Physiological, biochemical and behavioural responses of freshwater crayfish (*Paranephrops zealandicus*) to hypoxia and water nutrients

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Abstract

Hypoxia is a decline in water oxygen levels and is often caused by aquatic nutrient enrichment. It is recognised as one of the foremost environmental issues impacting and influencing species distribution. This population level effect is likely mediated by the physiological, biochemical and behavioural impacts associated with reduced oxygen. Most aquatic animals are able to maintain a constant oxygen uptake with small reductions in water oxygen, and are thus able to fuel the various processes driven by aerobic metabolism. However, as hypoxia persists or strengthens, regulation fails and aerobic metabolism is impaired leading to impacts such as the loss of acid-base homeostasis. The nutrient loading that often drives hypoxic episodes (through increased primary production) may impose additional stress that could also impact hypoxic responses and tolerance. The New Zealand freshwater crayfish *Paranephrops zealandicus* often inhabits streams that are in close proximity to farmland and are therefore prone to agricultural and pastoral run-off events. Consequently, crayfish will be faced with the challenge of energy homeostasis in conditions where aerobic capacity is compromised. The current study has investigated the physiological, biochemical and behavioural responses of *P. zealandicus* to hypoxia and high nutrients, to determine the potential impacts of agriculturally-impacted waters on this economically and culturally important species.

Freshwater crayfish were moderately tolerant of hypoxia, regulating oxygen consumption rates (MO_2) down to a critical oxygen tension of 45 mmHg. Contrary to the standard crayfish response, no distinct bradycardia developed under progressive hypoxia. Intraspecific variability in the MO_2 responses impacted the results, with individual *P. zealandicus* showing a variety of oxyregulating and oxyconforming patterns. Exposure to prolonged hypoxia (six hours) did not further affect MO_2 or heart rate. Both of these responses, however, were impacted by the severity of hypoxia. Crayfish under severe hypoxia (10 mmHg) showed a 79% decrease in MO_2, relative to the 50 % fall found under moderate hypoxia (30 mmHg). High ammonia (30 mg L^{-1}) and high nitrite (20 mg L^{-1}) did not further exacerbate the physiological responses of crayfish under severe hypoxia. Similarly, the addition of these nutrients did not alter markers of biochemical perturbation (acid-base status, anaerobic metabolism endpoints) relative to severe hypoxia alone, which induced a

significant acidosis (pH increase, carbon dioxide decrease), indicative of a loss in acid-base homeostasis. Analysis of anaerobic metabolism, as represented by haemolymph lactate accumulation, showed a heavy reliance of *P. zealandicus* on anaerobic metabolism under severe hypoxia, which may have driven the observed haemolymph acidosis.

Given the biochemical and physiological impacts of severe hypoxia (10 mmHg) on crayfish, it might have been anticipated that behavioural responses would be enacted that would permit crayfish to evade these consequences. There was a lack of hypoxia avoidance shown in a behavioural choice chamber experiment. There was, however, a strong emergence response observed under extreme hypoxia (4.2 mmHg). The avoidance and emergence experiments suggested that behavioural responses are only initiated at very low oxygen partial pressures (PO_2) in *P. zealandicus*. That the emersion response occurred at levels significantly lower than the calculated critical oxygen tension (P_{CRIT}; the point at which oxyregulation fails and the animals MO_2 falls in concert with falling PO_2) indicates that behavioural responses may only be used when absolutely necessary.

The ability of *P. zealandicus* to endure hypoxia through a variety of physiological and biochemical response, and only eliciting behavioural responses as a last resort, has important environmental implications. Tolerance will allow *P. zealandicus* to reduce any unnecessary risks associated with emergence (e.g. aerial desiccation and risk of predation) for as long as possible. Even if hypoxia worsens its ability to emerge will nevertheless provide it with a means of escape. The results of the current study are relevant for understanding and managing the population declines and recolonisation opportunities in this ecologically and culturally important species.

Chapter One

1. General introduction

1.1. Context

Although organisms are adapted to their natural habitat, and have the ability to acclimate to environmental change, they function most efficiently under a specific range of environmental parameters. Perturbations of the environment can push important physicochemical parameters outside of an animal's capacity to deal with them, which will cause deleterious effects such as impaired homeostasis, growth and survival (Meade and Watts, 1995; Remen *et al.,* 2008). In turn, these environmental changes, many of which are human-induced, can have devastating effects on ecosystem functioning and species biodiversity (Camargo and Alonso, 2006). It is consequently of great importance to understand ecosystem, population and individual resilience to environmental change (Tuomainen and Candolin, 2011).

One such environmental issue of growing national and international concern is eutrophication of aquatic systems. Eutrophication is the nutrient enrichment of water bodies, such as lakes, streams, and even oceans (Smith, 2003). The chief nutrients responsible for this effect are those that promote the growth of aquatic algae and bacteria, so include nitrogenous compounds (nitrates, nitrites, ammonia) and phosphorus. One consequence of this increased growth is extreme cycles of respiration (at night) and photosynthesis (by day), the former of which is responsible for drastic declines in water oxygen partial pressure (PO_2) (i.e. hypoxia) (Andersen *et al.,* 2006; Seibel, 2011). Changes in PO_2 have a significant effect on the biology of aquatic organisms (Taylor and Wheatly, 1980; Reiber, 1995; McMahon, 2001; da Silva-Castiglioni *et al.,* 2010), and subsequently on aquatic ecosystems as a whole (Smith, 2003, Diaz and Rosenberg, 2008).

1.1.1. Eutrophication: Causes and prevalence

Increased nutrient input is thought to be one of the foremost environmental problems for freshwater ecosystems (Bloxham *et al.,* 1999; McDowall *et al.,* 2009; Schindler, 2001). Agricultural and industrial run-off are considered the main sources of nutrient

enrichment, with waste streams such as farm and human effluents being the most prominent contributors (Bloxham *et al.,* 1999; Gillingham and Thorrold, 2000; Harris and Coley, 1991; Levin *et al.,* 2009; Jensen, 1996; McDowall *et al.,* 2009). In addition, nutrients can accumulate naturally due to the ammonification of organic substrates, atmospheric deposition, and from nitrogenous waste excretion by aquatic organisms (Alcaraz *et al.,* 1999; Camargo and Alonso, 2006). Excessive nutrient input enhances primary production and phytoplankton growth, increasing microbial respiration and depleting dissolved oxygen levels (Levin *et al.,* 2009). Furthermore, key nutrients, such as nitrite and ammonia, are also known to be potentially toxic to aquatic animals (Howarth, 1988; Chien, 1992; Rabalais, 2002), suggesting that freshwater species exposed to eutrophication-impacted waters may be compromised beyond just reduced oxygen availability.

In 2010, the Ministry of Environment classified 32% of New Zealand lakes greater than one hectare in area as eutrophic (Verburg *et al.*, 2010). This degradation in water quality is linked to the degree of pastoral and agricultural development. For example, Davis-Colley *et al.* (2011) found that 70% of the total nitrogen in 77 New Zealand rivers was from pastoral land use and 6.8% from dairying. These impacts have resulted in the reduction of stream fauna biodiversity (Quinn *et al*., 1997; Gillingham and Thorrold, 2000; Hamill and McBride, 2003; Larned *et al*., 2004; Wilcock *et al*., 2009; Abell *et al.,* 2011). Quality of New Zealand surface waters are of great importance because of the spiritual, economic and recreational benefits/uses of water (Gillingham and Thorrold, 2000). There are regulatory guidelines in place to achieve this. The ANZECC (2000) water quality guidelines state that a value of 700 μg L^{-1} of nitrate should be attained in order to protect 95% of species in freshwaters in New Zealand, while oxygen levels should not be lower than 80% saturation. With intensive farming increasing, recognising the negative impacts nutrient enrichment has on freshwater organisms is paramount (Gillingham and Thorrold, 2000).

Nitrite toxicity

The toxicity of nitrite in water is principally the result of its ability to convert oxygen-carrying pigments (e.g. in crustaceans; haemocyanin) into a pigment incapable of carrying oxygen (metheamocyanin). This in turn, causes hypoxia in tissues and impairs metabolism (Cheng and Chen, 1999; Sowers *et al.,* 2004; Camargo and Alonso, 2006). This will clearly

have widespread and significant effects on a range of body systems, and indeed impacts on endocrine, cardiovascular, ion regulatory and excretory processes have been noted (Kroupova *et al*., 2005). Importantly this hypoxia-inducing effect of nitrite would exacerbate the effects of reduced environmental dissolved oxygen that often coincide with nitrite-mediated eutrophication. However, this effect of nitrite on haemocyanin is only seen in some species (Camaro and Alonso, 2006). For example, the signal crayfish, *Pacifastacus leniusculus* (Harris and Coley, 1991) does exhibit this sensitivity, but the red swamp crayfish, *Procambarus clarkii*, does not (Gutzmer and Tomasso, 1985).

Nitrite also has another mode of toxicity. It can competitively inhibit branchial chloride uptake. As chloride concentrations in freshwater animals are significantly greater than those in the surrounding water, these ions are constantly lost through passive diffusion. Uptake of chloride is thus essential for maintaining chloride balance and if this process is blocked, as in the presence of nitrite, it can lead to chloride depletion, and an electrolyte imbalance (Gutzmer and Tomasso, 1985; Lewis and Morris, 1986; Harris and Coley, 1991; Jensen, 1996). In general, the greater the chloride loss rate, the higher the chloride uptake rate, and the more susceptible an animal is to nitrite accumulation and toxicity (Aggergaard and Jensen, 2001). Because increased water chloride concentrations both reduce diffusive chloride loss, and outcompete nitrite for uptake, the toxicity of nitrite is greater in freshwater than in seawater (Grosell and Jensen, 1999; Jensen, 2003).

Ammonia toxicity

Ammonia is generally considered more toxic to decapod crustaceans than nitrite (Chen and Lei, 1990; Chen *et al*., 1990a, b; Meade and Watts, 1995; Romano and Zeng, 2007a). In part this is because of the higher bioavailability of ammonia, which can more easily diffuse across the lipid bilayer into the haemolymph compared with nitrite (Weihrauch *et al*., 2004). However it may also substitute for H^+, Na^+ or K^+ in a number of transporters, including the basolateral Na^+/K^+-ATPase (DeVries *et al.,* 1994). There are also ammonia-specific transporters, Rhesus proteins (Rh), which are thought to play an important role in ammonia excretion (Weihrauch *et al*., 2009). The exact mechanism of ammonia uptake and excretion in crustaceans is widely debated and species seem to differ in ammonia transport mechanisms (Weihrauch *et al.,* 2004). Mode of transport also relates to mechanism of

toxicity as the inhibition of the Na^+/K^+ exchange, for example, can cause impaired osmotic and ionic regulation and acid-base balance at the gill, but can also cause neurological effects due to the role of this enzyme in establishing resting membrane potential (Lin *et al.,* 1993; Spaargaren, 1990; Young-Lai *et al.,* 1991; Chen and Chen, 1996; Harris *et al.,* 2001; Camargo and Alonso, 2006). Under long term ammonia exposure growth is often retarded and survival reduced (Chen and Kou, 1992; Lin and Chen, 2001).

1.1.2. Hypoxia: Causes and impacts

As stated above nutrient enrichment of waters causes the excessive growth of aquatic micro-organisms which results in an exponential uptake of the waters oxygen creating hypoxic conditions (Graham, 1990; Camargo and Alonso, 2006). This is especially prevalent in New Zealand where pastoral and agricultural run-off is common.

However, there are other factors that can lead to reduced dissolved oxygen levels and/or which can exacerbate the impacts of eutrophication-mediated hypoxia. For example, temperature is thought to be a key factor in determining the extent of hypoxia (Conley *et al.,* 2007). This is because as temperature increases, oxygen solubility diminishes, and therefore reduces oxygen availability (Carpenter, 1965; Garcia and Gordon, 1992; Keeling *et al*., 2010). In addition, higher temperatures increase the rate of metabolic processes (Q_{10} effect) (Iriberri *et al*., 1985; White *et al*., 1991; Burel *et al*., 1996) and thus the rate at which an organism consumes oxygen (Jones, 1952; Enquist *et al*., 2003). This reduction in oxygen solubility and increase in respiration rate can result in functional hypoxia.

Temperature often varies within a body of water creating stratification of oxygen levels (Sarmiento *et al*., 1998). Stratification can also be created from differential water densities and decomposition of organic matter, both of which lead to oxygen-poor bottom water (Sket, 1996; Bishop *et al*., 2004; Diaz, 2001; Breitburg, 2002). These vertical and horizontal oxygen gradients create situations where animals may encounter hypoxia, but have access to more oxygenated waters through migration (Chapman and McKenzie, 2009). Hypoxia also naturally arises in stagnant, poorly mixed, waters where water oxygen levels are consumed quickly (Seibel, 2011). Low dissolved oxygen in freshwater systems is most

commonly encountered in un-shaded, slow-flowing, lowland rivers where aquatic plants are abundant.

New Zealand streams that are most vulnerable to hypoxic conditions are those that pass through pastoral land (Levin *et al.,* 2009; McDowall *et al.,* 2009). This has led to some streams having excessive biological oxygen demands (Smith *et al.,* 1998; Taylor *et al.*, 1997). For example Wilcock *et al.* (1998) found in a report that daily dissolved oxygen levels of 50 mmHg in Waikato streams and an even lower oxygen level of 22 mmHg was found in Canterbury streams by Urbina and Glover (2012). These streams have lower biodiversity but contain an increase in dominant species that are hypoxic tolerant, altering the trophic structure of the ecosystem (Levin *et al.,* 2009; McDowall *et al.,* 2009).

Oxygen ultimately provides energy in the form of ATP (via oxidative metabolism) (Schmidt-Nielsen, 1997). ATP provides the energy for cellular processes (Schmidt-Nielsen, 1997). Therefore, hypoxia can reduce ATP production with immediate changes in biochemistry and physiology, followed by eventual effects on growth, reproduction and survival of aquatic animals (Landman *et al*., 2005; Camargo and Alonso, 2006; Levin *et al.,* 2009). Oxygen tolerances and thresholds (and thus sensitivity to hypoxia) differ among taxonomic groups and depend on many factors such as: activity level, life stage and body size (Levin *et al.,* 2009). When faced with stressful environmental conditions most animals can remove themselves by voluntary migration but when this is not possible they must compensate for the reduced oxygen levels (Reiber, 1995; Burnett and McMahon, 1987).

Globally, the detrimental effects of hypoxia have been shown by the many low oxygen "hotspots" that result in mass fish kills. For example Thronson and Quigg (2008) reviewed the past 55 years of fish kills in the USA and found the majority were due to low oxygen levels. The high biological oxygen demand of the Mary River in Australia reduced the oxygen concentration so drastically that it killed inhabitant fish (Townsend and Edwards, 2003). Nationally, low oxygen levels (13 mmHg) in New Zealand agricultural drains have been shown to result in fish kills (Rowe and Schipper, 1985).

1.1.3. New Zealand freshwater crayfish

New Zealand has two endemic freshwater crayfish species *Paranephrops planifrons* and *Paranephrops zealandicus*. These species are allopatric in terms of their distribution, but within each of their geographical settings they are widely dispersed (Hopkins, 1970). Both species inhabit streams, preferably trout free, with high silt cover and riparian zones that buffer run-off (Whitmore *et al.,* 2000). *P. zealandicus* is present from Canterbury to Southland in the South Island and is also found on Stewart Island (Hopkins, 1970), whereas *P. planifrons* is the more northern species. The southern species, the subject of the current study, is an opportunistic omnivore that feed on detritus, invertebrates, suspended particles and periphyton (Whitmore *et al.,* 2000). Their distribution and numbers have been reported as being in decline since 1921 (Hiroa, 1921), with the population in the Canterbury region being no exception (Hopkins, 1970; Usio and Townsend, 2000). The reasons for the declines in crayfish distributions are unclear. Suggested causes include deforestation, the introduction of exotic species, and an inability to disperse across the plains due to intensive pastoral development and wetland drainage over the past 150 years (McDowall, 1998). However, the predominant mechanism is believed to be due to the anthropogenic impact of pastoral land-use and subsequent environmental change (Usio and Townsend, 2000; McDowall, 2005).

Bioindicator and model species

Freshwater crayfish represent a wide diversity of different species capable of inhabiting a variety environmental niches. They have a well-characterised life history, and exhibit a number of unique physiological attributes. Because of these traits, freshwater crayfish have been utilised as model organisms in many areas of research including: phylogenetics, vision research, metal toxicology, neurobiology, conservation and evolution (Wald, 1967; Apte *et al.,* 2007; Grosell *et al.,* 2002; Crandall, 2006). For example, *P. leniusculus* has recently been used to model hypoxia-induced hypothermia because they exhibit an array of adaptations to deal with oxygen stress that increase the efficiency of oxygen transport systems (Gorr *et al.,* 2010).

A bioindicator can be defined as an animal that represents the effect of environmental change on a habitat by readily reflecting the state of its environment

(McGeoch *et al*., 2002; Kuklina *et al.,* 2013). Freshwater crayfish are utilised as bioindicators because they are physiologically adaptable to environmental perturbations while simultaneously being sensitive to stressors (Kuklina *et al.,* 2013). They also respond rapidly, both physiologically and behaviourally, to environmental stimuli, and are often ecologically-relevant to the system of interest (Li *et al.,* 2000; Kholodkevich *et al.,* 2008; Bierbower and Cooper, 2009).

How freshwater organisms respond to environmental change will determine the impacts that such changes have on these delicately-poised ecosystems. Freshwater crayfish provide a model organism to study these responses. There is a multitude of previous research on freshwater crayfish but studies using New Zealand species is limited. This provides the perfect opportunity to determine if New Zealand freshwater crayfish responses and tolerances to eutrophication and /or hypoxia are comparable to other species that have adapted under different environmental pressures and ecological conditions.

1.2. Background

1.2.1. Physiological responses of freshwater crayfish to environmental stressors

Environmental toxicology often quantifies biomarkers to examine the responses of organisms to environmental stressors such as hypoxia and increased nutrients (Adams *et al.,* 2001; Bonvillain *et al.,* 2012). Biomarkers are "a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)" (Depledge and Fossi, 1994). The most commonly used physiological responses are cardiac (heart rate) and respiratory (oxygen consumption) activity (Bierbower and Cooper, 2009; Romano and Zeng, 2013). This is because an imbalance in these systems indicates substandard environmental conditions (Depledge and Galloway, 2005; Burnett *et al.,* 2013; Kuklina *et al.,* 2013).

In response to hypoxia, freshwater crayfish exhibit a suite of physiological strategies and compensatory mechanisms to cope with oxygen stress (Mauro and Thompson, 1984; McMahon, 1986; Reiber, 1995; Morris and Callaghan, 1998; Reiber and McMahon, 1998;

McMahon, 2002; da Silva-Castiglioni *et al*., 2010). These include up-regulating oxygen delivery leading to enhanced oxygen transport (Booth and McMahon, 1982; Gorr *et al.,* 2010; McMahon, 1988; Reiber, 1995). Alternatively, crayfish can also decrease energy demands through the reduction of metabolic activities (Gorr *et al.,* 2010). An increase the frequency and stroke volume of the scaphognathite pump in an attempt to uptake more oxygen is also observed in crayfish (Burggren and McMahon, 1983).

Freshwater crayfish cardiac responses to hypoxia include a decrease in heart rate (bradycardia) which is compensated by an increase in cardiac stoke volume. This is energetically advantageous and allows for maximal perfusion of the respiratory system, and the tissues (Reiber and McMahon, 1998). This bradycardic response also facilitates increased diastolic filling time which would allow stroke volume to increase and thus increase arterial pressures generated by the heart (Reiber, 1994; Reiber and McMahon, 1998).

The only information about hypoxia tolerance in New Zealand freshwater crayfish is from Landman *et al.* (2005). These authors found that the median lethal (LC_{50}) dissolved oxygen level to *P. planifrons* was 0.77 ± 0.06 mg L^{-1}. However, this study did not investigate the physiological or behavioural responses of crayfish to hypoxia.

The effects of high levels of waterborne nutrients on freshwater crayfish species has been extensively studied (Booth and McMahon, 1982; McMahon, 1988; Chen and Cheng, 1993; Reiber, 1995; Racotta and Hernández-Herrera, 2000; Gorr *et al.,* 2010). However, most of the research has investigated the impacts on growth and survival and only a few have investigated the physiological effects of nutrients (Bermudes and Ritar, 2008). Oxygen consumption responses to nutrients are variable. For example, on exposure to ammonia a reduction is seen in *Penaeus monodon* (Chen and Cheng, 1993), *P. japonicus* (Chen *et al.,* 1994) and *P. vannamei* (Racotta and Hernandez-Herrera, 2000). Freshwater fish exposed to ammonia, nitrate and nitrite also showed a decrease in oxygen consumption (Tilak *et al.,* 2007). Conversely an increase in oxygen consumption is seen in the Chinese shrimp *P. chinensis* (Jiann-Chu and Chi-Yuan, 1992, 1995).

Similar differences are seen in responses to nitrite. The shrimp species *P. japonicas, Penaeus setiferus* and *P. chinensis* increase MO_2 (Alcaraz *et al.,* 1999), whereas *C. destructor* MO_2 rates were 50% of control MO_2 (Meads and Watts, 1995). A tachycardic response is common in animals exposed to nutrients as shown in rainbow trout (*Oncorhynchus mykiss*) (Aggergaard and Jensen, 2001) and in *Nephrops norvegicus* (Schmitt and Uglow, 1997).

Differences are thought to be due to exposure times and variations in species sensitivities (Jiann-Chu and Chi-Yuan, 1991; Lin *et al*., 1993). Knowledge of the combined effects of hypoxia and nutrients on the physiological responses of crustaceans is sparse. The studies that do exist show hypoxia increases the toxicity of nutrients and exacerbates effects on both MO_2 and heart rate (Mugnier *et al.*, 2008, Maguad, *et al.,* 1997). For example, Warren and Schenker (1960) reported the toxicity of ammonia was increased under hypoxic conditions in rainbow trout and minnows.

1.2.2. Biochemical responses of freshwater crayfish to hypoxia and high nutrients

As oxygen levels decline, crustaceans utilise anaerobic metabolism to sustain metabolic energy production as aerobic metabolism can no longer be maintained (Childress and Seibel, 1998; Hervant *et al*., 1995; Hill *et al*., 1991; Paschke *et al*., 2010; Spicer *et al*., 2002; Richards, 2009). However, ATP yield from anaerobic metabolism is around 5% of that generated from aerobic metabolism, meaning that energy use is less efficient and results in an exhaustion of fermentable fuels (Hochachka and Somero, 2002). In addition, the production of metabolic wastes such as lactate can be problematic if they accumulate excessively.

As anaerobic metabolism occurs a hyperglycaemic response is observed as fermentable fuels are mobilised to provide energetic substrate for anaerobic metabolism (Hervant *et al*., 1997; Maciel *et al*., 2008; Marqueze *et al*., 2006). The greater the stores of these fuels in the muscle the longer an animal can sustain anaerobic metabolism. Thus, because haemolymph and muscle lactate and glucose concentrations fluctuate in response to hypoxia (Gäde, 1984; Mauro and Thompson, 1984; Morris and Callaghan, 1998; da Silva-Castiglioni *et al*., 2010), many studies have quantified these biomarkers to investigate biochemical responses during hypoxic stress. For example, anaerobic metabolism is seen in freshwater crayfish such as *P. clarkii* and *Orconectes limosus* (Gäde, 1984; Mauro and Thompson, 1984) and is also observed in other crustaceans (Anderson *et al*., 1994; Zou *et al*., 1996; Hervant *et al*., 1995, 1997). However there have been no studies on the anaerobic metabolism responses of New Zealand freshwater crayfish to aquatic hypoxia. Titulaer (1995) investigated lactate accumulation in *P. zealandicus* but this was during aerial, not aquatic,

respiration. Titulaer (1995) showed an accumulation of lactate after 12 hours of aerial respiration which was metabolised after 48 hours.

On initial exposure to hypoxia many crustaceans show a hyperventilatory response. This brings more water over the gills and increases CO_2 washout which leads to haemolymph alkalosis (decreased CO_2 and increased pH; Taylor and Whiteley, 1989; Wilson and Taylor, 1992; Lutz and Storey, 1996; Rebelo *et al*., 1999; Wu, 2002). This alkalosis has been observed in lobsters, crayfish and crabs and is associated with an increase in oxygen affinity of the respiratory pigment haemocyanin, and thus an enhanced uptake and transport of oxygen (termed the "negative Bohr effect"; Butler *et al*., 1978; McMahon *et al*. 1978; Wilkes and McMahon; 1982b; Lallier and Truchot, 1989). However this phenomenon is short-lived, and as hypoxic exposure continues the enhanced oxygen affinity, and concomitant higher oxygen transport, increase haemolymph PO_2 decreasing the animals need to continue hyperventilation. The hypoventilation causes CO_2 to increase, and pH to decrease, causing an acidosis and a reduction in the negative Bohr Effect (Henry and Wheatly, 1992; Varley and Greenaway, 1992). This also means that the alkalosis-induced increase in oxygen affinity decreases. Therefore, acid-base balance is extremely important under hypoxic conditions and the measurement of these haemolymph parameters (PCO_2 and pH) can be used as indicators of stress (Morris and Callaghan, 1998; McMahon, 2001).

This has been seen in the freshwater crayfish *Cherax destructor* (Morris and Callaghan, 1998), the giant river prawn *Macrobrachium rosenbergi* (Chen and Kou, 1998) and in the crab, *Cancer productus* (Harris and Coley, 1991). The ability to avoid acidosis at such low oxygen levels will have important implications on acid-base status, enzyme function, and ability to survive hypoxia (Campbell, 1973; Henry and Wheatly, 1992; Pavasovic *et al*., 2004). The acid-base responses to hypoxia in New Zealand crayfish are unknown.

In contrast to hypoxia alone, less is known regarding the role of high nutrient exposure on the biochemical responses to hypoxia. The few studies that have been conducted suggest the toxic effects of ammonia and nitrite exacerbate the deleterious effects of low oxygen waters (Mugnier *et al.*, 2008). This is especially true for nitrite, which even in the absence of hypoxia can cause decreased haemolymph oxygen (Pedersen *et al.,* 2010). The blue shrimp *Litopenaeus stylirostris* was unaffected by either hypoxia or ammonia alone, but when combined an additive effect on lactate levels was observed (Mugnier *et al.*,

2008). There are no studies on the effects of nutrients on New Zealand freshwater crayfish let alone experiments investigating the combined effects of hypoxia and nutrients.

1.2.3. Behavioural responses of freshwater crayfish to hypoxia

Many organisms have the ability to detect and behaviourally avoid low oxygen waters by migrating away from hypoxia and/or by emerging out of the water (Pihl *et al*., 1991) Both of these behavioural responses allow the animal to take advantage of more oxygenated media (Taylor and Wheatly, 1980; McMahon and Wilkes, 1983; McMahon and Hankinson, 1993; McMahon and Stuart, 1999).

Avoidance

Most studies on hypoxia avoidance in aquatic species have been conducted on shrimp and fish such as salmon, and Atlantic cod (Das and Stickle, 1993; Herbert *et al.,* 2011). Avoidance studies on freshwater crayfish have examined predator avoidance (Alexander and Covich, 1991; Nystrom, 2005; Sherba *et al.,* 2000), and one study has investigated avoidance responses to CO_2 and the low pH and hypoxic conditions that come with hypercapnic environments (Bierbower and Cooper, 2010). However, there is no work on *Paranephrops* species.

In fish, oxygen-sensing chemoreceptors are located both internally and externally in the gill (Burleson *et al*., 1992). It is these chemoreceptors that allow for physiologically adaptive reflexes such as bradycardia, increased gill ventilation and stroke volume increase to be initiated, all aimed to regulate MO_2 and maintain aerobic metabolism (Gamperl and Driedzic, 2009; Perry *et al*., 2009). In crustaceans, the exact location of oxygen-sensing receptors is a matter of debate. Zinebi *et al*. (1990) suggested the receptors are located in the scaphognathites. Other authors believe they are located in the gills (Massabuau *et al*., 1980; Massabuau and Burtin, 1984; for review Reiber, 1997), while some argue they are internal, located within the arterial system, heart or central nervous system (Larimer, 1962; McMahon *et al.*, 1974). Regardless of the exact location, there is sufficient evidence to suggest that all studied crustacean species have the ability to detect oxygen levels and respond accordingly.

Many methods have been used to determine hypoxia avoidance in aquatic animals. The most common is the presentation of differing oxygen levels at opposite ends of an exposure chamber (Renaud, 1986; Das and Stickle, 1993) or chambers where different dissolved oxygen levels are generated alongside each other (Herbert *et al*., 2011 Poulsen *et al*., 2011; Cook *et al*., 2013). The former approach has been used on invertebrates such as shrimp and crabs where their smaller size does not disturb oxygen gradients. Unfortunately, uneven distribution in oxygen levels still occurs in such exposure conditions, and the oxygen level immediately in the vicinity of the animal needs to be measured to accurately determine the level of preference. Such measurements can themselves cause disturbance to the oxygen gradient and to the animal itself, leading to further complications. The use of an alternative chamber, that allows differing oxygen levels alongside each other, has been used for larger aquatic animals such as kingfish (Cook and Herbert, 2012), rainbow trout (Poulsen *et al*., 2011), Atlantic cod (Herbert *et al*., 2011) and snapper (Cook *et al*., 2013). The main advantage of this chamber is the ability to alter the oxygen levels of either side of the chamber with minimal mixing, even when animals are allowed to move freely. However, experimental design challenges still exist. For example, the most realistic measures of avoidance responses are anticipated when residence time is long and randomisation of oxygen levels (instead of a stepwise progressive hypoxia) is performed. However, this can generate logistical issues (e.g. with rapid changes in water oxygen).

