rate changes over time. This result contrasts the 25%, 50%, and 100% treatments in which there were no significant differences between the O_2 consumption rate changes rather a difference was found for the oxygen consumption rates (Fig 2.8). Furthermore, the 150% treatment showed a unique result in which both crab species appeared to consume oxygen at the same rate and had the same change in rate over time as one another (Table 2.7).

When comparing the two study species we find both similarities and differences between their oxygen consumption abilities. The results suggest that at more extreme salinities (2% and 150%) these species will perform in different ways than they would within environmentally normal ranges (between 25% and 100%). In hypo-salinity waters (2%) the two species differed in their consumption rates and consumption rate changes over time

with *H. sexdentatus* displaying a higher MO_2 than *H. crenulatus* (Fig. 2.8). In salinities within the species' normal range (25%, 50%, and 100%) there were no significant differences in O_2 consumption rate changes between species, however, there were differences in oxygen consumption rates (*H. sexdentatus* had higher rates than *H. crenulatus* for the 25% and 100% treatments and *H. crenulatus* had a higher rate for the 50% treatment). In hyper-salinity waters (150%) both species consumed oxygen at the same rate and had the same rate of change over time. These findings suggest that at low salinities these species perform differently and at high salinities these species utilise similar oxygen consumption strategies.

These findings are supported by previous work on osmoregulating decapod crustaceans, such as *Panopeus herbstii*, *Callinectes sapidus*, *Callinectes similis*, where increased respiration is found at salinities lower than the species environmental norms (Dimock and Groves, 1975; Findley et al., 1978; Guerin and Stickle, 1997; Hulathduwa et al., 2007). For example, investigations by Shumway (1983) found increased consumption rates in low salinities for four Brazilian crab species, *Panopeus herbstii*, *Callinectes danae*, *Petrolisthes armatus* and *Pachygrapsus transversus*. Here oxygen consumption for two of the species (*C. danae* and *P. transversus*) was unaffected by salinities between 40-100% (produced from a 34 ppt stock), however, a further dilution to 20% resulted in an increase in metabolic rate and a maximum metabolic rate was found at 0% salinity. *P. herbstii* responded similarly where salinity around 60% caused an increased metabolic rate, with a maximum MO_2 found at the lowest 2% salinity. Finally, *P. armatus* showed a gradual increase in O_2 consumption from 100 to 60% with a maximum found at the 20% salinity treatment (Shumway, 1983). These findings are consistent with the findings for *H. crenulatus* and *H. sexdentatus* which displayed different responses at the lowest 2% salinity.

At the lower 2% hyposalinity the two New Zealand species performed differently in their responses compared to the very similar responses they displayed at the 150% hypersalinity treatment. This suggests that these animals utilise different physiological, metabolic and/or behaviour coping mechanisms, or lack thereof, in hyposaline conditions. At the higher 150% salinity both species consumed oxygen at the same rate and magnitude suggesting these species have a similar mechanism or a similar evolutionary history that has allowed them to exploit and survive within environmental niches that have comparable hypersalinity fluctuations.

Differences in physiological and regulatory responses of aquatic animals can be a result of various evolutionary and adaptive abilities. The degree to which a species or individual within a species oxyregulates or oxyconforms in different salinities is dependent on several factors including body size, development, moult cycle, stress level, temperature and locomotory and behavioural abilities (Thompson and Pritchard, 1969; Pörtner and Grieshaber, 1993). These physiological adaptations can be attributed to the environmental niches in which the animals reside. *H. crenulatus* live in estuaries, whereas, *H. sexdentatus* is found in rocky boulder beaches with Waipara beach in particular having freshwater stream outlets directly onto the beach (Fig. 2.2). This may explain why the species displayed different O_2 consumption rates at hyposalinities. The sampled population of *H. sexdentatus* could be more likely to encounter niches with outflows of freshwater (and are forced to reside within them due to the rocky outcropping on the patch of otherwise sand-covered Waipara beach where the animals were collected (Fig. 2.2)), whereas the sampled *H. crenulatus* population lived within an estuary where they may be more able to move to areas with optimal environmental conditions. *H. crenulatus* was also much smaller than the sampled *H. sexdentatus* (Fig. 2.1) which could be another contributing factor in the different metabolic rates of these animals.

Chapter Three

3. Heart rate responses of crabs to hypoxia and salinity

3.1. Introduction and aims

Estuaries and intertidal waterways are not only characterised by fluctuations in hypoxia but are also subject to regular changes in salinity, temperature and aerial exposure. In order for animals to successfully exploit these regions they must not only employ behavioural mechanisms but must also have adaptive physiological and biochemical mechanisms as well. An adaptation by which crabs are able to successfully inhabit these environments is to regulate and modify their heart rate and cardiac stroke volumes (which is used as a homeostatic regulatory response). Variability of heart rate is commonly used as a physiological measure of stressor tolerance in crustaceans (Depledge and Lundebye, 1996; Bierbower and Cooper, 2009). This is because the heart rates of crabs are typically elevated (tachycardia) in response to stress. Certain species are able to rapidly increase their heart and respiration rates to survive physical perturbations or to avoid predation (Bamber and Depledge, 1997).

Under hypoxic conditions heart rate, stroke volume and total cardiac output are modified in fish (Shiels et al., 2002; Farrell, 2007) and crustaceans (Defur and Mangum, 1979; Henry et al., 1990; McMahon and Burnett, 1990; Harper and Reiber, 1999). Crustaceans can serve as good biomarkers of stress under hypoxic conditions because they are able to decrease their heart rate as hypoxia increases (McMahon and Burnett, 1990). This is known as a bradycardic response. In combination with this bradycardia response decapod crabs may also increase respiratory pumping (ventilation) in order to maintain O_2 perfusion of the gills (Reiber and McMahon, 1998). These physiological adaptations are used to enhance oxygen uptake and thus help compensate for external hypoxia (Reiber and McMahon, 1998). Hypoxia induced bradycardia is found in many decapod species, such as *Carcinus maenas* and *Homarus americanus* (Wilkens, 1993; Wilkens et al., 1996), however it has yet to be investigated in the two intertidal crabs *H. crenulatus* or *H. sexdentatus*.

Crabs are also able to alter their heart rate and cardiac outputs in response to changes in water salinity. Most species have a preference for and maintain their internal media close to 100% salinity, i.e. the natural seawater in which they reside (McGaw, 2001). An example is found in the lined shore crab, *Pachygrapsus crassipes*, which will avoid low salinities and show a preference toward salinities closest to their natural 100% seawater (Gross, 1957). When avoidance behaviour is not possible crabs will either osmoregulate their internal osmotic content or osmoconform to their surrounding environment in order to survive. Decapod crabs often employ the former strategy with some species, such as *Hemigrapsus nudus, Callinectes sapidus,* and *Carcinus maenus*, being hyperosmotic regulators that can survive in a range of salinities (Hume and Berlind, 1976; Taylor, 1977; Corotto and Holliday, 1996; McGaw and Reiber, 1998). Some species, such as *H. nudus*, can even survive at low salinities (6% seawater) for extended periods of time (2 days), however, they have a decreased capacity to osmoregulate in salinities below 12% (Dehnel, 1962).

Although most estuarine decapods can withstand a wide range of salinities some species, such as *Cancer magister*, are classified as weak hyperosmoregulators and conversely cannot survive prolonged salinity exposures under 36% (12 ppt) (McGaw and Reiber, 1998). Decapods increase their heart rates in low salinities in order to withstand the resultant homeostatic stress. Increased heart rate and cardiac output has been found in *Carcinus maenas* in salinities below 45% sea water; this contrasts a decreased heart rate in 45-80% sea water (Spaargaren, 1974). In comparison, Taylor et al. (1977) and McGaw and McMahon (1996) found no change in heart rate of *C. maenas* when exposed to 50% sea water. These results, which may seem like contradictions, may be due to crab heart rates being very variable and may involve an initial shock response of the animal at the beginning of experimentation (McGaw and McMahon, 1996). This suggests that decapods require substantial time (hours) to acclimate to the experimental apparatus upon handling and experimentation. Decapod crabs appear to increase their heart rates in order to maintain homeostatic balance by moving more water past their gills and through their respiratory and cardiovascular systems. This physiological response is used in order to uptake and regulate internal Na^+, Cl^- and osmotic balances.

Heart rate is a commonly used measure of stressor tolerance in crustaceans (Hume and Berlind, 1976; Cumberlidge and Uglow, 1977; Defur and Mangum, 1979; Wheatly and

Taylor, 1981; Ketpadung and Tangkrock-olan, 2006; Cooper and Bierbower, 2009). Heart rate was measured for both *H. crenulatus* and *H. sexdentatus* for each of the previously mentioned stressor treatments and combination of stressors (five salinity and four dissolved oxygen treatments refer to chapter two). The crabs were placed in individual 400 ml containers with one of the salinity treatments and one of the dissolved oxygen treatments (achieved by bubbling the water). Heart rate was measured for an acute exposure period (6 hours) and will be used in conjunction with the oxygen consumption data to further understand and identify the physiological tolerances of *H. crenulatus* and *H. sexdentatus* to salinity and dissolved oxygen.

3.2. Methods and Materials

Heart rate was measured using an infrared (IR) sensor attached non-invasively to the crab's carapace. In order to achieve this a rubber ring was superglued to the back of the crab's carapace where heart rate was measured best. The IR sensor was then fitted into this ring (Fig. 3.1). The sensor consists of both IR transmitter and detector units which shoot light through the crab and receive light that bounces back. The heart rate of the animal was recorded as differences in the reflected light. The differences result in a voltage change detected by the IR sensor. This is then fed to a custom made signal conditioning box connected to a PowerLab 4/25 and/or 4/25T units (AD Instruments). The animal's cardiac transcript is finally visually displayed on LabChart 7 computer software.

Figure 3.1: Photographs of H. crenulatus *(left) and* H. sexdentatus *(right) with a rubber ring glued to their carapace and an optical probe fitted within*.

Six animals were used for each trial where each individual was exposed to all of the trial treatments. For example, 6 *H. crenulatus* were each exposed to 2, 25, 50, 100, and 150% salinities and a further 6 *H. crenulatus* were exposed to 6, 25, 50, 100% DO. Each crab was given 48 hours recovery time between exposure treatments. For the salinity trials crabs were exposed to salinities in the order of 100, 50, 25, 150, and 2% salinity. For the DO trails

crabs were exposed to DO's in the order of 100, 50, 25, and 6% DO. This order was to minimise the stress on the crabs.

Heart rate experiments used a similar setup to the DO trials. The same containers (i.e. 10.5 x 6.2 x 6.2 cm plastic containers) and 15 ± 1 ^{o}C water baths were used. One difference was found in the lids for these experiments in which a smaller 5 mm hole was made rather than a 3 cm. Through this hole the IR sensor and an oxygen tube, with an air stone connected, were fitted before the crab was immersed into the trial solution. For all salinity trials the water was continuously aerated using Aqua One Stellar 380D air pumps in order to maintain the water at 100% oxygen saturation. For the DO trials a Wösthoff gas mixing instrument (DIGAMIX®) was used to create the desired dissolved oxygen level.

These trials used either 1, 3 or 6 animals at a time depending on available equipment. Trials using the Wösthoff ran a maximum of 1 animal at a time whereas salinity trials could run 6. Where trials ran 3 or more crabs small plastic obstructions were placed between each container in order to account for stress that might be induced by the animals viewing each other (Fig. 3.2).

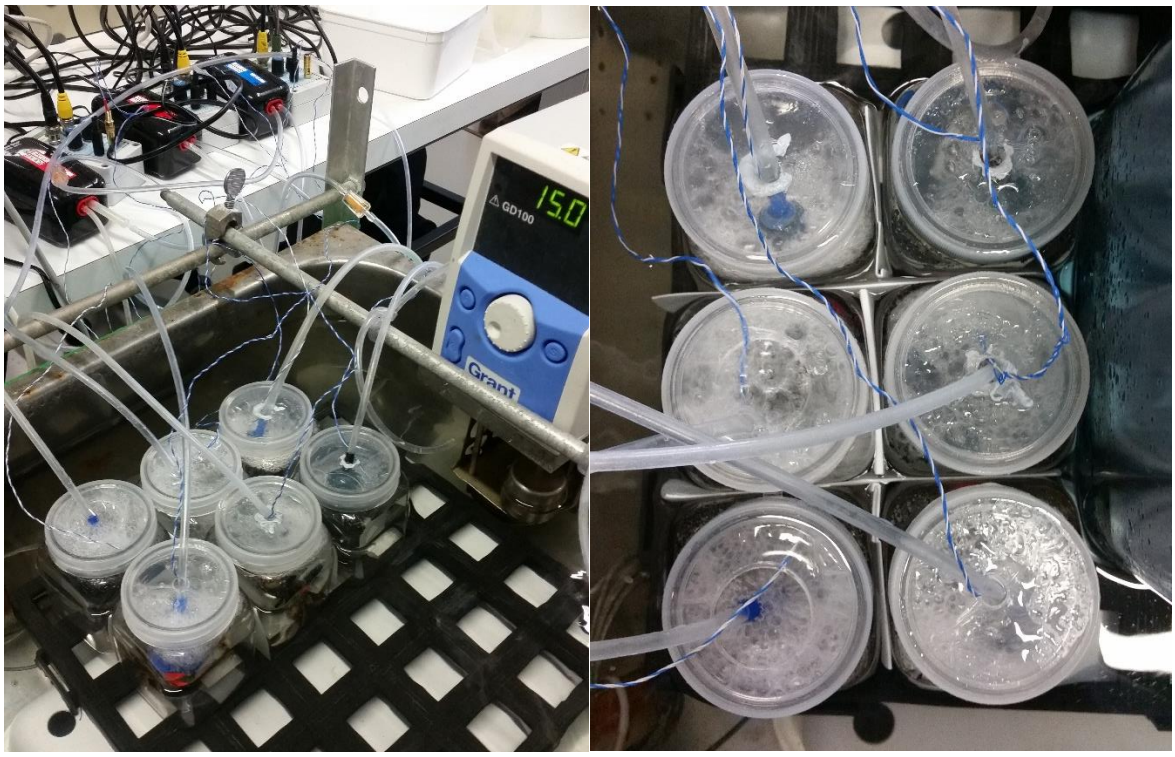

Figure 3.2: Photographs showing the container set up for 6 crabs in the water bath.

Statistical analysis was carried out using the R statistical programme. Statistical significance was taken at the level of $P < 0.05$. Normality of data was tested using a quantile-quantile normality plot as well as a Shapiro-Wilk normality test. Homogeneity of variance was tested by plotting the fitted against the residual values as well as a Levene's test for homogeneity of variance. Where treatment effects were shown to be significant post-hoc Tukey's HSD analyses were performed. Where the assumption of normality was violated a non-parametric Kruskal-Wallis one-way analysis of variance was performed. Following this a post-hoc Dunn's test with a Bonferroni adjustment was performed to account for and analyse the multiple comparisons. For the between species heart rate comparisons parametric data was analysed using one-way analysis of variance (ANOVA) and non-parametric data was analysed using a Kruskal-Wallis one-way analysis of variance.

3.3. Results

All heart rate experiments ran for a total of 6 hours per crab per treatment. For *H. crenulatus* heart rate ranged from 32.3 to 196.1 beats per minute (bpm). For *H. sexdentatus* heart rate ranged from 16.4 to 169.7 bpm. For all experiments time, from the first recording (at the one hour mark) onwards, did not significantly affect or alter heart rate ($p > 0.05$). This suggests that time was not a factor in relation to the animals' heart rates. The six hours of data were therefore combined for each treatment and analysed as one set of data. Only one exception to this result was found where, for *H. sexdentatus*, the hour one heart rate recordings for the 50% salinity treatment were significantly different to the other five hours of recordings ($p < 0.05$). Because of this the first hour of recordings for the *H. sexdentatus* 50% salinity trials were removed.

Using the accumulated heart rate data (n = 36 heart rate recordings for each experiment with the exception of n = 30 for the *H. sexdentatus* salinity trial) it was clear that hypoxia affected the heart rates of both crab species. At the lowest dissolved oxygen treatment both species appeared to display a bradycardic response in which their heart rates significantly decreased when compared to the higher DO levels (43.1 ± 7.6 bpm for *H. crenulatus* and 32.9 ± 10.1 bpm for *H. sexdentatus*).

For *H. crenulatus* heart rates significantly increased from 43.1 bpm at the 6% DO treatment to 108.5, 134.9 and 122.2 bpm for the 25%, 50% and 100% DO treatments respectively (Fig. 3.3). There were significant differences between the 6% and the other three treatments and similarly there were significant differences between the 25% and the other treatments (Table 3.1). At the higher DO treatments (50% and 100%) there was no significant difference between treatments.

A similar result was found for the *H. sexdentatus* DO trials where heart rates increased from a bradycardic 6% response (32.9 bpm) to higher 62.1, 76.6 and 102.9 bpm responses at the 25%, 50% and 100% DO treatments respectively (Fig. 3.4). There were significant differences between the 6% and the other three treatments, as well as, the 100% and the other three treatments (Table 3.2). There were no significant differences between the 25% and 50% treatments.

Results for the *H. crenulatus* salinity trials found no significant differences in heart rates between any of the five treatments (Kruskal-Wallis P = 0.2) (Fig. 3.5). The heart rates were 120.7, 116.5, 114, 106.9 and 119.2 bpm for the 2%, 25%, 50%, 100% and 150% salinity treatments respectively.

The heart rate salinity trials of *H. sexdentatus* exhibited significant differences between many of the treatments. *H. sexdentatus* heart rates were found to be highest at the 2% (123 bpm) and 150% (130.8 bpm) treatments, followed by lower heart rates for the 25% (97.8 bpm) and 100% (108 bpm), and a lowest mean heart rate for the 50% (78.6 bpm) treatment (Fig. 3.6). The 2% treatment was not significantly different from the 100% or 150% treatment heart rates. The 25% treatment was not significantly different from the 50% or 100% treatment heart rates and the 50% treatment was not significantly different to the 25% treatments mean heart rate (Table 3.3).

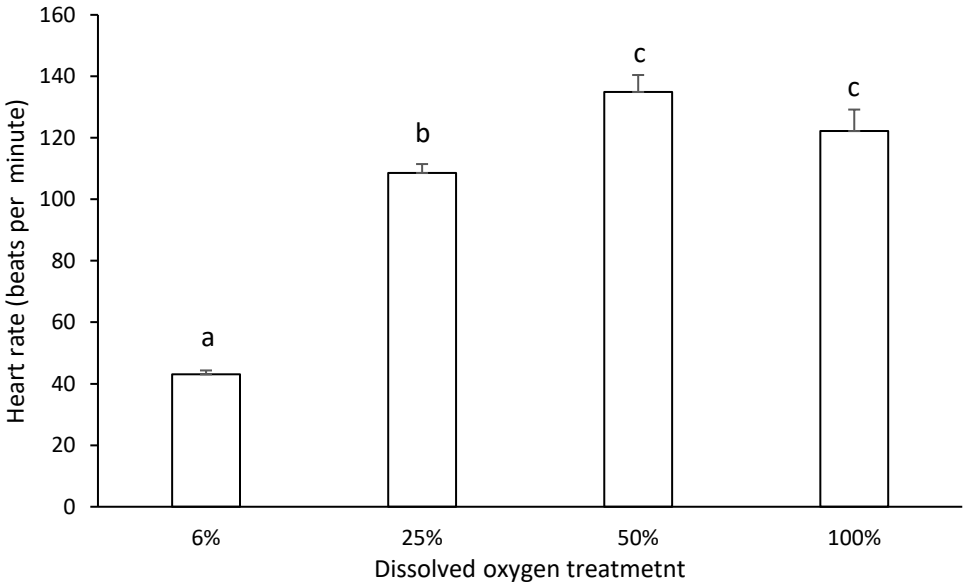

Figure 3.3: Mean heart rate (bpm) ± S.E.M. of H. crenulatus *at different dissolved oxygen levels. Plots sharing letters are not significantly different.*

The *H. crenulatus* dissolved oxygen data violated the assumptions of normality and homogeneity (Shapiro test P <0.05, Levene's test F value = 23.34, P <0.05). For this data (represented in Fig. 3.3) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of dissolved oxygen found (P <0.05). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 93.27. A Dunn's-test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 3.1).

Table 3.1: The P-values of the Dunn's-test for multiple comparisons for the H. crenulatus *dissolved oxygen heart rate data. The P-values were adjusted using the Bonferroni method.*

	100%	25%	50%
25%	0.002	-	-
50%	1.00	0.02	-
6%	<0.05	<0.05	<0.05

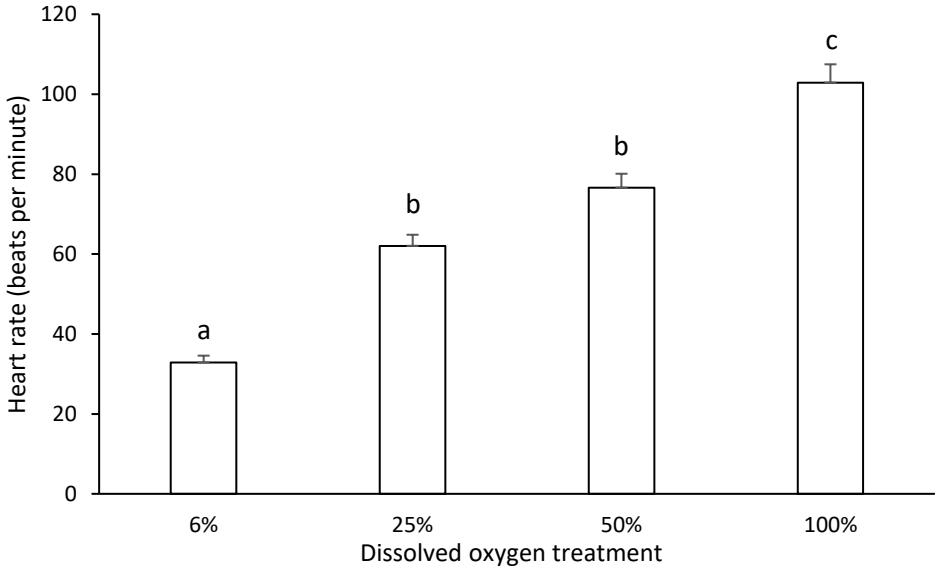

Figure 3.4: Mean heart rate (bpm) ± S.E.M. of H. sexdentatus *at different dissolved oxygen levels. Plots sharing letters are not significantly different.*

The *H. sexdentatus* dissolved oxygen data violated the assumptions of normality and homogeneity (Shapiro test P <0.05, Levene's test F value = 17.82, P <0.05). For this data (represented in Fig. 3.4) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of dissolved oxygen found (P <0.05). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 98.48. A Dunn's-test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 3.2).

Table 3.2: The P-values of the Dunn's-test for multiple comparisons for the H. sexdentatus *dissolved oxygen heart rate data. The P-values were adjusted using the Bonferroni method.*

	100%	25%	50%
25%	<0.05	-	-
50%	0.04	0.21	-
6%	<0.05	<0.05	<0.05

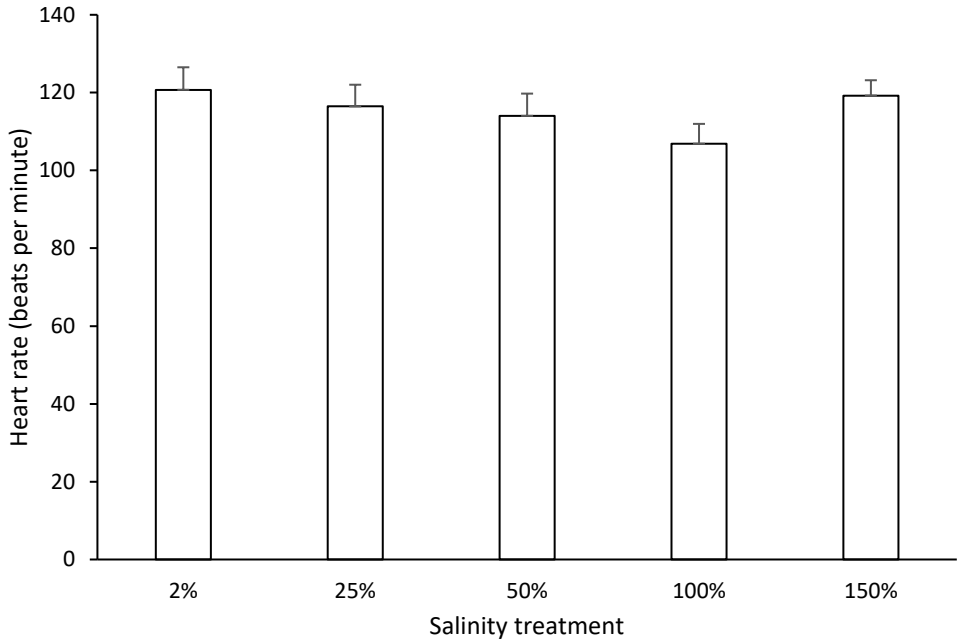

Figure 3.5: Mean heart rate (bpm) ± S.E.M. of H. crenulatus *in differing salinity treatments. There were no significant differences between treatments.*

The *H. crenulatus* salinity data violated the assumptions of normality and homogeneity (Shapiro test P <0.05, Levene's test F value = 2.87, P = 0.02). For this data (represented in Fig. 3.5) a Kruskal-Wallis one-way analysis of variance was performed, with no significant effect of salinity found (P = 0.2) meaning no post-hoc tests were necessary. Summary of the data: df = 4, Kruskal-Wallis chi-squared value = 5.98.

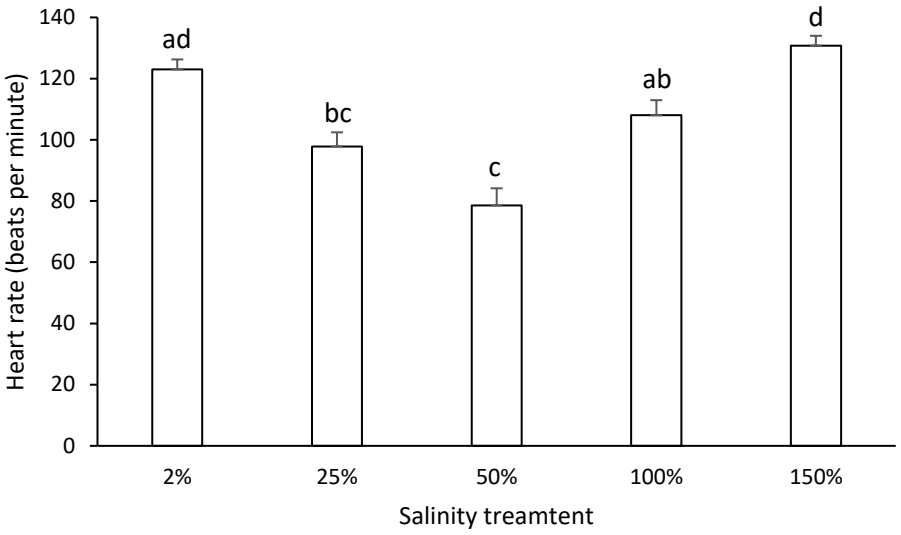

Figure 3.6: Mean heart rate (bpm) ± S.E.M. of H. sexdentatus *in differing salinity treatments. Plots sharing letters are not significantly different.*

The *H. sexdentatus* salinity data violated the assumptions of normality and homogeneity (Shapiro test P = 0.0001, Levene's test F value = 4.35, P = 0.002). For this data (represented in Fig. 3.6) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 4, Kruskal-Wallis chi-squared = 57.83. A Dunn's-test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 3.3).

Table 3.3: The P-values of the Dunn's-test for multiple comparisons for the H. sexdentatus *salinity heart rate data. The P-values were adjusted using the Bonferroni method.*

	100%	150%	2%	25%
150%	0.005	-	-	-
2%	0.17	1.00	-	-
25%	1.00	<0.05	0.002	-
50%	0.01	0.14	<0.05	0.54

Species heart rate comparisons

For the dissolved oxygen trials the heart rates of the two New Zealand crab species were analysed using a non-parametric Kruskal-Wallis test and were found to be different from one another. This was found at each of the four stressor levels (Fig. 3.7) with the Kruskal-Wallis test each having a P-value less than 0.05 (2% <0.05, 25% <0.05, 50% <0.05, and 100% = 0.02). *H. crenulatus* were found to have higher heart rates at all the DO levels in contrast to *H. sexdentatus*.

For the salinity trials the heart rate comparisons were analysed using a Kruskal-Wallis test where two of the treatments were not significantly different, at the 2% (P = 0.34) and 100% (P = 0.07) experiments (Fig. 3.8). The crab species displayed different heart rates at the other three salinities. In the lower 25% and 50% salinities *H. crenulatus* had a higher heart rate than *H. sexdentatus* (25% P = 0.009, 50% P <0.05) and at the highest 150% salinity (P = 0.04) the latter species had a higher heart rate than the former.

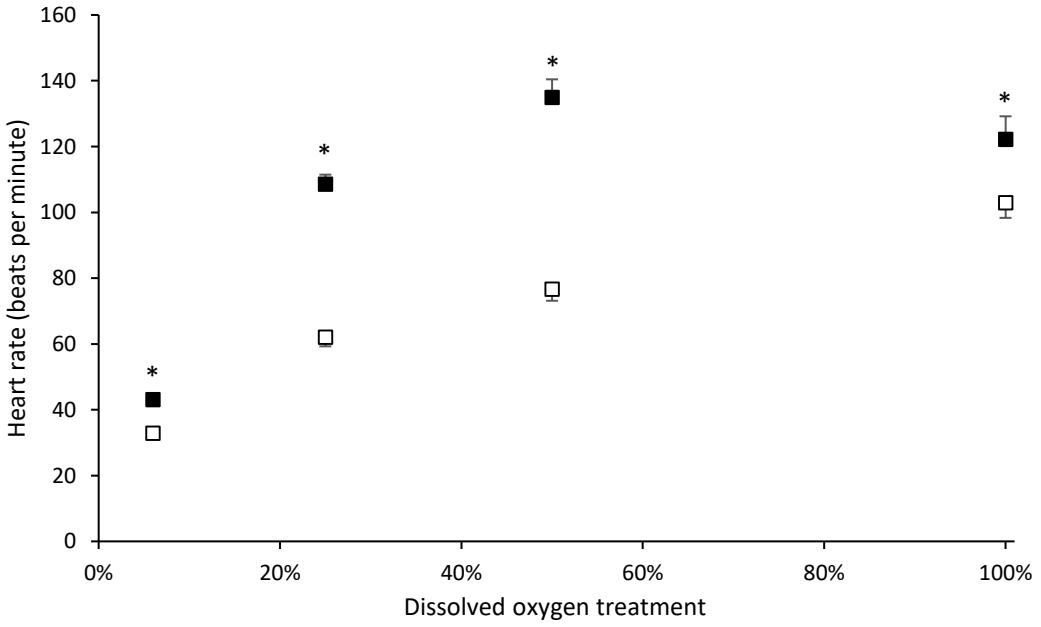

Figure 3.7: Heart rates of H. crenulatus *(■) and* H. sexdentatus *(□) ± S.E.M. in differing salinity treatments. * Significantly different from one another.*

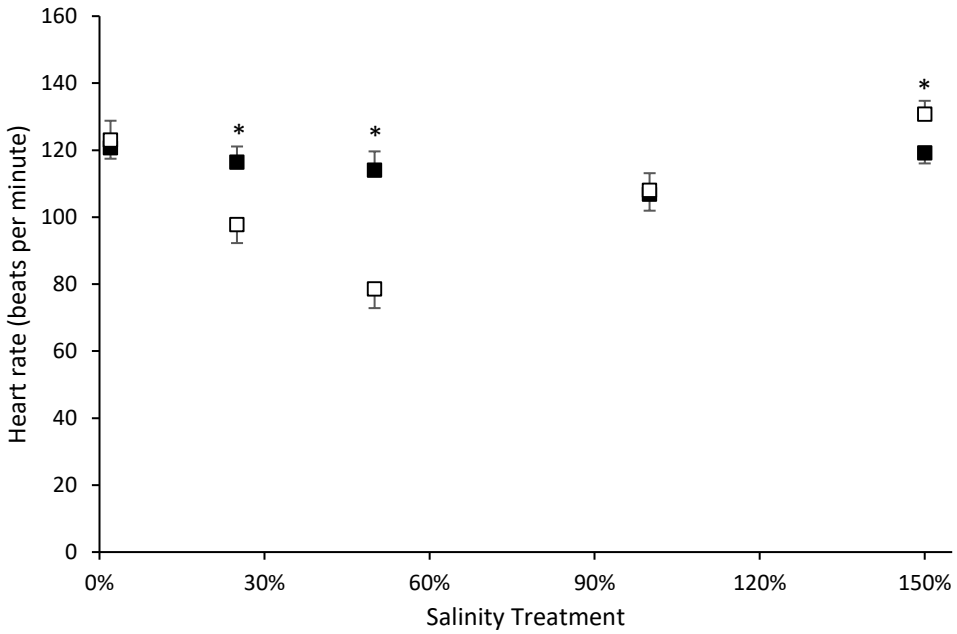

Figure 3.8: Heart rates of H. crenulatus *(■) and H. sexdentatus (□) ± S.E.M. in differing dissolved oxygen treatments. * Significantly different from one another.*

3.4. Discussion

Under hypoxic conditions most crustaceans are known to decrease their heart rate as a physiological response to manage stress (Wilkens, 1993; McMahon, 1999). For example, the heart rates of the marine Penaeid shrimp *Metapenaes ensis* decreased with decreasing dissolved oxygen concentrations (Wu et al., 2002). Heart rates of the freshwater crayfish's *Austropotamobius pallipes* and *Procambarus clarkii* are even found to decrease by half when exposed to hypoxic waters (from 63 bpm at 140 mmHg to 32 bpm at 30 mmHg for *A. pallipes* and from 125 bpm at 150 mmHg to 60 bpm at 25 mmHg for *P. clarkii*) (Wheatly and Taylor, 1981; Reiber and McMahon, 1998). As mentioned earlier in this chapter, under hypoxic conditions heart rates decrease with a concurrent increase in stroke volume (deFur and Mangum, 1989; Harper and Reiber, 1999). The energy-efficiency of this response results in an unchanged cardiac output which works by essentially slowing perfusion and allowing an increased time for the diffusion of oxygen to occur (Satchell, 1960; McMahon, 2001; Farrell, 2007).

A hypoxia-induced bradycardia is especially common below the P_{CRIT} of an animal due to insufficient oxygen supply to meet the aerobic demands of the circulatory and respiratory pumps (Airriess and McMahon, 1994). Hypoxia-induced bradycardia often occurs at or near the hypoxia tolerance limit of the animal (Mendonca and Gamperl, 2010). For example, the crab *Scylla serrata* maintains its heart rate in normoxic conditions, however, below a PO_2 of 50 mmHg heart rate rapidly drops (Hill and Koopowitz, 1975). Hypoxia does not always induce a bradycardic response where in some species a tachycardia (increased heart rate) is found. Increased ventilatory pumping induced by hypoxia is more commonly found in juveniles of a species rather than adults. For example, larval and early juvenile stages of the aforementioned shrimp *M. ensis* display a tachycardia in hypoxic conditions which occurs until the animal is in its mid-juvenile life stage, at which point its adult response is found (Spicer, 2001). This response may also be partially attributed to the size of the animal where smaller and larval crustaceans, for example *Daphnia magna*, commonly display a tachycardia (Pirow et al., 1999; McMahon, 2001).

Some animals are able to maintain their heart rates during hypoxia. This lack of bradycardia, or tachycardia, in hypoxia is due to either the animals not being under any apparent stress or because they have cardiac compensating parameters allowing them to sustain heart rate as DO declines. For example, the fish *Pagothenia bernacchii* did not display any increase or decrease in heart rate when exposed to 50 mmHg hypoxic waters (Axelsson et al., 1992). A lack of hypoxia induced bradycardia is also seen in other fish species such as the sea raven *Ciliata mustela*, five-bearded rockling *Hemitripterus americanus*, and the rockpool fish *Gobius cobitus* but rarely seen in crustaceans (Berschick et al., 1987; Fritsche, 1990; Fritsche and Nilsson, 1990). One crustacean example where a bradycardia is not found is seen in the freshwater crayfish *Orconectes rusticus* which compensated for a lack of bradycardia by an increase in both scaphognathite rate and cardiac output (Wilkes and McMahon, 1982).

In crustaceans stress may lead to different heart rates at the commencement and early stages (such as the first hour of recording) of experiments which may not reflect the stress imposed by the treatment condition the animals are in (McGaw and McMahon, 1996). Because of this, recordings of the current study did not start until after the first hour. This appeared to be enough time for the crabs to acclimate to the experimental setup as heart rates for each treatment were not significantly different at hourly intervals for the remainder of the 6 hour trial period for either species.

The current experiments clearly show hypoxia affecting the heart rates of both *H. crenulatus* and *H. sexdentatus*. At the lowest dissolved oxygen treatment (6%/~10 mmHg) both species appear to display a bradycardia where their heart rates were significantly decreased compared to the higher DO levels. For *H. crenulatus* the lowest 6% DO treatment facilitated heart rates that were significantly lower than the other DO treatments. Similarly there were significant differences between the 25% and the other treatments (Table 3.1). At the two highest treatments (80 mmHg and 160 mmHg) there was no significant difference in heart rates suggesting *H. crenulatus* maintains a normal heart rate at these oxygen levels. A similar result was found for *H. sexdentatus* in which heart rates at the 6% treatment were significantly lower than the other treatments (Fig. 3.2). However, in contrast to *H.* crenulatus, *H. sexdentatus* had a higher heart rate at the 100% treatment, compared to the others (Table 3.2), and showed no significant difference between the 25% and 50%

treatments. Having lower heart rates for the 25% and 50% treatments, compared to the 100% treatment, suggests that these animals either maintain a normal heart rate around 60-80 bpm in normoxic conditions (40-80 mmHg), or it is more likely that the normoxic environment is around the 100% (160 mmHg) and heart rates are kept at ~100 bpm. The latter is comparable to *H. crenulatus* where they maintain heart rates ~120-135 bpm between 80-160 mmHg. What can also be tentatively concluded is that *H. crenulatus* is able to maintain its heart rate in lower dissolved oxygens in contrast to *H. sexdentatus.* This conclusion is supported by the comparison data (Fig. 3.5.) in which *H. crenulatus* has significantly higher heart rates across all DO treatments compared to *H. sexdentatus*. In order to soundly conclude these differences we need to further examine the biochemical responses of these animals which will be described in chapter four.

When a crustacean is pushed towards its tolerance limits it often responds with physiological, cardiovascular and respiratory compensating mechanisms. In response to external salinity changes crustacea often increase their heart rates in an attempt to mitigate the stressful condition. This response is due to ion balance changes, involving Na^+ and Cl^-, which either efflux or influx ions depending on the salinity of the external media and/or the regulatory ability of the animal. In hyposaline waters crustaceans are often found to increase their heart rates. For example, the euryhaline decapod *C. maenas* shows a marked increase in heart rate when transferred from 100% seawater to more dilute solutions (below 75%-15% seawater) (Hume and Berlind, 1976). Other crustaceans, such as the lobster *Homarus americanus*, the blue crab *Callinectes sapidus*, and the Dungeness crab *C. maister* also display increased heart rates when exposed to low salinities (Taylor, 1977; Jury et al., 1994; McGaw and McMahon, 1996; McGaw and Reiber, 1998). A comparable increased heart rate response is found when crustacea are exposed to hypersaline conditions. This is seen in several crustaceans, such as the freshwater shrimp *Macrobrachium olfersii* and the decapod *C. maenas* (Freire and McNamara, 1992; Aagaard, 1996).

The two intertidal crab species being studied displayed different heart rate responses to varying salinities. For *H. crenulatus* heart rates were not significantly different across all salinity treatments (ranged from 106.9-120.7 bpm). This suggests that this species is well adapted to maintain its heart rate at various salinities over an environmentally

equivalent acute exposure period. For *H. sexdentatus*, heart rates differed depending on the salinity. At the hypo- and hyper-salinities heart rates were highest, 123 bpm for the 2% (0.7 ppt) and 130.8 bpm for the 150% (52.5 ppt) treatments. In contrast, for the intermediate salinities heart rate was reduced. This finding suggests that at salinities above or below environmental norms *H. sexdentatus* becomes stressed and attempts to alleviate this stress, to some degree, by increasing their heart rate which is likely to increase the mobility of cardiovascular and respiratory pumping of ions (i.e. to moderate the influx and efflux of Na^+ and Cl^- ions). When we compare the results of both species we find that heart rates are similar when treated to 2% and 100% salinity, *H. crenulatus* has a higher heart rate when exposed to 25% and 50% salinity, and *H. sexdentatus* has a higher heart rate when exposed to 150%.

This chapters findings suggest that *H. crenulatus* is well adapted to changing environments therefore has little need to alter heart rate, other than at the extreme hypoxia level (10 mmHg). In contrast, *H. sexdentatus* is less able to cope, with heart rate decreasing as PO_2 declines, and displaying a distinct tachycardia at the extremes of salinity.

Chapter Four

4. Biochemical responses of crabs to hypoxia and salinity

4.1. Introduction and aims

Crustaceans inhabit an array of environments many of which are highly variable in abiotic and biotic stressors. The physical and chemical makeup of these habitats have driven physiological, behavioural and biochemical adaptations in these species. In response to factors such as varying dissolved oxygen and salinity, crabs have developed specific biochemical and physiological adaptations. For example, in severe hypoxic waters mechanisms such as anaerobic metabolism (alternate pathways to produce ATP in contrast to aerobic metabolism) and reduced metabolic rates are employed (as was found for both *H. crenulatus* and *H. sexdentatus* in chapter 2) (Storey and Storey, 1990; Childress and Seibel, 1998). Biochemical strategies are also utilised by crustaceans where large fermentable fuel stores (such as glycogen) are synthesized to form glucose, through gluconeogenesis, which is used to account for the lower energy efficiency of anaerobic metabolism (Verri et al., 2001).

Lactate, or similar molecules, are the main end products of anaerobiosis in crustaceans which can result from hypoxia particularly once water oxygen levels drop below the animals P_{CRIT}. Crustaceans are rarely tolerant to severe anoxia or hypoxia and accumulate lactate at a high rate (Hervant et al., 1995). This is due to the utilisation of anaerobic glycolysis, fuelled mainly by glycogen, which leads to lactate accumulation (Hervant et al., 1996). The amount haemolymph lactate accumulated is dependent on the duration and severity of hypoxia (Hervant et al., 1995). Lactate is regularly used as an indicator of hypoxic stress and has been studied in various decapod crabs (Burke, 1979; Stillman and Somero, 1996; Webster, 1996; Zou et al., 1996; Lorenzon et al., 2008).

An Increase in haemolymph glucose concentrations is another commonly used indicator of stress in crustaceans and other aquatic animals (Bonga, 1997). In crustaceans, glucose haemolymph is regulated within a strict range (Verri et al., 2001). Haemolymph

glucose comes from two sources, it is either directly absorbed through hepatopancreatic and/or intestinal epithelia or it is stored in the hepatopancreas in the form of glycogen (Verri et al., 2001). Glycogen is the fuel source of glucose and is synthesized through the gluconeogenesis pathway to form glucose 6–phosphate (Hall and van Ham, 1998). Therefore, haemolymph glucose increases with a corresponding decrease in glycogen during anoxic and hypoxic events (Zou et al., 1996).

External salinity can also invoke physiological and biochemical responses in decapod crabs. One of the foremost influences salinity has on aquatic crustaceans is changes to extracellular acid-base homeostasis (Henry and Cameron, 1982; Wheatly, 1985). Crabs maintain their acid-base status and ion regulation through the catalysed hydration of CO_2 by carbonic anhydrase. This reaction results in the production of carbonic acid (H_2CO_3) and from this HCO_3^- and H^+ are produced (Henry and Wheatly, 1992). HCO_3^- and H^+ act as counter-ions and affect not only the acid-base equilibrium of the animal but also the transfer of Cl^- and Na^+ across plasma membranes, through electroneural ion transporters (Whiteley et al., 2001). This is most commonly found in the gills, the predominant gas exchange organ of the animal, where at the epithelia HCO_3^- is often exchanged for Cl^-, and H^+ for Na^+ at basolateral Na^+/K^+ pumps (Na^+/K^+ ATPase) (Towle, 1997; Castilho et al., 2001). This process generates an electrochemical gradient which determines cells' excitable properties, cell osmotic equilibrium and membrane resting potentials (Garcon et al., 2009).

The exchange process of branchial ion transfer through coupled acid/base equivalents is the principal mechanism of acid-base regulation in crabs (Whiteley et al., 2001). There are, however, other mechanisms to control and regulate pH of extracellular fluids. These can either involve the buffering of protons by inorganic and or organic buffers, or by the removal of proton buildup through respiratory control of haemolymph PCO_3 (Henry and Wheatly, 1992). The circulatory system and haemolymph are particularly important for these processes where flow rates and haemolymph compositions play key roles in regulating biological function, which includes maintenance of acid-base balances, membrane stability and enzymatic functioning (Pavasovic et al., 2004).