Emergence

Emergence is a type of avoidance and is observed if an organism cannot migrate away from the contamination source while remaining submerged, so instead emerges out of the water into the air. Emergence in freshwater crayfish has been studied more extensively than avoidance. In the natural environment freshwater crayfish have been seen to emerge and/or migrate and forage on land and under experimental conditions will voluntarily emerge from the water for long periods in response to hypoxia and increased population density (McMahon and Wilkes, 1983; Taylor and Wheatly, 1980; Williams and Hynes, 1976). This is of adaptive significance especially in small pools which can become stagnant and where water oxygen levels are depleted (McMahon and Wilkes, 1983; Taylor and Wheatly, 1980).

The type and extent of the emersion response varies among crayfish species. For example partial emergence is seen in the crab, *Carcinus maenas*, which has been observed raising its carapace anteriorly above hypoxic water and reversing the normal ventilatory current direction, pumping oxygenated air into its branchial cavities (Taylor and Butler, 1973; Taylor *et al.,* 1973; Wheatly and Taylor, 1979). Complete emersion is observed in the shrimp *Palaemon elegans* (Taylor and Spicer 1987), and the freshwater crayfishes *Austropotamobius pallipes* (Taylor and Wheatly, 1980) and *Procambarus clarkii* (Wheatly *et al*. 1996). However, *Orconectes rusticus* shows an intermediate response, surfacing periodically and rolling onto its side to ventilate its branchial cavities with air (McMahon and Wilkes, 1983). Some species may also completely emerge but do so only periodically (deFur, 1988; McMahon and Wilkes, 1983).

Migrating into air can be physiologically costly because on emerging the gills collapse, decreasing the amount of respiratory surface available for gas exchange, therefore inhibiting oxygen uptake, resulting in hypoxaemia (Taylor *et al.,* 1973; Taylor and Wheatly, 1980; deFur, 1988). This gill collapse also inhibits CO_2 excretion increasing haemolymph CO_2 and resulting in respiratory and metabolic acidosis which lowers the oxygen affinity of haemocyanin (deFur, 1988; Johnson and Uglow, 1985; McMahon and Wilkes, 1983; Morris and Callaghan, 1998; Stillman and Somero, 1996; Wheatly and Taylor 1979; Taylor and Wheatly, 1980). This acidosis can be detrimental if it is not buffered by an elevation of haemolymph HCO_3^-. (Wheatly and Taylor, 1979, Taylor and Wheatly, 1980; Burnett and McMahon, 1987; Burnett, 1988). As gills are also important sites of nitrogenous waste excretion (Weihrauch, et al., 2009), upon collapse these wastes will also accumulate, impacting processes such as nervous function (Durand and Regnault, 1998; Rebelo *et al.,* 1999). Some species such as *Nephrops norvegicus* (lobster) and *A. pallipes* (freshwater crayfish) (Morris *et al.,* 1986; Ridgeway *et al.,* 2006) resort to anaerobic metabolism during emersion (measured by an increase in lactate) but others such as *C. destructor* do not (Morris and Callaghan, 1998). However, it is unknown how *Paranephrops* responds biochemically to emersion, or even if emersion is a response to hypoxia in this species.

1.3. Previous physiological research on *Paranephrops zealandicus*

The research that has been conducted on New Zealand freshwater crayfish is minimal. Wong and Freeman (1976 a, b, c) investigated the osmoregulatory mechanisms at differing ionic concentrations and how haemolymph parameters fluctuated over different temperatures and seasons. These studies showed that net salt uptake varied with salinity, season did not impact haemolymph concentrations but temperature did. Parkyn *et al*., (2011) showed that the aluminium modified zeolite clay product did not impact the behaviour (shelter seeking, righting and escape) or physiology (oxygen consumption and ammonia production) of *P. planifrons*. Behavioural responses to chemical cues have also been investigated by Hazlett (2000) who examined the responses of *P. zealandicus* to food and alarm odours. *P. zealandicus* significantly increased its lowered posture (teslon under the abdomen and body against the ground) when alarm cues were present. In contrast, when exposed to food cues crayfish spent more time in the raised position executing feeding movements. When *P. zelandicus* were presented with a combination of both food and alarm cues an intermediary response was observed in lowered posture. That is, the lowered posture when exposed to both cues was higher than that observed in food only experiments but lower than that observed in alarm only experiments (Hazlett, 2000). This shows that *P. zealandicus* are able to adjust responses depending on the threat of predation and need to feed. Hypoxia tolerance of New Zealand freshwater crayfish has been investigated by Landman *et al.* (2005). This study showed the freshwater crayfish was very tolerant to hypoxia with a 48 hour LC_{50} value of 12.8 mmHg. However, this was using the North Island Species *P. planifrons* and also wild caught species not farmed. Other than these studies, the physiological, biochemical or behavioural responses to hypoxia have not been examined.

1.4. Aims

Little is known about the responses of freshwater crayfish *P. zealandicus* to hypoxia and how increased nutrients, such as those that occur under conditions of eutrophication, may impact hypoxic responses in this species.

Chapter three investigates how *P. zealandicus* respond physiologically to short- and long-term hypoxia and if, like other freshwater crayfish, they show a hypoxia-induced bradycardic response. Chapter three also investigates the oxygen consumption pattern of *P. zealandicus* under hypoxia, in particular whether they regulate MO_2 and the point at which MO_2 can no longer be maintained. This chapter also investigates if multiple stressors (the addition of nutrients) results in an exacerbated effect on these hypoxic responses. It is hypothesised that *P. zealandicus* will show an oxyregulation pattern followed by an oxyconforming pattern as hypoxia deepens, and that a hypoxia-induced bradycardic response similar to that of other freshwater crayfish species will be observed.

Chapter four investigates the biochemical tolerance of *P. zealandicus* to hypoxia with and without the addition of high nutrients. It investigates impacts on acid-base balance, and the degree to which anaerobic metabolism is activated. It is hypothesised that hypoxia will lead to haemolymph acidosis and lactate accumulation. It is predicted that these effects will be exacerbated by combined exposure to hypoxia and elevated nutrients.

Chapter five investigates whether *P. zealandicus* is able to detect and avoid hypoxia, and if the hypoxia severity and presence of a refuge (either aquatic or aerial) impacts this response. It is hypothesised that *P. zealandicus* will show avoidance and emergence responses that will correspond with measures of biochemical and physiological perturbance as seen in other chapters, and its responses will resemble those seen in other freshwater crayfish species.

Chapter six summarises and examines the findings and the implication of these results for *P. zealandicus.* Chapter six also investigates methodological implications of the approaches taken in the current study and considers what future work may be required to further add to our knowledge of the effects of eutrophied waters on New Zealand freshwater crayfish.

Chapter Two

2. General methods

2.1. Collection and maintenance

Adult male *Paranephrops zealandicus* were obtained from the Sweet Koura Enterprises freshwater crayfish farm, Alexandra, New Zealand, and held in the aquarium system at the School of Biological Sciences, University of Canterbury. This system consists of recirculating well water where the water temperature is maintained at 15 ± 2 °C and is subjected to a 12-hour light, 12-hour dark photoperiod. *P. zealandicus* are a burrowing species and seek shelter under logs or leaves (Hopkins *et al.*, 1969). Therefore, animals were provided foliage within their tanks along with plastic tubing and concrete blocks to allow shelter while in captivity. Temperature, water exchange rates, ammonia, nitrite and nitrate were monitored weekly (the latter via commercially available aquarium test kits). Water exchange rates were maintained at 4 L min^{-1}, while ammonia, nitrite and nitrate were always less than 0.1, 0.1 and 5 5 mg L^{-1}, respectively. All crayfish were acclimated under these conditions for at least a week prior to experimentation to allow recovery from transportation. Animals were fed twice weekly on Nutrafin fish pellets but were fasted 2 days prior to experimentation.

Only intermoult animals were used for experiments and animals with damaged chelipeds or walking legs were excluded. Animals were randomly selected from the tanks with used crayfish kept separate from the unused. All animal procedures were approved by the University of Canterbury Animal Ethics Committee.

2.2. Metabolic rate

Oxygen consumption (MO_2) was used as a measure of metabolic rate to investigate the tolerance of freshwater crayfish to hypoxia and high nutrients. Oxygen consumption was measured using closed-box respirometry techniques (Fig. 2.1, 2.2). Prior to experimentation, individual freshwater crayfish were weighed and then acclimated to 440 mL Perspex respirometers (15 x 15 x 7.5 cm) 12 hours before oxygen consumption measurements were made. Previous work has shown that 12 hours is a sufficient acclimation time for this species

to settle after disturbance (Titulaer, 1995). Each respirometer was immersed into a temperature controlled water bath which was maintained at 15 ± 1 °C. During the acclimation period, a continuously recirculating flow of aerated freshwater into the respirometers from the surrounding water in the bath was maintained using a 04301 Caravan pump (TMC Technology Corp.) (Fig. 2.1)

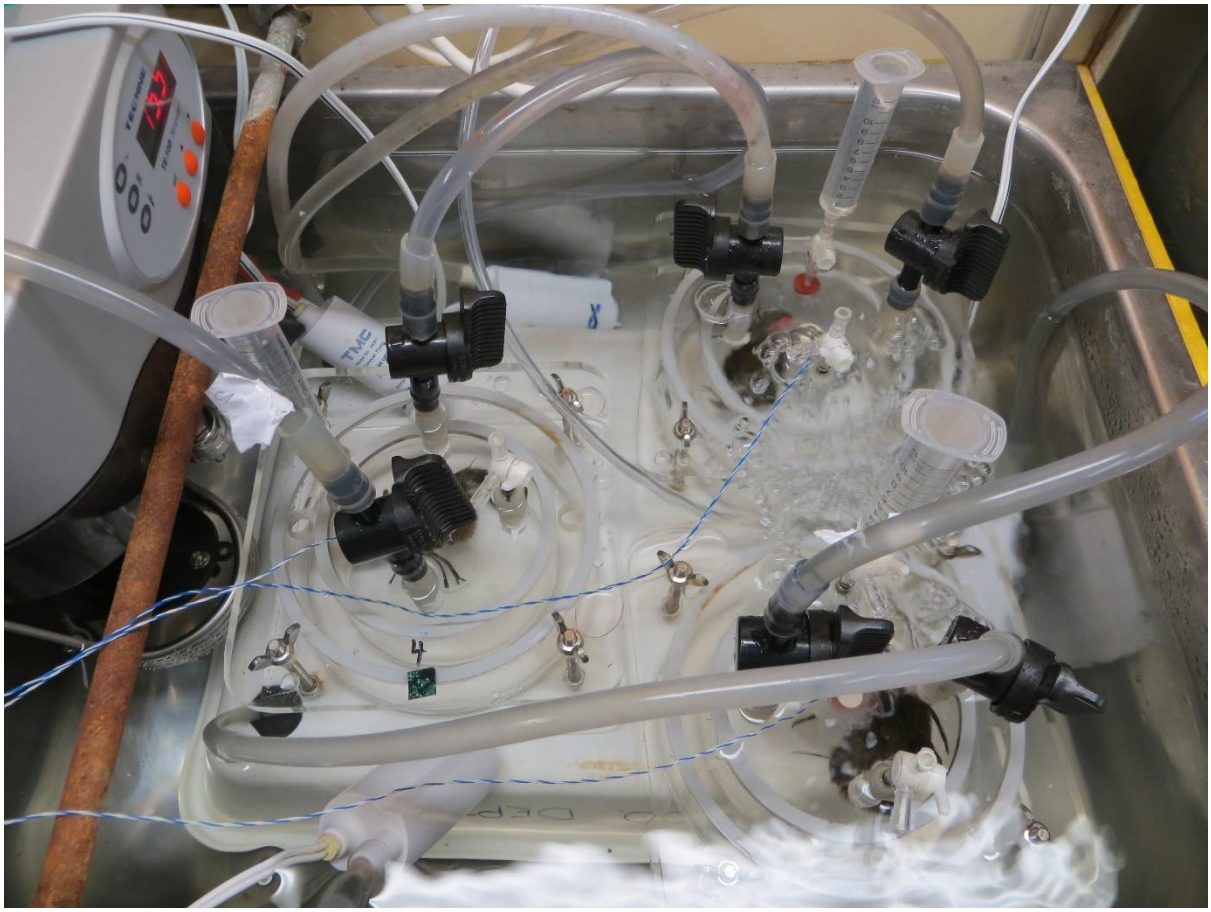

Figure 2.1: Photograph showing the respirometer set up in the water bath.

Immediately prior to measuring oxygen consumption all air bubbles were removed from the respirometers. Measurements were taken by closing the inlet and outlet valves and allowing the crayfish to deplete the oxygen in the respirometer. Dissolved oxygen was determined by extracting a 1 mL water sample from sampling ports using a syringe. The water taken from the sample was replaced by suction from a reservoir (an attached 10 mL syringe). To determine water PO_2, the water samples were injected into a MC100 oxygen microcell (Strathkelvin Instruments) which contained an IL 1302 oxygen electrode. Attached

to the electrode was a 781 oxygen meter (Strathkelvin Instruments) from which water PO_2 was recorded. Using sample readings at specific intervals (varied according to crayfish mass and respirometry protocol), the following equation (2.1) was used to calculate MO_2:

Equation 2.1: $$MO_2 = \frac{(a \times V \times \Delta PO_2)}{(w \times t)}$$

Where *a* is the oxygen capacitance of water in µmol L^{-1} $mmHg^{-1}$, *V* is the volume of the respirometer at 15 °C, ΔPO_2 is the change in oxygen partial pressure in mmHg, *w* is the crayfish weight in g, and *t* is the duration of respirometry in minutes. The weight of the crayfish was accounted for in the calculation of the volume.

The oxygen meter was calibrated (using air-saturated freshwater and correcting for atmospheric pressure) and zeroed (using a solution of 0.01 M of sodium tetraborate to which sodium sulphite was added and dissolved) before every experiment. Previous studies showed that the movement of the crayfish was sufficient to ensure adequate mixing of the water (Chech and Brauner, 2011), negating the need for mechanical mixing of chamber water. Measurements of oxygen consumption from a blank respirometer were used to determine microbial oxygen consumption. This showed negligible changes in oxygen tension over a 6-hour period ($P>0.05$). This indicated that the biological oxygen demand of freshwater was insignificant and did not affect the experimental readings.

2.2.1. Respirometry types

Three different types of closed-box respirometry (progressive, semi-closed and prolonged) were used to investigate the effects of hypoxia on freshwater crayfish. Progressive closed-box respirometry consisted of letting crayfish consume the oxygen from the normoxic freshwater over time to produce hypoxic conditions. Thus, in these exposures inlet and outlet valves were always kept shut with only the sampling ports and reservoir valves being opened and closed at every sampling interval. During semi-closed and prolonged closed box respirometry the initial PO_2 in the respirometers was reduced to 40, 30 or 10

mmHg, by introducing water from a reservoir which had been deoxygenated by bubbling nitrogen through it. A control was also run where the initial PO_2 was normoxic water (155 mmHg). Crayfish were left under these conditions for a short amount of time (semi closed-box respirometry 20-50 minutes) or for a 6-hour period (prolonged closed-box respirometry). Water PO_2 was measured regularly at 20 minute intervals. During semi-closed and prolonged closed box respirometry the inlet and outlet valves were closed during sampling but open in between sampling allowing renewed flow of fixed PO_2 water in from the reservoir, thus maintaining the exposure PO_2.

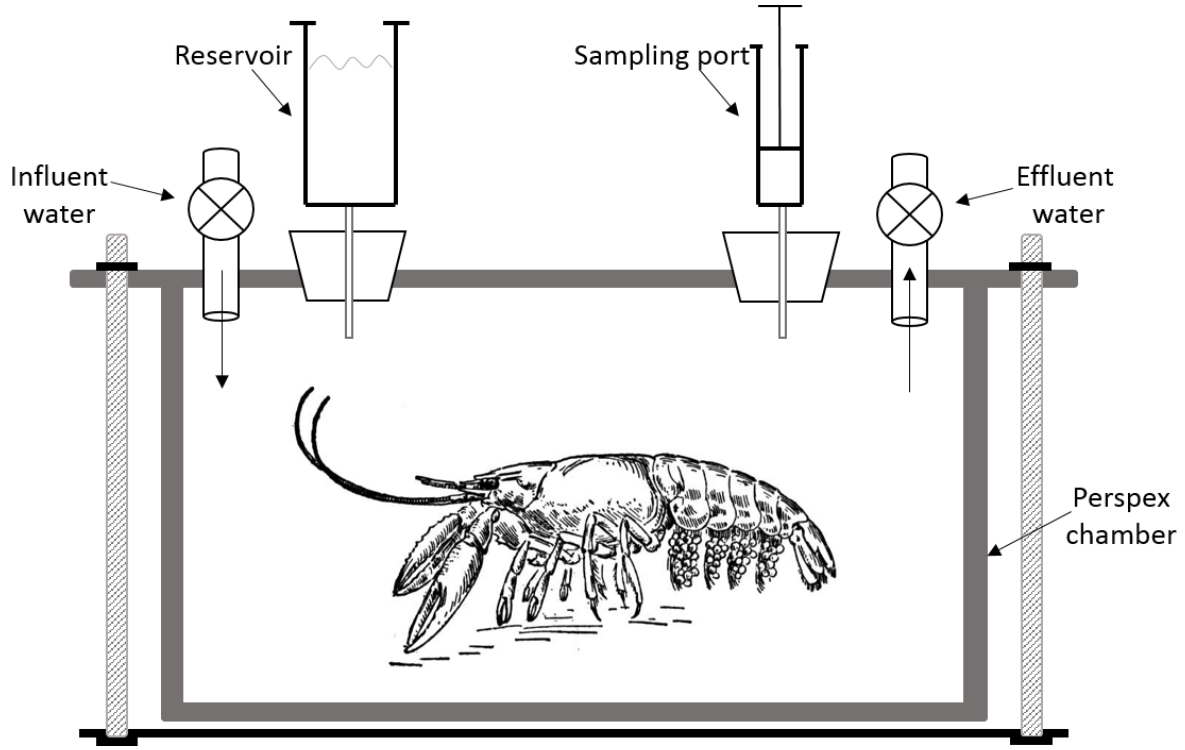

Figure 2.2: Diagrammatical representation of the closed-box respirometer.

2.3. Ammonia and nitrite

To assess the effect of elevated nutrients on hypoxic responses, crayfish were exposed to 6 hours of severe hypoxia (10 mmHg) in the presence of high ammonia (30 mg L^{-1}) or high nitrite (20 mg L^{-1}). These experiments were conducted using prolonged closed-box respirometry as described above, except with the addition of either ammonia chloride

(NH_4Cl; from a freshly made 1 M stock solution) or sodium nitrite ($NaNO_2$; from a freshly made 1 M stock solution) to the reservoirs prior to experimentation.

2.4. Measurement of heart rate

Heart rate was measured using a non-invasive infrared (IR) sensor. This was attached to the carapace of the crayfish at the rear of the cephalothorax directly above the heart (Fig. 2.3). A rubber ring was superglued onto the crayfish into which the IR sensor was fitted. The IR sensor contains a coupled IR transmitter and detector unit which beams infrared light onto the surface of the heart. As the conformation of the heart changes during each cardiac cycle the intensity of light that is reflected back to the detector fluctuates. This light affects the forward bias of the photo transistor, which in turn changes the current flow between the collector, emitter and load resistor. These result in a change in voltage which is fed to a PowerLab/425 unit (AD Instruments) via a custom made signal conditioning box. Cardiac pulses were visually identified and recorded using the Lab chart 7 computer software.

Figure 2.3: Photograph showing the IR sensor slotted into the rubber bung that was glued onto the carapace of the crayfish.

Crayfish were acclimated with the rubber ring and IR sensors connected. The IR heart rate sensors were fed through the lids of the respirometers through an opening and sealed with a rubber bung and glue to stop water leakage. This allowed both heart rate and MO_2 to be measured concurrently.

2.5. Haemolymph collection, analysis and storage

2.5.1. Haemolymph CO_2, pH, and PO_2 samples

Immediately after the prolonged closed-box respirometry experiments a gas-tight Hamilton syringe was used to remove two volumes of 0.5-1 mL of prebranchial haemolymph through the arthrodial membrane from a sinus at the base of the 3rd walking leg. The haemolymph was split between three 1 mL Eppendorf tubes, and was centrifuged at 5000 x *g* for 20-30 seconds. The centrifuged plasma was aspirated into three new Eppendorf tubes. One of these was capped and snap-frozen in liquid nitrogen, before being stored at -80 °C for further analyses (Section 2.6). The second was used to measure haemolymph total CO_2 and pH, and the third was used to measure haemolymph PO_2. Haemolymph PO_2 was measured immediately after centrifugation, via the same method described in Section 2.2.

Haemolymph total CO_2 was measured using a Corning Total CO_2 meter which was calibrated before every measurement using 0.5 µl of 0.1 M sodium bicarbonate. CO_2 partial pressure was calculated via rearrangement of the Henderson-Hasselbalch:

Equation 2.2: $$PCO_2 = \frac{CCO_2}{\propto CO_2(1+10^{(pH-pK1)}\ (1+10^{pH-pK2}))}$$

where PCO_2 is the partial pressure of carbon dioxide (mmHg), CCO_2 is total carbon dioxide (mmol L^{-1}); αCO_2 is the solubility co-efficient of carbon dioxide (0.0559 mmolL^{-1} $mmHg^{-1}$); pH is the measured pH of the haemolymph sample, pK_1 is the dissociation constant for reaction of carbonic acid to form bicarbonate and a proton (6.165); and pK_2 is the dissociation

constant for the reaction of bicarbonate to form carbonate and a proton (9.604). Values for *P. zealandicus* αCO_2, pK_1 and pK_2 were sourced from Titulaer (1995).

The remaining plasma was used to measure pH, using a microprobe which was connected to a Powerlab and Lab chart 7 software. The pH probe was calibrated using buffer solutions of pH 10 and 7, and was left in the sample until the reading stabilised for approximately 2 minutes.

2.5.2. Tissue collection and storage

Following haemolymph collection, crayfish were placed on ice for anaesthesia, and then euthanized by severing the ventral ganglia. The carapace was removed and the hepatopancreas was dissected and snap-frozen. Lastly the tail muscle was removed from the crayfish abdomen, and also snap-frozen. All samples were then stored in a -80 °C freezer.

2.6. Biochemical analysis of tissues

2.6.1. Tissue preparation

Each tissue sample was deproteinised with 0.5 mL of 70% perchloric acid before being homogenised. Homogenisation was conducted using an Omni Bead Ruptor system. Tissue (~0.5 g) was placed into a 2 mL Omni Bead Ruptor sample tube containing 5 ceramic beads, before being fastened into the machine and vigorously shaken for 20-30 seconds. Preliminary trials showed that subsequent transfer of the tissue and/or removal of the beads from the homogenised sample greatly reduced the amount of tissue able to be analysed. Thus, the homogenised tissue-filled bead ruptor sample tubes with the beads intact were placed in a centrifuge and spun for 15 minutes at 14,000 rpm (Beckman Coulter TM). The supernatants from these samples were then aspirated out into fresh, labelled Eppendorf tubes. These supernatants were used for the analysis of glucose and lactate.

2.6.2. Tissue analysis

Glucose

Muscle and haemolymph glucose was quantified using the hexokinase/ glucose-6-phosphate dehydrogenase enzymatic method (Sigma diagnostic kit GAHK-20), following the manufacturer's instructions. In this method the total amount of NADPH formed, catalysed by the action of glucose-6-phosphate dehydrogenase and hexokinase, which is directly proportional to the initial glucose concentration in the sample as shown by the following equations:

Equation 2.3: $$Glucose + ATP \xrightarrow{Hexokinase} Glucose - 6 - Phosphate + ADP$$

Equation 2.4: $$G6P \xrightarrow{G6PDH} 6 - Phosphogluconate + NADH$$

Glucose assays were conducted in a microplate format. The glucose assay reagent (hexokinase) were added to sample supernatants. The microplate was mixed and incubated at room temperature for 15 minutes. After which, the absorbances were read at 340 nm *versus* deionised water.

Lactate

Muscle supernatants and haemolymph lactate were quantified using an enzymatic kit (L-Lactate, Megazyme) following the manufacturer's instructions. In this method the lactate in the sample is proportional to the NADH produced (measured by absorbance at 340 nm) by the reactions catalysed by D-glutamate-pyruvate transaminase and L-lactate dehydrogenase, as shown by the following equations:

Equation 2.5:

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

Equation 2.6:

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

Lactate assays were conducted in a microplate format. The appropriate reagents (buffer, NAD^{+} and GTP), were added to samples and were then incubated for 3 minutes at room temperature, before absorbance was read on a 96 well plate reader at 340 nm (1st reading). Subsequently, L-LDH was mixed into the samples and the well plate was incubated again for 10 minutes and absorbance was reread (2nd reading). The values of the 2nd reading were subtracted from the 1st reading to give the final lactate absorbance. Absorbances were converted to lactate concentrations using a full calibration curve from standard values that were concurrently measured using the same batch of reagents for every plate.

Chapter Three

3. Oxygen consumption and heart rate responses of *Paranephrops zealandicus* to hypoxia and increased nutrients

3.1. Introduction

When extrinsic or intrinsic stimuli (referred to as stressors when outside of the animal's normal environmental range), perturb homeostasis, coordinated physiological responses are elicited to compensate for the disruption (Wendelaar-Bonga, 1997; Schreck, 2010). Most freshwater crustaceans are well adapted to moderate environmental fluctuations (e.g. short-term (3-4 hours) hypoxia) having evolved a multitude of physiological mechanisms that attempt to minimise this stress (see Reiber 1995, McMahon 2001 and Romano and Zeng 2013 for reviews). Thus, the measurement of these responses can represent how stressed and/or tolerant an animal is to environmental change. For example, a response often used to determine tolerance to stressors is metabolism. Metabolism is the total amount of chemical energy used by an organism and its metabolic rate is the energy per unit time (Schmidt-Nielsen 1997). When the metabolic rate of an animal changes there is an alteration in tissue oxygen demand. Because oxygen is required to produce ATP for aerobic metabolism, the rate at which oxygen is consumed (MO_2) can not only be used to measure metabolic rate but can also act as a useful indicator of an animal's response to stress (Schmidt-Nielsen, 1997; Taylor *et al*., 1997; Clarke and Fraser, 2004).

In the face of declining water oxygen levels, crustaceans generally attempt to maintain MO_2. This regulation of oxygen uptake allows for sustained oxygen conductance to tissues and results from a combination of physiological mechanisms such as modulation of haemolymph oxygen affinity, ventilatory parameters and cardiovascular functions (Dejours *et al.*, 1970; McMahon *et al*, 1974; Wheatly and Taylor, 1981; Wilkes and McMahon, 1982a; McMahon and Wilkens, 1983; Reiber, 1995;). This regulation of oxygen (oxyregulation) occurs until MO_2 can no longer be maintained (i.e. failure of physiological systems to take oxygen up from water and/or deliver oxygen to tissue). After this point MO_2 declines as water PO_2 declines, a phenomenon known as oxyconforming. This transition between oxyregulation and oxyconformation is termed the P_{CRIT} (critical oxygen tension). The activation of anaerobic

metabolism at oxygen levels below P_{CRIT} represented by the concomitant increases in lactate has been seen in many species (Pörtner and Grieshaber, 1993; Morris and Callaghan, 1998; Pörtner, 2010).

The P_{CRIT} value is an analytical tool used to determine the oxygen regulatory abilities of animals, and thus is an indication of hypoxia tolerance (Hill, 1976). However, it should be noted that the calculation, and use, of P_{CRIT} has been debated (Scott *et al*., 2008). The value of P_{CRIT} is somewhat limited by the fact that it is influenced by a number of physical and physiological parameters (Maynard, 1960; Bayne, 1971; Taylor *et al*., 1977; Herreid, 1980). These include temperature, pH, season, sex, moult stage and water chemistry (Taylor *et al.,* 1973). Nevertheless, in general an animal is said to be a strong regulator and more hypoxia tolerant the lower its P_{CRIT} value. Values for the P_{CRIT} of New Zealand freshwater crayfish are sparse. The only reported P_{CRIT} for *P. zealandicus* is from Titulaer (1995) who calculated a value of approximately 45 mmHg after exposing animals to 84 hours of hypoxia.

Heart rate, stroke volume, and therefore cardiac output, are all modified under environmental hypoxia (Taylor *et al*., 1977; Shiels *et al*., 2002; Farrell, 2007). Heart rate serves as a biomarker of stress in many animals, especially under hypoxic conditions (Bierbower and Cooper, 2009). This is because many crustaceans, including freshwater crayfish, decrease heart rate under hypoxic conditions in an attempt to enhance perfusion of the gills (McMahon and Burnett, 1990). This allows additional oxygen uptake, and thus transport to tissues, to compensate for the external hypoxia (Massabuau and Burtin, 1984; Reiber and McMahon, 1998). This hypoxia induced bradycardia is seen in many freshwater crayfish species (Larimer and Gold, 1961; Larimer, 1962; McMahon *et al*., 1974; deFur and Mangum, 1979; Wheatly and Taylor, 1981) but has not been studied in *P. zealandicus*.

Progressive hypoxia using closed box respirometry is a useful approach for creating hypoxic conditions and for investigating subsequent responses of the respiratory and cardiovascular systems to hypoxia (Wheatly and Taylor, 1981; Morris and Callaghan, 1998). In closed box respirometry animals consume the oxygen within a sealed chamber over time, gradually creating hypoxia. The speed at which hypoxia develops depends on the size of the chamber, but it usually occurs quickly, at least in crayfish. As such, the hypoxia generated using this method often constitutes an acute exposure. Some studies have, however, found longer exposures give a better idea of the impacts of hypoxia (Nielsen and Haggerman, 1998; Racotta and Hernández-Herrera, 2000; Harper and Reiber, 2006).