Haemolymph osmolarity, Na^+, K^+ and Cl^- are good indicators of an animal's ability to regulate to their external surroundings in particular to varying salinities. In addition to the membrane Na^+, K^+ ATPase ion transporter crustaceans also possess K^+ and Cl^- channels

located on the epithelial cells of the gills basal membrane. Na^+ channels, Cl^-/HCO_3^- exchangers, and V-type proton pumps are also present in the apical membranes of gills which underlie NaCl uptake by strong hyperosmoregulators (Kirschner, 2004; Freire et al., 2008). The ability of an animal to regulate these ions is indicative of its osmoregulatory abilities. For example, the haemolymph osmolarity of the hermit crab *Clibanarius vittatus* acclimated to 45% salinity was indicative of a weak hyporegulator in contrast to the ambient water salinity (1102.5 ± 22.1 mOsm kg^{-1} vs. 801.0 ± 40.2 mOsm kg^{-1}) (Lucena et al., 2012). Haemolymph Na^+, K^+ and Cl^- of this species is strongly hyporegulated in high salinities, which contrasts the overall haemolymph osmolarity. Lucena et al. (2012) suggest these findings show *C. vittatus* as being capable of modest hypo-osmotic/ionic regulation at increased salinities.

Haemocyanin concentrations are another measure of an animal's capacity to survive stressors as a higher haemocyanin concentration reflects a higher oxygen-carrying capacity (Hagerman and Weber, 1981). Measurements of haemocyanin, through haemolymph absorbances, is commonly used in crustacean studies (Nielsen and Hagerman, 1998; Pascual et al., 2003; Cheng et al., 2013), including decapod crabs (Depledge and Bjerregaard, 1989; Urbina et al., 2013).

The responses of the two New Zealand intertidal crabs to acute stressors, such as hypoxic and salinity stress, have yet to be thoroughly investigated with few studies undertaken on this species to date (e.g. Hicks, 1973; Depledge and Lundebye, 1996; Lee et al., 2010). The aim of this study was to investigate the biochemical and haematological responses of the decapods to various hypoxic and salinity exposures, in order to determine the effects and environmental tolerances of these species. In the future, studies could be expanded to include other stressors and allow these species to be used as indicators of environmental change. In this chapter the haematological impacts of hypoxia and external salinity were investigated for the two crab species. Studies looked at the haemolymph osmolarity, Na^+, K^+, Cl^-, glucose and haemocyanin concentrations in order to get a broad understanding of the haematological changes of the animals under differing stressors. The original aim was to also look at haemolymph lactate but this proved unsuccessful.

4.2. Methods and Materials

The biochemical trials used similar methods to that used for the oxygen consumption and heart rate trials, whereby crabs were acclimatised to the University of Canterbury's aquaria, fasted for 24 hours before trials and trials ran for 6 hours (detailed in chapter 2). The crabs from both the heart rate and oxygen consumption trials were reused for the biochemical trials, depending on the treatment and the duration the crab had been held within the facilities. This was implemented to minimise the number of animals used complying with the animal ethics requirements. Some crabs were used for multiple biochemistry trials. In these circumstances crabs were reused if they had recovered from their previous biochemical trial (i.e. had no residual problems, such as loss of limbs or mortality) for at least 14 days, and the initial trial was not one of the more stressful treatments (i.e. the 2% dissolved oxygen or the 6% or 150% salinity treatments). Due to some of the experiments requiring a lot of haemolymph the *H. crenulatus* trials required more crabs than *H. sexdentatus* due to their difference in size and haemolymph content. Six replicates were used for each trial and each species was tested at four levels of dissolved oxygen (6%, 25%, 50% and 100%) and five salinities (2%, 25%, 50%, 100% and 150%). Both species required 54 animals for these trials ((4 x 6) for the dissolved oxygen trials + (5 x 6) for the salinity trials) and the *H. crenulatus* trials required a further 54 animals in order to obtain enough haemolymph for the multiple biochemical tests.

Immediately following experimentation haemolymph was removed using a 1 ml syringe and 23G x 1 ½" needle. Haemolymph (0.2-0.4 mL) was removed from the base of the crabs' legs through the arthrodial membrane and placed into an Eppendorf tube. The amount of haemolymph obtained depended on the size of the animal. The haemolymph was used to measure total osmolarity, Cl^-, Na^+, K^+, haemocyanin concentration and glucose. Trials were conducted immediately following haemolymph removal.

Total haemolymph osmolarity was measured using WESCOR VAPRO vapor pressure osmometers (model 5520 or model 5600). Each machine was calibrated using 10 µl of a 290 mOsm, 1000 mOsm NaCl, and 100 µl NaCl solutions. Each calibration test solution, as well as

the following haemolymph tests, used 10 µl of solution placed onto a small paper disk which was then inserted into the osmometer. Each sample was measured three times and the results averaged.

Haemolymph Cl^- was measured using one of either a LABCONCO Digital Chloridometer or a Sherwood chloride analyser 926. The machines use an acid solution, consisting of 10% acetic and 1% nitric acid, and gelatin. Each instrument then titrates this solution which represents the 0 solution ($mmolL^{-1}$ Cl). The machine is then calibrated with 10 µl of 100 $mmolL^{-1}$ Cl standard solution. Once calibrated the haemolymph Cl is measured 10 µl samples for the LABCONCO chloride meter and 20 µl samples for the Sherwood chloride meter.

Na^+ and K^+ were quantified using a Sherwood flame photometer 410. The instrument was calibrated using 1000, 800, 600, 400, 200 and 0 $\mu molL^{-1}$ of either a Na^+ or K^+ solution (depending on what is being tested) in order to create a calibration curve, and a subsequent calibration equation, which is used to calculate the sample results. For the Na+ measurements 10 µl of haemolymph was diluted 600-800 times (i.e. 10 µl of haemolymph was added to 5990-7990 µl of distilled water creating a 1:600-800 dilution factor), whereas the K+ experiments only needed to be diluted with a 1:300 dilution factor (10 µl of haemolymph added to 2990 µl of distilled water). Due to the limited amount of haemolymph only 1 replicate was used per haemolymph sample per trial for both Na^+ and K^+.

Haemolymph glucose was measured using a Sigma diagnostic kit (GAHK-20). In this method an equimolar amount of NAD is reduced to NADH and the consequent increase absorbance at 340 nm from this reaction is directly proportional to glucose concentration. This is achieved through the catalytic reactions of glucose-6-phosphate dehydrogenase and hexokinase as shown in the following equations:

Equation 4.1: $$Glucose + ATP \xrightarrow{Hexokinase} Glucose - 6 - phosphate + ADP$$

Equation 4.2: $$G6P + NAD \xrightarrow{G6PDH} 6 - Phosphogluconate + NADH$$

Glucose assays were conducted using a 96 well microplate. Within each microplate two samples of haemolymph from each crab as well as five standard solutions, from which a calibration curve and equation were made, were added with the glucose assay reagent (hexokinase). Incubation occurred at room temperature for 15 minutes after which the absorbances were read at 340 nm on a SpectraMax M5 microplate reader. From these readings and the calibration equation glucose concentrations were calculated.

Haemolymph lactate was measured using an enzymatic L-Lactic acid kit (L-Lactate Megazyme) following the manufacturer's instructions. In this method sample lactate concentration is proportional to the NADH produced by the catalysed reactions of D-glutamate transaminase and L-lactate dehydrogenase as shown in the following equations:

Equation 4.3: $$L - Lactate + NAD^{+} \overset{(L-LDH)}{\Longleftrightarrow} pyruvate + NADH + H^{+}$$

Equation 4.4: $$Pyruvate + D - glutamate \xrightarrow{(D-GPT)} D - alanine + 2 - oxoglutarate$$

Lactate assays also used a microplate format, however, unlike the previous biochemical assays haemolymph was deproteinated before analysis. Here equal parts haemolymph and perchloric acid (70% perchloric acid) (in this case 30 µl haemolymph was added to 30 µl perchloric acid) were mixed in an Eppendorf tube. The solution was then centrifuged for 10 min at 14,000 rpm to separate the supernatant from the protein pellet. 10 µl of the supernatant was then removed from the Eppendorf tubes and pipetted onto a microplate. Following this, the appropriate reagents were added (buffer, NAD+ and GTP), they were then incubated for 3 minutes at room temperature, before the absorbances were read on a 96 well plate reader at 340 nm (1st reading). After this first reading L-LDH was mixed into the samples and the plate was again incubated for a 10 minute period before absorbance was read and recorded again (2nd reading). The total lactate content was calculated by subtracting the 1st reading values from the 2nd reading values. From these readings and the calibration equation lactate concentrations were calculated.

Haemolymph haemocyanin concentration was measured at 340 nm using a Unicam 8625 UV/VIS spectrophotometer. The instrument was calibrated using 1 mL of distilled

water pipetted into a 3.5 mL cuvette. This is inverted 10 times before being placed within the spectrophotometer, which is then zeroed. Following this, 1 mL of distilled water is pipetted into another cuvette in which 10 µl of haemolymph sample is added (this sample is immediately taken from the animal and placed into the cuvette), inverted 10 times and absorbance is then spectrophotometrically measured.

Following the previous chapters' statistical analysis was carried out using the R statistical programme. Statistical significance was taken at the level of $P < 0.05$. Normality of data was tested using a quantile-quantile normality plot as well as a Shapiro-Wilk normality test. Homogeneity of variance was tested by plotting the fitted against the residual values as well as a Levene's test for homogeneity of variance. Where treatment effects were shown to be significant post-hoc Tukey's HSD analyses were performed. Where the assumption of normality was violated a non-parametric Kruskal-Wallis one-way analysis of variance was performed. Following this a post-hoc Dunn's test with a Bonferroni adjustment was performed to account for and analyse the multiple comparisons.

4.3. Results

Like methods used in the previous chapters all biochemical experiments ran for a total of 6 hours per crab per treatment with the same setup as the heart rate trials. In this section the various dissolved oxygen and salinity treatments were tested to see their effects on haemolymph osmolarity, chloride, potassium, sodium, glucose and lactate. Haemolymph was obtained from each crab at the conclusion of each 6 hour trial. For the salinity trials, and each subsequent haemolymph parameter experiment, a graph containing an isosmotic line was designed. Here the chemical composition of the 100% salinity treatment, i.e. 35 ppt is equal to 1000 mOsm of major ions in the water. Therefore, the 100% salinity treatment contains 552.9 mOsm of chloride, 11.4 mOsm of potassium and 307.4 mOsm of sodium. These values were used to create each of the isosmotic lines for the salinity trial results for chloride, potassium and sodium.

4.3.1. Osmolarity

Effect of oxygen level

For the dissolved oxygen trials both *H. crenulatus* and *H. sexdentatus* maintained haemolymph osmolarity near 1000 mOsmol kg^{-1}. For *H. crenulatus* osmolarity ranged from 992.5 to 1149.8 mOsmol kg^{-1} with no significant differences between the 6%, 25% and 50% treatments. The 100% treatment was different in contrast to the 6% and 25% treatments but was not different when compared to the 50% treatment (Fig. 4.1). In comparison, *H. sexdentatus* displayed no difference in osmolarity across all tested dissolved oxygens within a range of 1017.3 – 1078 mOsmol kg^{-1} (Fig. 4.1). When osmolarity was compared between the two species we found no difference between the 6% trial but differences were found for the 25%, 50% and 100% trials (Fig. 4.2).

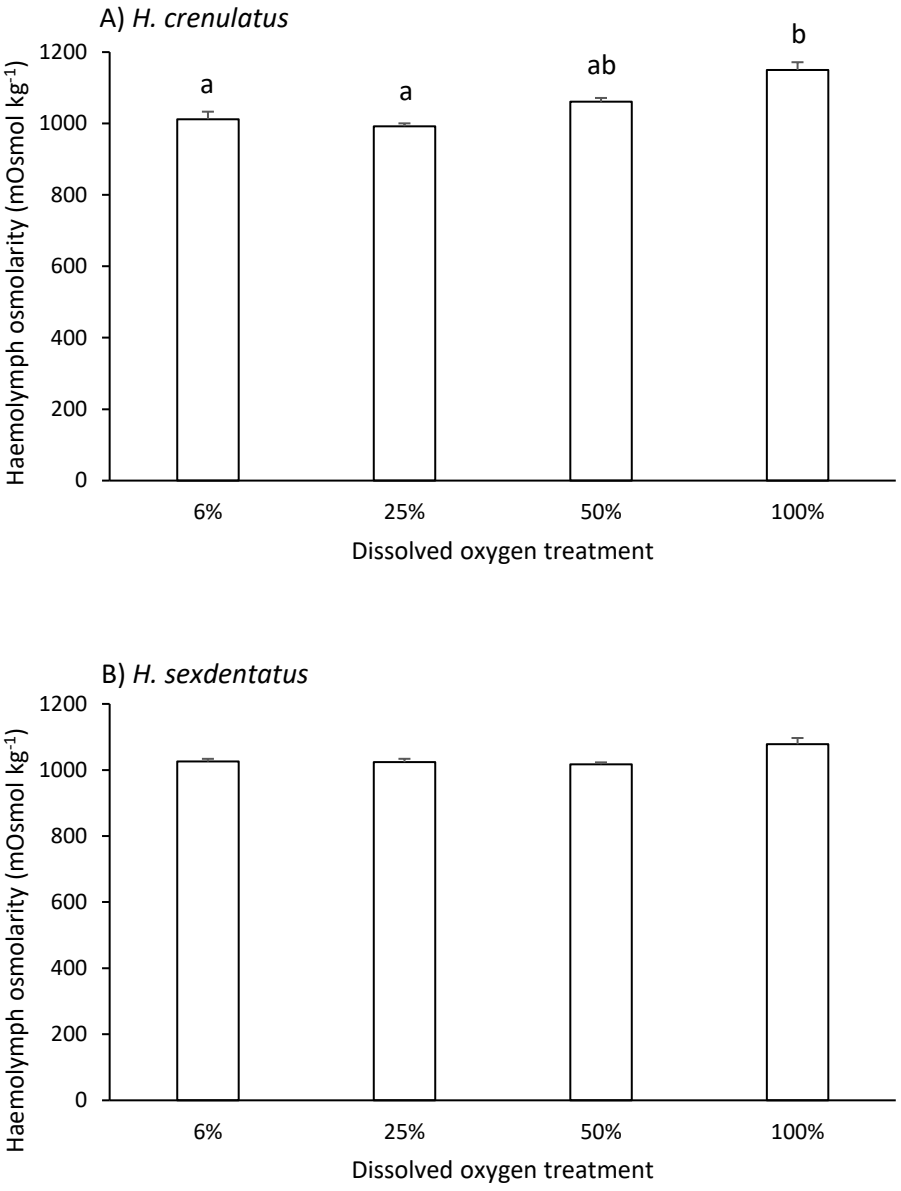

Figure 4.1: Haemolymph osmolarity (mOsmol kg^{-1}) ± S.E.M. of H. crenulatus *(A) and* H. sexdentatus *(B) at different dissolved oxygen levels. Plots sharing letters are not significantly different.*

The *H. crenulatus* haemolymph osmolarity DO data did not violate the assumption of normality but did violate the assumption of homogeneity (Shapiro test P = 0.08, Levene's test F-value = 3.55, P <0.05). For the data (Fig. 4.1) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of dissolved oxygen found (P <0.05) Summary of the data: df = 3, Kruskal-Wallis chi-squared = 17.45. A Dunn's-test for multiple

comparisons of independent samples was then used to compare the differences between treatments.

The *H. sexdentatus* haemolymph osmolarity DO data violated the assumptions of normality and homogeneity (Shapiro test P <0.05, Levene's test F-value = 5.95, P <0.05). For the data (Fig. 4.1) a Kruskal-Wallis one-way analysis of variance was performed, with no significant effect of dissolved oxygen found (P = 0.07). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 6.95.

Comparing the haemolymph osmolarities for the DO trials at the 6% DO treatment no difference was found (P = 0.7), however, at the other three treatments the animals were different (25% P = 0.02, 50% P <0.05, 100% P = 0.02) (Table 4.1). *H. sexdentatus* were found to have a higher haemolymph osmolarity at 25% DO, whereas *H. crenulatus* had a higher osmolarity at 50% and 100% DO (Fig. 4.2).

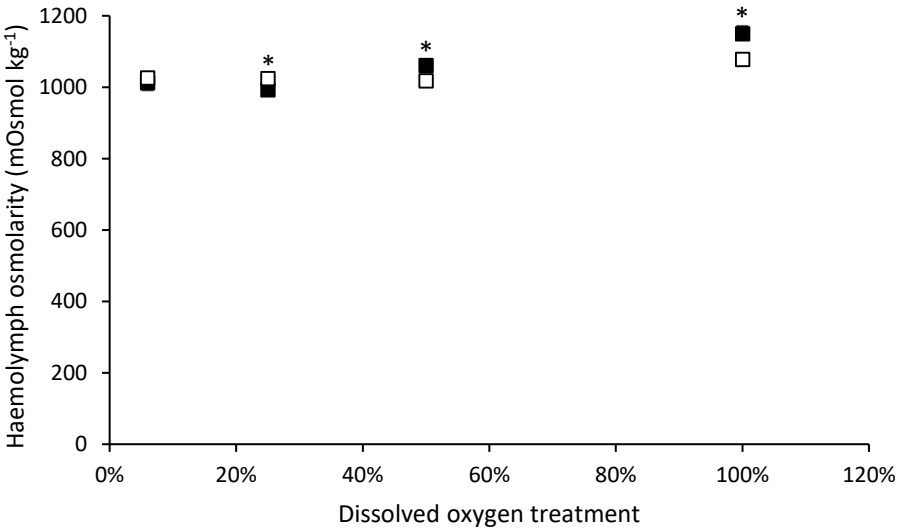

Figure 4.2: Haemolymph osmolality of H. crenulatus *(■) and* H. sexdentatus *(□) (mOsmol kg^{-1}) ± S.E.M. in different dissolved oxygen levels. * Significantly different from one another.*

Table 4.1: The P-values, Kruskal-Wallis chi-squared (non-parametric) or F-value (parametric), and Degrees of freedom of the species dissolved oxygen haemolymph osmolarity comparisons.

Dissolved oxygen level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
6% (non-parametric)	0.75	0.11	1
25% (parametric)	0.02	6.99	10
50% (parametric)	<0.05	15.51	10
100% (parametric)	0.02	7.47	10

Effect of salinity

The crabs responded to salinity by hyperregulating their haemolymph osmolarity between the 2% and 100% treatments. For *H. crenulatus* an increase in haemolymph osmolarity is seen as salinities increase (from 707.3 mOsmol kg^{-1} at 2% to 1269.7 mOsmol kg^{-1} at the 150% treatment), however as the animal approached the 100% salinity and above (150%) the haemolymph osmolarity was hyporegulated and reduced below the isosmotic line (Fig. 4.3). A post-hoc Tukey's test found a difference between all treatments except for the 25% and 50% treatments (Table 4.2). For *H. sexdentatus* an increase in haemolymph osmolarity was also found between treatments (from 795.3 mOsmol kg^{-1} at 2% to 1711.8 mOsmol kg^{-1} at the 150% treatment). Similarly for *H. sexdentatus* salinities between 2% and 100% salinity were hyperregulated whereas salinities approaching the 100% treatment and above resulted in a change in haemolymph osmolarity. However, for this species above 100% salinity haemolymph osmolarity conformed to the external media in contrast to being hyporegulated as was found for *H. crenulatus* (Fig. 4.3). A post-hoc Dunn's test found a difference between the 150% and the other 2%, 25%, and 50% treatments (Table 4.2). When comparing the species haemolymph osmolarity we found no difference between the 2% ($P = 0.07$), 25% ($P = 0.4$), 50% ($P = 0.4$), or 100% ($P = 0.1$) treatments with a single difference found for the 150% treatment ($P < 0.05$) (Table 4.3). *H. sexdentatus* was found to

have a higher haemolymph osmolarity at the 150% salinity in contrast to *H. crenulatus* (Fig. 4.3).

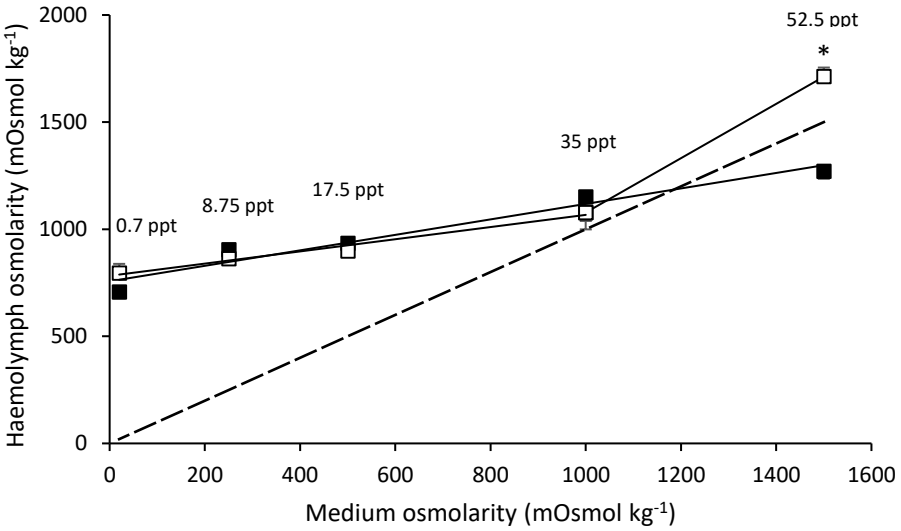

Figure 4.3: Haemolymph osmolality of H. crenulatus *(■) and* H. sexdentatus *(□) (mOsmol kg^{-1}) ± S.E.M. in different salinity treatments. The dotted line represents the isosmotic line. * Significantly different from one another.*

The *H. crenulatus* haemolymph osmolarity salinity data did not violate the assumptions of normality or homogeneity (Shapiro test P = 0.35, Levene's test F-value = 2.16, P = 0.1). For the data (Fig. 4.4) a one-way ANOVA was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 25, F-value = 88.89. A post-hoc Tukey's test for multiple comparisons of means was then used to compare the differences between treatments (Table 4.2).

The *H. sexdentatus* haemolymph osmolarity salinity data violated the assumption of normality but did not violate the assumption of homogeneity (Shapiro test P <0.05, Levene's test F-value = 1.66, P = 0.19). For the data (Fig. 4.4) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 4, Kruskal-Wallis chi-squared = 22.79. A Dunn's-test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 4.2).