The current study aimed to investigate the tolerance of *P. zealandicus* to hypoxia by measuring its physiological responses (MO_2 and heart rate). Changes in MO_2 were monitored as a function of declining PO_2 to determine whether *P. zealandicus* switches from oxyregulation to oxyconforming under hypoxic conditions and if so, to identify the critical oxygen tension at which this switch occurs. This study also aimed to investigate whether exposure time affects these responses. These data will be useful in assessing the environmental resilience of *P. zealandicus* to environmental hypoxia, which has been shown to in inhabit hypoxia-prone waters

Eutrophic waters are not only low in oxygen but by definition are also high in nutrients such as ammonia and nitrite. These elevated levels of nutrients may themselves have physiological impacts on aquatic biota, and could thus alter responses to hypoxia. Consequently, it is environmentally-relevant to know how an animal such as *P. zealandicus*, responds to the multiple stressors of hypoxia combined with elevated nutrients.

In fish and crayfish, nitrites bind to blood pigments and form molecules that have a lower oxygen binding affinity, and thus impair normal oxygen transport to tissues (Tahon *et al*., 1988; Meades and Watts, 1995). As a result, this internal hypoxia causes a reduction in MO_2 (Tilak *et al.*, 2007). Conversely, the main mechanism of toxicity for ammonia is the disruption it causes to ion transport. This causes significant issues in the central nervous system, which results in a reduction of oxygen consumption and increase in heart rate (Sanders *et al* 1992; Bloxham *et al*, 1999; Weihrauch *et al.,* 2004). Nitrite also increases heart rate because it has vasodilatory effects which leads to a reduction in arterial blood pressure (Laustiola *et al*., 1991; Vleeming *et al*., 1997). Reestablishment of blood pressure through a compensatory increase in cardiac pumping causes a tachycardia (Aggergaard and Jensen, 2001). As for hypoxia alone, there are many environmental and physiological factors that magnify or lessen the toxicity of high nutrients (Schmitt and Uglow, 1997). For example, another mechanism of toxicity for nitrite is its ability to competitively inhibit chloride uptake, owing to nitrite mimicry of chloride ion uptake pathways. Consequently the uptake of nitrite, and thus its subsequent toxic impacts, is often determined by the chloride uptake rate (Aggergaard and Jensen, 2001; Jensen, 2003).

Although many studies have focused on the individual toxicity of nutrients or hypoxia there have been minimal studies on the combined effects of these factors on the

physiological responses of crustaceans. However, the few studies that have been conducted show responses are exacerbated. For example, under hypoxia and high nutrient exposures, heart rate and oxygen consumption were significantly lower than they were in rainbow trout *Oncorhynchus mykiss* and the blue shrimp *Litopenaeus stylirostris* exposed to only one stressor (Maguad *et al*, 1997; Mugnier *et al.*, 2008). A chronic decrease in MO_2 induced by low oxygen/high nutrient concentrations could result in an inability to sustain adequate physiological condition, growth and survival because of an insufficient metabolism (Alcaraz *et al*., 1999). This could have significant consequences for an ecologically and economically important species such as *P. zealandicus*. Therefore, to determine the effects of eutrophication the addition of nutrients to low oxygen levels and their physiological impacts on *P. zealandicus* were also investigated.

3.2. Methods and Materials

P. zealandicus were obtained from a crayfish farm and held in the aquarium system (see Section 2.1), and experiments were subsequently conducted in temperature controlled rooms in the Biological Sciences building at the University of Canterbury. Experiments were conducted at the same time each day to avoid complications associated with diel cycles. See Section 2.1 for further details on the acclimation procedures and use of animals.

3.2.1. Physiological responses to hypoxia under progressive closed box respirometry

Experiments on oxygen consumption and heart rate were conducted on 26 and 17 crayfish, respectively (mean mass 30.80 ± 0.93 g). There were no significant differences in weights between the crayfish ($P > 0.05$). The water bath could accommodate four respirometers, three of which contained experimental crayfish and the other was left blank, as a control. Experimental measurements commenced once the inlet and outlet valves were closed. Measurements of oxygen consumption were taken every 20 minutes, while heart rate was continuously measured. Heart rates were thereafter calculated for every 20-minute interval. Experimental measurements ceased once the water oxygen content within the respirometers fell below 10 mmHg or after 6 hours, which ever occurred first. After this

period, the respirometers were flushed with fully oxygenated water and the crayfish were allowed to recover for a minimum an hour before being returned to the aquarium. See Section 2.2 for further details on the experimental design and measurement of MO_2 and heart rate.

3.2.2. Physiological responses to hypoxia under semi-closed box respirometry

Experiments on oxygen consumption and heart rate under semi-closed respirometry conditions were conducted on eight crayfish exposed to control (fully oxygenated) conditions (mean mass 46.6 ± 8.5 g); eight crayfish exposed to moderate hypoxic (40 mmHg) conditions (mean mass 41.1 ± 2.3 g); and eight crayfish exposed to severe hypoxic (10 mmHg) conditions (mean mass 44.1 g ± 6 g). See Section 2.2 for further details on the production of hypoxic water and measurements of MO_2.

Experimental measurements commenced once the respirometer pumps were transferred to the reservoirs and the reservoir water allowed to completely mix with the water from the respirometer. Measurements of oxygen consumption were taken every 10 minutes while heart rates were continuously measured and then calculated for every 10-minute interval. Experimental measurements ceased once the water oxygen content within the respirometers fell by 20% of the initial PO_2 or after 1 hour, which ever occurred first. After this period, the respirometers were flushed with fully oxygenated water and the crayfish allowed to recover for at least an hour before being returned to the aquarium.

3.2.3. Physiological responses to hypoxia and/or high nutrients under prolonged flow-through respirometry

Experiments on oxygen consumption and heart rate under prolonged flow-through conditions were conducted on a total of 26 crayfish. Sample number and average weights for each treatment type is shown in Table 3.1. There were no significant differences in weights between the crayfish ($P > 0.05$).

Table 3.1: The sample number and average weight (± S.E.M) of *P. zealandicus* used in the prolonged closed-box respirometry to measure heart rate and MO_2

Treatment type	n value	Mean mass ± S.E.M (g)
Control	5	39.3 ± 8.3
H30	5	40.9 ± 5.6
H10	6	44.8 ± 4.2
H10A30	5	36.9 ± 5.3
H10N20	5	46.5 ± 12.9

MO_2 and heart rates were measured as mentioned as described in detail in Section 2.2 and 2.4, and as briefly described above (Section 3.2.1). The only differences between the protocol described for closed box respirometry and the current protocol were that heart rates and MO_2 were calculated hourly. Experimental measurements ceased after six hours. After this period, the respirometers were flushed with fully oxygenated water and the crayfish allowed to recover for a minimum of an hour before being returned to the aquarium.

3.2.4. Data analysis and statistical methods

Statistical analysis was carried out using the R statistical programme. Statistical significance was taken at the level of $P < 0.05$. All data are presented as the mean ± standard error of the mean (SEM), unless otherwise stated. In the progressive hypoxia experiments individual exposure PO_2 values were grouped at every 5 mmHg (± 2.5 mmHg). Homogeneity of variance was tested by plotting the fitted values against the residual values. Normal distribution was tested with a quantile-quantile normality plot and histogram of the residuals. A residual analysis test showed that homogeneity of variance was not met, thus, values were log transformed and grouped means were compared using a one-way analysis of variance (ANOVA). Where a treatment effect was shown to be significant, post-hoc Tukeys HSD analyses were performed. After a residual analysis test confirmed homogeneity of variance, MO_2 and heart rate values from semi-closed box respirometry were compared with the values obtained from progressive closed box respirometry using a two-way ANOVA, followed

by Tukey HSD post-hoc tests. The same was done comparing progressive closed-box respirometry with prolonged closed box respirometry.

Three different methods were conducted for determining P_{CRIT}. First, a graphical interpretation was used. Second the 'brokenstick' statistical R code (see Appendix A) was applied to the data. Third ANOVA and Tukey HSD results (mentioned above) were used to identify the PO_2 that was significantly different from 155 mmHg. These analyses were conducted on both raw and grouped values to ensure that the method of analysing the data did not influence the outcomes of the experiments.

3.3. Results

3.3.1. Physiological responses to hypoxia under progressive closed box respiromentry

The progressive closed box experiments ran for an average of 280 ± 13 minutes. MO_2 ranged from 5.85 to 0.00 μmol g^{-1} h^{-1}, and heart rate ranged from 129.1 to 32.6 beats per minute. There was no significant change in PO_2 of the control blank respirometer ($P > 0.05$) indicating that microbial oxygen consumption did not impact the readings. Some crayfish showed signs of stress (rolling onto their sides) at the lower PO_2 levels, however, there were no mortalities recorded during this experiment.

Oxygen consumption

Using the pooled means at every 5 mmHg interval it was clear that hypoxia affected oxygen consumption of freshwater crayfish (Fig. 3.1). During progressive hypoxia the oxygen consumption of freshwater crayfish decreased in a curvilinear manner with decreasing external PO_2. This pattern was the same regardless of whether individual data points (Fig. 3.2) or grouped averages were examined. However, some individuals showed a linear pattern (sometimes with a strong slope and sometimes without) (Fig. 3.3).

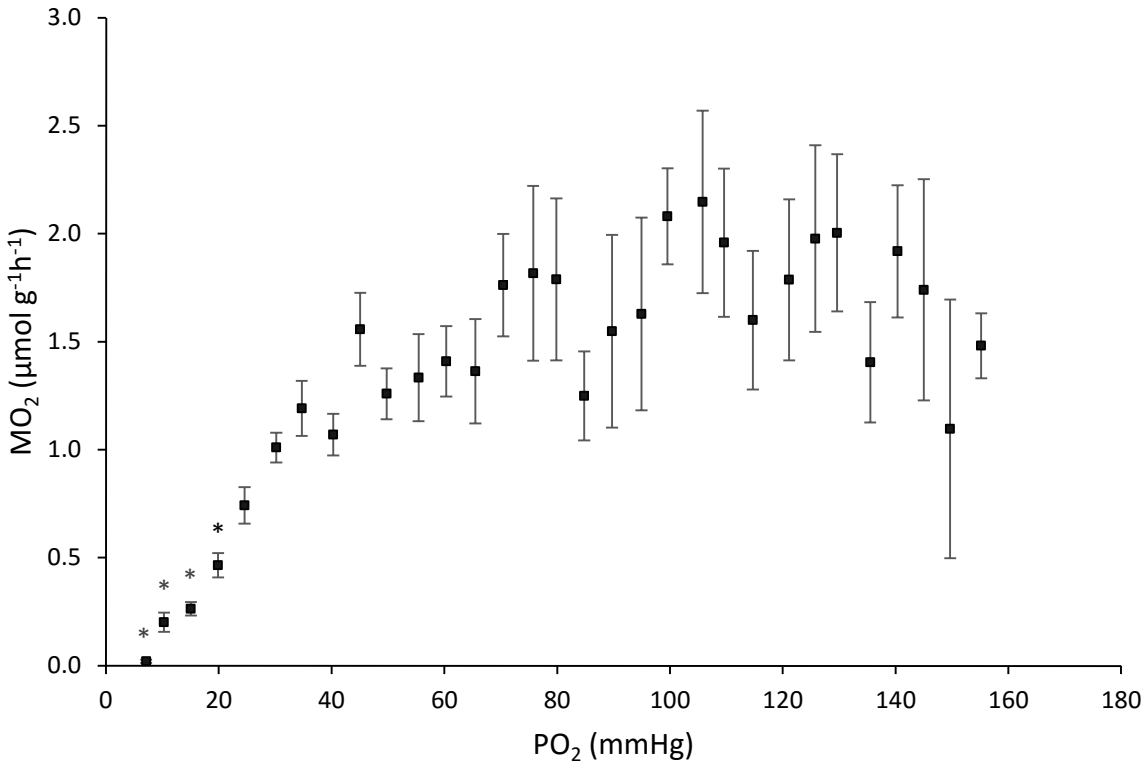

Figure 3.1: Average oxygen consumption rate (MO_2) at 5 mmHg intervals of *P. zealandicus* under progressively declining water oxygen levels (PO_2) (n=26). * significantly different from the normoxic MO_2.

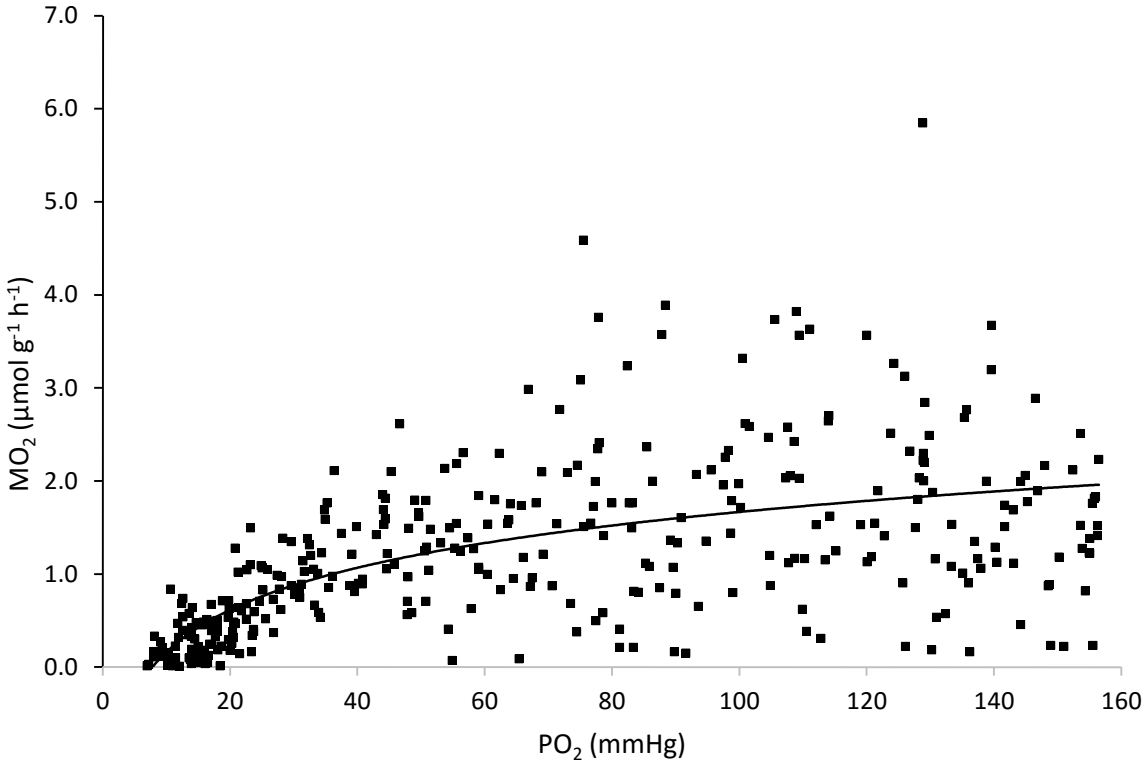

Figure 3.2: Individual oxygen consumption (MO_2) values of *P. zealandicus* (squares) and trend line of all points (solid line) under under progressively declining water oxygen levels (PO_2)(n=26).

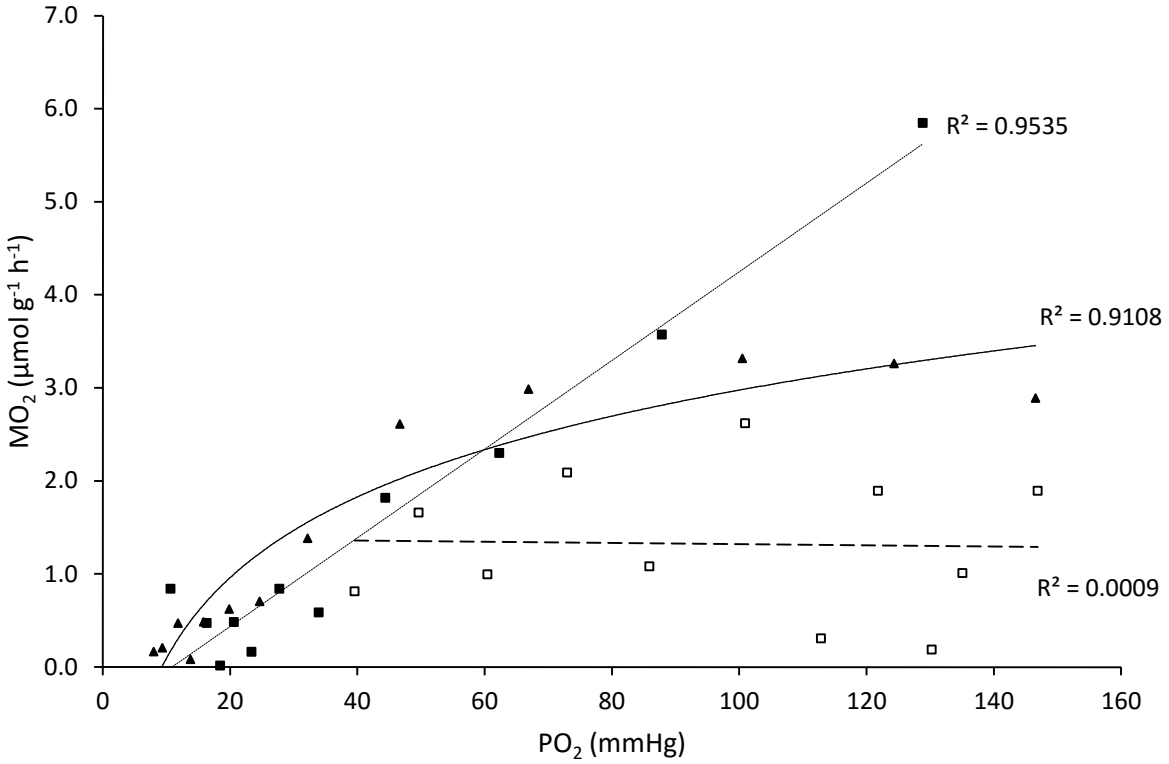

Figure 3.3: *P. zealandicus* individual oxygen consumption (MO_2) values of a strong oxyconformer (■; dotted line) a strong oxyregulator (▲; solid line) and a crayfish with a linear relationship with no slope (□; dashed line) under progressively declining water oxygen levels (PO_2).

Oxygen consumption showed an initial increase after the onset of progressive hypoxia from 1.48 ± 0.15 µmol g^{-1} h^{-1} at 155 mmHg to 2.10 ± 0.36 µmol g^{-1} h^{-1} at 105 mmHg (Fig. 3.1). After this, oxygen consumption slowly decreased to almost half of the peak value, to 1.07 ± 0.10 µmol g^{-1} h^{-1} at 40 mmHg. Oxygen consumption then decreased drastically to 50% of the normoxic value at 20 mmHg ($P < 0.05$). Thereafter, crayfish MO_2 continued to decline as PO_2 was decreased, and at 5-10 mmHg, MO_2 had decreased to 1-13% of the normoxic value ($P < 0.05$, Fig. 3.1).

Using the R program, the "brokenstick" statistical analysis was run on the raw (Fig. 3.4) and grouped (at every 5 mmHg, Fig. 3.5) oxygen consumption data and P_{CRIT} was calculated to be 44 mmHg and 45 mmHg, respectively. To double check this value a scatterplot was used to separate data points (those under 45 mmHg and those over 45

mmHg). Trend lines were then forecasted forward and backward (respectively) to create a cross-over point. This was conducted on both the raw data (Fig. 3.6) and the pooled data (Fig. 3.7), both showed similar results to that of the "brokenstick" value (45 and 47 mmHg, respectively).

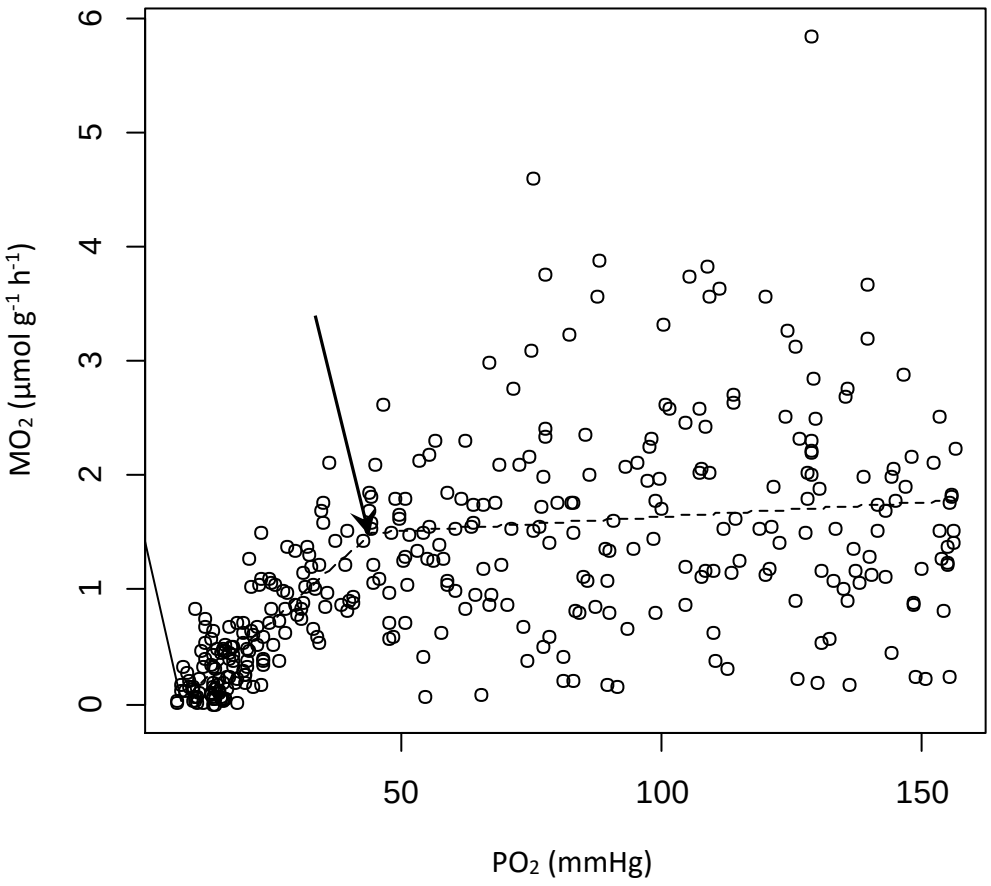

Figure 3.4: "Brokenstick" statistical analysis of the raw data of *P. zealandicus* oxygen consumption (MO_2) under progressive hypoxia (PO_2). Dashed line shows the mean values and arrow indicates the calculated P_{CRIT} value.

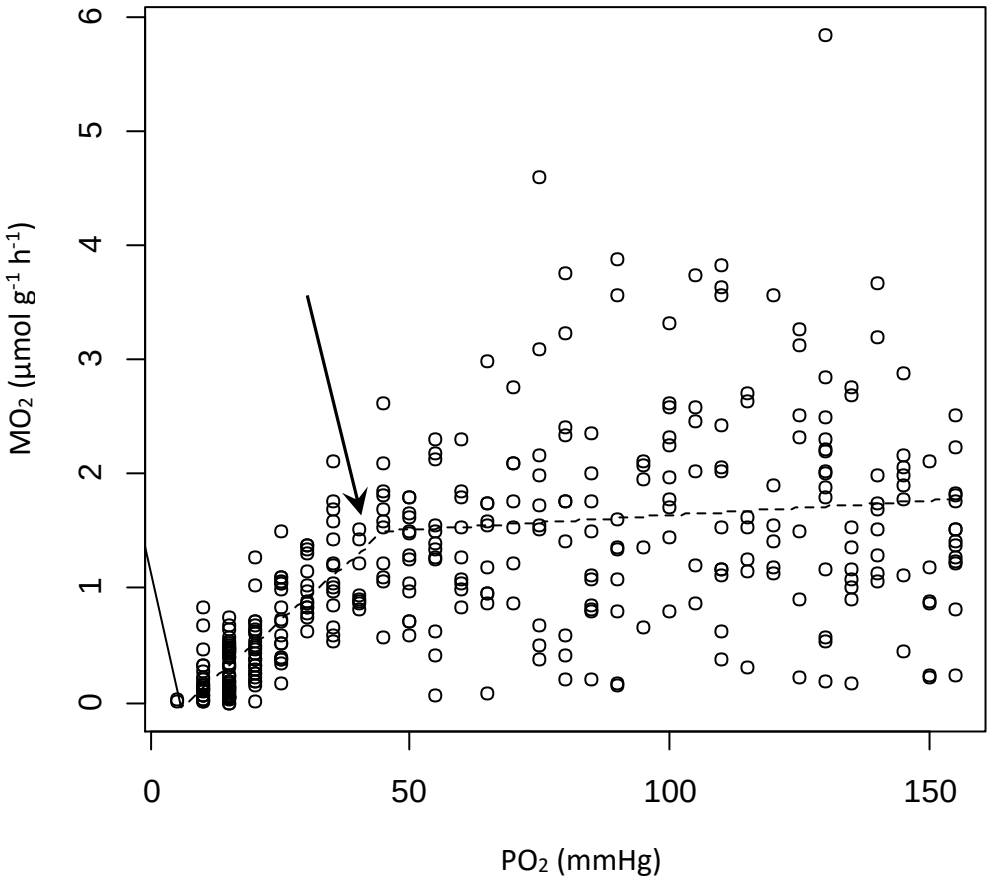

Figure 3.5: "Brokenstick" statistical analysis of the grouped data of *P. zealandicus* oxygen consumption (MO_2) under progressive hypoxia (PO_2). Dashed line shows the mean values and arrow represents the calculated P_{CRIT} value.

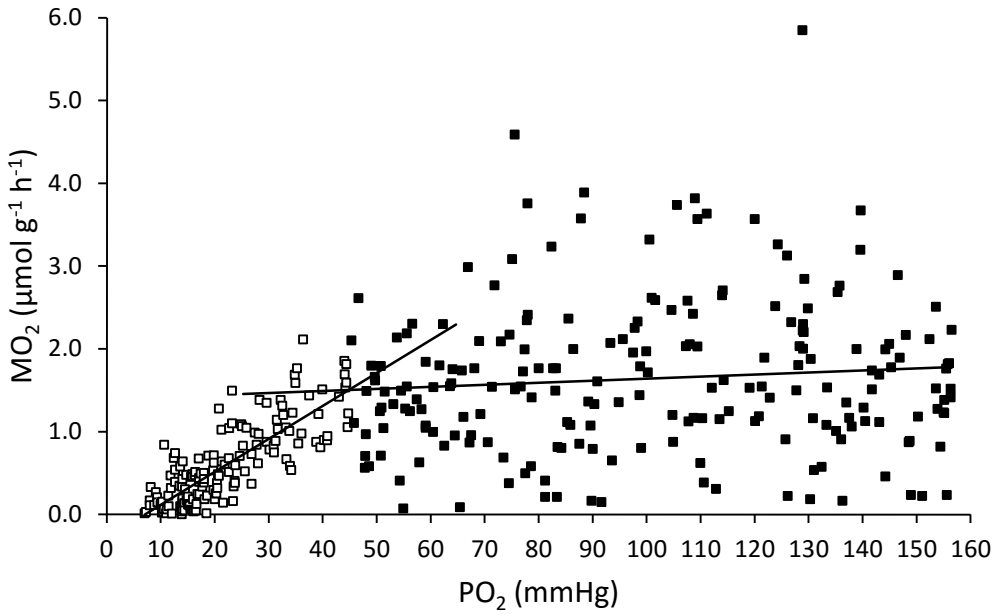

Figure 3.6: Raw oxygen consumption (MO_2) values of *P. zealandicus* under progressively declining oxygen levels (PO_2). Data is split up into values below 45 mmHg (□) and values above 45 mmHg (■). Solid lines represent the trend lines for each data set. The point at which the trend lines intersect is the P_{CRIT} value.

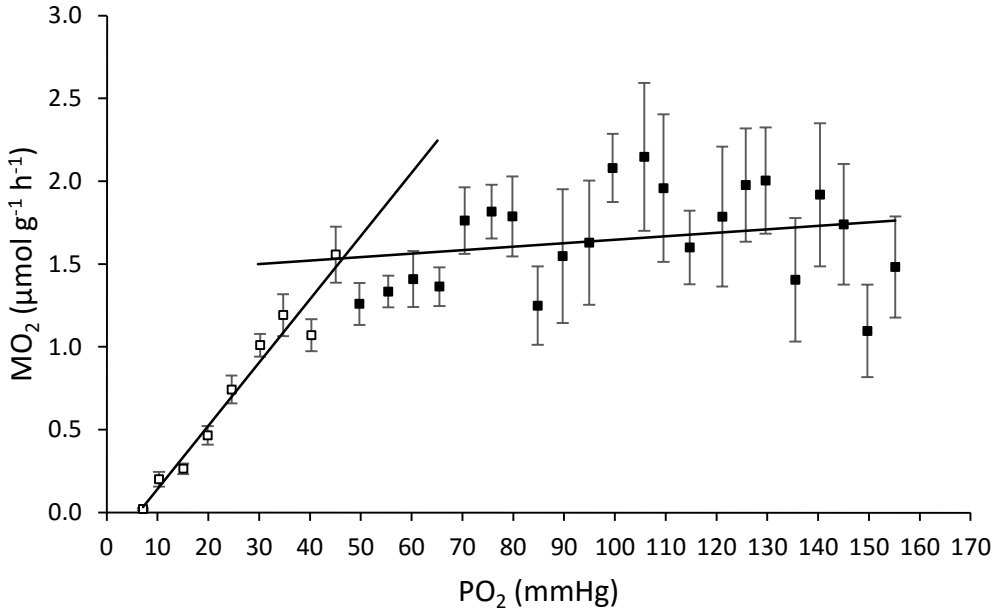

Figure 3.7: Grouped (at 5 mmHg intervals) oxygen consumption (MO_2) values of *P. zealandicus* under progressively declining oxygen levels (PO_2). Data is split up into values

below 45 mmHg (□) and values above 45 mmHg (■). Solid lines represent the trend lines for each data set. The point at which the trend lines intersect is the P_{CRIT} value.

Heart rate

The heart rates of animals exposed to progressive hypoxia were unaffected by reduced PO_2 ($P > 0.05$). This outcome was the same for both the raw (Fig. 3.8) and pooled data (Fig. 3.9). Using the pooled data heart rate was determined to be 57.2 (± 5.6) beats per minute at 155 mmHg, a value that decreased, but not significantly, to 48.4 (± 2.7) beats per minute at 10 mmHg.

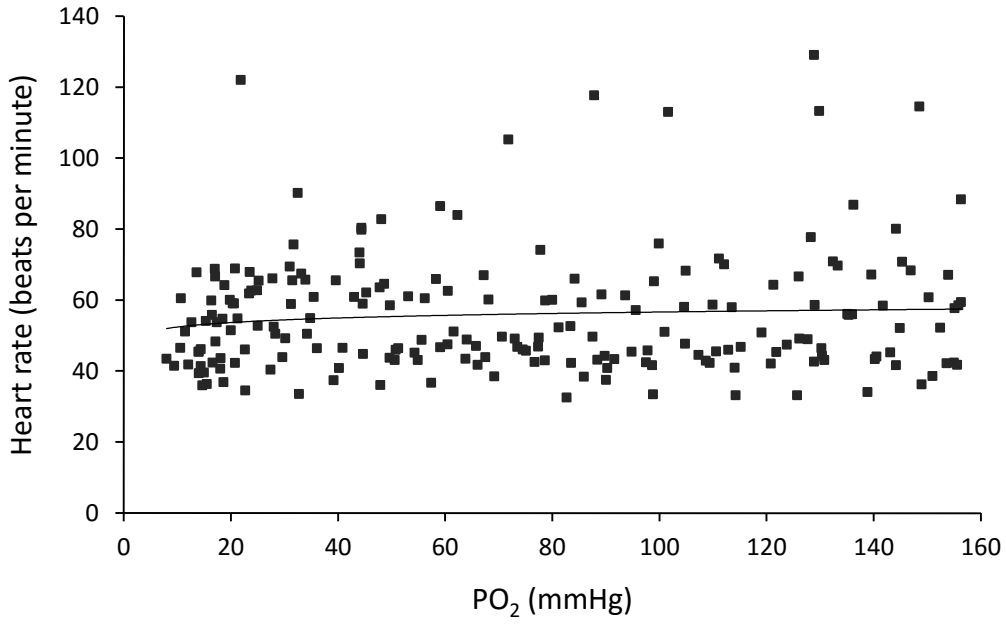

Figure 3.8: Raw data of heart rate of *P. zealandicus* under progressively declining water oxygen levels (PO_2). The solid line shows the trend line of the data (n=17).

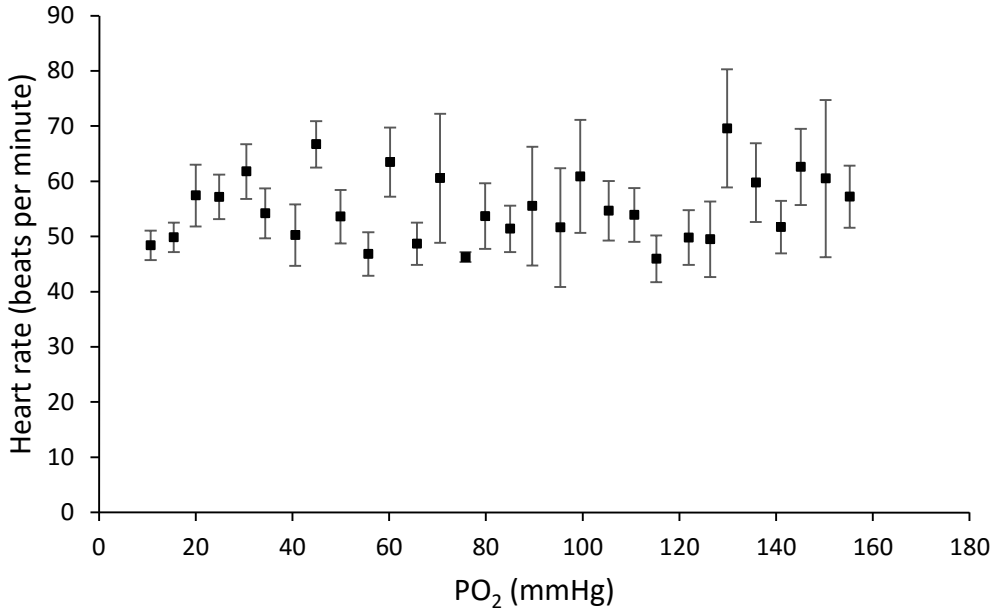

Figure 3.9: Grouped (at 5 mmHg intervals) data of the heart rate of *P. zealandicus* under progressively declining water oxygen levels (PO_2).

3.3.2. Physiological responses to hypoxia under semi closed box respirometry

Oxygen consumption

Oxygen consumption was statistically unchanged between crayfish exposed to 130 and 40 mmHg ($P > 0.05$; Fig. 3.10). In contrast, a reduction in oxygen consumption was seen at the lowest PO_2 level of 20 mmHg where the mean value recorded was approximately half the normoxic value, and significantly different from both 130 and 40 mmHg. Measured PO_2 levels throughout experimentation were found to be 130.2 (± 5.71), 39.2 (± 1.72), and 18.17 (± 4.28) mmHg. The data for semi-closed respirometry thus match the results from the progressive closed box respirometry experiments (where a significant difference from normoxia was only seen at 20 mmHg). The MO_2 of crayfish measured by semi-closed box respirometry did not differ from the MO_2 obtained by progressive closed box respirometry irrespective of PO_2 ($P < 0.05$, Fig.3.10).

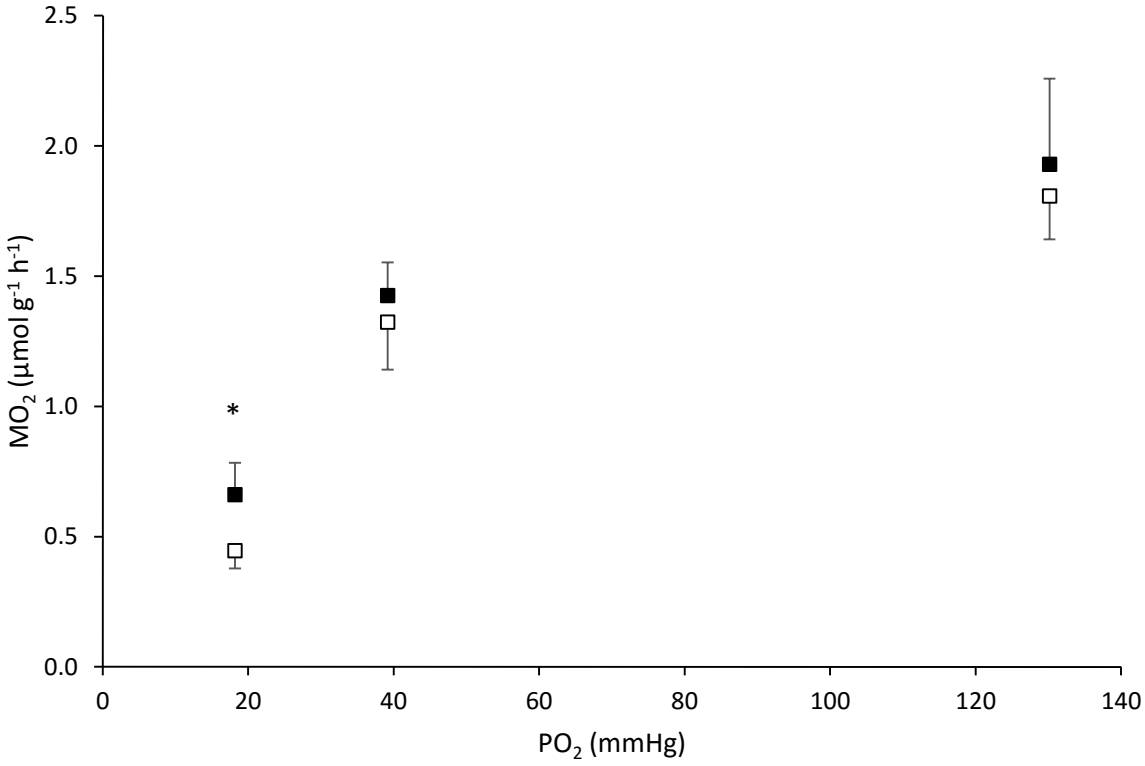

Figure 3.10: Oxygen consumption (MO_2) of *P. zealandicus* in differing oxygen levels using semi closed box respirometry (■) and closed box respirometry (□) techniques. * significantly different from the normoxic MO_2.

Heart rate

The heart rate of crayfish measured during semi-closed box respirometry did not differ from the heart rate values obtained during progressive closed box respirometry ($P < 0.05$, Fig. 3.11). Heart rate did not differ significantly between animals exposed to 130 and 40 mmHg ($P > 0.05$), and although it was not significant, there was a decline heart rate between 40 and 20 mmHg ($P > 0.05$).

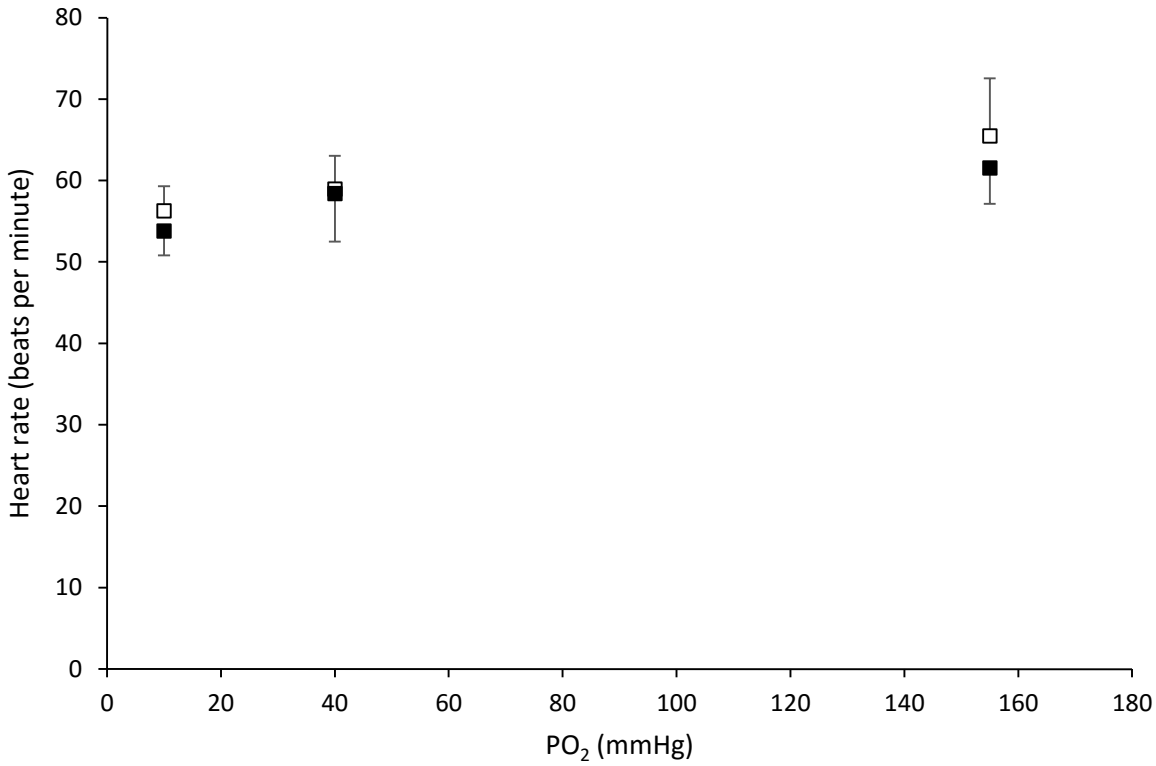

Figure 3.11: Heart rate of *P. zealandicus* in differing oxygen levels using semi-closed box respirometry techniques (■) and closed box respirometry techniques (□).

3.3.3. Physiological responses to hypoxia and/or high nutrients under prolonged flow -through respirometry

Oxygen consumption

Oxygen levels throughout the experiments for control, moderate hypoxia, severe hypoxia, nitrite and severe hypoxia, and ammonia and severe hypoxia were found to be 145 (± 1.3), 25.6 (± 0.9), 13.1 (± 0.5), 14.7 (± 0.5) and 12.2 (± 0.8) mmHg, respectively. Oxygen consumption in animals exposed to control conditions decreased from 2.07 (± 0.13) µmol g^{-1} h^{-1} to 1.80 (± 0.29) µmol g^{-1} h^{-1} from hour one to hour six ($P > 0.05$, Fig. 3.12). However, this decrease was not significant. MO_2 in animals exposed to moderate hypoxia (H30) decreased significantly from hour one (1.33 ± 0.03 µmol g^{-1} h^{-1}) to hour 3 (0.56 ± 0.04 µmol g^{-1} h^{-1}) ($P < 0.0001$). This was followed by a significant increase at hour 5 (0.973 ± 0.13 µmol g^{-1} h^{-1})(P

<0.05). However, even with this increase, MO_2 significantly decreased over the 6 hour exposure from 1.33 (± 0.03) µmol g^{-1} h^{-1} to 0.60 (± 0.14) µmol g^{-1} h^{-1} (P <0.0001, Fig.3.13). Interestingly, MO_2 in animals exposed to severe hypoxia (H10) decreased from 0.51 (± 0.08) µmol g^{-1} h^{-1} to 0.30 (± 0.05) µmol g^{-1} h^{-1} from hour one to hour six (P >0.05, Fig. 3.14) but this decline was not significant. MO_2 in animals exposed to severe hypoxia and nitrite (H10N20) decreased significantly from 0.92 (± 0.40) µmol g^{-1} h^{-1} to 0.22 (± 0.09) µmol g^{-1} h^{-1} (P <0.05)(Fig. 3.15). MO_2 in animals exposed to severe hypoxia and ammonia (H10A30) conditions decreased significantly from 1.03 (± 0.21) to 0.25 (± 0.05) µmol g^{-1} h^{-1} (P<0.05)(Fig. 3.16). Thus, as exposure time increased there was a decrease in MO_2 across all exposure conditions, except for in severe hypoxia alone.

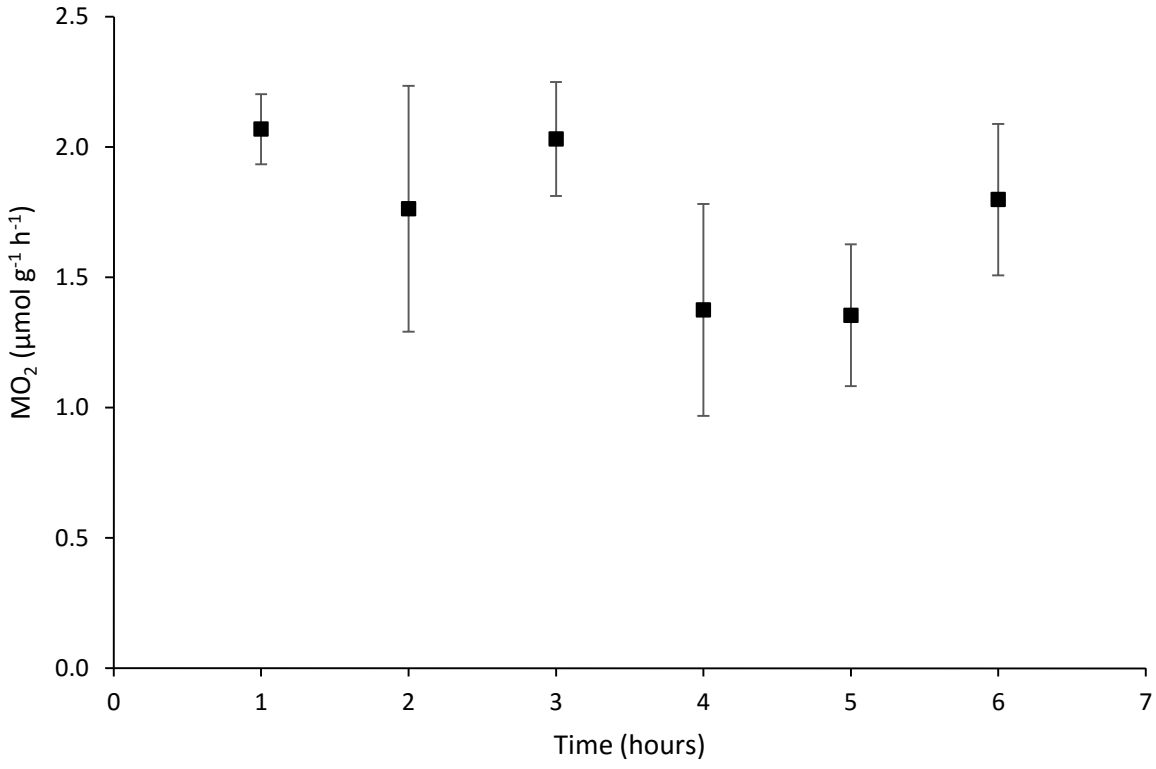

Figure 3.12: Oxygen consumption (MO_2) of *P. zealandicus* under normoxic (145 mmHg) conditions over a six hour period. There were no significant differences between the plotted points.

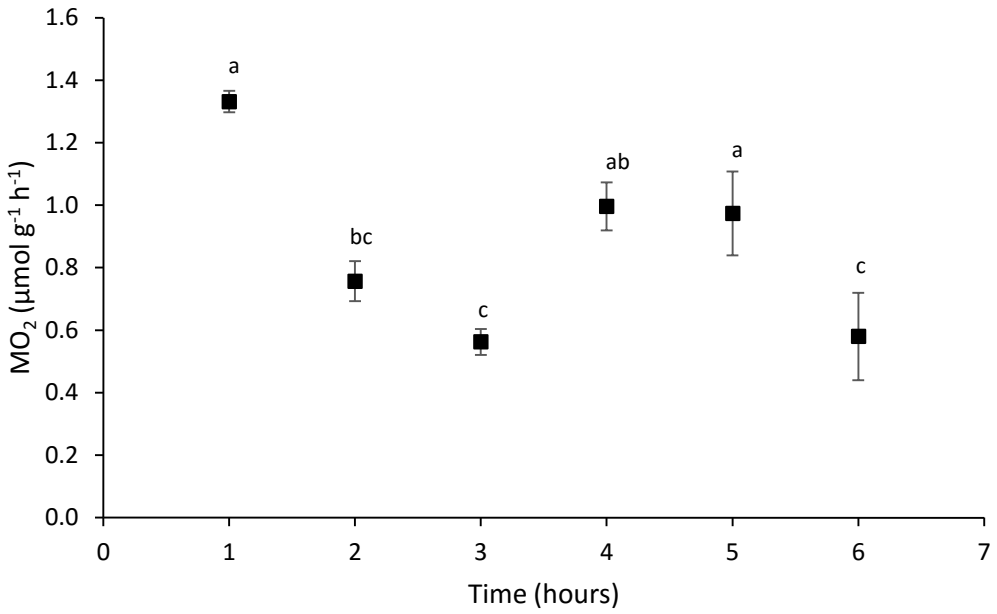

Figure 3.13: Oxygen consumption (MO_2) of *P. zealandicus* under moderate hypoxic (25.6 mmHg) conditions (H30) over a six hour period. Plotted points sharing letters are not statistically significantly different.

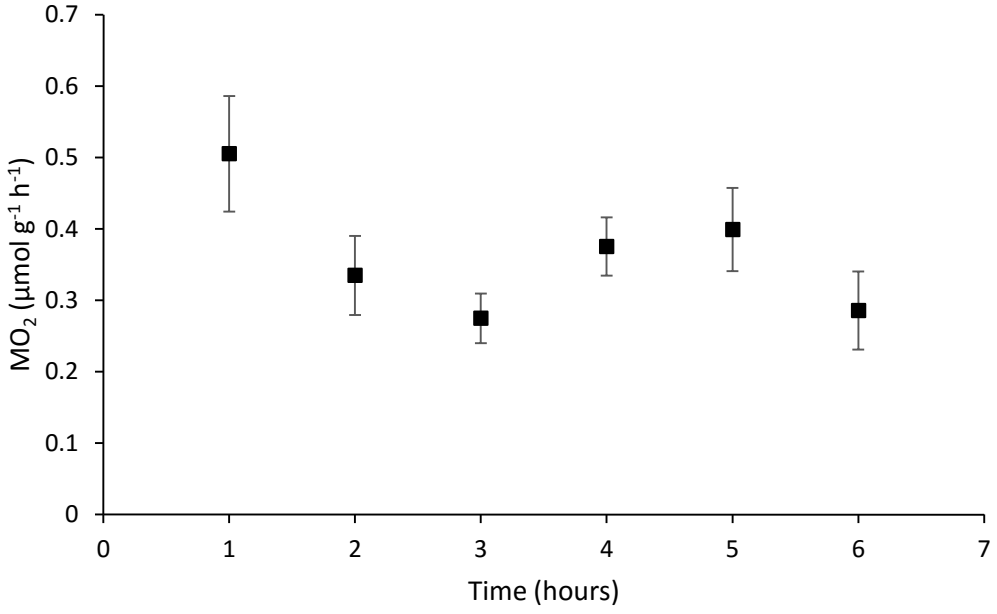

Figure 3.14: Oxygen consumption (MO_2) of *P. zealandicus* under severe hypoxic (13.1 mmHg) conditions (H10) over a six hour period. There were no significant differences between the plotted points.

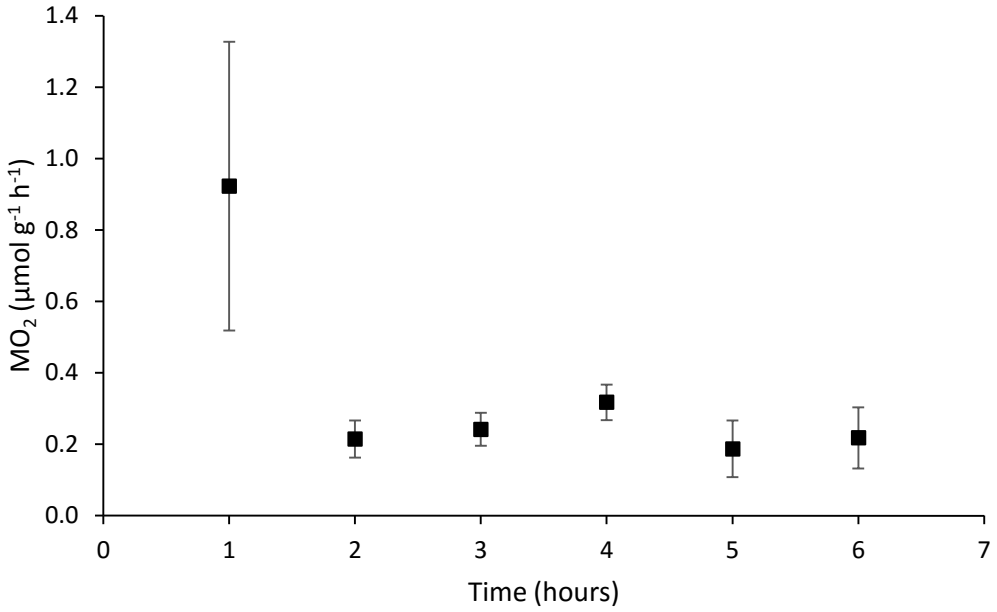

Figure 3.15: Oxygen consumption (MO_2) of *P. zealandicus* under severe hypoxia (14.7 mmHg) and high nitrite (20 mg L^{-1}) conditions (H10N20) over a six hour period. There were no significant differences between the plotted points.

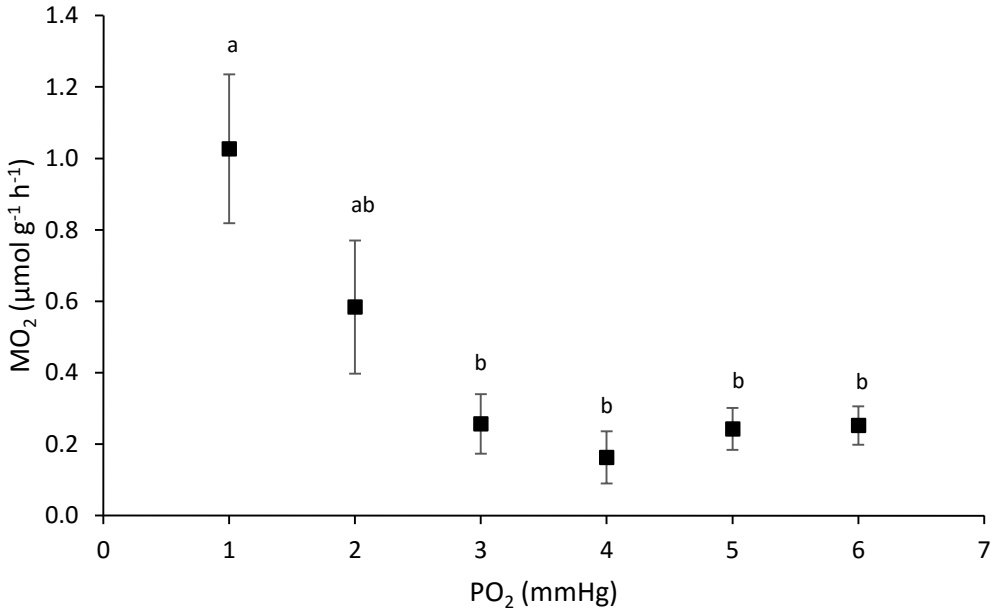

Figure 3.16: Oxygen consumption (MO_2) of *P. zealandicus* under severe hypoxia (12.2 mmHg) and high ammonia (30 mg L^{-1}) conditions (H10A30) over a six hour period. Plotted points sharing letters are not statistically significantly different.

The mean MO_2 over the six hours was highest in animals exposed to control conditions (1.73 ± 0.30 µmol g^{-1} h^{-1}). This was significantly higher than that found in all other treatments (P <0.0001, Fig. 3.17). The MO_2 of crayfish exposed to H30 was significantly higher than the MO_2 of crayfish exposed to H10 (P <0.05) but not significantly higher than H10N20 or H10A30 exposed crayfish (P >0.05) (Fig. 3.17). There were also no significant differences between the MO_2 of crayfish exposed to any of the three severe hypoxia treatments (P>0.05) (Fig. 3.15). Thus, prolonged hypoxia negatively affected freshwater crayfish MO_2. However, the addition of nutrients did not alter their response.

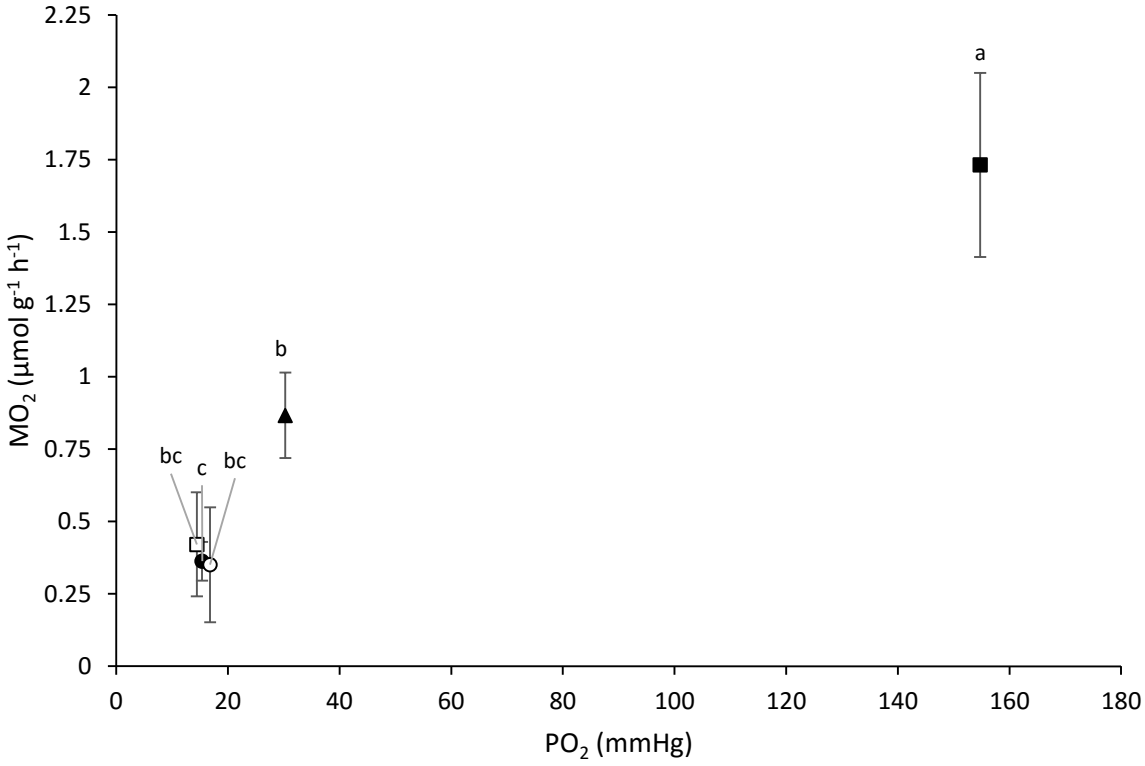

Figure 3.17: Mean oxygen consumption (MO_2) of the six hours of exposure of *P. zealandicus* under normoxic (■), moderate hypoxic (H30) (▲), severe hypoxic (H10) (●), severe hypoxic and high nitrite (H10N20) (○) and severe hypoxic and high ammonia conditions (H10A30) (□).Plotted points sharing letters are not statistically significantly different.