Table 4.2: The P-values of the post-hoc Tukey's multiple comparisons of means for the H. crenulatus *haemolymph osmolarity salinity trials (A) and the P-values of the Dunn's-test for multiple comparisons for the* H. sexdentatus *haemolymph osmolarity salinity trials (B). The P-values were adjusted using the Bonferroni method.*

A) *H. crenulatus*	100%	150%	2%	25%
150%	0.011	-	-	-
2%	<0.05	<0.05	-	-
25%	<0.05	<0.05	<0.05	-
50%	<0.05	<0.05	<0.05	0.9
B) *H. sexdentatus*				
150%	1.00	-	-	-
2%	0.075	0.001	-	-
25%	0.15	0.003	1.00	-
50%	0.47	0.016	1.00	1.00

Table 4.3: The P-values, Kruskal-Wallis chi-squared (all data were non-parametric), and Degrees of freedom of the species salinity haemolymph osmolarity comparisons.

Salinity level	P-value	Kruskal-Wallis chi-squared value	Degrees of freedom
2%	0.08	3.10	1
25%	0.42	0.64	1
50%	0.38	0.78	1
100%	0.11	2.56	1
150%	<0.05	8.31	1

4.3.2. Chloride

Effect of oxygen level

Haemolymph chloride was unaffected by dissolved oxygen for both species. *H. crenulatus* maintained chloride within the range 474.2 – 527.2 mmol L^{-1} (Fig. 4.4) and *H. sexdentatus* maintained chloride within the range 510.7 – 526.8 mmol L^{-1} (Fig. 4.4). When chloride was compared we found no statistically significant difference between the two species (Table 4.4).

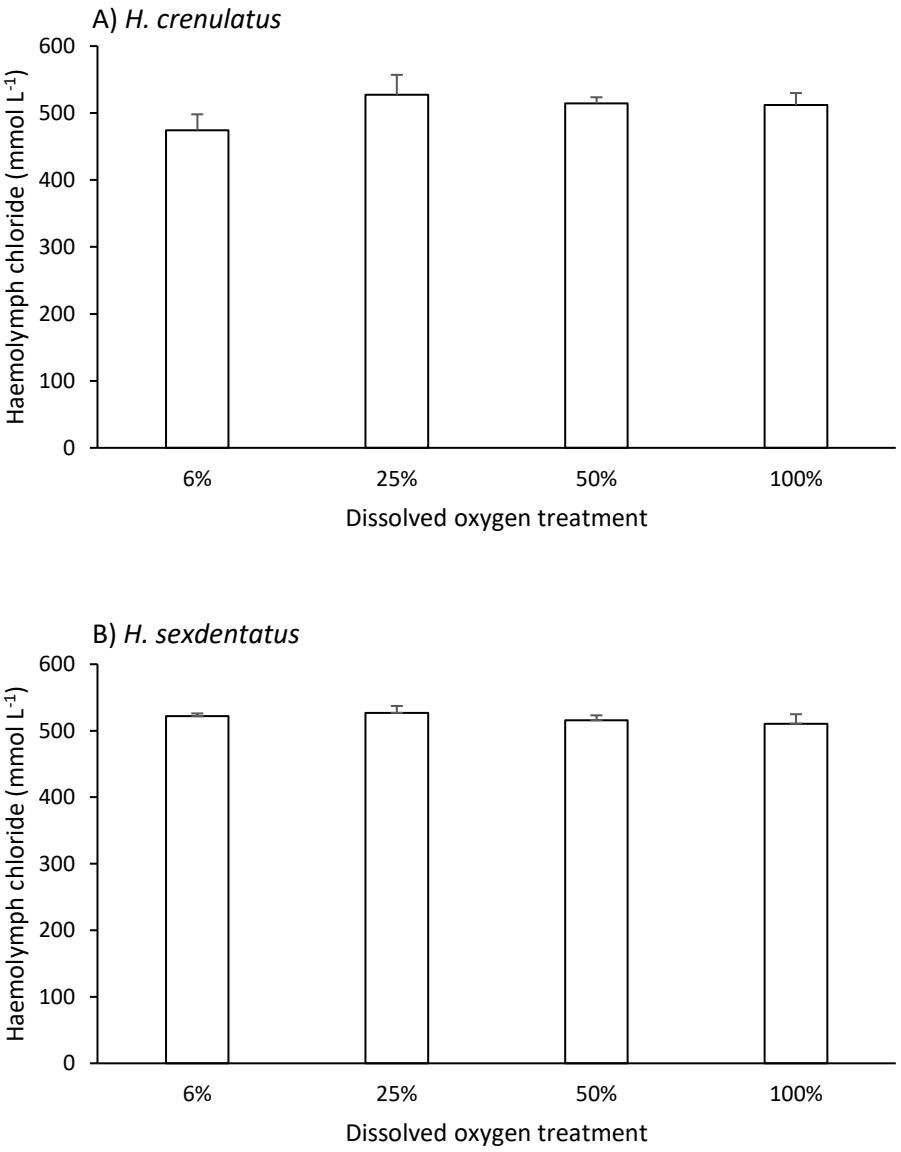

Figure 4.4: Haemolymph chloride (mmol L^{-1}) ± S.E.M. of H. crenulatus *(A) and* H. sexdentatus *(B) at different dissolved oxygen levels. Plots sharing letters are not significantly different.*

The *H. crenulatus* haemolymph chloride DO data did not violate the assumption of normality but did violate the assumption of homogeneity (Shapiro test P = 0.97, Levene's test F-value = 6.08, P <0.05). For the data (Fig. 4.4) a Kruskal-Wallis one-way analysis of variance was performed, with no significant effect of dissolved oxygen found (P = 0.44). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 2.73.

The *H. sexdentatus* haemolymph chloride DO data did not violate the assumption of normality but did violate the assumption of homogeneity (Shapiro test P = 0.51, Levene's

test F-value = 3.33, P = 0.04). For the data (Fig. 4.4) a Kruskal-Wallis one-way analysis of variance was performed, with no significant effect of dissolved oxygen found (P = 0.77). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 1.14.

Comparing the haemolymph chloride for the DO trials there were no differences between the two species (6% P = 0.08, 25% P = 0.1, 50% P = 0.87, 100% P = 0.69) (Fig. 4.5). The data was analysed using a non-parametric Kruskal-Wallis test.

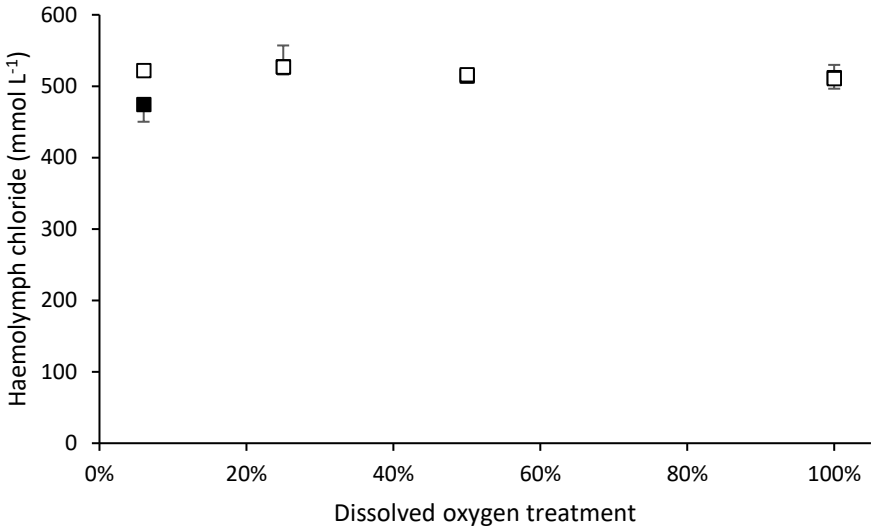

Figure 4.5: Haemolymph chloride of H. crenulatus *(■) and* H. sexdentatus *(□) (mmol L^{-1}) ± S.E.M. in different dissolved oxygen levels. * Significantly different from one another.*

Table 4.4: The P-values, Kruskal-Wallis chi-squared (all data were non-parametric), and Degrees of freedom of the species dissolved oxygen haemolymph chloride comparisons.

Dissolved oxygen level	P-value	Kruskal-Wallis chi-squared value	Degrees of freedom
6%	0.08	3.10	1
25%	1	0	1
50%	0.87	0.03	1
100%	0.69	0.16	1

Effect of salinity

Much like total osmolarity, as external salinity increased so too did haemolymph chloride for both species. For *H. crenulatus* haemolymph chloride increased from 349.5 mmol L^{-1} at 2% salinity to 636.7 mmol L^{-1} at 150% salinity (Fig. 4.6). A post-hoc Tukey's test found differences between most treatments except between the 2% and 25%, 25% and 50%, and 50% and 100% treatments (Table. 4.5). For *H. sexdentatus* haemolymph chloride increased from 310.5 mmol L^{-1} at 2% salinity to 762.2 mmol L^{-1} at 150% salinity (Fig. 4.6). Similar to the osmolarity results, a post-hoc Dunn's test found differences between the 150% and the 2%, 25%, and 50%, but not the 100%, treatments for *H. sexdentatus* (table 4.5). The species comparisons also found a comparative result seen for osmolarity in which the crabs showed no difference in haemolymph chloride except at the 150% treatment. Here at the 2% ($P = 0.51$), 25% ($P = 0.99$), 50% ($P = 0.11$), and 100% ($P = 0.69$) treatments no differences were found between the two crabs, however, at the 150% treatment ($P < 0.05$) a difference was found (Table 4.6). *H. sexdentatus* was found to have higher haemolymph chloride concentrations at the 150% salinity in contrast to *H. crenulatus* (Fig. 4.6). This finding is similar to that found for the osmolarity trials.

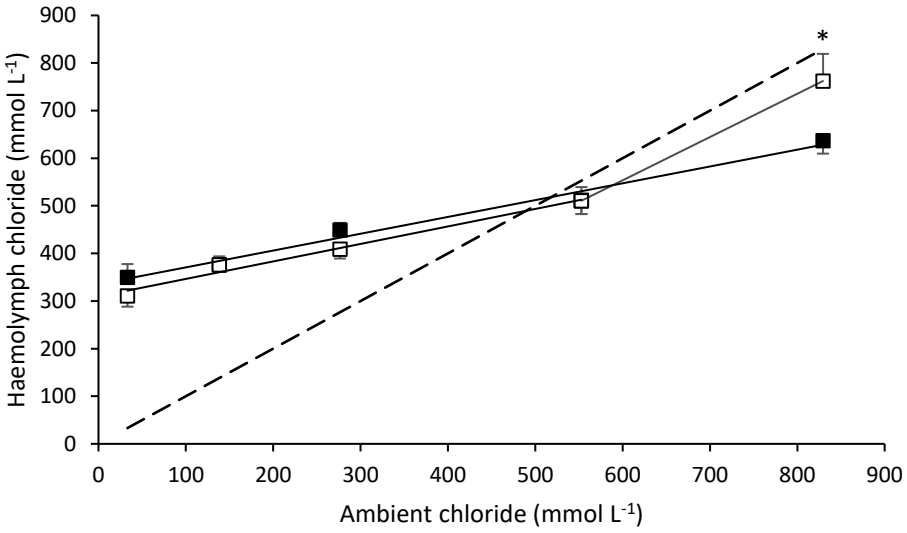

*Figure 4.6: Haemolymph chloride of H. crenulatus (■) and H. sexdentatus (□) (mmol L^{-1}) ± S.E.M. in different salinity treatments. The dotted line represents the isosmotic line. * Significantly different from one another.*

The *H. crenulatus* haemolymph chloride salinity data did not violate the assumptions of normality or homogeneity (Shapiro test P = 0.51, Levene's test F-value = 1.05, P = 0.4). For the data (Fig. 4.6) a one-way ANOVA was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 25, F-value = 28.39. A post-hoc Tukey's test for multiple comparisons of means was then used to compare the differences between treatments (Table 4.5).

The *H. sexdentatus* haemolymph chloride salinity data did not violate the assumption of normality but did violate the assumption of homogeneity (Shapiro test P = 0.06, Levene's test F-value = 5.72, P <0.05). For the data (Fig. 4.6) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 4, Kruskal-Wallis chi-squared = 22.72. A Dunn's-test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 4.5).

Table 4.5: The P-values of the post-hoc Tukey's multiple comparisons of means for the H. crenulatus *haemolymph chloride salinity trials (A) and the P-values of the Dunn's-test for multiple comparisons for the* H. sexdentatus *haemolymph chloride salinity trials (B). The P-values were adjusted using the Bonferroni method.*

A) *H. crenulatus*	100%	150%	2%	25%
150%	<0.05	-	-	-
2%	<0.05	<0.05	-	-
25%	<0.05	<0.05	0.91	-
50%	0.27	<0.05	0.02	0.15
B) *H. sexdentatus*				
150%	1.00	-	-	-
2%	0.23	0.004	-	-
25%	0.66	0.02	1.00	-
50%	0.06	<0.05	1.00	1.00

Table 4.6: The P-values, Kruskal-Wallis chi-squared (non-parametric) or F-value (parametric), and Degrees of freedom of the species salinity haemolymph chloride comparisons.

Salinity level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
2% (parametric)	0.51	0.46	10
25% (parametric)	0.99	0	10
50% (parametric)	0.11	3.12	10
100% (non-parametric)	0.69	0.16	10
150% (parametric)	<0.05	14.61	10

4.3.3. Potassium

Effect of oxygen level

Both *H. crenulatus* and *H. sexdentatus* maintained haemolymph potassium near the ambient 11.4 mmol L^{-1} for all of the DO trials. The haemolymph potassium levels of *H. crenulatus* ranged from 10.23 to 10.89 mmol L^{-1} with no significant differences between treatments (Fig. 4.7). *H. sexdentatus*, however, did display differences between DO treatments which ranged from 10.53 to 12.77 mmol L^{-1}. Here the 25%, 50% and 100% trials were all comparable whereas the 6% trial was only comparable to the 100% trial (Fig. 4.7). Species' potassium comparisons found no difference between the 6% trial but differences were found for the 25%, 50% and 100% trials (Fig. 4.8).

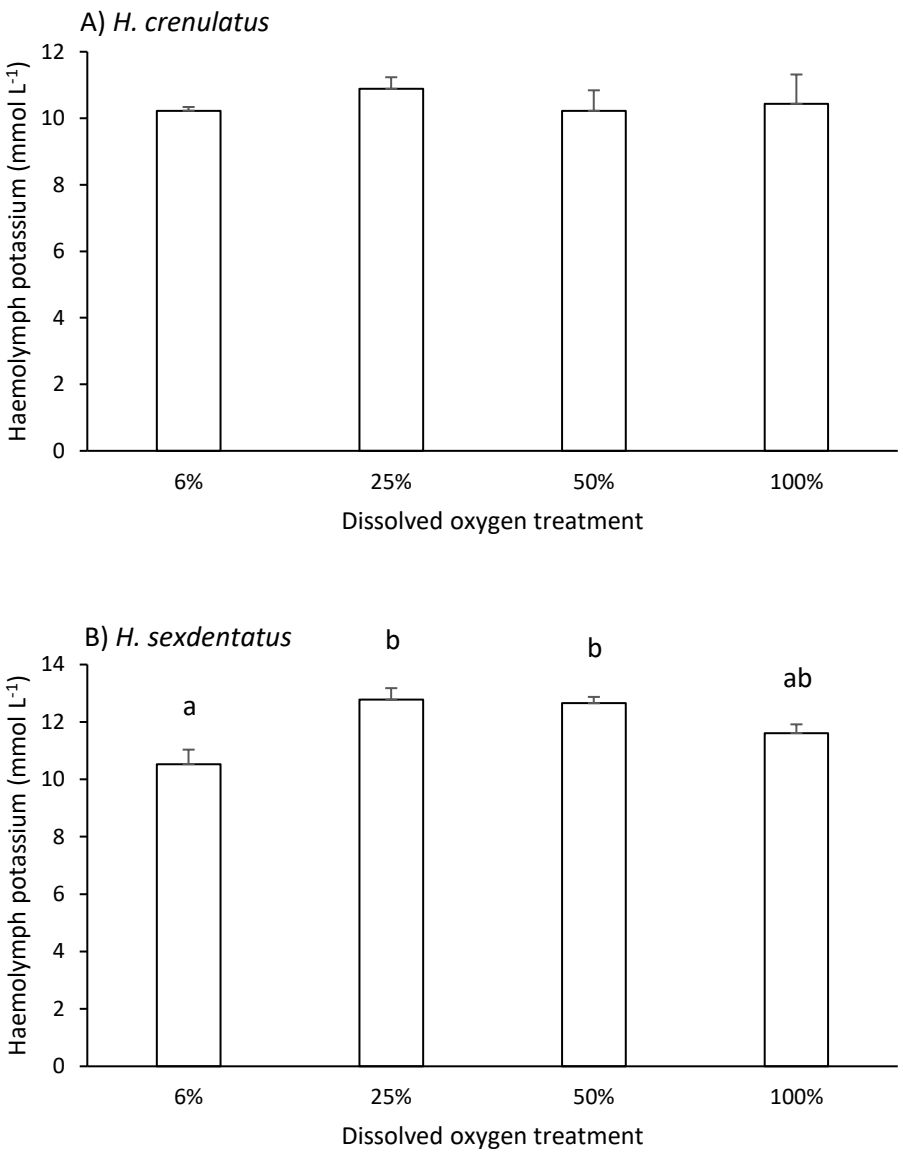

Figure 4.7: Haemolymph potassium (mmol L^{-1}) ± S.E.M. of H. crenulatus *(A) and* H. sexdentatus *(B) at different dissolved oxygen levels. Plots sharing letters are not significantly different.*

The *H. crenulatus* haemolymph potassium DO data did not violate the assumptions of normality or homogeneity (Shapiro test P = 0.09, Levene's test F-value = 2.89, P = 0.06). For the data (Fig. 4.7) a one-way ANOVA was performed, with no significant effect of dissolved oxygen found (P = 0.78). Summary of the data: df = 20, F-value = 0.36.

The *H. sexdentatus* haemolymph potassium DO data did not violate the assumptions of normality or homogeneity (Shapiro test P = 0.51, Levene's test F-value = 0.58, P = 0.63).

For the data (Fig. 4.7) a one-way ANOVA was performed, with a significant effect of dissolved oxygen found ($P < 0.05$). Summary of the data: df = 20, F value = 9.46. A post-hoc Tukey's test for multiple comparisons of means was then used to compare the differences between treatments.

Comparing the haemolymph potassium for the dissolved oxygen trials, at the 6% ($P = 0.87$) and 100% ($P = 0.2$) DO treatments no difference was found whereas at the 25% ($P < 0.05$) and 50% ($P < 0.05$) treatments differences were found (Table 4.7). *H. sexdentatus* were found to have higher, or equivalent haemolymph potassium concentrations at all of the DO treatments (Fig. 4.8).

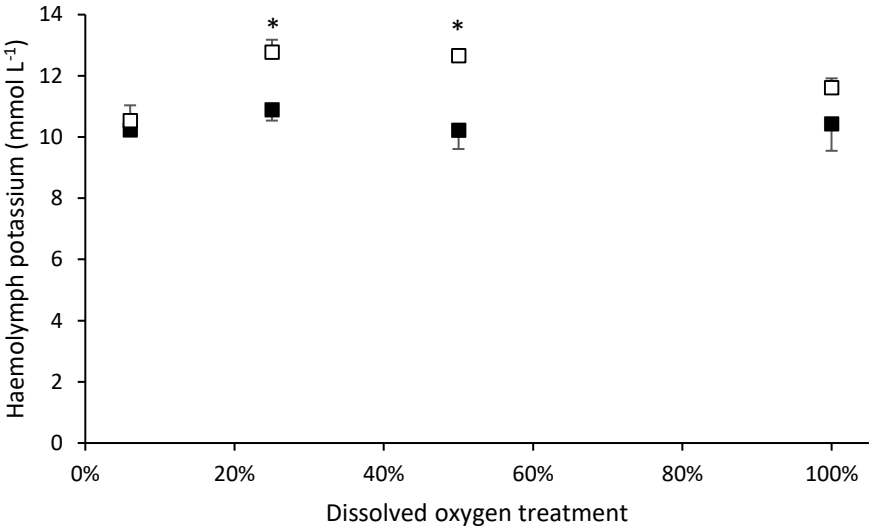

Figure 4.8: Haemolymph potassium of H. crenulatus *(■) and* H. sexdentatus *(□) (mmol L^{-1}) ± S.E.M. in different dissolved oxygen levels. * Significantly different from one another.*

Table 4.7: The P-values, Kruskal-Wallis chi-squared (non-parametric) or F-value (parametric), and Degrees of freedom of the species dissolved oxygen haemolymph potassium comparisons.

Dissolved oxygen level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
6% (non-parametric)	0.87	0.03	1
25% (parametric)	<0.05	15.10	10
50% (parametric)	<0.05	16.58	10
100% (parametric)	0.2	1.89	10

Effect of salinity

When exposed to varying salinities both species increased their haemolymph potassium. For *H. crenulatus* haemolymph potassium increased as salinities increased (from 6.73 mmol L^{-1} at 2% to 14.06 mmol L^{-1} at the 150% treatment) (Fig. 4.9). A post-hoc Tukey's test found a difference between all treatments except for between the 2% - 25%, and 50% - 100% treatments (Table 4.6). For *H. sexdentatus* a similar increase in haemolymph potassium was found (from 5.87 mmol L^{-1} at 2% to 11.84 mmol L^{-1} at the 150% treatment) (Fig. 4.9). A post-hoc Dunn's test found differences between the 150% and the 2%, 25%, and 50% treatments as well as between the 2% and 100% treatments (Table 4.8). When comparing the species' haemolymph potassium no difference was found for the 2% ($P = 0.2$) or 100% ($P = 0.4$) treatments with differences found for the 25% ($P = 0.03$), 50% ($P < 0.05$) and 150% ($P = 0.01$) treatments (Table 4.9). *H. crenulatus* was found to have higher or equivalent haemolymph potassium concentrations at all of the salinity treatments in contrast to *H. sexdentatus* (Fig. 4.9).