Heart rate

The heart rate decreased over time in animals exposed to all treatments, but this effect was not significant. The highest heart rates were in the normoxic control, and the lowest in severe hypoxia (Fig. 3.18).

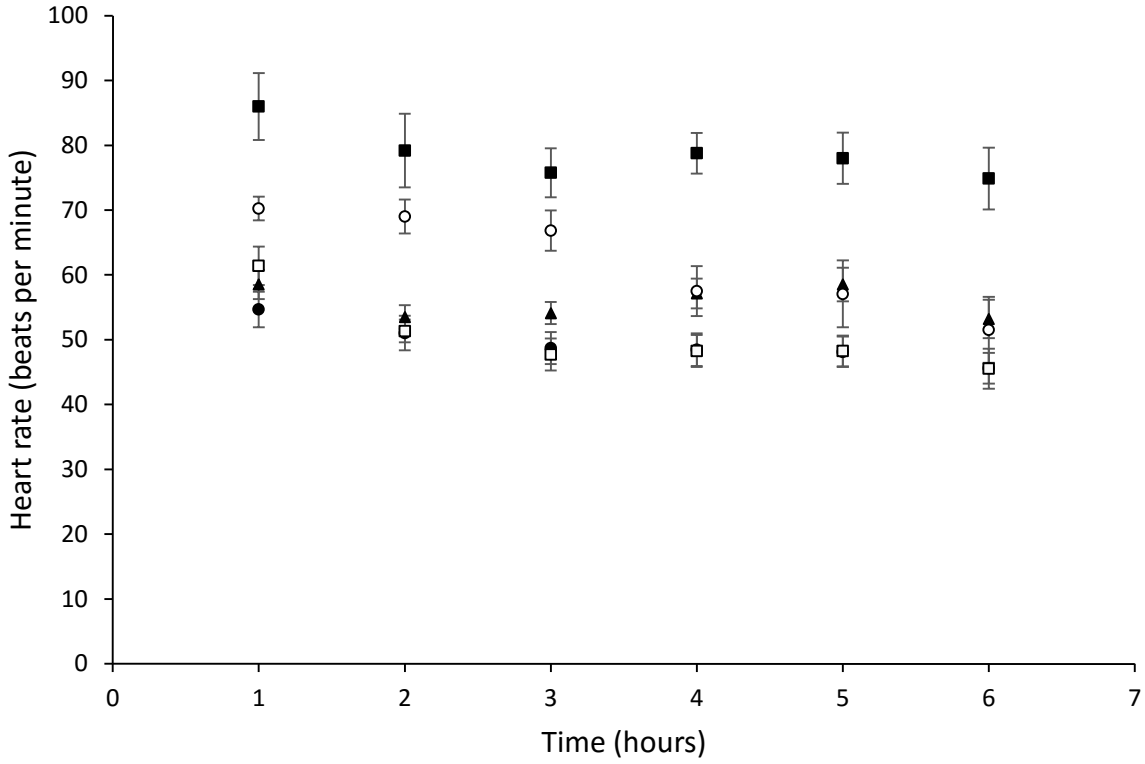

Figure 3.18: Mean heart rate of *P. zealandicus* from the six hours of exposure under normoxic (n=5) (■), moderate hypoxic (H30, n=5) (▲), severe hypoxic (H10, n=5) (●), severe hypoxic and high nitrite (H10N20, n=5) (○) and severe hypoxic and high ammonia conditions (H10A30, n=5) (□).There were no significant differences in heart rate within each treatment over the 6 hour period.

The mean heart rate of crayfish calculated over the 6 hours of exposure in shown in Fig. 3.19. Under control conditions the heart rate (78.8 ± 7.8 beats per minute) was significantly higher than that of crayfish exposed to H30 (55.8 ± 3.7), H10A30 (50.4 ± 4.4 beats per minute) and H10 (49.4 ± 4.6 beats per minute) ($P < 0.05$). There were no significant

differences in heart rate between crayfish exposed to any of the severe hypoxia treatments (P >0.05) (Fig. 3.19). Thus, prolonged hypoxia and the addition of ammonia, but not nitrite, impacted crayfish heart rate.

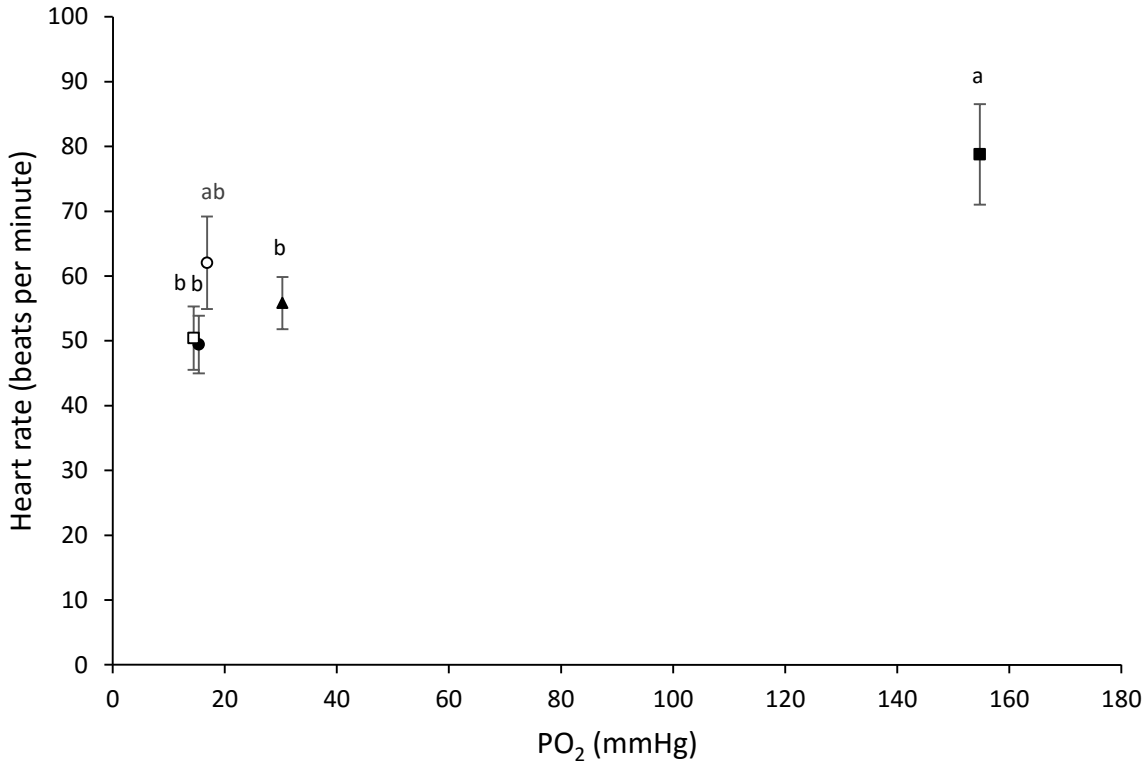

Figure 3.19: Mean heart rate of the six hours of exposure of *P. zealandicus* under normoxic (■), moderate hypoxic (H30) (▲), severe hypoxic (H10) (●), severe hypoxic and high nitrite (H10N20) (○) and severe hypoxic and high ammonia conditions (H10A30) (□). Plotted points sharing letters are not statistically significantly different.

3.4. Discussion

Crayfish have developed physiological mechanisms to maintain homeostasis in low oxygen water. These include hyperventilation, modulating haemocyanin, hypometabolism and bradycardia (Bonvillain *et al*., 2012). The latter two mechanisms were investigated in the New Zealand freshwater crayfish *Paranephrops zealandicus*. Although mechanisms

underlying hypoxia tolerance have been widely researched in freshwater crayfish this is the first study to investigate the response of the New Zealand freshwater crayfish to a combination of hypoxic and high nutrient waters.

3.4.1. Physiological responses to hypoxia- oxygen consumption

Aquatic animals, including crayfish, typically attempt to maintain MO_2 as water oxygen levels are reduced (see Reiber, 1995 and McMahon, 2001). They are able to do so down to a critical oxygen tension after which MO_2 is no longer maintained and consumption of oxygen drastically declines. The average MO_2 for freshwater crayfish under normoxic conditions in the current study was 1.48 ± 0.15 µmolg h^{-1}, within the range reported for other freshwater crayfish (0.67 to 2.32 µmol g^{-1} h^{-1}; Wheatly and Taylor 1981; Wilkes and McMahon 1982; Massabuau and Burtin, 1984). This normoxic MO_2 was maintained, after an initial overshoot, down to a PO_2 of 45 mmHg at which point MO_2 declined with declining PO_2. This value represents the P_{CRIT}, the critical oxygen tension at which MO_2 was no longer able to be maintained, and the point at which the crayfish switched from being an oxyregulator to being an oxyconformer. These values are in agreement with previous reports on *P. zealandicus*, which showed an MO_2 of 1.32 µmol g^{-1} h^{-1} at normoxia, and a P_{CRIT} of 45 mmHg (Titulaer, 1995). The calculated P_{CRIT} of 45 mmHg in *P. zealandicus* suggests this species is of similar hypoxia tolerance to other freshwater crayfish species. For example, *Austropotamobius pallipes* maintains MO_2 down to 40 mmHg, and *Procambarus clarkii* down to 45 mmHg (McMahon*et al.,* 1975; Taylor and Wheatley, 1980; Reiber and McMahon, 1998).

The MO_2 of crayfish exposed to short-term progressive hypoxia did not differ from the MO_2 of crayfish exposed to prolonged hypoxia. This was unexpected as previous studies have shown that increasing exposure time to hypoxia negatively impacts physiological responses (Banchero, 1987; Racotta and Hernández-Herrera, 2000; Petersen and Gamperl, 2009). However, in these cited studies animals were exposed for weeks. A shorter exposure time of six hours was selected in the current study because the tolerance of *P. zealandicus* to such low oxygen levels was unknown, and a 6-hour time frame is environmentally realistic for hypoxia based on photosynthesis/respiration cycles in eutrophied waters. During the 6-hour exposure, crayfish under moderate hypoxia and severe hypoxia, did significantly decrease their MO_2, showing that responses are time-dependent. However, these decreases were

insufficient to make them significantly different from the short term exposure responses. The statistical outcome was similar even if the data over the last hour, rather than over the entire 6 hour exposure, were used.

The value of 45 mmHg for P_{CRIT} was supported by all the calculation methods used in the current study, except one. When based on the point where MO_2 in hypoxic conditions became significantly different from normoxic values a P_{CRIT} of 20 mmHg was reported. This suggests this means of calculating P_{CRIT} may not be as reliable as other techniques, although the difficulty in identifying a similar P_{CRIT} by all trialed methods may have an experimental basis. Factors that can prevent the establishment of a definitive P_{CRIT} for oxyregulators include the rate of decline in PO_2, acclimation time, or variability between individuals of the same species (Herried, 1980). In the present study the experimental time averaged over a period of 280 minutes (range 180-420 minutes) with a rate of PO_2 decline equal to 30 mmHg per hour. Studies have shown that if PO_2 levels fall too rapidly the ability to marshal full compensatory responses can be limited (Herreid, 1980). However, McMahon *et al.,*(1974) exposed lobster, *Homarus americanus,* to progressively deepening hypoxia over a period of 60-120 minutes. This species was able to respond and showed a definitive oxyregulatory behaviour in half the amount of time as that in the present study. It is possible this difference relates to differences between *H. americanus* and *P. zealandicus*, with the former species being significantly larger, and thus having a smaller surface area to volume ratio, which may better promote regulation. In another study, Wheatly and Taylor (1981) exposed crayfish *Austropotambius pallipes,* to hypoxia by reducing water PO_2 in 7-8 steps of 15-30 mmHg at 90 minute intervals, making the duration of the experiment between 360 and 600 minutes. This is a slightly slower decline in PO_2 compared with the present study, but they were also able to more easily establish a P_{CRIT}. It therefore seems unlikely that the rate of decline in PO_2 is what caused this high variability in MO_2 and difficulty in pinpointing P_{CRIT}.

C. destructor, A. pallipes and *H. americanus* are all considered oxyregulators in that at least over part of the PO_2 exposure range they maintain a near-constant MO_2. In contrast, an oxyconformer is a species that does not maintain MO_2 as PO_2 declines, even when the PO_2 is above hypoxic levels. Whether *P. zealandicus* is a true oxyregulator is unknown. Categorising species as oxyregulators or oxyconformers is problematic because there is no clear phylogenetic or ecological pattern in the response to hypoxia (Tang, 1933; Bayne, 1973; Mangum and van Winkle, 1973; van Winkle and Mangum, 1975; Taylor and Brand, 1975;

Herreid, 1980). Indeed, oxyregulators and oxyconformers are scattered amongst different taxonomic groups and ecological habitats. Furthermore, a continuum of responses exists with strong oxyregulators on one end and oxyconformers on the other (Mangum and van Winkle, 1973; Taylor and Brand, 1975). For example, *Homarus gammarus, Orconectes virilise, Cancer magister*, *Homarus vulgaris, Astacus leptodactylus* and *Astacus pallipes* all maintain a constant MO_2 down to a low water PO_2 of ~30-40 mmHg (Johansen *et al*., 1970; Spoek, 1974; McMahon *et al*., 1974; Butler *et al*., 1978; Massabuau *et al*., 1980; Massabuau and Burtin, 1984) and could be classed as strong oxyregulators. Conversely, the crab *Callinectes danue*, shows a weaker regulation, becoming dependent on water oxygen availability below 97 mmHg, while the crab *Paralithodes kamtschatica* is completely oxy-dependent and oxyconforms no matter the PO_2 (Cameron, 1989; Rantin *et al*, 1996).

Several studies have also shown that oxyregulatory behaviour does not just vary between species but among individuals in the same species (Bayne, 1971; Duke and Ultsch, 1990). The degree to which individual in a species either oxyregulates or oxyconforms is dependent on factors such as salinity, moult cycle, stress, body size, development and temperature (Thompson and Pritchard, 1969; Potner and Grieshaber, 1993). For example, the lobster *Homarus vulgaris* was an oxyconformer at high environmental temperatures whereas at lower temperatures it was a regulator (Spitzer *et al.,*1969; Butler and Taylor, 1975). This individual variability can be seen in the present study where some individuals show a clear linear oxyconforming pattern from normoxia to severe hypoxia, others a clear oxyregulation, and a few with patterns intermediate to these two responses (Fig. 3.3). This variability between individual crayfish could explain the irregular pattern of the MO_2 response as all the individual values were pooled. It could also explain the two different P_{CRIT} values obtained from the different analysis methods.

3.4.2. Physiological responses to hypoxia- heart rate

Most crustaceans exposed to hypoxia exhibit a decreased heart rate under hypoxic conditions (Larimer and Gold, 1961; Larimer, 1962; McMahon *et al*., 1974; deFur and Mangum, 1979; Wheatly and Taylor, 1981; Reiber *et al*, 1992). For example, Wheatly and Taylor (1981) showed that the freshwater crayfish *A. pallipes* heart rate decreased by half, from 63 beats per minute at 140 mmHg to 32 beats per minute at 30 mmHg. The heart rate

of *Procambarus clarkii* also decreased by over half, from 125 beats per minute at 150 mmHg to 60 beats per minute at 25 mmHg (Reiber and McMahon, 1998). This hypoxia-induced bradycardia is especially prominent below the animals P_{CRIT} as there is insufficient oxygen to meet the aerobic demands of the circulatory pump (Airriess and McMahon, 1994). The decrease in heart rate is usually accompanied by an increase in stroke volume, meaning that cardiac output is unchanged, but cardiac work becomes more energetically-efficient (Wheatly and Taylor, 1981; Reiber, 1995; Reiber and McMahon, 1998; McMahon, 2001). By essentially slowing perfusion it allows the animal to increase time for diffusion and thus oxygen extraction, yet reduces the energetic costs of cardiac pumping (Satchell, 1960; Randall and Shelton, 1963; Short *et al*., 1979; Taylor and Barrett, 1985; McMahon, 2001; Farrell 2007). However, *Parenephrops zealandicus* did not show this normal hypoxia-induced bradycardic response. Heart rate under normoxic conditons (57 beats per minute) did not differ from the heart rate under severe hypoxia (10 mmHg) (48.4 beats per minute).

It is possible that the progressive hypoxia experiments were too short a period for cardiac responses to ensue. Prolonged hypoxia experiments were conducted to investigate if longer exposure to hypoxia would elicit a bradycardic response. Control crayfish had a significantly higher heart rate than those exposed to all experimental conditions except low oxygen and high nitrite. However, heart rates did not differ between progressive hypoxia and prolonged (6 hours) hypoxia experiments. The heart rate of crayfish under prolonged exposure did not change significantly over the six hours in all treatments. This suggests that the lack of bradycardia was not an effect of a short exposure period.

A hypoxia-induced bradycardia is not, however, observed in all animals. Juvenile and larval shrimp, *Metapenaeus ensis*, and the cladoceran, *Daphnia magna*, employ a different compensatory mechanism, and instead heart rate is increased (McMahon 2001: Paul *et al*., 1998; Pirow *et al*., 1999). This tachycardia is also seen in the grass shrimp, *Palaemonetes pugio,* where, similar to the bradycardic response, the purpose is to maintain cardiac output (Harper and Reiber, 1999). Similar to the present study, some animals also maintain heart rate under hypoxic conditions. For example in fish, the heart rate of *Hemitripterus americanus, Ciliata mustella and Pagothenia bernacchii* are unresponsive to hypoxia (Saunders and Sutterlin, 1971; Fritsche, 1990; Axelsson *et al*., 1992). This maintenance of heart rate is either due to other cardiac parameters compensating for the lack of bradycardia or because the animal is not under any undue stress. Mendonca and Gamperl (2010)

concluded that hypoxia-induced bradycardia is most likely to occur near the limit of hypoxia tolerance. Thus, animals that lack alterations in heart rate are suggested to be more tolerant of hypoxia (Bierbower and Cooper, 2009). This is observed in the hypoxia-tolerant air breathing lungfish where bradycardia is absent in low oxygen conditions (Fritsche *et al*., 1993; Sanchez *et al*., 2001; Perry *et al*., 2005). Among freshwater crustaceans, the absence of bradycardia and maintenance of heart rate has also been seen in *Orconectes rusticus* subjected to 144 hours of hypoxia. *O. rusticus*, however, did show a 3-4-fold increase in cardiac output (Wilkes and McMahon, 1982). Cardiac output is adjusted in animals to maintain blood flow and thus perfusion of the gill and adequate oxygenation (Farrell, 2007; McKenzie *et al*., 2009; Mendonca and Gamperl, 2010; Guadagnoli *et al*.,2011).This compensatory increase in cardiac output has been shown in many studies (Morris *et al*, 1996; Paul *et al*., 1998; Pirow *et al*., 1999). Whether this was the case for the *P. zealandicus* is unknown, as only heart rate was measured in the current study. Ventilation volume and haemocyanin oxygen affinity have also been shown to compensate for the lack of bradycardia in the lobster, *Homarus vulgaris* (Butler *et al*., 1978). Consequently, heart rate alone may be an insufficient measure to fully elucidate the cardiac responses of *P. zealandicus* to hypoxia.

Many early measurements of heart rate in crustaceans were extremely invasive and involved the implantation of electrode wires into the pericardial cavity (Maynard, 1960; Blatchford, 1971). For this reason, and because a lot of studies did not allow for sufficient acclimation time, published data are often maximal values and could provide an inaccurate perspective of the physiological response. For example, the lobster *Homarus americanus*, showed a decrease in heart rate from 93 to 30 beats per minute (McMahon *et al.*, 1974). However, when another species of lobster was tested, but given a much longer acclimation period (1 week instead of 2 hours), the hypoxia-induced bradycardic response was much less substantial (Butler *et al.,* 1978). This suggests that using non-invasive techniques and allowing sufficient acclimation time produces a weaker hypoxia-induced bradycardic response. In the current study, a light-weight non-invasive infrared sensor was placed onto the carapace of the crayfish. The lack of disturbance this technique provided could perhaps help explain the lack of bradycardia.

3.4.3. Physiological responses to severe hypoxia and high nutrients- nitrite

On exposure to nitrite a reduction in MO_2 is often seen due to the ability of nitrite to interrupt oxygen transport (Tahon *et al*. 1988; Meades and Watts, 1995). In the current study, *P. zealandicus* were exposed to both hypoxia and high nitrite. Therefore, it would be expected that the crayfish exposed to multiple stressors would have a significantly lower MO_2 than those exposed to hypoxia alone. This additive effect was seen in the shrimp *Penaeus setiferus* which reduced MO_2 on exposure to 15 mmHg but further reduced MO_2 when also exposed to 17 mg L^{-1} of nitrite (Alcaraz *et al.,* 1999). In contrast to the shrimp, the MO_2 of *P. zealandicus* was not significantly different in conditions of severe hypoxia and nitrite compared to those exposed to severe hypoxia alone. This suggests the addition of nitrite did not impact MO_2 in *P. zealandicus* and that perhaps this species is not as sensitive to nitrite as *P. setiferus*. Variation in nitrite sensitivities between species is common. For example, in *Cherax quadricarinatus* MO_2 was unimpacted by the addition of nitrite whereas *Oncorhynchus mykiss* displays a significantly reduced MO_2 (Mead and Watts, 1995; Jensen, 1995; Pedersen *et al*., 2010). It is generally considered that haemocyanin is less impacted by nitrite than haemoglobin, and this could drive differences in sensitivities between fish and crustaceans (Jensen, 1995). More tolerant species will also exhibit a lack of MO_2 reduction. For example, the goldfish (*Carassius auratus*), which can survive long periods of anoxia, was unimpacted by 100 µmol L^{-1} nitrite (Pedersen *et al.,* 2010)

Other species exposed to nitrite have shown a tachycardic response. For example, rainbow trout (*O. mykiss*) showed a tachycardic response to nitrite (1 mmol L^{-1}) with heart rate increasing from 50 to 87 beats per minute (Aggergaard and Jensen, 2001). In the current study, although there was no overall effect of nitrite on heart rate, significant individual variation in responses was seen. For example, in the last hour of exposure one individual had a heart rate of 20 beats per minute and another 70 beats per minute. In a study by Aggergaard and Jensen (2001), interspecific differences were seen within the test subjects where one group was affected much more than the other. This was ascribed to individual differences in branchial chloride uptake rates as nitrite competitively inhibits this process in fish (Perry *et al.*, 1992). This suggests that branchial chloride uptake rate will affect toxicity, a hypothesis supported by the literature. For example, the eel *Anguilla rostrata,* has very low branchial chloride uptake rates making them more tolerant to nitrite than other species such

as the rainbow trout (Hyde and Perry, 1989). Whether the variation in heart rate of *P. zealandicus* exposed to nitrite is explained by differences in branchial chloride handling (see Section 6.6.1.) is unknown and requires further study.

3.4.4. Physiological responses to severe hypoxia and high nutrients- ammonia

The addition of ammonia in severe hypoxia treatments did not impact the significantly reduced MO_2 of *P. zealandicus*. Ammonia alone is known to have impacts on MO_2. The shrimp species*, Penaeus japonicas* and *Litopenaeus schmitti* exhibit an increase in MO_2 when exposed to 5 and 40 mg L^{-1} ammonia, respectively (Chen and Lai 1992; Barbieri, 2010). An increase in oxygen consumption was also observed in the seahorse, *Hippocampus abdominalis*, after exposure to 14.8 mg L^{-1} of ammonia (Adams *et al*., 2001). In contrast, the shrimp *Penaeus setiferus* significantly reduces MO_2 in response to ammonia (Alcaraz *et al.,* 1999).

Similarly, and in contrast to the results observed here for *P. zealandicus*, other studies have shown interactions between ammonia and hypoxia by measuring MO_2. For example, *Penaeus semisulcatus* is two times more sensitive to ammonia when exposed to 40 mmHg than to normoxia (Wajsbrot *et al*., 1991). A similar effect was also seen in *Litopeneus stylirostris* which was exposed to 32 mmHg and 2.0 mg L^{-1} ammonia (Mugnier *et al.*, 2008), and by Alcaraz *et al*. (1999) in the shrimp *Penaeus setiferus*, where MO_2 under the combined exposure of hypoxia (16 mmHg) and ammonia was 10 times lower than the MO_2 of animals exposed to only low oxygen levels. Consequently, the literature consensus is that duel exposure to ammonia and hypoxia is more deleterious to an organism than singular exposures (Thurston *et al*., 1981; Maguad *et al*., 1997). The lack of difference in MO_2 of *P. zealandicus* when nutrients were added to severe hypoxia suggests a tolerance to eutrophication.

The effects of ammonia on heart rate have been studied by Tonapi and Varghese (1984, 1987) who showed an increased heart rate in cladocerans (*Moina rectirostris, Daphnia carinata* and *Simocephalus* sp.) and crab*, Berytelphusa cunnicularis*. A tachycardic response to ammonia has also been recorded for the freshwater crayfish *Pacifastacus leniusculus* (Bloxham *et al*., 1999). In a study by Schmitt and Uglow (1997) a hypoxia- and nutrient-

tolerant prawn only showed an increase in heart rate after experiencing progressive increases in water ammonia levels and not a sudden increase. This increase in heart rate was also only observed above ammonia concentrations of 2000 µM, a concentration unlikely to occur naturally. However, in the present study this tachycardic response was not observed for *P. zealandicus*. Instead relative to severe hypoxia, where the heart rate was already significantly decreased relative to normoxia, the addition of ammonia had no further impact. It seems that the impacts of hypoxia were more dominant than those of nutrients suggesting a high tolerance to nutrient levels. The interactive effects of nutrients and hypoxia on *P. zealandicus* heart rate have not previously been studied. However, it could be presumed that like other physiological responses, an exacerbation of the effect would have occurred from the combination of nutrients and low oxygen levels within heart rate responses. This was not the case in the current study and heart rate was unaffected by the addition of nutrients. However, it should be noted that the current study did not test the effects of ammonia and nitrite on their own which would have aided interpretation.

3.5. Summary

The presented results of MO_2 and heart rate under conditions of hypoxia, with and without the presence of nutrients differ from those of other studies. The MO_2 response of *P. zealandicus* to declining PO_2 was not as clear as the majority of studies and produced two different P_{CRIT} values (20 and 45 mmHg). The standard hypoxia-induced bradycardic response was not observed under progressive hypoxia and an exposure time of 6 hours did not alter MO_2 or heart rate responses. The maintenance of MO_2 and heart rate down to a low PO_2 is critical to sustain aerobic metabolism, ensuring adequate oxygen supply to tissues under hypoxic conditions. Although many animals are adapted to live in anaerobic conditions, at least in the short term, this is not sustainable over long periods, and can impact survival. The typical additive effect of the nutrient and hypoxia combination was not observed in either MO_2 or heart rate and *P. zealandicus* seemed unaffected by the addition of nutrients, suggesting an unexpected tolerance to eutrophication. The maintenance of aerobic metabolism would reduce anaerobic metabolism. This in turn, would prevent the build-up of toxic end products such as lactate and increase the ability of *P. zealandicus* to survive in low

oxygen and high nutrient environments. Whether *P. zealandicus* used anaerobic metabolism under hypoxic conditions is the subject of the next chapter. The ability to regulation MO_2 down to a low PO_2 and not elicit a bradycardic response suggests *P. zealandicus* still had enough aerobic capacity to maintain relatively normal functioning of biological processes suggesting they are hypoxia tolerant.

Chapter Four

4. Biochemical responses of *Paranephrops zealandicus* to hypoxia and increased nutrients

4.1. Introduction

Crustaceans inhabit a diverse range of habitats, many of which are highly variable in terms of the physical and chemical environment. As such, survival in such variable settings has necessitated the adoption of a suite of behavioural, physiological and biochemical responses that allow them to adjust to changes in factors such as the levels of oxygen in the water (McMahon *et al*., 2002). Low dissolved oxygen (i.e. hypoxia) is particularly problematic in that if oxygen levels are too low, aerobic metabolism cannot be maintained. The most common organismal responses that allow survival in environmental hypoxia are: 1) reducing metabolic rate (which was observed for hypoxia-exposed *P. zealandicus* in Chapter 3); 2) utilisation of alternative pathways to produce ATP such as anaerobic metabolism; 3) having large fuel stores such as glycogen in the muscle and hepatopancreas to accommodate the lower energy efficiency of anaerobic metabolism; and 4) minimising metabolic acidosis by enhancing buffering capacity and altering haemolymph properties such as PCO_2, pH and PO_2 (Storey and Storey, 1990; Hervant *et al*., 1995; Lutz and Storey, 1996; Childress and Seidel, 1998; Hochachka and Lutz, 2001).

The circulatory system plays a particularly important role in hypoxic responses. Not only do changes in cardiovascular flow occur (see Section 2.4.2.), but the composition of the haemolymph may also play a role. Crustacean haemolymph functions as a regulator of biological function (e.g. through its transport of hormones), and it also transports nutrients and wastes. As such, changes in haemolymph are often a reflection of the physiological state of the animal (Lorenzon *et al*., 2001). Perhaps the most important function of the haemolymph is its ability to regulate acid-base balance, which is required for the optimisation/maintenance of membrane stability and enzymatic functions (Campbell, 1973; Henry and Wheatly, 1992; Pavasovic *et al*., 2004). Hypoxic conditions can, however, disturb acid-base balance as an accumulation of CO_2 and lactate result in acidosis (see Section 1.2.2.).

Unlike many other invertebrates, crustaceans only utilise one pathway of anaerobic glycolysis; the fermentation of glycogen to lactate (Bridges and Brand, 1980; Van Aardt 1988; Hervant *et al*., 1995). Thus, lactate is the main end product of anaerobiosis for crustaceans and increases once water oxygen levels fall below P_{CRIT}. Accumulation of haemolymph lactate is dependent on the severity and duration of hypoxia (Albert and Ellington, 1985), and has been previously used as an indicator of hypoxic stress in crayfish (Gäde, 1984; Mauro and Thompson, 1984; Morris and Callaghan, 1998; Jackson *et al*., 2001; da Silva-Castiglioni *et al*., 2010, 2011).