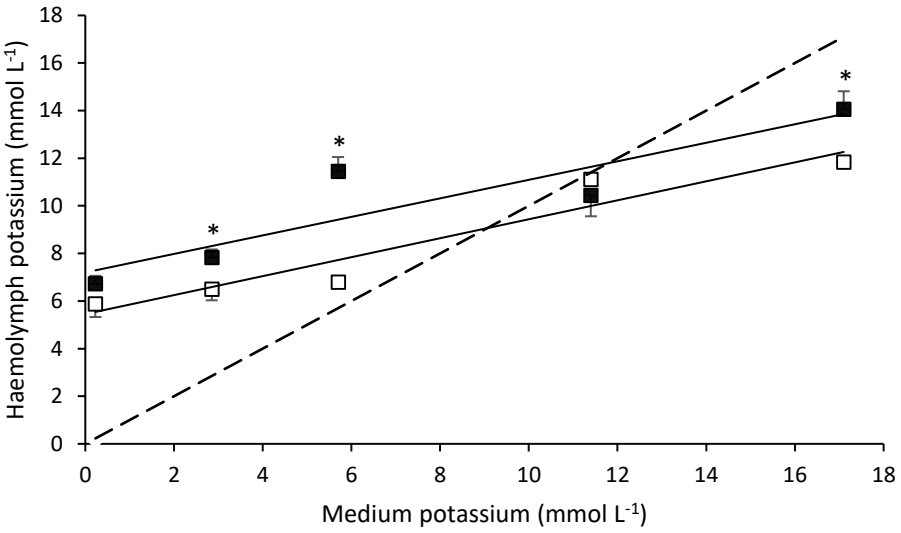

Figure 4.9: Haemolymph potassium of H. crenulatus *(■) and* H. sexdentatus *(□) (mmol L^{-1}) ± S.E.M. in different salinity treatments. The dotted line represents the isosmotic line. * Significantly different from one another.*

The *H. crenulatus* haemolymph potassium salinity data did not violate the assumptions of normality or homogeneity (Shapiro test P = 0.13, Levene's test F-value = 1.69, P = 0.18). For the data (Fig. 4.9) a one-way ANOVA was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 25, F-value = 26.55. A post-hoc Tukey's test for multiple comparisons of means was then used to compare the differences between treatments (Table 4.8).

The *H. sexdentatus* haemolymph potassium salinity data violated the assumption of normality but did not violate the assumption of homogeneity (Shapiro test P <0.05, Levene's test F-value = 0.81, P = 0.53). For the data (Fig. 4.9) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 4, Kruskal-Wallis chi-squared = 22.88. A Dunn's-test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 4.8).

Table 4.8: The P-values of the post-hoc Tukey's multiple comparisons of means for the H. crenulatus *haemolymph potassium salinity trials (A) and the P-values of the Dunn's-test for multiple comparisons for the* H. sexdentatus *haemolymph potassium salinity trials (B). The P-values were adjusted using the Bonferroni method.*

A) *H. crenulatus*	100%	150%	2%	25%
150%	<0.05	-	-	-
2%	<0.05	<0.05	-	-
25%	0.02	<0.05	0.65	-
50%	0.72	0.02	<0.05	<0.05
B) *H. sexdentatus*				
150%	1.00	-	-	-
2%	0.01	<0.05	-	-
25%	0.14	0.01	1.00	-
50%	0.42	0.04	1.00	1.00

Table 4.9: The P-values, Kruskal-Wallis chi-squared (non-parametric) or F-value (parametric), and Degrees of freedom of the species salinity haemolymph potassium comparisons.

Salinity level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
2% (parametric)	0.17	2.15	10
25% (parametric)	0.03	6.51	10
50% (non-parametric)	<0.05	8.37	1
100% (parametric)	0.45	0.63	10
150% (parametric)	0.01	9.39	10

4.3.4. Sodium

Effect of oxygen level

The haemolymph sodium levels of *H. crenulatus* ranged from 462.8 to 534.6 mmol L^{-1} with significant differences between the 6% and 25% treatments (Fig. 4.10). The haemolymph sodium levels of *H. sexdentatus* ranged from 415 to 588 mmol L^{-1} with significant differences between the 25% and 100% treatments. (Fig. 4.10). Comparing the sodium haemolymph concentrations we found no difference between the 6% trial with differences found for the 25%, 50% and 100% trials, similar to the findings for potassium (Table 4.10).

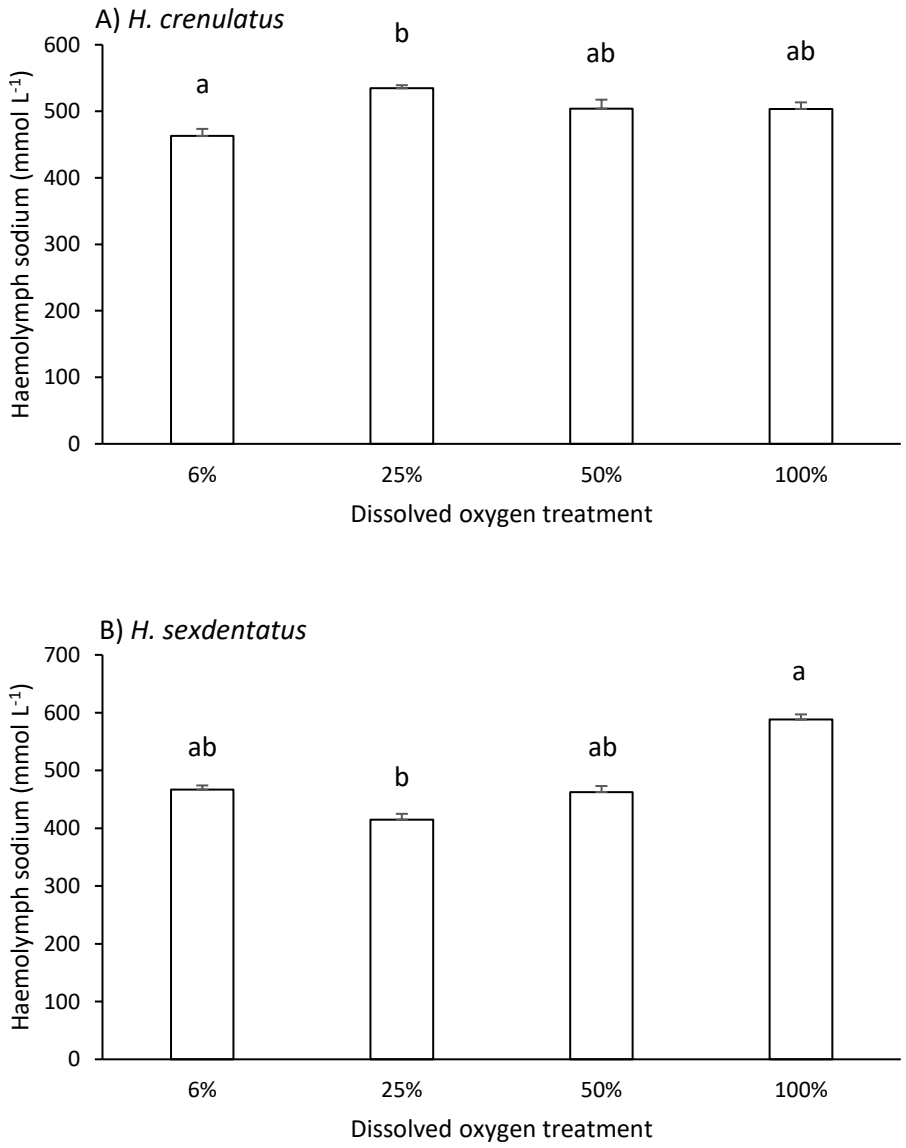

Figure 4.10: Haemolymph sodium (mmol L^{-1}) ± S.E.M. of H. crenulatus *(A) and* H. sexdentatus *(B) at different dissolved oxygen levels. Plots sharing letters are not significantly different.*

The *H. crenulatus* haemolymph sodium DO data did not violate the assumption of normality but did violate the assumption homogeneity (Shapiro test P = 0.15, Levene's test F-value = 4.05, P = 0.02). For the data (Fig. 4.10) a Kruskall-Wallis one-way analysis of variance was performed, with a significant effect of dissolved oxygen found (P <0.05). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 13.25. A post-hoc Dunn's test for multiple comparisons of independent samples was then used to compare the differences between treatments.

The *H. sexdentatus* haemolymph sodium DO data violated the assumption of normality but did not violate the assumption homogeneity (Shapiro test P <0.05, Levene's test F-value = 0.1, P = 0.96). For the data (Fig. 4.10) a Kruskall-Wallis one-way analysis of variance was performed, with a significant effect of dissolved oxygen found (P <0.05). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 17.97. A post-hoc Dunn's test for multiple comparisons of independent samples was then used to compare the differences between treatments.

Comparing the species' haemolymph sodium levels at the 6% (P = 0.74) DO treatment no difference was found whereas at the 25% (P <0.05), 50% (P = 0.02) and 100% (P <0.05) treatments differences were found. *H. crenulatus* were found to have higher, or equivalent haemolymph sodium concentrations at the 6% and 100% DO treatments, whereas *H. sexdentatus* had higher DO concentrations at the 25% and 50% treatments (Fig. 4.11).

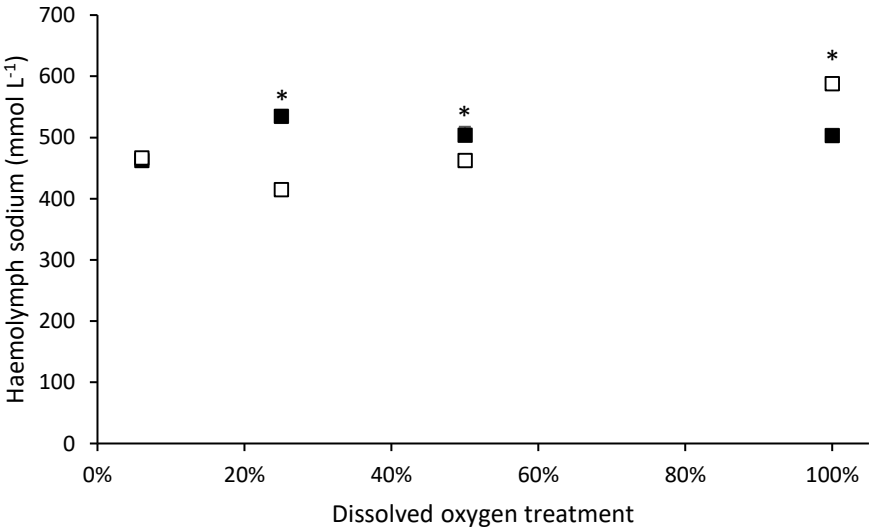

Figure 4.11: Haemolymph sodium of H. crenulatus *(■) and* H. sexdentatus *(□) (mmol L^{-1}) ± S.E.M. in different dissolved oxygen levels. * Significantly different from one another.*

Table 4.10: The P-values, Kruskal-Wallis chi-squared (non-parametric) or F-value (parametric), and Degrees of freedom of the species dissolved oxygen haemolymph sodium comparisons.

Dissolved oxygen level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
6% (parametric)	0.74	0.12	10
25% (non-parametric)	<0.05	8.31	1
50% (parametric)	0.02	7.02	10
100% (parametric)	<0.05	47.81	10

Effect of salinity

Much like was found for the previous haemolymph parameters when exposed to varying salinities both species, to some extent increased their haemolymph sodium as external seawater was increased. For *H. crenulatus* haemolymph sodium increased from 381.8 mmol L^{-1} at 2% to 633.3 mmol L^{-1} at the 150% treatment (Fig. 4.12). A post-hoc Dunn's test found significant differences between the 150% treatment and the other four treatments (Table 4.7). For *H. sexdentatus* haemolymph sodium increased from 371.8 mmol L^{-1} at 2% to 767 mmol L^{-1} at the 150% treatment (Fig. 4.12). A post-hoc Dunn's test found differences between the 150% and the other treatments, excluding the 50% treatment, as well as a difference between the 2% and 100% treatments (Table 4.7). When comparing the species haemolymph sodium no difference was found for the 2% or 25% treatments with differences found for the 50%, 100% and 150% treatments (Fig. 4.12).

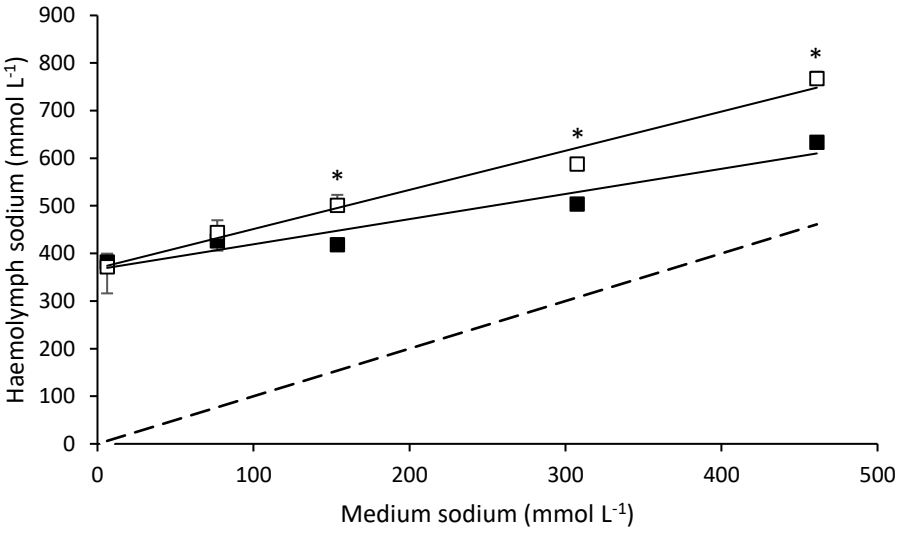

Figure 4.12: Haemolymph sodium of H. crenulatus *(■) and* H. sexdentatus *(□) (mmol L^{-1}) ± S.E.M. in different salinity treatments. The dotted line represents the isosmotic line. * Significantly different from one another.*

The *H. crenulatus* haemolymph sodium salinity data violated the assumptions of normality and homogeneity (Shapiro test P = 0.01, Levene's test F-value = 2.84, P = 0.04). For the data (Fig. 4.12) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 4, Kruskal-Wallis chi-squared = 22.74. A post-hoc Dunn's test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 4.11).

The *H. sexdentatus* haemolymph sodium salinity data did not violate the assumption of normality but did violate the assumption of homogeneity (Shapiro test P <0.05, Levene's test F-value = 8.35, P <0.05). For the data (Fig. 4.12) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 4, Kruskal-Wallis chi-squared = 24.37. A Dunn's-test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 4.11).

Comparing the haemolymph sodium for the salinity trials, at the 2% (P = 0.34) and 25% (P = 0.57) treatments no difference was found whereas at the 50% (P <0.05), 100% (P <0.05), and 150% (P <0.05) treatments a difference was found between the crabs (Table.

4.12). *H. sexdentatus* was found to have higher or equivalent haemolymph sodium concentrations at all of the salinity treatments in contrast to *H. crenulatus* (Fig. 4.12).

Table 4.11: The P-values of the post-hoc Dunn's test for multiple comparisons of independent means for the H. crenulatus *(A) and* H. sexdentatus *(B) haemolymph sodium salinity trials. The P-values were adjusted using the Bonferroni method.*

A) *H. crenulatus*	100%	150%	2%	25%
150%	1.00	-	-	-
2%	0.04	<0.05	-	-
25%	0.71	0.02	1.00	-
50%	0.28	<0.05	1.00	1.00
B) *H. sexdentatus*				
150%	1.00	-	-	-
2%	0.02	<0.05	-	-
25%	0.22	<0.05	1.00	-
50%	1.00	0.06	1.00	1.00

Table 4.12: The P-values, Kruskal-Wallis chi-squared (non-parametric) or F-value (parametric), and Degrees of freedom of the species salinity haemolymph sodium comparisons.

Salinity level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
2% (non-parametric)	0.34	0.92	1
25% (parametric)	0.57	0.35	10
50% (non-parametric)	<0.05	7.41	1
100% (parametric)	<0.05	47.81	10
150% (non-parametric)	<0.05	8.31	1

4.3.5. Glucose

Effect of oxygen level

H. crenulatus and *H. sexdentatus* maintained haemolymph glucose levels between 0.27 to 0.31 mmol L^{-1} for the DO trials. The haemolymph glucose levels of *H. crenulatus* ranged from 0.28 to 0.31 mmol L^{-1} with significant differences found between the 6% and 100%, 25% and 50%, and 25% and 100% treatments (Fig. 4.13). *H. sexdentatus* haemolymph glucose levels also ranged from 0.27 to 0.31 mmol L^{-1} with significant differences found between the 6% and 50%, 6% and 100%, 25% and 100%, and 50% and 100% treatments (Fig. 4.13). Species' glucose comparisons found no difference between the 6%, 25%, or 50% trials with a difference found for the 100% trial (Fig. 4.14).

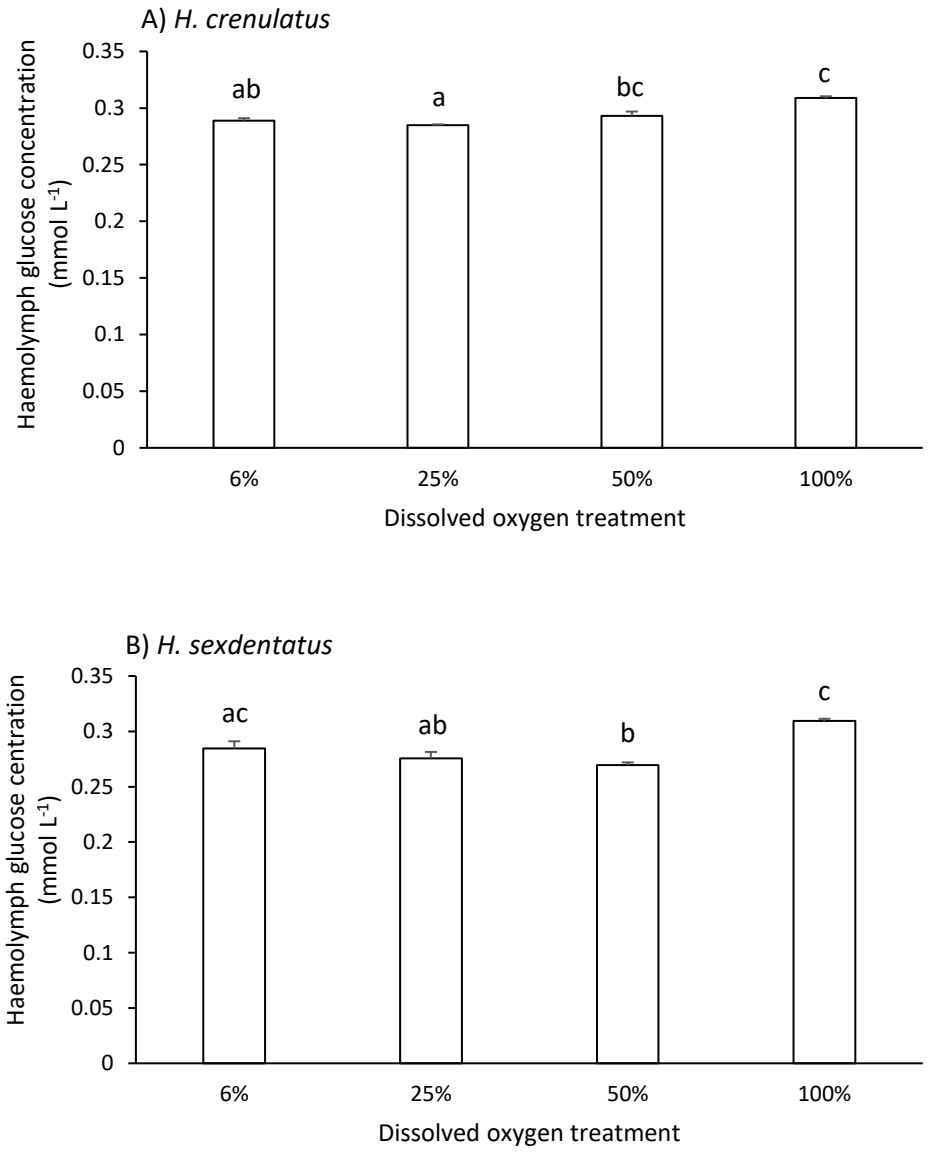

Figure 4.13: Haemolymph glucose (mmol L^{-1}) ± S.E.M. of H. crenulatus *(A) and* H. sexdentatus *(B) at different dissolved oxygen levels. Plots sharing letters are not significantly different.*

The *H. crenulatus* haemolymph glucose DO data violated the assumptions of normality and homogeneity (Shapiro test P <0.05, Levene's test F-value = 3.58, P = 0.03). For the data (Fig. 4.13) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of dissolved oxygen found (P <0.05). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 21.6. A post-hoc Dunn's test for multiple comparisons of independent samples was then used to compare the differences between treatments.

The *H. sexdentatus* haemolymph glucose DO data violated the assumption of normality but did not violate the assumption of homogeneity (Shapiro test P <0.05, Levene's test F-value = 2.27, P = 0.11). For the data (Fig. 4.13) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of dissolved oxygen found (P <0.05). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 21.6. A post-hoc Dunn's test for multiple comparisons of independent samples was then used to compare the differences between treatments.

Comparing the haemolymph glucose levels for the dissolved oxygen trials, at the 6% (P <0.05), 25% (P <0.05), and 50% (P <0.05) DO treatments differences were found whereas at the 100% (P = 0.15) treatment no difference was found (Table 4.13). *H. crenulatus* were found to have higher, or equivalent haemolymph haemocyanin concentrations at all of the DO treatments (Fig. 4.14).

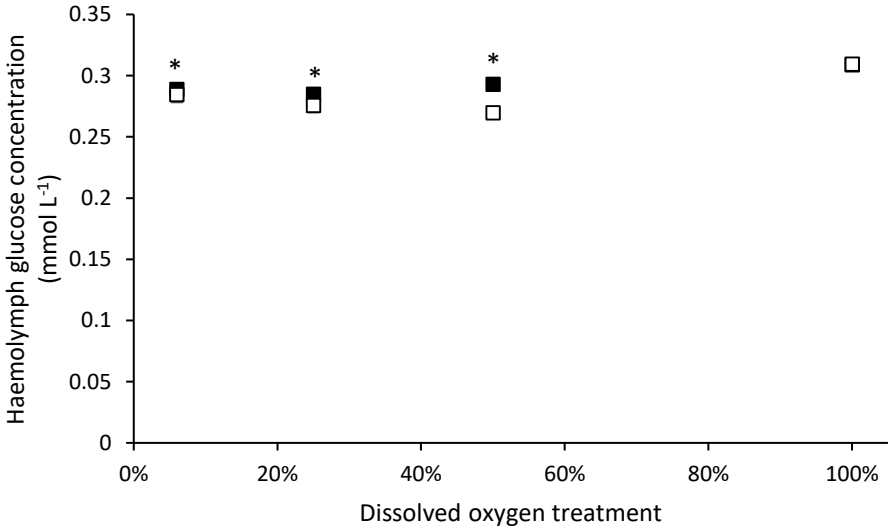

Figure 4.14: Haemolymph glucose of H. crenulatus *(■) and* H. sexdentatus *(□) (mmol L^{-1}) ± S.E.M. in different dissolved oxygen levels. * Significantly different from one another.*

Table 4.13: The P-values, Kruskal-Wallis chi-squared (non-parametric) or F-value (parametric), and Degrees of freedom of the species dissolved oxygen haemolymph haemocyanin comparisons.