An increase in haemolymph glucose concentrations is also commonly used as an indicator of stress in aquatic animals (Barton and Iwama, 1991; Wendelaar Bonga, 1997) including crayfish (Telford, 1974; D'Agaro *et al.,* 2003). Haemolymph glucose reflects the dynamic equilibrium between the synthesis of glycogen (glycogenesis) and the breakdown of glycogen to glucose 6-phosphate (gluconeogenesis) (Randall *et al.,* 1967; Hall and van Ham, 1998). A reduction in glycogen is expected under hypoxia as it is broken down into glucose for fuel, which is therefore followed by an increase in haemolymph glucose (Taylor and Spicer, 1987; Van Aardt, 1988; Zou *et al*., 1996). Changes may also be observed in tissue glucose (either from changes in glycogen synthesis/break down), or because of increased/decreased plasma delivery.

In freshwater crayfish habitats, low oxygen levels are usually associated with eutrophication (Section 1.1.1). Consequently, hypoxia is likely to occur in concert with high ammonia and nitrite. Generally, ammonia is considered more toxic to decapod crustaceans than nitrite, as shown by its generally lower LC_{50} values (Chen and Lei, 1990; Chen *et al*., 1990a, b; Meade and Watts, 1995; Romano and Zeng, 2007a). This is mainly due to the disruption of ion transporters cause by ammonia (Section 1.1.1.) For example, the effect of ammonia on Na^+/K^+ ATPase can lead to an accumulation of haemolymph K^+ and reduction in haemolymph Na^+ (Needham, 1961; Sanders *et al.,* 1992; Remen *et al*., 2008).

In aquatic animals, the main mechanism of toxicity is by causing internal hypoxia (Section 1.1.1.). The ability of nitrite to competitively inhibit chloride uptake also reduces intracellular and extracellular chloride resulting in electrolyte imbalance (Lewis and Morris, 1986). Therefore, an animal's chloride uptake rate can impact nitrite toxicity (Section 1.1.1.). Nitrite toxicity also depends on many other factors such as the rate of nitrite accumulation, duration of exposure, temperature, and the presence of other stressors (Jensen, 2003).

Increased nutrients are stressors. Thus, animals exposed to high nutrient waters often respond to ammonia and nitrites as they would to stressors such as hypoxia. For example, nutrient-exposed animals show a lowered pH, reduced haemolymph PO_2, increase in lactate and an initial hyperglycaemic response followed by a decrease in glucose levels (Woo and Chiu, 1997; Jensen 2003; Yildiz and Benli, 2004; Romano and Zeng, 2007).

The responses to hypoxia and high nutrients have been investigated in many freshwater crayfish species (Woo and Chiu, 1997; Jensen, 1990; Hong *et al.,* 2009; Jiang *et al.,* 2014). By comparison, there has been little research in New Zealand on the impacts of hypoxia and high nutrients to freshwater crayfish (Titulaer, 1995: see Section 1.3.2). Thus, the aim of the study was to investigate the haematological and biochemical responses of hypoxia with and without exposure to high nutrients, in order to determine the effects of environmental eutrophication and resulting decrease in environmental oxygen on *P. zealandicus*. In this chapter, the impacts of low O_2 and high nutrients on haemolymph acid-base status were studied. In addition anaerobic capacity was investigated in *P. zealandicus*, as represented by measurements of muscle and haemolymph lactate and glucose.

4.2. Methods and Materials

4.2.1. Haemolymph parameters

P. zealandicus were held in laboratory conditions as described in Chapter 2. A total of 32 crayfish were exposed (some used for multiple assays) for 6 hours under different experimental treatments (weights and sample numbers in Table 4.1). There were no significant differences between the mass of crayfish assigned across experimental treatments ($P = 0.64$). Animals were exposed to five different treatments for six hours these were: control (155 mmHg), moderate hypoxia (30 mmHg: H30), severe hypoxia (10 mmHg: H10), severe hypoxia and high ammonia (10 mmHg and 30 mg L^{-1} of ammonia chloride: H10A30) and severe hypoxia and high nitrite (10 mmHg and 20 mg L^{-1} sodium nitrite: H10N20) All treatments were conducted in respirometers (see Section 2.3), and crayfish were acclimated for a minimum of 12 hours in the respirometer prior to experimentation. Immediately after animals had been exposed to each treatment (each exposure lasted for six hours)

haemolymph was removed from the base of the walking leg, and PO_2, PCO_2 and pH were measured following the procedures detailed in Chapter 2 (Section 2.5.1.). Haemolymph samples were snap-frozen in liquid nitrogen and stored at -80 °C for later analysis of lactate and glucose concentration.

4.2.2. Glucose and lactate concentrations

After euthanizing the crayfish the hepatopancreas and tail muscle were removed and immediately frozen as described in Section 2.5.2. To attain accurate readings of glucose and lactate levels, some initial testing was performed. During this testing stage it was shown that the hepatopancreas samples were unusable due to their colouration. This issue persisted even after adding polyvinylpolypyrrolidone (a decolourising chemical). Subsequently, hepatopancreas samples were not assayed for glucose and lactate. Following initial testing, glucose and lactate concentrations were measured in the tail muscle and haemolymph samples as described in Section 2.6.2. Some haemolymph samples and tail muscle samples were not able to be used (due to clotting or insufficient tissue). Thus, each exposure and sample type had different sample numbers as shown in Table 4.1.

Table 4.1: The average weights (g) and n values of crayfish for which haemolymph and muscle lactate and glucose analyses were conducted. Treatment codes defined above.

		Lactate		Glucose	
	Treatment code	n value	Average weight (g)	n value	Average weight (g)
Haemolymph	Control	3	38	4	37
	H30	5	45	6	44
	H10	4	46	3	46
	H10A30	7	41	8	42
	H10N20	5	46	5	46
Tail muscle	Control	5	40	5	39
	H30	6	44	5	45
	H10	4	43	4	43
	H10A30	6	42	5	40
	H10N20	5	46	5	46

4.2.3. Data analysis and statistical methods

Statistical analysis was carried out using the R statistical programme. Statistical significance was taken at the level of P value lower than 0.05. All data are presented as the mean ± standard error of the mean (SEM). Homogeneity of variance was tested by plotting the fitted values against the residual values. Normal distribution was tested with a quantile-quantile normality plot and histogram of the residuals. Grouped mean values that met these assumptions were compared using a one way ANOVA followed by a Tukeys HSD post hoc test. A residual analysis test showed that homogeneity of variance was met in all experiments except in the haemolymph lactate and PCO_2 experiments, thus, values were log transformed and grouped means were compared using a one way analysis of variance (ANOVA). Where a treatment effect was shown to be significant, post-hoc Tukeys HSD tests were performed.

4.3. Results

4.3.1. Haemolymph parameters

Haemolymph PO_2 in control crayfish was 32.22 ± 3.10 mmHg. In hypoxia, a significant reduction in PO_2 was observed, an effect exacerbated in the presence of high nitrite, which exhibited the lowest PO_2 (3.52 ± 0.66 mmHg) ($P < 0.05$). No statistically significant differences were seen between animals exposed to moderate hypoxia, severe hypoxia and those exposed to severe hypoxia in conjunction with high ammonia ($P > 0.05$)(Fig. 4.1).

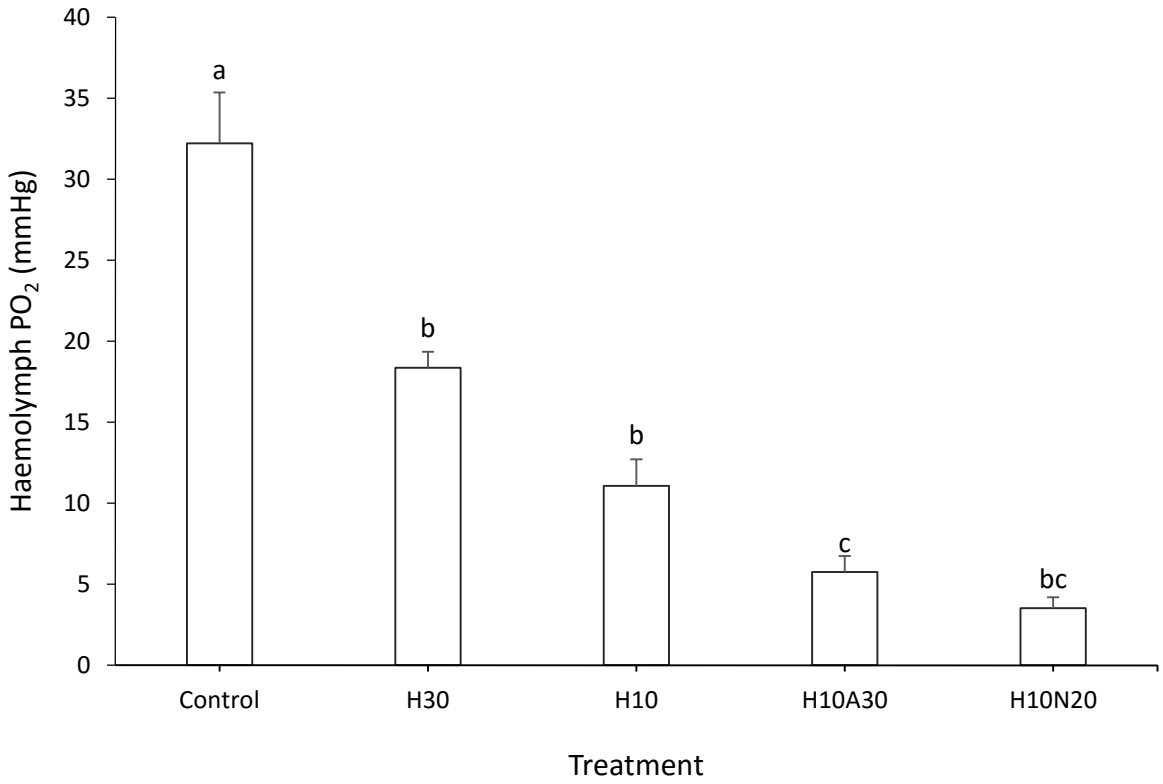

Figure 4.1: PO_2 levels in haemolymph of *P. zealandicus* from control, moderate hypoxia (H30, n=6), severe hypoxia (H10, n=6), severe hypoxia and high nitrite (H10N20, n=6) and severe hypoxia and high ammonia (H10A30, n=6) exposures. Plotted points sharing letters are not significantly different.

Haemolymph pH in control crayfish was 7.73 ± 0.27. There was a significant acidosis as a result of severe hypoxia alone (6.19 ± 0.40, Fig. 4.2). The addition of high nutrients

(ammonia and nitrite) to severe hypoxia resulted in haemolymph pH values that were not significant from either control or severe hypoxia alone values (6.88 ± 0.21 for severe hypoxia and ammonia, 6.93 ± 0.24 for severe hypoxia and nitrite) (Fig. 4.2).

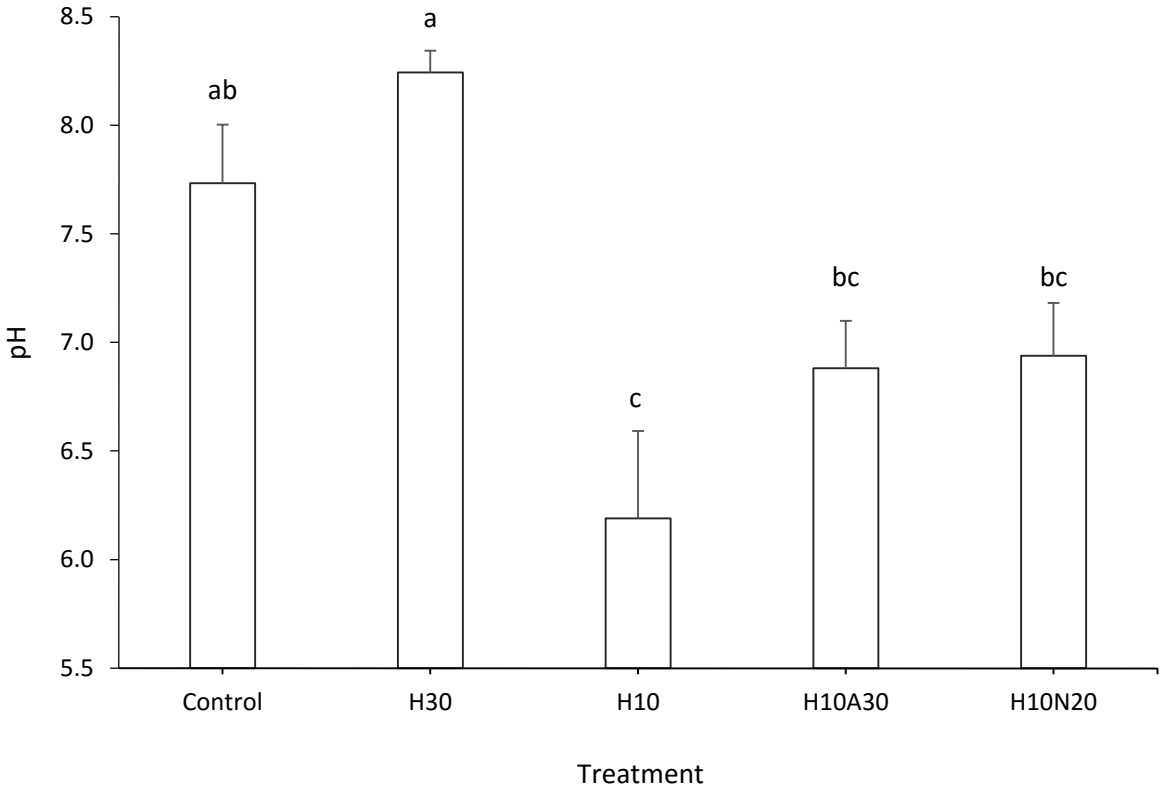

Figure 4.2: pH levels in haemolymph of *P. zealandicus* from control (n=6), moderate hypoxia (H30, n=6), severe hypoxia (H10, n=6), severe hypoxia and high nitrite (H10N20, n=6) and severe hypoxia and high ammonia (H10A30, n=6) exposures. Plotted points sharing letters are not significantly different.

Haemolymph PCO_2 of control animals (5.2 ± 1.7 mmHg) was not significantly different from any of the experimental exposures (Fig. 4.3). Crayfish exposed to moderate hypoxia and severe hypoxia in association with nitrite did, however, have significantly lower haemolymph PCO_2 values than those exposed to severe hypoxia ($P < 0.05$).

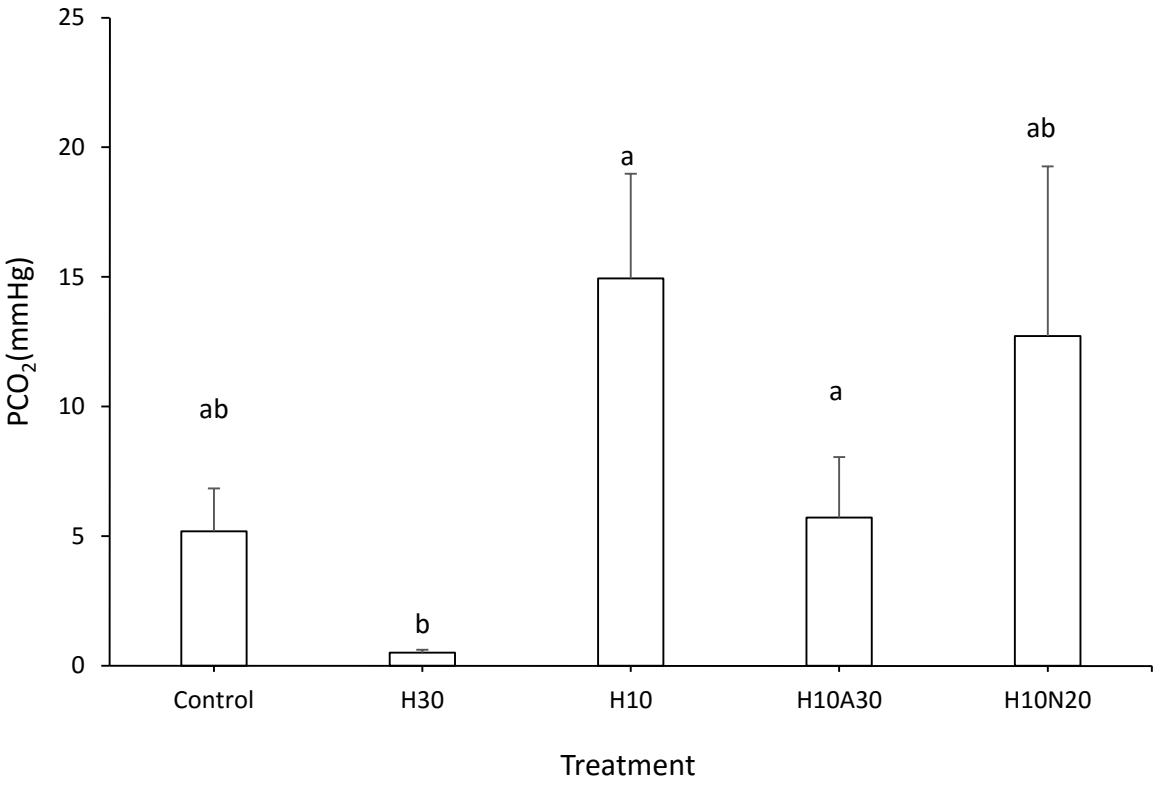

Figure 4.3: PCO_2 levels in haemolymph of *P. zealandicus* from different exposure types. Plotted points (n = 6) sharing letters are not significantly different.

4.3.2. Lactate and Glucose

Haemolymph lactate increased as severity of hypoxia increased (Fig 4.4). Significant increases were observed in *P. zealandicus* exposed to moderate (5-times increase) and severe (50-times increase) hypoxia, compared to the normoxic control. The greatest increase in lactate was observed in the severe hypoxia and nitrite treatments which showed a 60-times increase over haemolymph from control crayfish ($P < 0.05$). No differences in haemolymph lactate were observed between crayfish exposed to different severe hypoxia treatments (i.e. no effect of nutrients; Fig. 4.4).

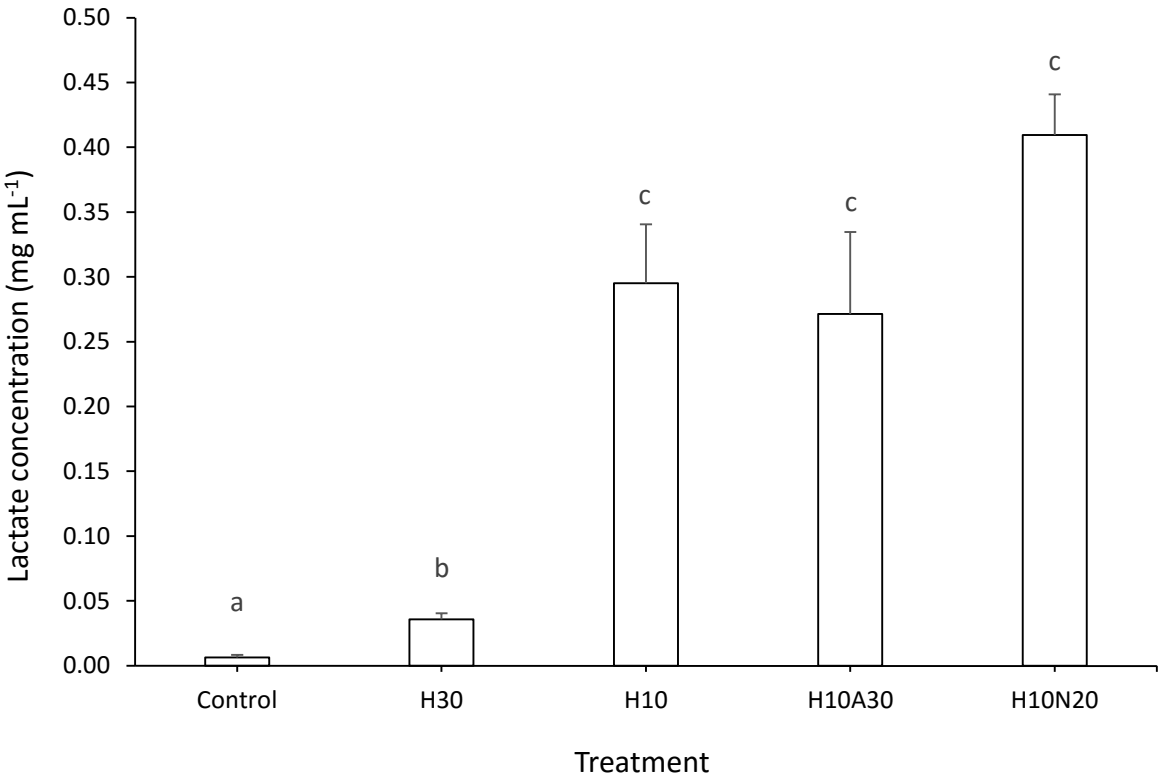

Figure 4.4: Lactate levels in haemolymph of *P. zealandicus* from different exposure types. Plotted points sharing letters are not significantly different. See Table 4.1 for n values.

In hypoxia, *P. zealandicus* haemolymph glucose levels significantly decreased, with the lowest concentration found in crayfish exposed to severe hypoxia and high nitrite (Fig. 4.5). This levels was approximately one third of those recorded in the control group ($P < 0.05$). No differences were observed between the other treatments (Fig. 4.5), but all were significantly lower than the control.

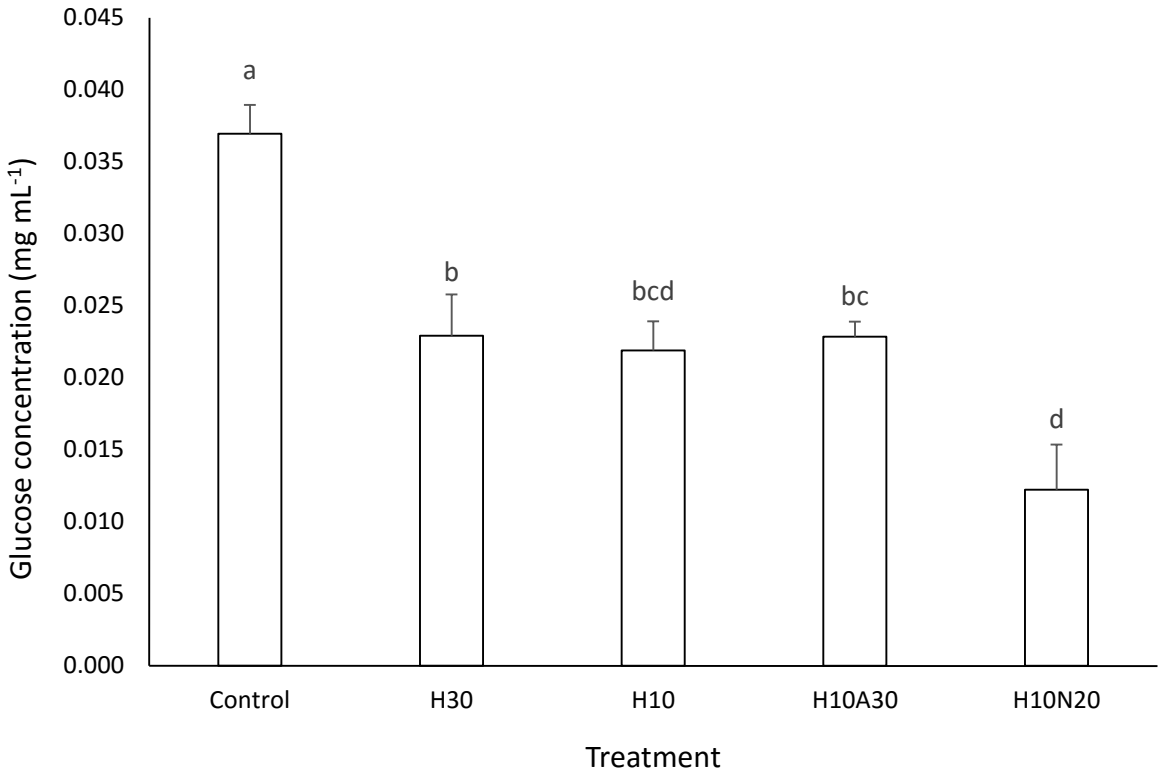

Figure 4.5: Glucose levels in haemolymph of *P. zealandicus* from different exposure types. Plotted points sharing letters are not significantly different. See Table 4.1 for n values.

Tail muscle lactate of *P. zealandicus* did not vary significantly between the treatments (P >0.05) (Fig. 4.6). A similar effect was seen for glucose (P >0.05) (Fig. 4.7). The muscle glucose levels of crayfish exposed to H30 was almost significantly higher than controls (P =0.057).

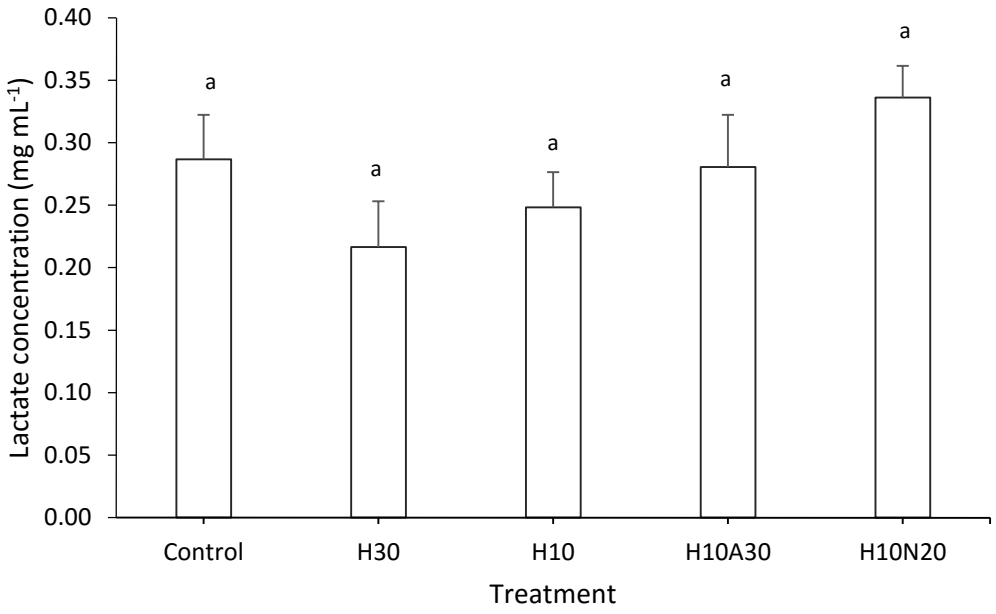

Figure 4.6: Lactate levels in tail muscle of *P. zealandicus* from different exposure types. Plotted points sharing letters are not significantly different. See Table 4.1 for n values.

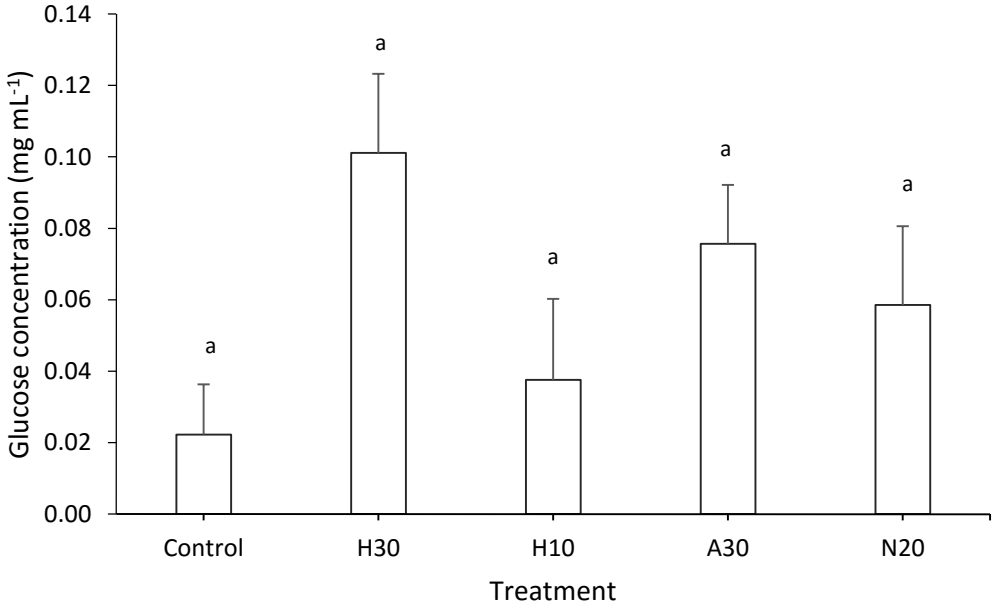

Figure 4.7: Glucose levels in tail muscle of *P. zealandicus* from different exposure types. Plotted points sharing letters are not significantly different. See Table 4.1 for n values.

4.4. Discussion

4.4.1. Haemolymph gases

In the current study haemolymph PO_2 dropped as low as 3.5 mmHg in response to severe hypoxia. Such a response is consistent with the majority of crustacean literature (Taylor, 1976; McMahon, 1986 for reviews). In many crustaceans, this reduction increases the utilisation of the venous oxygen reserve in an attempt to increase the oxygen transfer factor by creating a favourable gradient for oxygen diffusion (McMahon *et al.,*, 1974; McMahon, 1988).