Dissolved oxygen level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
6% (non-parametric)	<0.05	8.31	1
25% (non-parametric)	<0.05	8.31	1
50% (non-parametric)	<0.05	8.31	1
100% (parametric)	0.15	2.49	10

Effect of salinity

For the salinity trials *H. crenulatus* haemolymph glucose again ranged between 0.27and 0.31 mmol L^{-1} (Fig. 4.15). A post-hoc Dunn's test found several statistically significant differences between treatments (2% and 25%, 2% and 100%, 100% and 150%) (Table 4.14). For *H. sexdentatus* haemolymph glucose once again ranged between 0.27 and 0.31 mmol L^{-1} (Fig. 4.15). A post-hoc Dunn's test found several statistically significant differences between treatments (2% and 100%, 25% and 100%, 25% and 150%) (Table 4.14). Comparing the species' glucose levels differences were found between all of the treatments ($P < 0.05$) (Fig. 4.16).

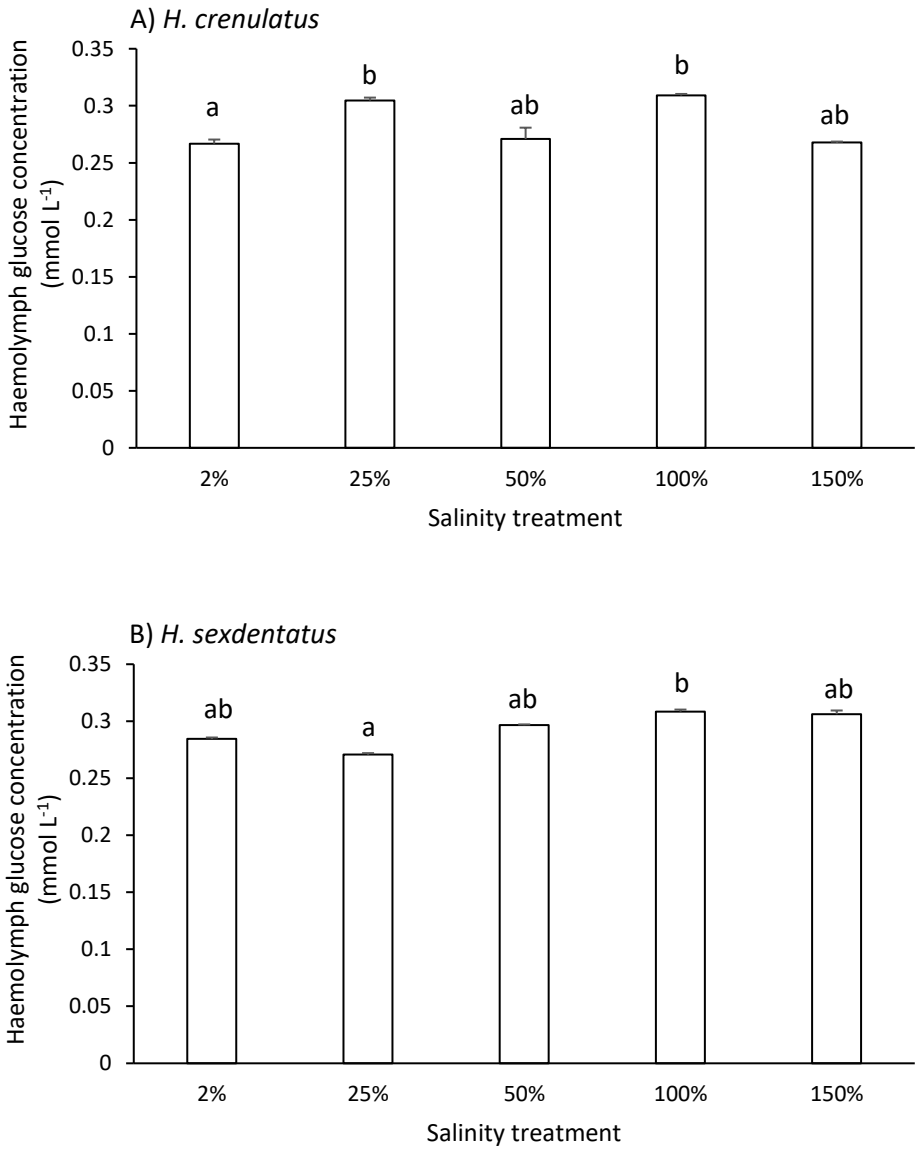

Figure 4.15: Haemolymph glucose (mmol L^{-1}) ± S.E.M. of H. crenulatus *(A) and* H. sexdentatus *(B) at different salinity treatments. Plots sharing letters are not significantly different.*

The *H. crenulatus* haemolymph glucose salinity data violated the assumption of normality but did not violate the assumption of homogeneity (Shapiro test P <0.05, Levene's test F-value = 2.63, P = 0.06). For the data (Fig. 4.15) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 4, Kruskal-Wallis chi-squared = 27. A post-hoc Dunn's test for multiple

comparisons of independent samples was then used to compare the differences between treatments (Table 4.14).

The *H. sexdentatus* haemolymph glucose salinity data violated the assumptions of normality and homogeneity (Shapiro test P <0.05, Levene's test F-value = 5.88, P <0.05). For the data (Fig. 4.15) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 4, Kruskal-Wallis chi-squared = 27.88. A post-hoc Dunn's test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 4.14).

Comparing the haemolymph glucose for the salinity trials, at all treatments (P <0.05) differences were found (Table. 4.15).

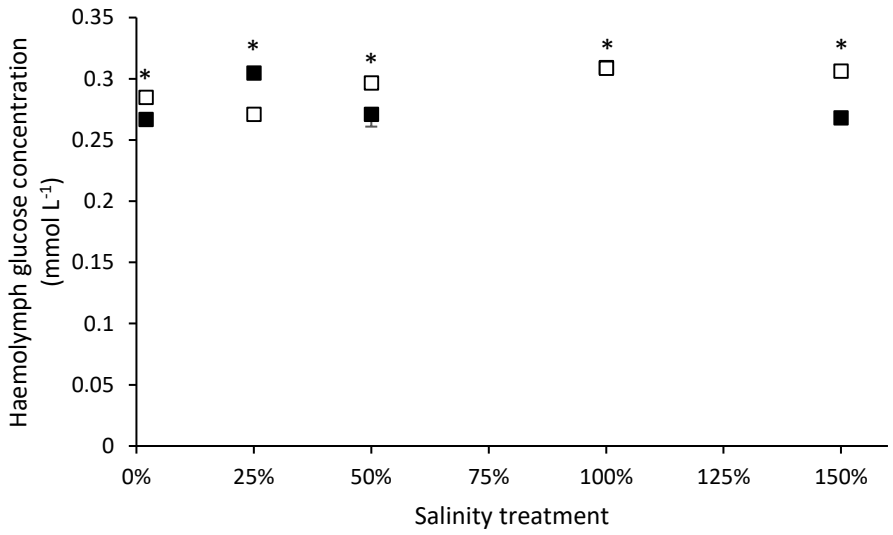

Figure 4.16: Haemolymph glucose concentrations of H. crenulatus *(■) and* H. sexdentatus *(□) (mmol L^{-1}) ± S.E.M. in different salinity treatments. * Significantly different from one another.*

Table 4.14: The P-values of the post-hoc Dunn's test for multiple comparisons of independent means for the H. crenulatus *(A) and* H. sexdentatus *(B) haemolymph glucose salinity trials. The P-values were adjusted using the Bonferroni method.*

A) *H. crenulatus*	100%	150%	2%	25%
150%	<0.05	-	-	-
2%	<0.05	1.00	-	-
25%	1.00	0.10	<0.05	-
50%	0.18	1.00	0.33	1.00
B) *H. sexdentatus*				
150%	1.00	-	-	-
2%	<0.05	0.18	-	-
25%	<0.05	<0.05	1.00	-
50%	0.18	1.00	1.00	0.18

Table 4.15: The P-values, Kruskal-Wallis chi-squared (non-parametric) or F-value (parametric), and Degrees of freedom of the species salinity haemolymph sodium comparisons.

Salinity level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
2% (non-parametric)	<0.05	8.34	1
25% (non-parametric)	<0.05	8.31	1
50% (non-parametric)	<0.05	8.31	1
100% (parametric)	0.01	9.18	10
150% (non-parametric)	<0.05	8.31	1

4.3.6. Haemocyanin

Effect of oxygen level

H. crenulatus and *H. sexdentatus* maintained haemolymph haemocyanin levels between 0.12 and 0.27 mmol L^{-1} for the DO trials. The haemolymph haemocyanin levels of *H. crenulatus* ranged from 0.13 to 0.27 mmol L^{-1} with significant differences found between the 100% and the other (6%, 25%, and 50%) treatments (Fig. 4.17). *H. sexdentatus* haemolymph haemocyanin levels ranged from 0.12 to 0.16 mmol L^{-1} with no significant differences found between treatments (Fig. 4.17). Species' haemocyanin comparisons found no difference between the 6%, 25%, or 50% trials with a difference found for the 100% trial (Fig. 4.18).

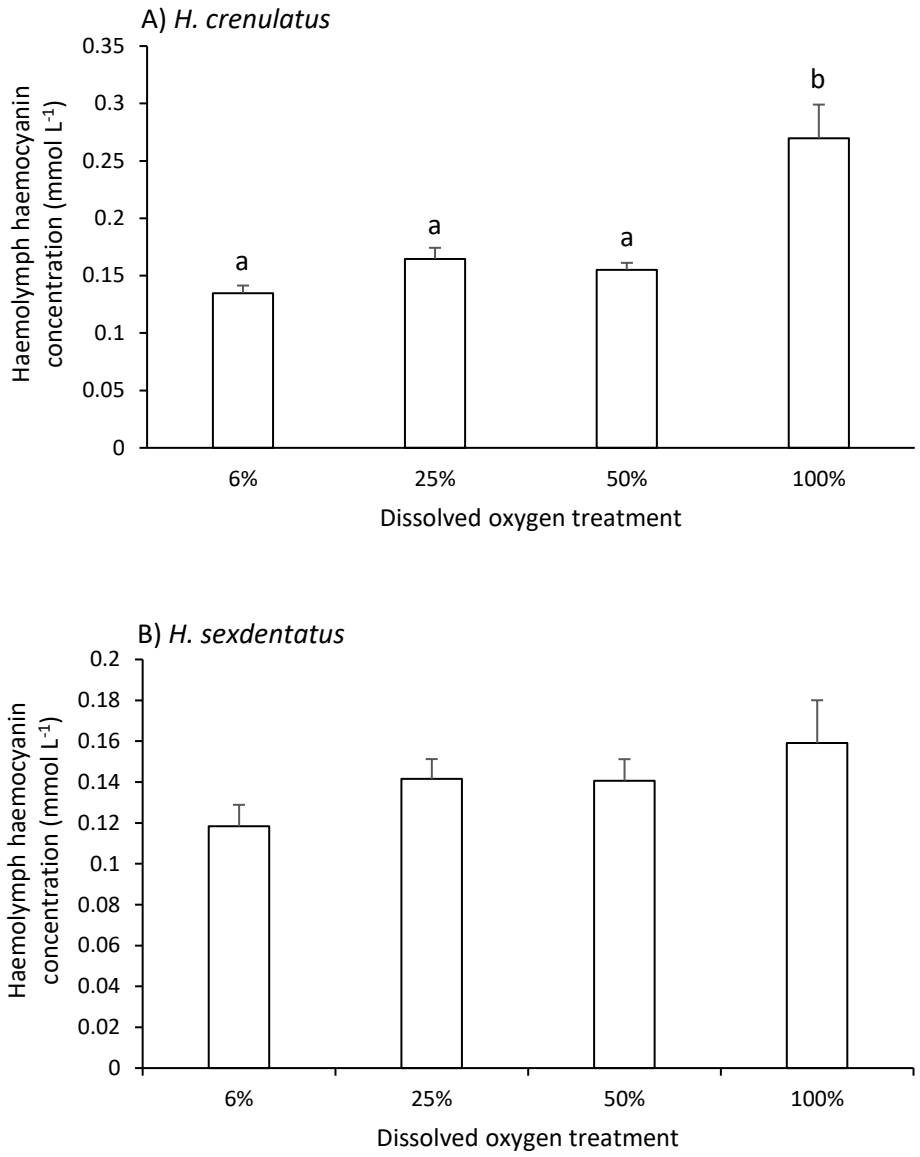

Figure 4.17: Haemolymph haemocyanin (mmol L^{-1}) ± S.E.M. of H. crenulatus *(A) and* H. sexdentatus *(B) at different dissolved oxygen levels. Plots sharing letters are not significantly different.*

The *H. crenulatus* haemolymph haemocyanin DO data violated the assumptions of normality and homogeneity (Shapiro test P <0.05, Levene's test F-value = 5.16, P <0.05). For the data (Fig. 4.17) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of dissolved oxygen found (P <0.05). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 16.46. A post-hoc Dunn's test for multiple comparisons of independent samples was then used to compare the differences between treatments.

The *H. sexdentatus* haemolymph haemocyanin DO data violated the assumptions of normality and homogeneity (Shapiro test P = 0.03, Levene's test F-value = 3.28, P = 0.04). For the data (Fig. 4.17) a Kruskal-Wallis one-way analysis of variance was performed, with no significant effect of dissolved oxygen found (P = 0.15). Summary of the data: df = 3, Kruskal-Wallis chi-squared = 5.32.

Comparing the haemolymph haemocyanin levels for the dissolved oxygen trials, at the 6% (P = 0.18), 25% (P = 0.09), and 50% (P = 0.42) DO treatments no difference was found whereas at the 100% (P <0.05) treatment a difference was found (Table 4.16). *H. crenulatus* were found to have higher, or equivalent haemolymph haemocyanin concentrations at all of the DO treatments (Fig. 4.18).

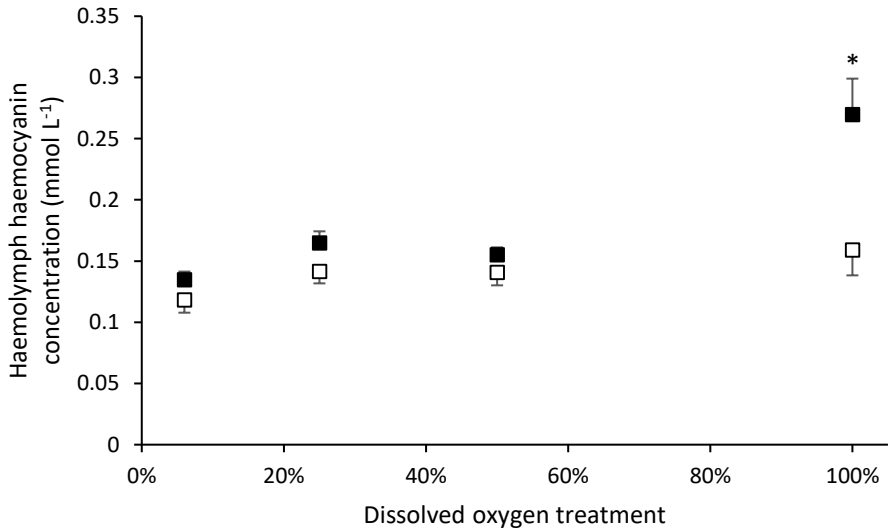

Figure 4.18: Haemolymph haemocyanin concentrations of H. crenulatus *(■) and* H. sexdentatus *(□) (mmol L^{-1}) ± S.E.M. in different dissolved oxygen levels. * Significantly different from one another.*

Table 4.16: The P-values, Kruskal-Wallis chi-squared (non-parametric) or F-value (parametric), and Degrees of freedom of the species dissolved oxygen haemolymph haemocyanin comparisons.

Dissolved oxygen level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
6% (parametric)	0.18	2.13	10
25% (parametric)	0.09	3.44	10
50% (non-parametric)	0.42	0.64	1
100% (parametric)	<0.05	11.29	10

Effect of salinity

For the salinity trials *H. crenulatus* haemolymph haemocyanin ranged between 0.072 and 0.18 mmol L^{-1} and appeared to increase between the 2% and 100% treatments and then decreased thereafter between 100% and 150% salinity (Fig. 4.19). However, a post-hoc Dunn's test only found statistically significant differences between the 2% and 100% treatments (Table 4.17). For *H. sexdentatus* haemolymph haemocyanin ranged between 0.12 and 0.18 mmol L^{-1} (Fig. 4.19). A Kruskal-Wallis one-way analysis of variance found no statistically significant differences between any of the salinity treatments (Table 4.17). Comparing the species' haemocyanin levels differences were found between the 2%, 25%, and 150% treatments, whereas at the 50% and 100% treatments no differences were found (Fig. 4.20).

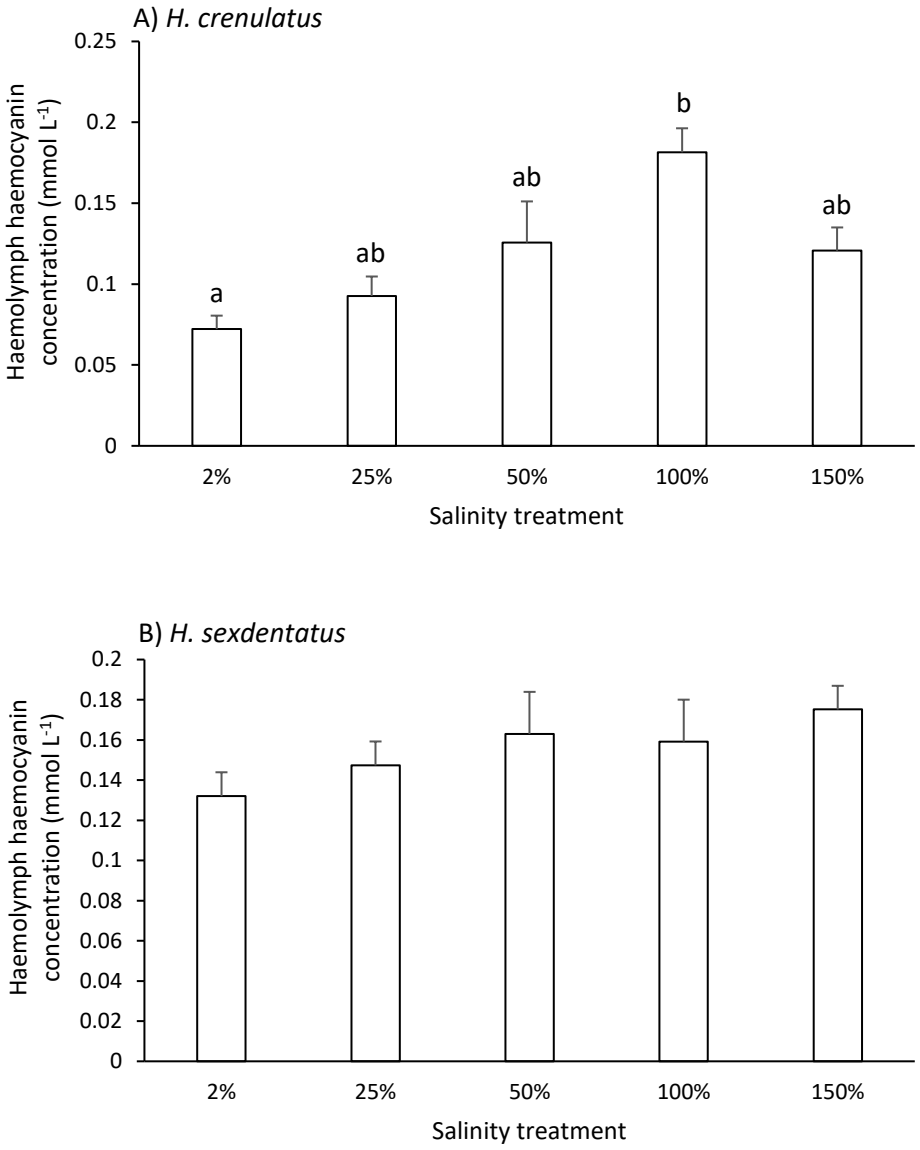

Figure 4.19: Haemolymph haemocyanin (mmol L^{-1}) ± S.E.M. of H. crenulatus *(A) and* H. sexdentatus *(B) at different salinity treatments. Plots sharing letters are not significantly different.*

The *H. crenulatus* haemolymph haemocyanin salinity data violated the assumptions of normality and homogeneity (Shapiro test P = 0.03, Levene's test F-value = 3.59, P = 0.02). For the data (Fig. 4.19) a Kruskal-Wallis one-way analysis of variance was performed, with a significant effect of salinity found (P <0.05). Summary of the data: df = 4, Kruskal-Wallis chi-squared = 15.86. A post-hoc Dunn's test for multiple comparisons of independent samples was then used to compare the differences between treatments (Table 4.17).

The *H. sexdentatus* haemolymph haemocyanin salinity data did not violate the assumptions of normality or homogeneity (Shapiro test P = 0.34, Levene's test F-value = 1.48, P = 0.24). For the data (Fig. 4.19) a one-way ANOVA was performed, with no significant effect of salinity found (P = 0.32). Summary of the data: df = 25, F-value = 1.25.

Comparing the haemolymph haemocyanin for the salinity trials, at the 2% (P = 0.34), 25% (P = 0.57), and 150% treatments differences were found whereas at the 50% (P <0.05) and 100% (P <0.05) treatments no differences were found between the crabs (Table. 4.18). *H. sexdentatus* was found to have higher or equivalent haemolymph haemocyanin concentrations at all of the salinity treatments in contrast to *H. crenulatus* (Fig. 4.20).

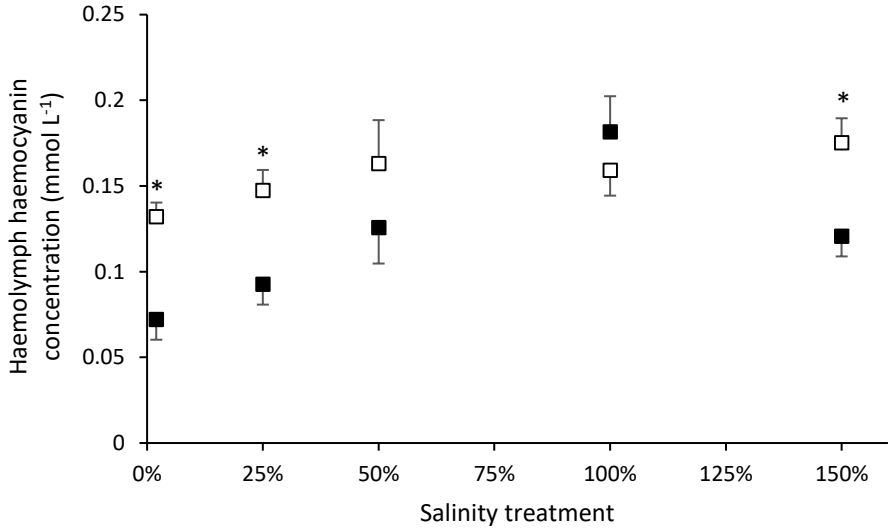

Figure 4.20: Haemolymph haemocyanin concentrations of H. crenulatus *(■) and* H. sexdentatus *(□) (mmol L^{-1}) ± S.E.M. in different salinity treatments. * Significantly different from one another.*

Table 4.17: The P-values of the post-hoc Dunn's test for multiple comparisons of independent means for the H. crenulatus *haemolymph haemocyanin salinity trials. The P-values were adjusted using the Bonferroni method.*

	100%	150%	2%	25%
150%	1.00	-	-	-
2%	<0.05	0.23	-	-
25%	0.07	1.00	1.00	-
50%	0.74	1.00	0.47	1.00

Table 4.18: The P-values, F-value (parametric), and Degrees of freedom of the species salinity haemolymph haemocyanin comparisons.

Salinity level	P-value	Kruskal-Wallis chi-squared value/F-value	Degrees of freedom
2%	<0.05	20.4	10
25%	<0.05	12.55	10
50%	0.24	1.54	10
100%	0.36	0.91	10
150%	<0.05	10.35	10

4.4. Discussion

As an animal becomes stressed it can undergo both physiological and biochemical processes to maintain normal functioning. Aquatic organisms are able to cope with external dissolved oxygen and salinity stress through osmoregulation or osmoconformation of various physiological processes and haemolytic parameters. Adaptive responses to hypoxia and salinity stress include reductions in metabolic rate, modifications of haemocyanin binding capacity and acid-base balance, and regulation of haemolymph osmolarity and ion concentrations (such as Cl^-, K^+, and Na^+) (Johnson and Uglow, 1985; Charmantier et al., 1994; Chen and Kou, 1998; Paschke et al., 2010). The two primary ions that make up 90% of the total haemolymph osmolarity are Na^+ and Cl^-, because of this have received most of the research attention (e.g. Cameron, 1978; Riestenpatt et al., 1996; Henry et al., 2003). Glucose is another commonly studied haemolymph constituent of crustaceans as it is regularly increased as a response stress (Bonga, 1997).

Osmolarity

Haemolymph osmolarity is a good indicator of stress including that found when animals encounter salinity and/or dissolved oxygen levels exceeding (or below) environmental norms. This haemolymph parameter is therefore commonly investigated in conjunction with physiological research. During hypoxic experiments haemolymph osmolarity of crabs is not often found to change. For example, experiments by DeFur et al. (1990) found no coherent trends or changes in blood osmolality or Cl^- ions of *Callinectes sapidus* over short (1-7 days) and long term (23-25 days) hypoxia exposures (140-155 mmHg vs. 50 mmHg). Blood osmolarities were also found to be unaffected by hypoxia for the shrimp *Crangon crangon*, which remains hyperosmotic to the external medium at 60, 30 and 10 mmHg, and the decapod crab *Carcinus maenas* which maintains its osmolarity down to 20 mmHg (Spaargaren, 1977; Hagerman and Uglow, 1982).

These findings reflect those found for *H. crenulatus* and *H. sexdentatus* where both species maintained their haemolymph osmolarities near the ambient 1000 mOsmol kg^{-1} salinity across all dissolved oxygen exposures. *H. crenulatus* maintained haemolymph osmolarity between 993 ± 8 and 1150 ± 22 mOsmol kg^{-1} with a difference seen between the 6% and 25% treatments and the 100% treatment. *H. sexdentatus* maintained haemolymph osmolarity between 1017 ± 6 and 1078 ± 19 mOsmol kg^{-1} with no difference between any of the DO treatments. When comparing the two New Zealand crabs we found no difference for the 6% treatments with differences found for the 25%, 50%, and 100% treatments. *H. crenulatus* were also found to have higher haemolymph osmolarities for the 50% and 100% treatments with *H. sexdentatus* having a higher osmolarity for the 25% treatment.

In contrast to dissolved oxygen, crustaceans often regulate and occasionally osmoconform haemolymph osmolarity in various salinities (For a review see McGaw and McMahon, 1996). For example, *C. maenas* is able to regulate haemolymph osmolarity at a near constant level in changing external salinities (Weeks et al., 1993). When *C. maenas* inhabit dilute waters it hyper-regulates its blood osmolarity, whereas in marine environments with higher salinities blood osmolarity is kept nearer the ambient medium (Siebers et al., 1982). *C. sapidus* also regulate haemolymph osmolarity, and other body fluids, contrasting the results found for this species under hypoxic stress. *C. sapidus* are found to hyper-regulate body fluids through varying salinities across 24 hours (25%, 50%, 75%, 100% salinities made from a 33 ppt 100% seawater stock) (McGaw et al., 1999). *Cancer magister* are classed as weak osmoregulators where haemolymph osmolarity levels are relatively close to the ambient salinity (i.e. close to the isosmotic line) (Hunter and Rudy, 1975; McGaw et al., 1999). In other circumstances certain decapod species will osmoconform maintaining haemolymph osmolarities almost equal to the external salinity (i.e. following the isosmotic line). *Libinia emarginata* are a species that osmoconform as adults, with osmolarity and other ion levels closely following the external salinity (Kalber, 1970; McGaw et al., 1999).

H. crenulatus and *H. sexdentatus* responded to salinity by hyper-regulating their haemolymph osmolarity between 2% and 100% seawater. *H. crenulatus* appears to be a strong hyper-osmoregulator maintaining haemolymph osmolarity above the ambient media in dilute sea waters. However, at higher seawaters (i.e. 150%) the regulatory response

changed and the crab hypo-regulated osmolarity (230 mOsmol kg^{-1} below the seawater at 150%). For this species osmolarity ranged from 707 ± 30 mOsmol kg^{-1} at 2% salinity (20 mOsmol kg^{-1} salt water) to 1270 ± 34 mOsmol kg^{-1} at 150% salinity (1500 mOsmol kg^{-1} salt water). Much like *H. crenulatus*, *H. sexdentatus* is a hyper-osmoregulator in osmolarities between 2% and 100%, however, at salinities above 100% this species utilises a different regulatory response. *H. sexdentatus* are found to osmoconform above this level which is reflective of the environment they inhabit (high shore rocky beaches being less likely to fluctuate in salinities above 100% in contrast to brackish and estuarine waters). For this species osmolarity ranged from 795 ± 79 mOsmol kg^{-1} at 2% salinity and 1712 ± 39 mOsmol kg^{-1} at 150% salinity.

These findings are supported by previous work which further suggest *H. crenulatus* and *H. sexdentatus* as being euryhaline, strong hyper-osmoregulators in dilute sea waters and weak hypo-osmoregulators in high salinities (Hicks, 1973; Jones, 1976; Taylor and Seneviratna, 2005). Findings for *H. sexdentatus* in the current study are slightly different to that suggested in previous work, however, as they appear to osmoconform at high salinities rather than hypo-osmoregulate. This could be due to a number of factors such as the high maximum salinity treatment (52.5 ppt).

Chloride

Much like osmolarity, haemolymph chloride is not commonly found to change when a crustacean is exposed to hypoxic waters. This was found for *C. sapidus* in studies by DeFur et al. (1990) (mentioned above) and many other species such as *C. maenas* which regulated chloride ~500 mmol L^{-1} in full strength 35% (ppt) salinity over 50 hours of hypoxia exposure (Burnett and Johansen, 1981). Studies on freshwater crayfish are also in support of this claim in which three species (*Austropotambius pallipes*, *A. italicus*, and *A. torrentium*) all exhibited no decreases in haemolymph chloride concentrations when exposed to hypoxia. One species (*A. italicus*) even displayed a slight increase in haemolymph chloride when kept in 30% oxygen saturation (Demers et al., 2006). Some species are, however, variable in their abilities to regulate chloride where some are able to maintain Cl^- to lower dissolved oxygen

levels than others. For example, the Baltic prawn *Palaemon adspersus* maintains a stable regulation of Cl^- down to 80 mmHg with lower O_2 tensions resulting in impaired regulation and the animals eventually become isosmotic with the external medium (Hagerman and Uglow, 1981). Other species are able to maintain Cl^- at much lower DO levels, for example *C. crangon* remained hyperosmotic regulating Cl^- in DO levels as low as 30 mmHg and salinity levels as low as 10% (ppt) (Hagerman and Uglow, 1982). Finally, some species such as the ditch shrimp *Palaemonetes varians* are able to maintain Cl^- regardless of salinity (as low as 0.8%) or oxygen tension (<10 mmHg) regulating between 200 and 300 mmol L^{-1} (Hagerman and Uglow, 1984).

For the two New Zealand intertidal crabs haemolymph chloride was regulated at all the experimental dissolved oxygen levels. *H. crenulatus* maintained chloride within a 474 ± 24 to 527 ± 30 mmol L^{-1} range and *H. sexdentatus* maintained chloride within a 511 ± 14 to 527 ± 10 mmol L^{-1}, similar to that found for *C. maenas*. The species were also found to have no significant differences between any of the DO levels suggesting they maintain Cl^- at a similar concentration being very close to the medium 500 mmol L^{-1}.

Following osmolarity, chloride is also commonly regulated across different salinities in crustaceans as it is a common blood constituent and makes up ~55% of the chemical composition of sea water (35 ppt). Many species hyper-regulate Cl^- and other inorganic blood constituents (Na, K, Ca) in dilute seawaters, hypo-regulate in sea waters exceeding the environmental norm and in environmentally normal waters these constituents are often passively moved between the blood and the external medium (Zanders, 1980; Siebers et al., 1982). For example, *C. maenas* maintain Cl^- ~500 mmol L^{-1} in 35 ppt/500 mmol L^{-1} waters, whereas at a lower 17 ppt/240 mmol L^{-1} sea water haemolymph Cl^- was hyper-regulated (~300-350 mmol L^{-1}) (Burnett and Johansen, 1981). *Pachygrapsus crassipes* regulate their haemolymph chloride, here between ~250 to 550 mmol L^{-1} in a wide range of salinities (between 10 and 50 ppt seawater, i.e., ~200 to 800 mmol L^{-1}) (Burnett et al., 1981). This species also hyper-regulates in dilute waters, appear to passively move ions in environmentally normal waters and hypo-regulate in waters with salinities above the norm.

Much like total osmolarity, as external salinity increased so too did haemolymph chloride for both species. For *H. crenulatus* haemolymph chloride increased from 350 ± 27 mmol L^{-1} at 2% salinity (well above the ambient 30 mmol L^{-1}), 512 ± 18 mmol L^{-1} at 100%

(similar to the ambient 500 mmol L^{-1}), to 637 ± 28 mmol L^{-1} at 150% salinity (well below the ambient 750 mmol L^{-1}). Supporting regulatory patterns found in other crabs. For *H. sexdentatus* haemolymph chloride increased from 311 ± 57 mmol L^{-1} at 2% salinity (again above the ambient chloride), 511 ± 14 mmol L^{-1} at 100% (similar to the ambient 500 mmol L^{-1}), to 762 ± 22 mmol L^{-1} at 150% salinity (similar to the ambient 750 mmol L^{-1}). The findings for *H. sexdentatus* are different to those found for *H. crenulatus*, and many of the aforementioned species, in which this species appear to conform above 100% salinity rather than hypo-regulate. This follows the *H. sexdentatus* results where the species osmoconformed above 100%. When comparing the two New Zealand species we find no differences between all of the salinities excluding the 150% treatment.

Potassium

When compared to normoxic conditions, various haemolymph parameters of crustaceans under hypoxia, or anoxia, are often altered. For example, Cl^- and Na^+ of the brackish water isopod *Sauduria entomon* exposed to anoxic conditions decreased over time (Hagerman and Szaniawska, 1991). However, in this species one haemolymph parameter (K+) remained unchanged when exposed to anoxia. On the other hand, in the freshwater giant prawn *Macrobrachium rosenbergii* haemolymph potassium decreased under hypoxic conditions after 6 hours of exposure, from ~4.0-4.5 mmol L^{-1} at high oxygen levels (between 2.75 and 7.75 mg O_2 L^{-1}) to ~3.55 mmol L^{-1} K^+ at lower hypoxic conditions (1.75 mg O_2 L^{-1}) (Cheng et al., 2003).

In the current study both *H. crenulatus* and *H. sexdentatus* maintained haemolymph potassium near the ambient (11.4 mmol L^{-1}) level across all dissolved oxygen treatments. No differences were found between the haemolymph potassium levels of *H. crenulatus* which ranged from 10.23 to 10.89 mmol L^{-1}. *H. sexdentatus* displayed subtle differences where at the lowest 6% DO treatment potassium levels were comparable to the 100% but not the other 25% or 50% treatments. Here *H. sexdentatus* haemolymph potassium ranged from 10.53 to 12.77 mmol L^{-1}. When comparing these two species potassium no difference was

found between the 6% treatment but differences were found for the 25%, 50% and 100% treatments.

Potassium in the gills of crustaceans is actively moved through $Na^+ + K^+$ -ATPase transporters. In response to varying salinities potassium concentrations in the range of 5 – 10 mmol L^{-1} maximally activate these transporters (*Km*). In crustacean species that inhabit brackish waters this *Km* value ranges somewhere between 1.1 to 14.3 mmol L^{-1} (Lucu and Towle, 2003). As salinities decrease below environmental norms some decapod species are able to hyper-regulate K^+, such as in *C. maenas* (7.1 ± 1 mmol L^{-1} at 9 ppt vs. 12 ± 2 at 35 ppt) and *C. magister* (Siebers et al., 1982; Siebers et al., 1983; Brown and Terwilliger, 1992). This is also found in other species. Findley and stickle (1978) found when implementing an intertidal simulation of varying salinities (30, 20, or 10 ppt), *C. sapidus* haemolymph potassium significantly decreased from ~10-11.2 at 30 ppt, to ~7.3-12.3 at 20 ppt, and finally ~6.5-8.2 mmol L^{-1} at 10 ppt salinity. Similar to the above findings, and those for chloride and osmolarity, K^+ is maintained at levels near that of the external medium of the species' normal salinity range, hyper-regulated below, and hypo-regulated at salinities above. This was found for *C. similis* and *C. sapidus* where *C. similis* regulated/maintained K^+ between ~5-7, 7-9, and 7-10 mmol L^{-1} at 10, 20, and 30 ppt respectively. Similarly *C. sapidus* regulated K^+ between ~6.5-9, 7-9.5, and 7-11 mmol L^{-1} at 10, 20, and 30 ppt respectively (Piller et al., 1995).

Regulation of potassium at different salinities in the two New Zealand crabs occurred within the range found for other crustaceans (1.1 to 14.3 mmol L^{-1}). Similar to other crustacean species these decapods displayed a hyper-regulatory response at low, maintenance at environmentally equivalent, and hypo-regulatory response at high salinities. *H. crenulatus*' K^+ level at 2% (6.7 mmol L^{-1}) salinity was much higher than the ambient 0.23 mmol L^{-1}, in contrast K^+ levels at 100% salinity (10.4 mmol L^{-1}) were similar to the ambient 11.4 mmol L^{-1} salinity, and lower (14.1 mmol L^{-1}) at 150% 17.1 mmol L^{-1} salinity. For *H. sexdentatus* K^+ was again much higher (5.9 mmol L^{-1}) at 2% salinity, a similar 11.1 mmol L^{-1} at 100%, and a higher 11.8 mmol L^{-1} at 150% salinity. Comparing these species we find no differences at the 2% and 100% treatments with *H. crenulatus* having higher K^+ levels at the 25%, 50%, and 150% treatments.

Sodium

Sodium is another common inorganic blood constituent of crustaceans and as of such follows a similar to pattern to that of osmolarity, chloride and potassium in relation to hypoxia and salinity stress. Under hypoxic conditions crustaceans commonly regulate sodium maintaining haemolymph levels throughout a range of dissolved oxygen concentrations. This has been found for species such as *C. crangon* which maintained haemolymph Na^+ at a steady state (between 150-250 mmol L^{-1}) in hypoxic conditions as low as 30 mmHg and 10 mmHg over 5+ hours (Hagerman and Uglow, 1982). In contrast some species haemolymph sodium concentrations decrease with hypoxia such as in the freshwater crayfishes *Austropotamobius pallipes* and *A. torrentium* (between 85%, 55%, and 30% O_2 saturation). In the same experiments *A. italicus* exhibited no change in sodium levels regardless of hypoxia (Demers et al., 2006). Similarly the brackish water isopod displayed no differences in Na^+ levels in hypoxia only being affected when DO concentrations decreased to anoxia (Hagerman and Szaniawska, 1991).

The experimental crabs in the present study maintained haemolymph sodium as hypoxia progressed. There were, however, some differences found between DO treatments. Haemolymph sodium levels of *H. crenulatus* ranged from 463 ± 10 to 535 ± 10 mmol L^{-1} with significant differences between the 6% and 25% treatments and *H. sexdentatus* ranged from 415 ± 10 to 588 ± 9 mmol L^{-1} with significant differences between the 25% and 100% treatments. Comparing the crabs' sodium haemolymph concentrations no differences were found for 6% DO with differences found for the 25%, 50% and 100% trials, similar to the findings for potassium.

Sodium concentrations of crabs in various salinities are again hyper-regulated at low salinities, comparable/maintained near environmentally normal salinities, and hypo-regulated at high salinities. For example, in the decapods *Callinectes similis* and *C. sapidus* this response is found. Both of these crabs inhabit estuarine waters between 30% and 15% salinity with *C. sapidus* inhabiting dilute waters as low as 0% whereas *C. similis* is rarely found below 15% (Perry, 1984; Hseuh et al., 1993; Piller et al., 1995). *C. similis* hyper-regulates Na^+ at lower salinities (~220 mmol L^{-1} at 10% salinity) maintained Na+ similar at

environmentally normal salinities (~300 mmol L^{-1} at 20% salinity) and hypo-regulated at high salinities (~350 mmol L^{-1} at 30% salinity). A similar response was found for *C. sapidus* which, reflecting its preferred environment, displayed a greater ability to regulate at lower salinities (~290 mmol L^{-1} at 10%) and had very similar Na^+ levels to *C. similis* at the other salinities (~300 mmol L^{-1} at 20% and ~350 mmol L^{-1} at 30% salinity) (Piller et al., 1995). *C. maenas* also hyper-regulate Na^+ at low salinities (9 ppt) at nearly twice the ambient Na^+ level (ambient Na^+: ~100-150 mmol L^{-1}, haemolymph Na^+: ~300 mmol L^{-1}), whereas at normal salinities (35 ppt) blood Na^+ was isoionic with the ambient Na^+ (ambient Na^+: 518 mmol L^{-1}, haemolymph Na^+: 531 ± 62 mmol L^{-1}) (Siebers et al., 1983).

At low salinities *H. crenulatus* and *H. sexdentatus* had much higher Na^+ levels than that of the ambient water. This was also found for the environmentally normal salinities (100%) and high salinities (150%), albeit not to the same extent found for the low 2% salinity. For *H. crenulatus* haemolymph sodium was hyper-regulated at all salinities which increased from 382 ± 18 mOsm at 2% (6 mmol L^{-1}) to 504 ± 10 mmol L^{-1} at 100% (307 mmol L^{-1}) and 633 ± 8 mmol L^{-1} at the 150% (461 mmol L^{-1}) treatment. For *H. sexdentatus* haemolymph sodium was also hyper-regulated and increased from 372 ± 55 mmol L^{-1} at 2% (6 mOsm) to 588 ± 10 mmol L^{-1} at 100% (307 mmol L^{-1}) and 767 ± 15 mmol L^{-1} at the 150% (461 mmol L^{-1}) treatment. When comparing the species haemolymph sodium no difference was found for the 2% or 25% treatments with differences found for the 50%, 100% and 150% treatments. This contrasts with previous findings for *C. sapidus*, *C. simlis*, and *C. maenas*.

Glucose

Unlike the previous haematological parameters, which are good indicators of an animals regulatory abilities, glucose can also be used as an indicator of stress. During hypoxic stress certain genes and their products (mRNA and proteins) that respond to hypoxia can be indicative of an animal becoming stressed (Brouwer et al., 2005). Enzymes of the glycolytic pathway (which increase anaerobic ATP production), glucose transporters and gluconeogenesis, which maintain blood glucose levels, are among the stress responses involved (Semenza et al., 1994; Hochachka et al., 1996; Gracey et al., 2001; Brouwer et al.,

2005). The resting glucose values of both *H. crenulatus* and *H. sexdentatus* (0.27 mmol L^{-1}) are comparable to those of other crustacean species (between 0.18 and 0.78 mmol L^{-1}) such as the crayfish *C. destructor* (0.18 mmol L^{-1}) (Onnen and Zebe, 1983; Taylor and Spicer, 1987; Van Aardt 1988; Morris and Callaghan, 1988).

Hyperglycaemia (blood glucose above resting glucose levels) is a common response of crustacea to hypoxic stress. Hypoxia-induced hyperglycaemia is found in shrimp where *Penaeus vannamei* experienced increases of haemolymph glucose from 0.72 ± 0.4 mmol L^{-1} in normoxia to 3.19 ± 1.69 mmol L^{-1} after 3 days, and to 2.54 ± 1.1 mmol L^{-1} after two-weeks of hypoxia exposure (Racotta et al., 2002). In the crayfishes *Parastacus defossus* and *P. brasiliensis* glucose increased after 4 hours of hypoxia and subsequently decreased, but still at a higher level than normoxia, after 8 hours of exposure (*P. defossus* between ~0.7-2.0 mmol L^{-1} and *P. brasiliensis* between ~0.7-3.0 mmol L^{-1}) (da Silva-Castiglioni et al., 2010). Hyperglycaemia is also commonly found in lobsters (such as *Panulirus interruptus* which varied between 0.55 and 1.98 mmol L^{-1}) (Ocampo et al., 2003) and crabs (such as the freshwater species *Eriocheir sinensis* which rapidly increased blood glucose after hypoxia exposure peaking at around the tenth hour) (Zou et al., 1996). The hyperglycaemia found for these species is suggested to function as a pre-emptive physiological response to the high substrate demands of anaerobic glycolysis (Oliveira et al., 2001; Marqueze et al., 2011).