Control crayfish had close to three times the haemolymph PO_2 (32 mmHg) than crayfish exposed to severe hypoxia (11 mmHg). Although these absolute values for PO_2 in the haemolymph of *P. zealandicus* are much lower than recorded in other species, the ratio between the two exposure conditions is comparable to previous studies. Although a decrease in haemolymph PO_2 is the standard crustacean response, an increase in haemolymph PO_2 or no change at all under hypoxic conditions has also been observed in some species (Butler *et al*., 1978. These differences in haemolymph PO_2 responses have been attributed to differences in exposure time, the severity of hypoxia and other respiratory and haemolymph adjustments that occur in concert with hypoxia.

Exposure of *P. zealandicus* to severe, but not moderate, hypoxia resulted in a significant disturbance of the haemolymph acid-base balance (Fig. 4.2). Many animals exposed to prolonged hypoxia experience a deleterious reduction in haemolymph pH known as acidosis. In most cases this is thought to be mainly due to the inability to excrete CO_2 due to a reduction in gas exchange (Lutz and Storey, 1996). This disturbance in haemolymph acid-base status can impair normal enzymatic functioning and regulation of metabolic processes. Corroborating the results of the current study, Mauro and Thompson (1984) also showed a drop in pH at 15 mmHg in the crayfish, *Procambarus clarkii*. Hypoxia-induced haemolymph acidosis has also been seen in *Macrobrachium rosenbergii* (Chen and Kou 1998) and *Cancer productus* (McMahon *et al*., 1984) where it was not only attributed to the absence of hyperventilation, but also to the accumulation of lactate. As discussed below (Section 4.4.2.),

haemolymph lactate levels were elevated significantly in severe hypoxia, and so were likely to be a significant driver of the acidosis observed in *P. zealandicus* at very low PO_2.

The extent of acid-base disturbance has been shown to be dependent of the severity of hypoxia. Acidosis is usually seen only in hypoxia-stressed animals that have lost the ability to buffer against this decrease in pH. This suggests *P. zealandicus* exposed to moderate hypoxia at 30 mmHg, which is below this species $P_{CRIT,}$ (Section 3.3.1.) were not significantly impacted, and the cascade of biochemical effects leading to acidosis were not induced. Exposure concentration-dependent acid-base disturbance has also been observed in *Palaemon elegans* and *P. serratus* (Taylor and Spicer, 1987).

In the current study, crayfish haemolymph parameters under severe hypoxia were minimally impacted by the addition of nitrite and ammonia. In fact the only significant difference between severe hypoxia alone and the addition of nutrients to severe hypoxia was that the haemolymph PO_2 in the presence of nitrite was significantly lower. An effect of nitrite was anticipated owing to the disruptive effect of this nutrient on oxygen transport and animal oxygen status (Section 1.1.1.1.).

The relative lack of effect of nutrients on haemolymph PCO_2 and pH was surprising as the addition of stressors usually exacerbates impacts. However, the acid-base disturbances of animals exposed to nutrients and hypoxia is not well researched. Variability in the data could explain the lack of significance, thus, a greater sample number may have been required to elucidate effects. Previous research has shown a reduction in haemolymph pH in *M. rosenbergii* (Chen and Lee, 1997), *P. monodon* (Cheng and Chen, 1999), *P. leniusculus* (Harris and Coley, 1991), and *M. japonicus* (Cheng and Chen, 2002a) exposed to elevated nitrite levels. Conversely, *A. astacus* showed an increase in pH when exposed to nitrite (Jensen, 1990). This increase was attributed to a maintained hyperventilation that led to reduced haemolymph CO_2 levels.

Studies on how ammonia exposure alters haemolymph pH are sparse. However, Chen and Cheng (1993) showed decreased pH on exposure to ammonia levels of 20 mg L^{-1} in *Penaeus monodon*. This same study showed a significant decrease in PCO_2 in animals exposed to ammonia. Similarly, nitrite has also been shown to elicit a decrease in PCO_2 (Jensen, 1990). Cheng *et al.*(2013) showed both nitrite and ammonia reduce PCO_2 and pH in kuruma shrimp (*M. japonicus*). These findings were linked to ability of ammonia to competitively inhibit H^+ in the Na^+/H^+ exchanger (Weihrauch *et al.,* 2004) which increases haemolymph H^+

concentration, thus decreasing pH (Wash *et al*., 1989; Cheng and Chen, 2013). All of these data are based on exposing animals to hypoxia or high nutrients alone and not on combined exposures, as in the current study. It is possible that the haemolymph values measured under severe hypoxia represent the fullest extent of change in this parameter given that crustacean blood is relatively well buffered owing in part to the role that haemocyanin has in this process (Paul and Pirow, 1997), and thus addition of ammonia could have no further impact.

4.4.2. Lactate

Crustaceans show increases in haemolymph lactate concentrations in response to hypoxic conditions (Gade, 1984; Morris and Callaghan, 1998; Fujimori and Abe, 2002; da Silva-Castiglioni *et al*., 2010). These increases are seen below P_{CRIT} and are an indication of anaerobic metabolism (Bridges and Brand, 1980; Albert and Ellington, 1985; Taylor and Spicer, 1987; Henry *et al*., 1990; Hagerman *et al*., 1990; Zou *et al*., 1996). In the current study, haemolymph lactate increased under both moderate (0.03 mg mL^{-1}) and severe (0.3 mg mL^{-1}) hypoxia relative to control levels (0.006 mg mL^{-1}) indicating anaerobic metabolism was being employed. The general response in crustaceans is that moderate hypoxia, similar to that employed in the current study, does not cause lactate accumulation and an increase is usually only observed in severe hypoxia (Taylor and Spicer, 1987; Hagerman *et al*., 1990). In a similar experiment to the current study, haemolymph lactate levels in *Procambarus clarkii* showed a much larger increase from 0.06 mg mL^{-1} to 0.8 mg mL^{-1} after exposure to 15 mmHg for 5 hours (Mauro and Thompson, 1984). In the shrimp *P. vannamei* a 4-fold increase in lactate levels was seen after 3 days at 20 mmHg (Racotta and Hernández-Herrera, 2000).

Most species regulate MO_2 until PO_2 levels reach a critical point (P_{CRIT}). At this point they start to oxyconform, whereby oxygen consumption drops with declining PO_2. Unless the animal takes measures to decrease metabolic needs, this drop in MO_2 will coincide with the generation of energy aerobically, leading to lactate accumulation. Thus, the observed increase in *P. zealandicus* haemolymph lactate concentrations from both moderate and severe hypoxia treatments suggests the PO_2 is below the animals P_{CRIT}. Morris and Callaghan (1998) also observed an increase in haemolymph lactate in the freshwater crayfish *P. clarkii* in oxygen levels below $P_{CRIT.}$ That the onset of lactate build-up in *P. zealandicus* occurs at

higher dissolved oxygen levels than in other species, suggests a relatively poor ability to maintain MO_2, and coincides with the findings from the physiology experiments (Chapter 4).

In the present study, haemolymph lactate of animals exposed to high nutrients and severe hypoxia was elevated above control crayfish but did not increase any more than lactate levels of crayfish exposed to severe hypoxia alone. Although not performed in the current study, exposure to only ammonia or nitrite appears to result in similar lactate accumulation patterns as in hypoxia-exposed animals. For example, increases in haemolymph lactate are observed in the goldfish (*Carassius auratus*), the sea bass (*Lates calcarifer*), and the freshwater prawn (*Macrobrachium nipponense*) exposed to nitrite (Woo and Chiu, 1997; Sinha *et al*., 2012; Jiang *et al*., 2014). The lack of difference in haemolymph lactate between the severe hypoxia treatments with or without nutrients suggests the addition of nutrients did not exacerbate anaerobic metabolism.

Muscle lactate accumulation has been seen in many animals exposed to hypoxia (Zou *et al.,* 1996; Marqueze *et al.,* 2006). However, in the current study there was no significant increase in muscle lactate. Although lactate is stored in the muscles, it diffuses to the haemolymph to be metabolised (Paterson, 1993). This could explain the lack of change in muscle lactate but high lactate accumulation in the haemolymph. However, the results of the current study are not without literature support, as muscle of the shrimp *Penaeus vannamei* also did not accumulate lactate when exposed to three days under hypoxic conditions (2–2.6 mg L^{-1}) (Racotta and Hernández-Herrera, 2000). The reduced accumulation of lactate could also be explained by diffusive loss of lactate into the water.

4.4.3. Glucose

The resting haemolymph glucose concentrations of *P. zealandicus* (0.037 mg mL^{-1}) were comparable to those of the freshwater crayfish *C. destructor* (0.03 mg mL^{-1}) (Morris and Callaghan, 1998) and other crustacean species (0.05- 0.13 mg mL^{-1}) (Taylor and Spicer 1987; Onnen and Zebe 1983;Van Aardt 1988). Under hypoxic conditions in the current study, a hypoglycaemic response was observed. This differs to the standard response to hypoxia in crustaceans, with shrimp (Racotta and Hernández-Herrera, 2000), crab (Zou *et al*., 1996), lobster (Ocampo *et al*., 2003) and crayfish (da Silva-Castiglioni *et al*., 2010) all showing hypoxia-induced hyperglycaemia. This increase is thought to function as physiological

preparation to the impending high substrate demands of anaerobic glycolysis (Zou *et al*., 1996; Oliveira *et al*., 2001; da Silva-Castiglioni *et al*., 2010, 2011; Marqueze *et al*., 2011).

While uncommon, others have reported deviations in this standard hyperglycaemia response to hypoxia. For example, in a study by Zou *et al.* (1996) haemolymph glucose initially increased to a peak level and then decreased in the hypoxia-exposed freshwater crab, *Eriocheir sinensis*. Similarly, after an initial increase, haemolymph glucose levels returned to those during normoxia in the hypoxic crayfish *Parastacus defossus* (da Silva-Castiglioni *et al*., 2010) and intertidal crab *Chasmagnathus granulata* (Santos and Colares, 1986). These differences in responses to the standard hyperglycaemia have been attributed to differences in exposure conditions and species (Das *et al.,* 2004; Bonvillain *et al.,* 2012). These studies do show, however, that the temporal pattern of haemolymph glucose is fluid, and indicates that spot sampling after six hours of exposure as in the current study may be insufficient to attain a true picture of haemolymph glucose dynamics.

Hyperglycaemia is often associated with a reduction of tissue glycogen stores (Taylor and Spicer, 1987; Van Aardt, 1988; Zou *et al*., 1996). Unfortunately, glycogen concentrations were not measured in the current study. However, this would be a useful analysis as it would provide a broader picture of glucose metabolism. The data generated from the current study could be interpreted as a rapid mobilisation of glycogen to glucose, followed by an equally rapid conversion of glucose to lactate via anaerobic metabolism. Supporting this concept, Muusze *et al*. (1998) also suggested the observed hypoxia induced hypoglycaemia in the cichlid, *Astronotus ocellatus,* indicated substrate mobilisation was unable to keep up with glycolysis. However a decline in tissue glycogen would be required to confirm this hypothesis. Alternatively, it has also been suggested (Bonvillain *et al*., 2012) that decreases in haemolymph glucose in animals exposed to moderate hypoxia may be a consequence of glycogen resynthesis. However, the utility of such a response in a hypoxic animal is unclear.

In response to stress animals often show an increase in muscle glucose (Jiang *et al.,* 2014). However, similar to the muscle lactate results in the present study, hypoxia did not alter the amount of glucose in the muscle. This is also consistent with the findings of unaltered muscle glucose in hypoxia-exposed *P. defossus* (da Silva-Castiglioni *et al.,* 2010). Glucose is produced in the muscle from glycogen and diffuses into the haemolymph for further use and transportation (Bridges and Brand, 1980; Gade, 1984). If lactate and glucose

had already diffused into the haemolymph this could explain the lack of difference, again indicating the importance of examining tissue responses over time.

Previous reports on the changes in haemolymph glucose in response to nutrients are contradictory, showing either an increase (Yildiz and Benli, 2004; Hong *et al*., 2009; Jiang *et al*., 2014), or decrease (Woo and Chiu, 1997; Remen *et al*., 2008). In the present study, both hypoxic exposures alone and with the addition of nutrients resulted in a reduction in haemolymph glucose. This reduction was particularly prominent in nitrite-exposed crayfish, which had half the amount of glucose (0.01 mg mL^{-1}) compared to other non-control groups. A 50% reduction in haemolymph glucose relative to control animals has been previously observed in ammonia-exposed Atlantic cod (*Gadus morhua*; Remen *et al.,* 2008).

The impact of aquatic nutrients on glucose levels appears to depend on exposure time and concentration. For example, Park *et al*. (2007) observed a reduction in haemolymph glucose in rockfish (*Sebastes inermis*) exposed to 200-700 mg L^{-1} of nitrite for 48 hours. However, this was followed by an increase after 96 hours. Whether an increase in glucose would have eventually occurred under longer exposure in *P. zealandicus* is unknown. This rockfish exposure concentration is, however, also much greater than that of the current study. Lower levels of 50 mg mL^{-1} of nitrite were also tested in rockfish, and showed the opposite pattern (an increase after 6 hours followed by a decrease from hour 12-96; Park *et al*., 2007). A similar study to that of Park and colleagues (2007) was conducted on fingerlings of mrigal (*Cirrhinus mrigala*) using the same exposure times but much lower nitrite concentrations (1-10 mg L^{-1}; Das *et al*., 2004). In this study, all exposure concentrations showed an initial decrease in blood glucose followed by an increase. However, it should be noted that glucose concentrations after 96 hours in both studies (Das *et al*., 2004 and Park *et al*., 2007) were not significantly different from controls. Das *et al*. (2007) suggested that the depletion of glucose through metabolism was greater than the production in glucose that would have occurred through glycogenolysis on initial exposure to high nitrite waters. This could explain why, in the current study, haemolymph glucose was lowest in crayfish exposed to low oxygen and high nitrites.

Ammonia concentration has also been seen to alter haemolymph glucose. Chetty and Indira (1995) observed a reduction in haemolymph glucose after exposing the mussel *Lamellidens marginalis* to 10 mg L^{-1} of ammonia for 48 hours. This is similar to the response to ammonia in the presence of severe hypoxia in the current study. However, when ammonia

concentrations were increased to 176 mg L^{-1} a significant hyperglycaemia was observed in *L. marginalis*. This confirms again that exposure concentration is likely to be a significant factor shaping responses of haemolymph glucose to stressor exposure.

The combined effects of hypoxia and nutrients on haemolymph glucose of freshwater crayfish in the current study were minimal. In a previous investigation (Remen *et al*., 2008) haemolymph glucose concentrations of *Gadus morhua* were not affected by the combined effects of ammonia and hypoxia, supporting the presented findings.

4.5. Summary

In response to low oxygen levels *P. zealandicus,* like many other crustaceans, appear to employ biochemical mechanisms to compensate for the deleterious conditions. These responses were much less exaggerated in animals exposed to moderate hypoxia compared with severe hypoxia. Acidosis was observed with severe hypoxia, likely a failed response to regulate gas exchange. Anaerobic metabolism was activated under hypoxic conditions as shown by an increase in haemolymph lactate, likely fuelled by the rapid conversion of glycogen-derived glucose to lactate, resulting in a hypoglycaemia. High ammonia and nitrite appear to have a minimal effect on the hypoxic responses of *P. zealandicus.* The competence of the biochemical responses, along with the physiological responses (Chapter 3), suggest a high tolerance of the New Zealand freshwater crayfish to moderate hypoxia. These experiments are, however, conducted in an environment where crayfish were unable to avoid these deleterious conditions. If an animal could avoid these responses it could possibly tolerate even lower oxygen levels.

Chapter Five

5. Behavioural responses of freshwater crayfish *Paranephrops zealandicus* to hypoxia.

5.1. Introduction

Aquatic animals engage in a multitude of physiological and biochemical mechanisms to combat low oxygen (as seen in Chapter 3 and 4, respectively). However behavioural strategies may also be employed to counter deleterious effects of hypoxia (Richards, 2009). The ability to detect and avoid hypoxic waters has been seen in many aquatic species including fish (Jones, 1952; Spoor, 1990; Wannamaker and Rice, 2000; Brady *et al*., 2009; Herbert *et al*., 2011; Lefevre *et al*., 2011; Poulsen *et al*., 2011), shrimp (Renaud, 1986; Das and Stickle, 1993) and crabs (Pihl *et al*., 1991). Not only can an animal avoid hypoxia by migrating away from such waters, some species, especially crustaceans, emerge out of the water and into the air (Taylor and Butler, 1973; McMahon and Wilkes, 1983). Avoiding hypoxic conditions is adaptively significant because aerobic metabolism is much more efficient in terms of the cellular energy generated and because anaerobic metabolism, the consequence of not avoiding hypoxia, leads to the potential build-up of toxic waste products (Hochachka and Somero, 2002). It has been postulated that there is a link between tolerance to hypoxia and the number of times and oxygen level at which compensatory behavioural responses are initiated (Richards, 2010). The basis for this assumption is that if an animal can elicit responses that give them access to more oxygen the animal would be subjected to less hypoxia-induced stress. Many studies have shown that animals will elicit behavioural responses such as avoidance when oxygen levels fall to a level at which the animal can no longer maintain metabolic rate (Schurmann and Steffensen, 1997; Claireaux *et al*., 2000; Chabot and Claireaux, 2008). Neither the degree to which behavioural responses are elicited under hypoxia, nor whether these responses alter hypoxia tolerance is known for *P. zealandicus*.

Closed-box respirometry was used to examine physiological and biochemical responses to hypoxia (Chapter 3 and 4). Under these conditions crayfish were unable to escape hypoxia as they did not have access to any refuge (e.g. more oxygenated waters, or

air). In contrast, in their natural habitats animals may have the opportunity to escape hypoxia (Schurmann and Steffensen, 1994; Herbert and Steffensen, 2005; Johansen *et al*., 2006; Chapman and McKenzie, 2009). There is, however, variation between the type of avoidance response and when it is elicited. For example, shrimp (*Penaeus* sp.) presented with hypoxic conditions will either: 1) burrow into the substratum; 2) swim above or around the hypoxic water mass; or 3) delay their migration until the hypoxic episode has passed (Egusa and Yamamoto, 1961; Renaud, 1986). In fish, Herbert *et al*. (2011) found no hypoxia avoidance in Atlantic cod, *Gadus morhua*, when they were presented with a free choice of normoxia and progressively declining oxygen. However, when given a choice between the same progressively declining oxygen *versus* a refuge with water of lower oxygen, cod avoided the most hypoxic water.

In particular circumstances migrating away from hypoxic waters may not be an option. This will occur, for example, where animals are stranded in small stagnant pools of water, or caught in waters with excessive microorganism respiration and oxygen levels are quickly consumed (Naylor, 1962; Wheatly and Taylor, 1981). In such cases, some aquatic animals have adaptations that allow them to leave the water and use atmospheric oxygen. Crustaceans, for example, have the ability to exploit aerial respiration by either partially or fully emerging out of the water (Batterton and Cameron 1978; Taylor and Wheatly 1980; Greenaway *et al*. 1983). The variations between emergence responses (see Section 1.2.3) are thought to be due to anatomical and habitat differences. Irrespective of the degree of emergence, this behavioural response likely enables an animal to obtain adequate oxygen from air, relative to its oxygen uptake abilities in hypoxic waters (Taylor *et al.,* 1973; Wheatly and Taylor, 1979; McMahon and Wilkes, 1983).

The issue with emerging into air is that, unless a species is adapted for both aerial and aquatic respiration, efficiency of oxygen uptake is impaired due to the collapse of the gills and acidosis ensues (see Section 1.2.3.) (deFur and McMahon 1984). Along with the risk of acidosis there is also the risk of predation and desiccation (Taylor and Butler, 1973; McGaw, 2009; Herbert *et al*., 2011). Thus, emergence is often a last resort in an attempt to escape hypoxia. However, there are cases where emerging onto land is a voluntary venture despite its consequences. For example, freshwater crustaceans voluntarily emerge onto land to avoid aquatic predation (McGaw, 2009), escape increased population density (Titulaer, 1995), to forage on land (Huxley, 1884) or to migrate overland to colonise new bodies of water

(Williams and Hynes, 1976). Emergence has been observed in *P. zealandicus* in response to hypoxic conditions (Titulaer, 1995), albeit over a chronic 84 hour exposure period.

The experimental design used in the current study was based on that from Cook and Herbert (2012), Herbert *et al.* (2011), Cook *et al.* (2013) and Poulsen *et al.* (2011) where fish were placed into a choice chamber with varying PO_2 on either side. However adjustments were made to exposure times and PO_2 combinations in an attempt to give more realistic measurements of avoidance. Behavioural responses to hypoxia could have important ecological implications for *P. zealandicus* which are exposed to frequent hypoxic episodes as a result of eutrophication (Hamill and McBride, 2003; Usio and Townsend, 2001). The populations of this important species are declining and environmental hypoxia could be a factor driving this phenomenon. However, the hypoxia avoidance behaviour of freshwater crayfish in general, let alone *P. zealandicus*, has been minimally studied (Bierbower and Cooper, 2010). The aim of the present study was to investigate the ability of *P. zealandicus* to choose between differing oxygen levels. Whether the presence of an oxygen refuge altered behavioural responses to hypoxia (e.g. *G. morhua*; Herbert *et al*., 2011) was also investigated. Finally, acute emergence responses of *P. zealandicus* to exponentially declining PO_2 were explored. In this experiment emergence was defined as the complete emergence of the crayfish out of the water for more than 4 minutes. Full, rather than partial, emergence suggests the animal is prepared to tolerate the predation, desiccation and physiological implication that comes with emersion. Thus, the level at which the crayfish emerges should represent the lowest oxygen level it can tolerate.

5.2. Methods and materials

Behavioural observations were made on 41 *P. zealandicus* males (average mass of 39.4± 3.4 g). All animals were obtained from Sweet Koura Enterprises crayfish farm. Experiments were conducted in a 15 °C temperature controlled room located in the School of Biological Sciences, University of Canterbury in March 2014. Each crayfish was only used once and individual crayfish were acclimated to individual chambers 12 hours prior to experimentation. The water in the chamber was replaced after each experiment.

5.2.1. Avoidance test chamber

A hypoxia choice chamber similar to that described in Cook *et al*. (2011) and Cook and Herbert (2012) was used to examine the behavioural responses of individual *P. zealandicus* to various low oxygen protocols (Fig. 5.1 and 5.2). The chamber (100 cm long x 50 cm wide x 30 cm deep) was filled with fresh well water up to a height of 20.75 cm before the water flowed out of two openings at the end of the tank. This water was not recycled as in similar experiments (Cook *et al*., 2011; Cook and Herbert, 2012), making it a flow-through system. This precluded any stress or waste chemicals from altering the behavioural choice. The behavioural arena (BA) (50 cm long x 30 cm wide x 30 cm deep) was supplied with water from reservoirs with two parallel rectilinear flows of water. The BA was not separated by a partition like the rest of the chamber which allowed the crayfish to move freely between each side. Flow rate was controlled by taps in the reservoir containers and was monitored before experimentation. Water in the reservoirs was supplied from a bucket which was constantly being filled from a well reserve. Water was siphoned through plastic tubing from the bucket to the reservoirs. Water flowing from each of the two reservoirs into the experimental chamber passed through baffles and honeycomb diffusers. The baffles were made from a series of shortened straws placed into a Perspex square frame which was placed in front of the diffusers (Fig. 5.2 In preliminary tests, the addition of food colouring to the inflowing water showed distinct separation of flows of each side (Fig. 5.3). These preliminary tests determined the rate of flow (5.88 L min^{-1}) which was fast enough to have water separation but slow enough to allow the reservoirs to be continuously full. At the end of the chamber, two outflows led exiting water through plastic tubing into a drain in the floor. A webcam that streamed video to a computer was placed on a tripod over the BA. The webcam was run through Microsoft movie maker and all behaviour was continuously recorded.

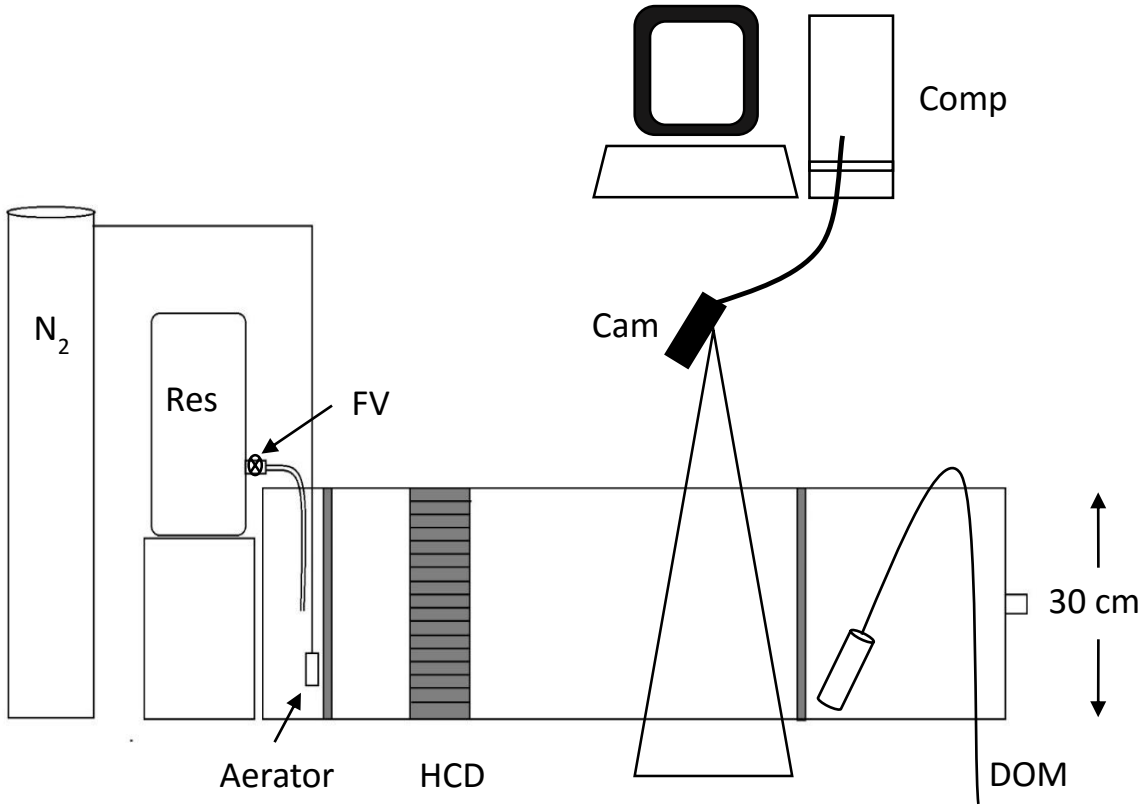

Figure 5.1: Diagrammatical representation of the avoidance chamber apparatus- side view. Water flow passing through reservoirs (Res) into the top end of the chamber was controlled using the flow valve (FV) where water was then either aerated or deoxygenated with nitrogen gas (N_2). A video camera (Cam) placed above the behavioural arena was connected to a computer (Comp) enabled observation throughout the experimental procedure. A dissolved oxygen meter (DOM) placed at the bottom end of the chamber allowed for measurement of water PO_2.

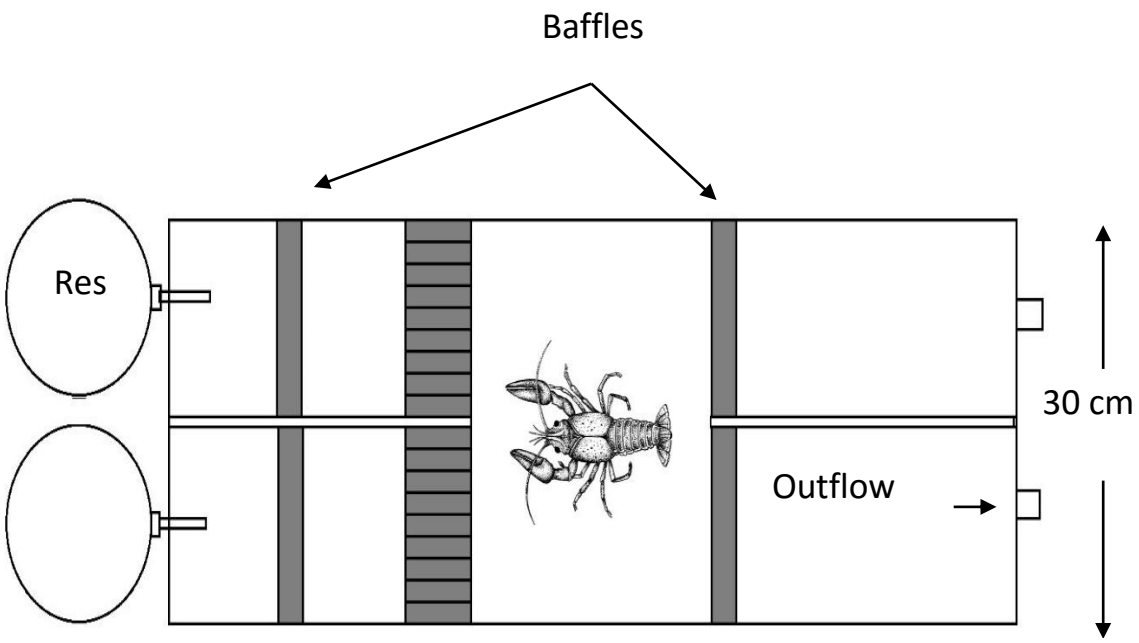

Figure 5.2: Diagrammatical representation of the avoidance chamber apparatus- birds eye view. Water flowed from the reservoir (Res) into the top end of the chamber initially passing through a set of baffles and honeycomb diffusers (HCD) creating laminar flow within the behavioural arena (BA) and out via the two outflow openings. Separate water flows were maintained by a plastic division at the top and bottom ends of the chamber.