A hyperglycaemic response was not found for either New Zealand crab species. Alternatively these species displayed a slight hypoglycaemic response. *H. crenulatus* maintained glucose between 0.28 and 0.31 mmol L^{-1} and *H. sexdentatus* maintained glucose between a similar 0.27 and 0.31 mmol L^{-1}. Differences between the 6% and 25%, 25% and 50%, and 25% and 100% treatments were found for *H. crenulatus*, and differences between 100% and the other DO treatments, and between 6% and 50% treatments were found for *H. sexdentatus*. When comparing the species a single difference between treatments was found (100% treatment), with no differences between the 6%, 25%, or 50% treatments.

The hypoglycaemia found for the two New Zealand crab species, and other responses opposed to hyperglycaemia, are less common, however, they are not unheard of. The aforementioned crab *E. sinensis,* for example, displayed a decrease in haemolymph glucose past the 10 hour peak for the remainder of the study (30 hours) (Zou et al., 1996). Similar results were found for the crayfish *P. defossus* which, after an initial increase, had

haemolymph glucose return to levels comparable to normoxia (da Silva-Castiglioni et al., 2010). The different responses of glucose mobilisation and regulation in hypoxia can be suggested to be attributed to species differences as well as different exposure conditions (Bonvillain et al., 2012).

Glucose concentrations of crustaceans are also found to increase in response to other stressors including handling (Paterson et al., 1997; Bergmann et al., 2001), pollutants and disease (Lorenzon et al., 1997; Lorenzon et al., 2000), emersion (Chang et al., 1998; Speed et al., 2001) and variations in salinity (Spaargaren and Haefner, 1987; Lorenzon et al., 2008). For example, acute salinity stress increased haemolymph glucose in the shrimp *Penaeus monodon*. Investigations by Annies and Rosamma (2007) found *P. monodon* increasing haemolymph glucose from 1.36 + 0.24 mmol L^{-1} at 0 ppt salinity, to 1.93 + 0.36 mmol L^{-1} at 15 ppt salinity, and 2.31 + 0.36 mmol L^{-1} at 35 ppt salinity. In hyper- and hyposalinities (0 ppt and 40 ppt) *Chasmagnathus granulata* haemolymph blood levels increased but did not significantly change in salinities between 10 and 30 ppt. *C. granulata* exposed to varying salinities decreased haemolymph glucose, after an initial increase (similar to findings for *E. sinensis* and *P. defossus* exposed to hypoxia), over an extended exposure period (over 24 hours) (Santos and Nery, 1987).

Salinity affected *H. crenulatus* and *H. sexdentatus* glucose levels in the same way as the hypoxia treatments where it ranged between 0.27and 0.31 mmol L^{-1} for both species. There were also no apparent directional trends or patterns displayed by either species. For *H. crenulatus* differences were found between 2% and 25%, 2% and 100%, 100% and 150% treatments, whereas for *H. sexdentatus* differences were found between the 2% and 100%, 25% and 100%, 25% and 150%. Comparing the species' glucose levels differences were found between all of the treatments. In comparison to previous works (for example, Santos and Nery, 1987; Annires and Rosamma, 2007) the differences between treatments and between species were minor, with a maximum difference of 0.04 mmol L^{-1}.

The findings of this research may, however, relate to the nature of haemolymph glucose which can change rapidly over a short time period. Because of this spot sampling after 6 hours may be insufficient in attaining a sound picture of crab haemolymph glucose changes in responses to hypoxia and salinity.

Haemocyanin

Several factors are known to affect haemocyanin concentrations in the haemolymph of crustaceans including, but not limited to, hypoxia and salinity (Taylor and Anstiss, 1999). In decapods hypoxia stimulates haemocyanin synthesis which commonly increases when a species is subjected to prolonged moderate hypoxia. For example, in the species *Crangon crangon* exposed to moderate hypoxia (40% saturation) haemocyanin concentrations in the haemolymph increased from 0.5-0.6 mmol L^{-1} in normoxia to 0.8 mmol L^{-1} (after 1 week) and 1.3 mmol L^{-1} (after two weeks) (Hagerman, 1986). Similarly haemocyanin synthesis was also suggested to be found for the burrowing decapod *Nephrops norvegicus* which over 10-13 days of moderate hypoxia exposure resulted in a biosynthesis of 0.024 mmol per day of haemocyanin (Hagerman and Uglow, 1985). Haemocyanin concentration increases in the haemolymph of crabs subjected to prolonged hypoxia has also been found for *Macrobrachium rosenbergii* (Chen and Kou, 1998) and *Callinectes sapidus* (deFur et al., 1990).

In the current study hypoxia did not elicit an increase in haemocyanin as is commonly found for decapods. Instead what was found for *H. crenulatus* was a decrease from the initial normoxic 100% condition (0.27 mmol L^{-1}) to between 0.16 and 0.13 mmol L^{-1} at the other conditions. No differences were found between any of the DO exposures for *H. sexdentatus* (ranging between 0.12 and 0.16 mmol L^{-1}). These concentrations are low in comparison to other species (such as for *C. crangon* i.e. 0.5-0.6 to 1.3 mmol L^{-1}) which may be a result of the acute 6 hour exposure period or due to the specific methodologies used in the present study. Comparing the two species a single difference was found at the 100% treatment with similar haemocyanin concentrations found for the 6%, 25%, and 50% treatments.

Salinity changes have also been found to induce increases in haemolymph haemocyanin concentrations in crustaceans. For example, the estuarine crab *C. maenas* acclimated to hypo-osmotic waters (50% sea water) resulted in large increases of haemolymph haemocyanin concentrations (Boone and Schoffeniels, 1979). Previous work on *H. crenulatus* has also found haemocyanin increasing in hypo- and hyper-osmotic waters

in contrast to normoxic conditions. Here Lee et al. (2010) found increases of haemocyanin from 0.4-0.5 mmol L^{-1} in normoxic (i.e. ~34 ppt: 100% sea water) to 0.8-1.0 mmol L^{-1} in hypo-osmotic (50% sea water), and ~0.6-0.7 mmol L^{-1} in hyper-osmotic (120% sea water) prolonged 96 hour exposures.

In the current study *H. crenulatus* and *H. sexdentatus'* haemolymph haemocyanin concentrations were similar in both hypo- or hyper-osmotic treatments. For *H. crenulatus* a single difference was seen between the 2% and 100% treatments whereas *H. sexdentatus* had no differences in haemolymph haemocyanin concentrations between treatments.

In a previous study *H. crenulatus* exposed to similar salinity treatments showed markedly higher haemocyanin concentrations than found in the present study (Lee et al., 2010). This may be a result of the exposure period (acute 6 hour vs. prolonged 96 hour) or it is likely due to deviations in the studies methodologies. In the current study 10 µL of haemolymph was simply inverted several times (after being pipetted into a cuvette containing 1 ml distilled water) before haemolymph was determined spectrophotometrically. Lee et al. (2010) instead diluted samples with a glycine buffer (50 mM glycine; 10 mM EDTA; pH 8.8) in order to prevent coagulation and evenly mix the sample and solution. Other studies have also employed different methods, such as those employed by Chen et al. (1994), which measured haemocyanin of the tiger shrimp *Penaeus monodon* by diluting 100 µL of haemolymph with 900 µL of distilled water in an Eppendorf tube and mixing it with a type 37,600 mixer (Barnstead/Thermolyne Company, Dubuque, IA, USA) preventing clotting and dissolving haemolymph equally throughout the water. In light of this information it would be useful to include one or both of the above methods in order to better attain haemocyanin readings of the New Zealand crab species, and other crustaceans, that have rapidly coagulating blood.

Because of the lack of comparable findings between the current studies and previous studies haemocyanin concentrations we cannot conclusively state what concentrations are found at any of dissolved oxygen or salinity treatments without further investigations. Instead we can tentatively conclude that there are differences seen within and between the two New Zealand crabs depending on the stressor and treatment level. For *H. crenulatus* there were differences found between the varying DO treatments and between salinity treatments (stated above). For *H. sexdentatus* no differences were found between any of

the DO or salinity treatments. When comparing the two New Zealand species we find differences between DO (only for the 100% treatment) and salinity treatments (6%, 25%, and 150% treatments). For the DO trials *H. crenulatus* had comparable or higher haemocyanin concentrations for all treatments and for the salinity trials the crabs were comparable for the 6%, 25%, and 150% treatments with *H. sexdentatus* found to have a higher haemocyanin level at 50% and *H. crenulatus* with a higher haemocyanin level at 100% salinity.

Summary

In response to low oxygen levels *H. crenulatus* and *H. sexdentatus* appear to be able to regulate haemolymph osmolarity and the other main haemolymph constituents, including chloride, potassium, and sodium, at near the levels found at environmentally normally conditions. Glucose levels were slightly hyporegulated suggesting the spot sampling after the acute 6 hour trials was insufficient in attaining a clear stress response or that the treatments did not sufficiently stress the animals as to cause an increase in haemolymph glucose. In response to varying salinities both species displayed similar responses to those of other crustaceans in which blood osmolarity and chloride were hyper-regulated below the animals environmental norm, maintained near the norm, and hypo-regulated above the norm (except for *H. sexdentatus* which appeared to conform osmolarity and chloride at levels above 35 ppt salinity). The other blood constituents (potassium and sodium) were hyper-regulated at all salinities. As was found for the dissolved oxygen trials haemolymph glucose displayed no apparent trends or changes in response to the various salinities indicative of a lack in stress or that the 6 hour spot sampling was inefficient in elucidating the response in these crabs. Further work is needed in order to gage at what level haemolymph haemocyanin concentrations are maintained by these animals in response to the varying stressors. Haemolymph lactate is another blood parameter that would have been a useful indicator of stress here and as of such should be included in future studies as well.

Chapter Five

5. General Discussion

5.1. Review

Not only is hypoxia a natural occurrence that is common in marine, benthic and coastal regions but it is also an increasing environmental disturbance as a result of global change. Hypoxia as a result of anthropogenic eutrophication is increasingly becoming one of the foremost threats to marine species distribution worldwide (Nixon, 1995; Turner et al., 2005; Diaz and Rosenberg, 2008; Turner et al., 2008). Salinity changes are also becoming more common due to industrialisation and human population growth. With this often comes river diversions, agricultural water shifts and intensifications, and other industrial activities which can lead to increases or decreases in freshwater outflows to oceans and estuaries (Nichols et al., 1986). Because of this salinity levels can drastically change over short time periods (within the lifetime of some species) which do not allow for evolutionary responses in surviving shifts that exceed an organism's tolerance.

When shifts in stressor levels change an organism is forced to adapt and attempt to survive the stress or avoid the condition through behaviour mechanisms. When stress is unavoidable an organism may cope with the stress or face mortality. The current study tested the abilities of two New Zealand crab species to acute, unavoidable, stress. The current study showed that *H. crenulatus* and *H. sexdentatus* responded to both hypoxia and salinity changes with a series of coordinated metabolic (consumption rate), heart rate changes, and biochemical (regulation of osmolarity, major ions, and maintenance of glucose and haemocyanin) responses, to similar, however, varying degrees depending on the treatment.

5.2. Physiological responses to hypoxia and salinity: Oxygen consumption

H. crenulatus and *H. sexdentatus* responded to decreasing dissolved oxygen levels, and eventual hypoxia, by maintaining MO_2 until a critical oxygen tension (P_{CRIT}) was reached. After this point regulation failed for both species and MO_2 declined as PO_2 dropped. This response is common among crustaceans (Taylor and Wheatly, 1980; Massabuau and Burtin, 1984; Morris and Callaghan, 1998; Reiber and McMahon, 1998; Paschke et al., 2010) and is suggested as a mechanism to minimise the activation, and consequently toxic effects, of anaerobic metabolism. The P_{CRIT} values for the two species were 52.54 + 0.11 mmHg for *H. crenulatus* and 47.76 + 0.14 mmHg with no significant effect of salinity (with one exception for *H. sexdentatus*). This suggests that under the acute salinity exposures the hypoxia level alone stimulated the shift from oxyregulating MO_2 to oxyconforming below the P_{CRIT}.

Metabolic rates and rate changes of crustaceans are, however, affected by salinities at high oxygen tensions (levels above the P_{CRIT}) (Stickle and Sabourin, 1979). For *H. crenulatus* salinity did not alter MO_2 rate change over the experimental trial with few exceptions. Similar results were found for *H. sexdentatus* with MO_2 rate changes over time similar between all trials with a single exception. The results further suggest that at hypo- and hyper-salinities MO_2 is significantly altered which may be a result of the animal becoming stressed.

The metabolic rates of the crabs were also significantly altered by salinity. For *H. crenulatus* metabolic rates were different between all salinities with few exceptions. *H. sexdentatus* displayed a different response in which MO_2 was similar between most treatments with exceptions found between the hypo-salinities (2% and 25% treatments) which had higher MO_2 rates than the higher salinity treatments. These findings suggest that *H. crenulatus* alters MO_2 rates in various salinities and that *H. sexdentatus* alters their MO_2 rates in low salinities. This may reflect the environments where these species are found. *H. crenulatus* from an estuarine environment encounter large fluctuations in salinity over the course of several hours therefore altering MO_2 might be advantageous. In contrast, *H. sexdentatus* are found in a tidal environment and are commonly subject to high salinities as tide pools form and waters evaporate.

5.3. Physiological responses to hypoxia and salinity: Heart rate

The heart rates of *H. crenulatus* and *H. sexdentatus* decreased upon exposure to hypoxic dissolved oxygen levels. This response, as well as the oxygen consumption findings in chapter two, further suggest these crabs possess a bradycardia response in the event of hypoxia as is usually found for crustaceans (McMahon et al., 1974; Wheatly and Taylor, 1981; Bradford and Taylor, 1982; Taylor, 1982; Wu et al., 2002). For the dissolved oxygen trials it was clear at the 10 mmHg treatment that there was a reduction in heart rate. It was not clear, however, at what point the bradycardia was initiated.

In waters above or below the environmental salinity level an increase in heart rate is often found in crabs (Hume and Berlind, 1976; Jury et al., 1994; McGaw and Reiber, 1998). What we can conclude about the salinity heart rate trials is that *H. crenulatus* is able to modulate its heart rate at a similar bpm when exposed to various salinities and *H. sexdentatus* were less able to maintain heart rates under acute salinity exposures. *H. sexdentatus* exhibited a tachycardia in hypo- and hyper-salinities (i.e. 2% and 150% treatments) as is commonly found in crabs (Aagaard, 1996; McGaw and McMahon, 1996).

5.4. Biochemical responses to hypoxia and salinity

Osmolarity, chloride, potassium, and sodium

In response to decreasing dissolved oxygen the haemolymph parameters of *H. crenulatus* and *H. sexdentatus* (total osmolarity, chloride, potassium, and sodium), were mostly unaffected. This is consistent with findings for other crustaceans (Burnett and Johansen, 1981; Hagerman and Uglow, 1982; DeFur et al., 1990; Hagerman and Szaniawska, 1991) which often maintain haemolymph osmolarity, chloride, potassium, and sodium concentrations similar to those in sea water (Mantel and Farmer, 1983; Wittman et al., 2010). Overall both species maintained haemolymph osmolarity, chloride, potassium, and

sodium in response to an acute hypoxia exposure. As dissolved oxygens decreased, and hypoxia increased, there were no apparent trends or shifts of the animals' haemolymph constituents. When comparing the species they appear to possess haemolymph regulatory abilities similar to one another across the various levels of dissolved oxygen with only minor differences.

Unlike hypoxia, variations in salinity often significantly affect haemolymph osmolarity and major blood ions in crabs (Findley and Stickle, 1978; Zanders, 1980; Siebers et al., 1982; Piller et al., 1995; McGaw and McMahon, 1996). Both the New Zealand intertidal crab species are good regulators of haemolymph ion levels at hyposalinities and *H. crenulatus* is also a good regulator in hyper-salinities. *H. sexdentatus* appears to switch to a conforming response (for total osmolarity and chloride), however, when salinities are increased above the environmental norm (i.e. 100% salinity).

Glucose and Haemocyanin

Like the above haemolymph parameters glucose and haemocyanin also reflect an animal's ability to regulate internal functioning under stressful conditions. However, these parameters are more reflective of an organism becoming stressed. The production of glucose, through gluconeogenesis, is a common response of crustaceans to an increase in stress (Randall et al., 1967; Hall and van Ham, 1998). Haemolymph haemocyanin levels of crustaceans are also commonly increased as a result of stress including hypoxia and salinity changes (Boone and Schoffeniels, 1979; Hagerman, 1986; Taylor and Anstiss, 1999). In hypoxia a hyperglycaemic response is often found (Racotta et al., 2002; Ocampo et al., 2003), however, this is not always the case (Zou et al., 1996; da Silva-Castiglioni et al., 2010). A lack of hyperglycaemia was found for *H. crenulatus* and *H. sexdentatus*. The response found in this study may be indicative of a lack in stress as hypoxia may have yet to become sufficiently stressful as to induce an increase in glucose production. This could be indicative of these two crab species being well adapted to tolerating acute hypoxia exposures. Salinity may also result in a hyperglycaemic response in crustaceans (Spaargaren and Haefner, 1987; Annies and Rosamma, 2007; Lorenzon et al., 2008). But this was not the result for either New Zealand decapod.

Haemolymph haemocyanin followed the results for glucose in that hypoxia did not elicit an increase in haemocyanin as is regularly expected. Alternatively haemocyanin decreased as dissolved oxygens decreased for *H. crenulatus*, and no differences were found between treatments for *H. sexdentatus*. Hyper- and hypo-salinities are generally found to increase haemocyanin concentrations of crustaceans (Boone and Schoffeniels, 1979). Salinity in the current study did not appear to significantly alter haemocyanin concentrations of either New Zealand species. The results here were also not comparable to that of previous work on *H. crenulatus* by Lee et al. (2010) which may be a result of the methodologies employed (more on this below). When comparing the species we find differences for the hyper- and hypo-salinities which suggests that in these salinities the crabs possess different haemocyanin concentrations compared to environmentally normal conditions.

In summary, these crab species appear able to survive acute hypoxia and are good regulators in various salinities. Differences between these may be owing to the environments in which each species resides. Both species were tolerant of low dissolved oxygen and eventual hypoxia displaying little stress response which is commonly manifested as glucose and haemocyanin increases. This may be due to both species being commonly subject to hypoxia and aerial exposure in their respective environments. Furthermore, *H. crenulatus* appeared better at maintaining heart rate, oxygen consumption, and regulating haemolymph parameters more so than *H. sexdentatus*, in the various salinities. This is likely attributed to the estuarine environment being characteristic of salinity fluctuations occurring on a larger scale in comparison to rocky shore environments.

5.5. Evaluation of methodologies

In the current study data were solely obtained from adult male crabs. Because of this future work should include females, as well as juvenile animals, as they often have a lower body mass and size, thus greater oxygen consumption and ionoregulatory demands compared to males (Thompson and Pritchard, 1969; Pörtner and Grieshaber, 1993). Laboratory housed animals and experiments should also be cautiously extrapolated to field

situations due to the differences in environments. In laboratory settings feeding, water flow, and temperature are kept constant and this does not reflect the intertidal nature of these species' natural environment. In the tanks the animals were housed with only other males all in close proximity to one another which may also increase or decrease the amount of stress and/or exercise.

Heart rate trials in the current study should also be interpreted with some caution. Other studies on crustacean heart rates also included stroke volume and cardiac output in order to further understand an animals cardiovascular capacities and how they are affected by stress (such as in the form of a bradycardia) (Farrell, 1993; Reiber 1995; McMahon, 2001). In these works heart rate was not always the best indicator of cardiac output where stroke volume of some crustaceans increased, rather than heart rate, which resulted in a similar total cardiac output.

5.6. Future studies and research implications

The current study measured the acute effects of hypoxia and salinity changes on two New Zealand intertidal crabs. This is reflective of a rapidly changing environment and is commonly studied in crustacean research (Freire and McNamara, 1992; McGaw and Reiber, 1998). However, in order to further understand these animals' physiological, biochemical, and overall regulatory abilities future work needs to include prolonged exposures as well. Prolonged exposures can be vastly different to that of short-term studies when measuring stress effects, such as hypoxia (Dalla Via et al., 1994), and are also common among crustacean studies (ranging from 24 hour to several weeks) (Hume and Berlind, 1976; Jury et al., 1994; Aargaard, 1996; McGaw and Reiber, 1998). Therefore, to better evaluate the tolerances of *H. crenulatus* and *H. sexdentatus* experiments longer than 6 hours need to be tested. Such studies may alter growth and reproduction in these animals because the physiological and biochemical responses come at an energy-demanding cost (Diaz and Rosenberg, 1995). Salinity may also impede the biological processes of these animals more so than has been found for the 6 hour experiments. Haematological parameters are particularly susceptible to change for some crustacea depending on their regulatory

abilities. For example, sodium and chloride in some crustaceans is unaffected by different salinities over 6 hours, however, over a prolonged 24 hours exposure haemolymph composition changes occur (Vitale et al., 1999).

In light of climate change it would also be useful to investigate the effects of different water temperatures, which can be coupled with salinity and DO, and the effects of aerial exposure on the stress and regulatory responses of the crabs. Behavioural studies may also be useful where investigations could see whether the crabs emerge onto land or towards different water conditions (i.e. less stressful waters) to avoid stress. Emergence is a typical avoidance response of crustaceans that are unable to migrate or regulate in the waters they currently inhabit. The extent to which crabs emerge/move away from stressful waters has been documented for other crustaceans in the past (Taylor and Butler, 1973; Wheatly and Taylor, 1979; McMahon and Wilkes, 1983).

Future studies should also include lactate and amended haemocyanin analyses. In the current study lactate was unable to be analysed due to complications with coagulation and the limited amount of haemolymph extracted from each animal versus the large haemolymph requirements of the various biochemical analyses (more haemolymph required than could be feasibly removed from each animal without risk of mortality). Haemocyanin analyses should test other methodologies used in previous work (such as Chen et al., 1994 and Lee et al., 2010), in order to better understand the dynamic responses of this haemolymph blood protein and whether or not it is increased as hypoxic and salinity stress is increased. The above confounding variables, as well as only including laboratory based studies, should be accounted for in future work to obtain further insight into the responses of *H. crenulatus* and *H. sexdentatus* to salinity and hypoxia.

The present and future studies mentioned will give us a clearer insight into the physiological and biochemical tolerances of *H. crenulatus* and *H. sexdentatus* to hypoxia and varying hyper- and hypo-salinities. The current findings, including the P_{CRIT}, bradycardia heart rate responses, and haematological changes in response to hypoxic and salinity changes can all be used as indicators of the animals' environmental tolerance. These tolerances, as well as future works on temperature and aerial exposure, can also represent a much needed indicator of biological stress in response to climate change. The current findings suggest these animals can be used as biological indicators of environmental

degradation in light of global change. These species can therefore be implemented in conservation and risk assessment analyses of the ecologically important environments in which they are found.

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