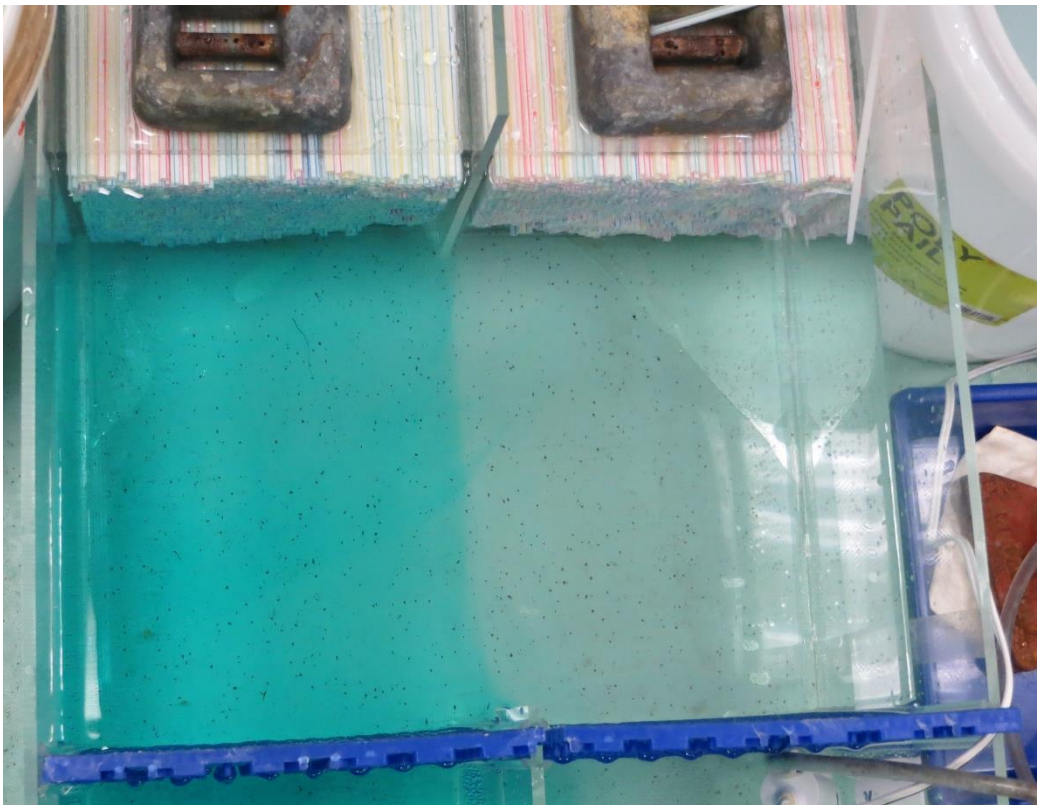

Figure 5.3: Photograph showing the dye test and distinct separation of recti-linear flows on each side.

During acclimation and in control experiments, water on both sides of the chamber was supplied with compressed air for full aeration through fine bubble diffusers. However, during experimental hypoxia this was replaced with a flow of compressed nitrogen. Preliminary tests were completed to determine the flow of nitrogen required to create the 3 hypoxia levels (90, 60 and 30 mmHg). However, it was found that even at the same flow rate, different gas tanks and gas regulators altered the water PO_2 levels differently. Therefore, the water PO_2 was monitored before experimentation and 30 minutes into experimentation and the flow rate changed accordingly. Furthermore, polystyrene blocks were fitted into the gaps between the baffles and honeycomb diffusers to reduce gas exchange between the water and air interface, allowing the lowest tested hypoxia level (30 mmHg) to be maintained.

5.2.2. Experimental design of avoidance experiments

Behavioural observations were conducted on eight crayfish exposed to control (fully oxygenated) conditions on both sides of the tank. Previous studies had shown that hypoxia avoidance differs depending on the water PO_2 it is paired with (Cook and Herbert, 2012; Herbert *et al*., 2011). Therefore, a number of experimental protocols were tested: 155 *vs.* 90, 155 *vs.* 60, 155 *vs.* 30, 90 *vs.* 60, 90 *vs.* 30 and 60 *vs.* 30 mmHg. Each individual crayfish (n = 8) was exposed to all 6 protocols in one day. The protocol order was randomised and no crayfish was exposed in the same order. This controlled for the effect of any daily rhythms that may have impacted the results. To avoid the chance of crayfish preferring a particular side, each oxygen treatment protocol was tested twice, alternating the PO_2 levels between each side. The combination of every oxygen level for each crayfish is shown in Table 5.1. Half the replicates were tested with particular PO_2 levels on the left side and the other half were tested to that same PO_2 on the opposite side.

Based on the flow rate and the chamber dimensions it was calculated it would take 30-46 minutes for the hypoxic water to flow from one end of the chamber to the other. Therefore, each protocol ran for 2 hour (1 h for the water PO_2 to equilibrate and a 1 hour testing period). Cook *et al.* (2013) found that a 1 hour testing period was long enough for fish to show behavioural preferences due to physiological (stress) responses, but short enough that they could not acclimate to hypoxia. Prior to each protocol change the BA water PO_2 levels were measured and then the nitrogen gas flow rate was changed. The water PO_2 levels

were measured again 30 minutes into the equilibration hour and the flow rates were altered if the PO_2 was incorrect. At the 30 minute point the webcam was tuned on and the crayfish were filmed. This meant that the crayfish were undisturbed for 90 minutes. At the end of the 2 hours the webcam was turned off, the water PO_2 levels were measured and the process started again for the new hypoxia protocol. Thus, it took a total of 12 hours to run through each of the protocols for each individual crayfish (6 protocols x 2 hours each). Recordings always commenced at the same time of day (9am).

Table 5.1: The testing protocol for each crayfish, including on which side each PO_2 level (mmHg) was employed. Protocols were, however, tested in random order.

Protocol	Left side	Right side
155/90	155	90
	90	155
155/60	155	60
	60	155
155/30	155	30
	30	155
90/60	90	60
	60	90
90/30	90	30
	30	90
60/30	60	30
	30	60

5.2.3. Emersion test chamber

The emersion response to declining oxygen levels was observed in unrestrained animals in freshwater at 15 °C. The emersion chamber is represented in Fig. 5.4. One corner of the chamber along with the sides of the chamber were shaded with black plastic to minimise visual disturbance and to ensure crayfish were not emerging in response to external cues. A Perspex platform was secured in the tank and allowed crayfish to climb above the water level. Similar to the avoidance experiments a camera was connected to a computer, and was attached to a tripod placed over the tank.

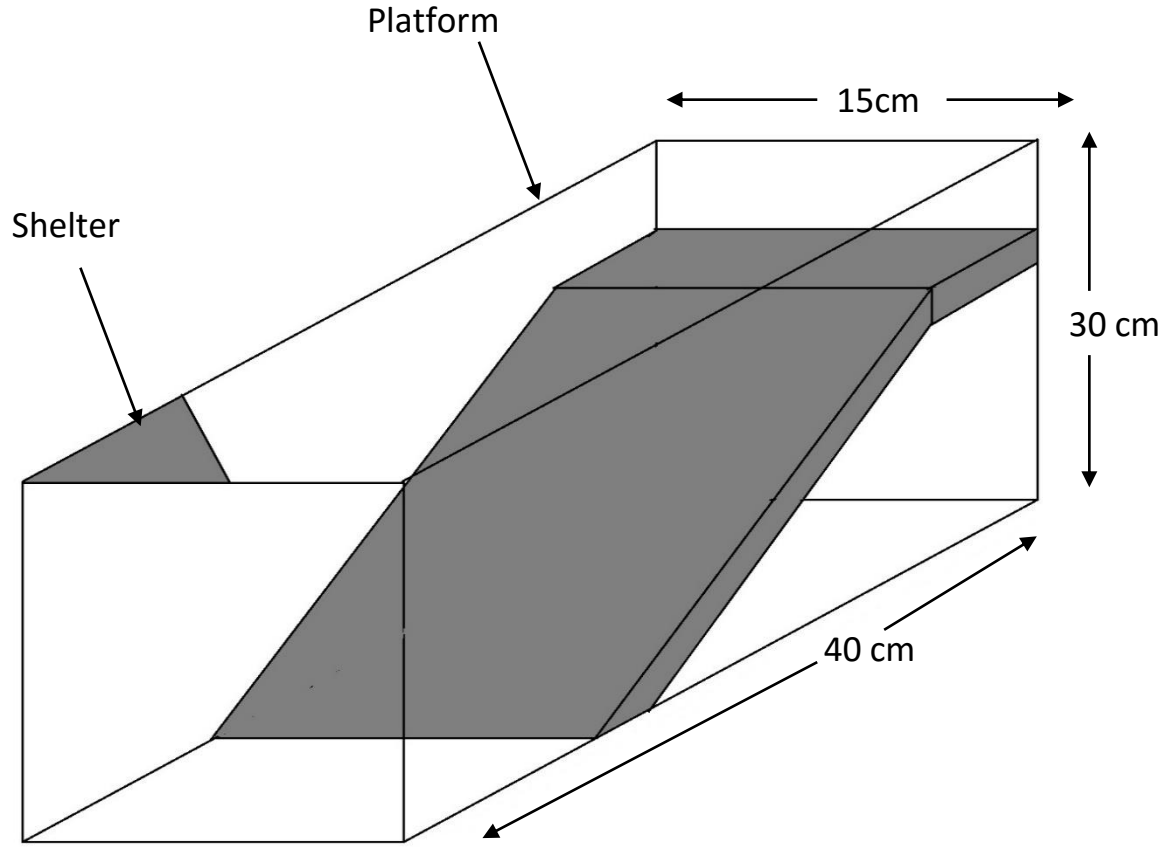

Figure 5.4: Diagrammatical representation of the emergence chamber apparatus. The platform allowed movement of *P. zealandicus* between water and air. The shelter was located at the opposite end to the platform covering the corner.

5.2.4. Experimental design of emersion experiments

A control experiment was conducted to ensure the emergence of crayfish was driven by the hypoxic conditions and not because of time or other factors. In this experiment crayfish were exposed to normoxic conditions and their movements between the air and water measured the same as during hypoxia experiments.

For observation of emergence, crayfish were subjected to progressive hypoxia to determine the PO_2 at which an emergence response was initiated. Each trial ran for an average of 54.35 ± 3.34 minutes. After animals had been acclimated in normoxic conditions for 12 hours the water in the chamber was rendered hypoxic by bubbling nitrogen through an aerator placed at one end of the chamber. The nitrogen flow was increased by 2 L per minute every 5 minutes to exponentially decrease PO_2. The PO_2 was monitored every minute using an oxygen probe which was left in the chamber for the entirety of the experiment. Crayfish were monitored through the live feed from the webcam. This meant the crayfish were undisturbed at all times. The water PO_2 and time at which the crayfish emerged was recorded. Preliminary experiments showed that the emersion response of *P. zealandicus* to aquatic hypoxia consisted of the animal using the ramp to leave the water completely. Emergence was defined as the movement of the crayfish completely out of the water for more than 4 minutes. Crayfish would sometimes climb the sides of the tank or to the top of the ramp and rest at the water/air interface temporarily to escape the hypoxic conditions. However, this was not considered full emergence under the definition above. Experiments ceased once emergence occurred, and the PO_2 was recorded.

Following this progressive hypoxia experiment a second manipulation was performed. Five crayfish were transferred from a chamber under normoxic conditions into a chamber which water PO_2 level was maintained at the lowest average PO_2 that crayfish emerged from in the previous experiment (3.2 mmHg). The time at which they emerged from this extreme hypoxia was recorded. In addition, crayfish were also exposed to intermediate hypoxia where water PO_2 was maintained at the same dissolved oxygen level as was achieved during the progressive hypoxia experiment after 30 minutes (7.6 mmHg). Again the time at which crayfish emerged was recorded.

5.2.5. Data analysis and statistical methods

Statistical analysis was carried out using the R statistical programme. Statistical significance was taken at the level of P value lower than 0.05. All data are presented as the mean ± standard error of the mean (SEM). Homogeneity of variance was tested by plotting the fitted values against the residual values. Normal distribution was tested with a quantile-quantile normality plot and histogram of the residuals.

To analyse avoidance experiments first percentages were converted to proportions by dividing by 100 and then arcsine transformed. After which grouped means were compared using a two factor ANOVA with treatments and side as factors. To analyse emergence experiments, grouped mean values that met these assumptions were compared using a one way ANOVA followed by a Tukeys HSD post hoc test to test for differences between experiments.

5.3. Results

5.3.1. Avoidance

Under all experimental conditions crayfish did not preferentially favour one side of the tank ($P > 0.05$, Fig. 5.5) with 50.2 ± 6.9% of time being spent on the left side and 49.8 ± 5.9% of time being spent on the right side. Data from each side were therefore combined. Crayfish distribution was not affected by the level of hypoxia ($P > 0.05$, Fig. 5.6), regardless of the tested PO_2 levels. Although crayfish spent the most time in the highest PO_2 under the 60/30 (71.6 ± 15.3%) treatment regime and unexpectedly spent the least amount of time in the highest PO_2 under the 90/60 (23.1 ± 14.5% time) treatment regime, these effects were not significant.

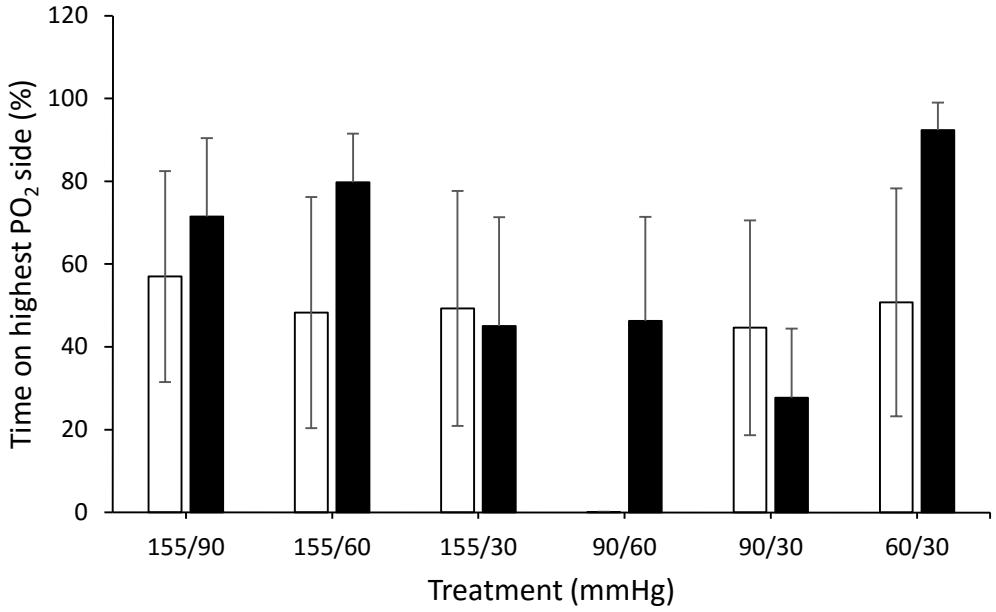

Figure 5.5: Comparing the percent of time spent on the highest PO_2 under each treatment on both the left (□) and right sides (■).Plotted values are means ± standard error of the mean.

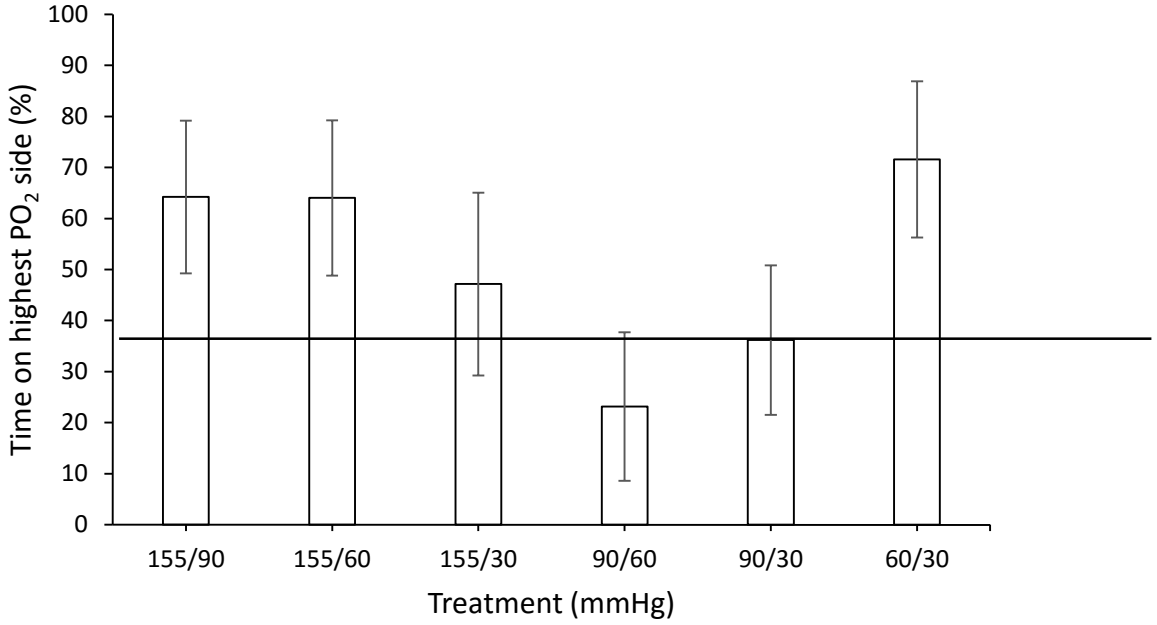

Figure 5.6: Percent of time *P. zealandicus* spent on the choice chamber side with the highest oxygen level (PO_2) under each treatment. Plotted values are means ± standard error of the mean.

5.3.2. Emergence

PO_2 in the experimental chamber decreased at an exponential rate reaching an average of 151.70 ± 3.26, 10.28 ± 0.40, 5.36 ± 0.22, 3.66 ± 0.03 mmHg at 0, 20, 40 and 60 minutes, respectively (Fig. 5.7).Emersion behaviour was exhibited at an average PO_2 of 4.20 ± 0.33 mmHg (range = 2.88-5.91 mmHg) after 54.25 ± 3.34 minutes (range = 42-71 minutes)(Fig. 5.8). All eight crayfish emerged out of the water. Crayfish exposed to the experimental progressive hypoxia emerged significantly more slowly than those crayfish exposed to the intermediate and extreme emergence experiments (155 mmHg) ($P < 0.05$).

The average PO_2 at emergence (2-4 mmHg) and the average PO_2 after 30 minutes (5-8 mmHg) were selected for the extreme and intermediate hypoxia experiments, respectively. The extreme hypoxia experiment PO_2 was measured to be 3.20 ± 0.18 mmHg. These crayfish emerged out of the water after 21.80 ± 3.79 minutes. The intermediate hypoxia PO_2 was measured to be 7.60 ± 0.25 mmHg. These crayfish emerged after 38.13 ± 3.50 minutes. These values are presented in Table 5.2. Crayfish exposed to extreme hypoxia emerged significantly faster than those exposed to intermediate hypoxia ($P < 0.05$). In control experiments (normoxia) 4 out of the 5 crayfish voluntarily emerged within 45 minutes.

In the current study, nitrogen gas was used to decrease oxygen levels. Maximal nitrogen flow was required to bring PO_2 down to the lowest levels, cooling the tank and gas. This cooled gas reduced water temperature in the chambers progressively from 15 °C down to 10 °C.

Table 5.2: Average PO_2 (mmHg) and time (minutes) with respective SEM values recorded for each emergence experiment.

	Progressive hypoxia	Intermediate hypoxia	Extreme hypoxia
Average PO_2 emerged ± SEM (mmHg)	4.2 ± 0.3	7.6 ± 0.2	3.3 ± 0.1
Average time emerged ± SEM (minutes)	54.3 ± 3.3	38.1 ± 3.5	21.8 ± 3.8

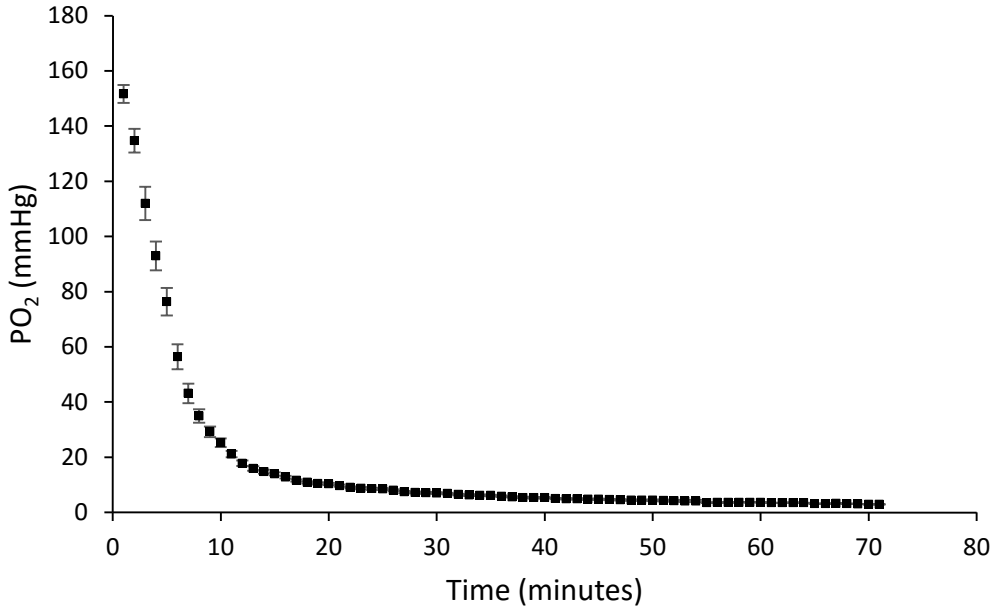

Figure 5.7: Average rate of water oxygen level (PO_2) decline over the course of the progressive hypoxia experiment. Plotted values are means ± standard error of the mean (n=8).

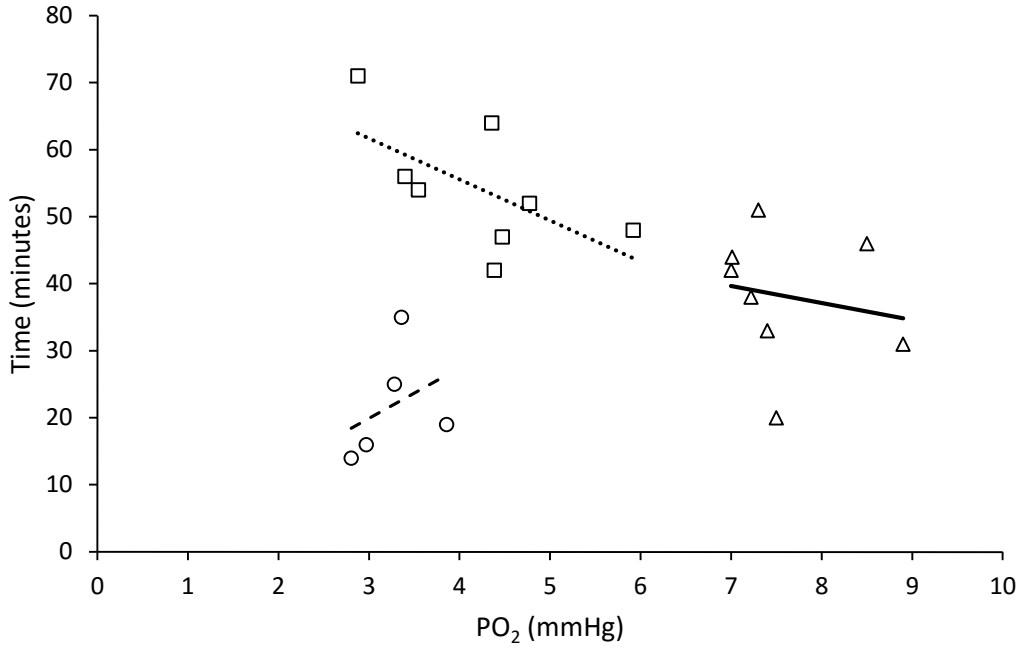

Figure 5.8: The time of emergence and oxygen level (PO_2) at which individual *P. zealandicus* emerged out of the water under the progressive hypoxia experiment (□; dotted trend line) the extreme hypoxia experiment (○; dashed trendline) and the intermediate hypoxia experiment (△; solid trendline).

5.4. Discussion

5.4.1. Hypoxia avoidance

Survival of animals exposed to stressful conditions such as hypoxia is dependent on their ability to detect these deleterious conditions and physiologically adjust and/or alter their behaviour accordingly. Research has demonstrated many aquatic species have the capacity to actively avoid hypoxic waters and that this avoidance is likely to be initiated below the animals P_{CRIT} (see Section 1.2.1.). Avoidance behaviour helps to offset the negative effects associated with habitation of hypoxic water, including dealing with anaerobic stress, and the limitations of energy available for growth, reproduction and other processes and behaviours (Brett and Groves, 1979; Das and Stickle, 1994; Skjæraasen*et al*., 2008). Many factors influence behavioural responses to hypoxia, with one key factor being the presence of an oxygen refuge.

Oxygen refuges allow aerobic metabolism to be maintained while animals periodically move into hypoxic waters to forage and avoid predation. However, if this refuge is removed or its PO_2 reduced, time spent in hypoxia is unavoidable and animals face severe metabolic stress (Herbert and Steffensen, 2005). Aquatic animals have been shown to avoid hypoxia more readily when the PO_2 of the oxygen refuge is reduced. For example, the Atlantic cod and rainbow trout do not avoid hypoxia if a fully oxygenated refuge is present but show greater avoidance responses as the PO_2 of this refuge declines (Herbert *et al.,* 2011; Poulsen *et al*., 2011). However, this was not the case for *P. zealandicus* which spent equal amounts of time between both sides of the choice chamber irrelevant of the PO_2 levels. This is a response that has been observed previously in the kingfish, *Seriola lalandi*, and Atlantic cod, *Gadus morhua* (Herbert *et al*., 2011; Cook and Herbert, 2012). Avoidance threshold differences between species may be attributed to species-specific tolerances, access to food, or predation (Claireaux *et al*., 1995; Burleson *et al*., 2001; Eby and Crowder, 2002; Skjæraasen*et al*. 2008; Stierhoff *et al*., 2009; Herbert *et al*., 2011). Nevertheless, this lack of hypoxia avoidance of *P. zealandicus* could suggest that either: 1) they do not have the ability to detect oxygen levels and therefore cannot avoid hypoxia; 2) the oxygen levels tested were not low enough to be physiologically stressful and therefore a behavioural response was not

required; 3) the exposure duration was insufficient to cause a behavioural avoidance; or 4) there were factors in the experimental design that influenced behaviour.

In fish, oxygen-sensing chemoreceptors are located both internally and externally in the gill (Burleson *et al*., 1992). It is these chemoreceptors that allow for physiologically adaptive reflexes such as bradycardia, increased gill ventilation and stroke volume increase to be initiated, all aimed to regulate MO_2 and maintain aerobic metabolism (Gamperl and Driedzic, 2009; Perry *et al*., 2009). In crustaceans, the exact location of oxygen-sensing receptors is a matter of debate (Section 1.2.3.). Regardless of the exact location, there is sufficient evidence to suggest that all studied crustacean species have the ability to detect oxygen levels and respond accordingly. Thus, it seems likely that *P. zealandicus* is able to detect low oxygen levels, and that this does not explain their lack of avoidance.

A key factor determining the onset and extent of avoidance behaviours is hypoxia tolerance (Claireaux *et al*., 1995; Burleson *et al*., 2001; Eby and Crowder, 2002; Skjæraasen*et al*., 2008; Stierhoff *et al*., 2009). The lower the P_{CRIT} the more hypoxia tolerant and the longer an animal can spend in hypoxia without impacting its metabolism. Atlantic cod have a P_{CRIT} of 32 mmHg and when exposed to 32 mmHg on one side and declining PO_2 on the other side of a choice chamber, they exhibit an avoidance response (Herbert *et al*., 2010). In some species such as the New Zealand snapper and rainbow trout avoidance is initiated below their P_{CRIT} (Poulsen *et al*., 2011; Cook *et al*., 2013). The positive relationship between hypoxia tolerance and avoidance thresholds has also been shown experimentally. For example, acclimating snapper to hypoxic conditions before subjecting them to hypoxia behavioural choice experiments showed a higher tolerance, with avoidance becoming apparent at lower PO_2 levels (Cook *et al*., 2013). Two P_{CRIT} values were calculated for *P. zealandicus* in the current study (20 and 45 mmHg; see Section 3.3.1.). The lowest oxygen level tested in the current study was 30 mmHg. The lack of avoidance supports the value of 20 mmHg as being the true P_{CRIT} value. This is because if 45 mmHg was the P_{CRIT} 30 mmHg should have been sufficiently low enough to cause avoidance.

It is possible that the experimental design could have influenced avoidance behaviour, a factor that also relates to whether the duration of exposure was sufficient. The experimental chamber, flow rates, and testing protocols were all carefully chosen in the current study. The side-by-side design of chamber facilitates discrete flows of water with different oxygen levels and minimal mixing (see Fig. 5.3). This is crucial as mixing of oxygen

flows has been shown to influence avoidance (Jones, 1952; Hoglund, 1961). However the nature of experimental design, with multiple testing protocols (to account for oxygen refuge effects; see above), the need to account for spatial effects (e.g. preferential sides of testing apparatus), and the need to limit hypoxia exposure to avoid effects of acclimation (see Section 5.2.1.2; Cook *et al*., 2013), meant that testing intervals needed to be short. Exposure time has been shown to be important in avoidance responses, however (Jones, 1952; Hoglund, 1961), and it is possible that insufficient time was given to generate the behavioural response to hypoxia. Arguing against this, however, is that other experiments have shown avoidance responses within much shorter time frames than those used in the current study (Herbert *et al*., 2011; Poulsen *et al*., 2011). Consequently, it seems unlikely that the lack of avoidance behaviour was a result of insufficient exposure duration. Instead it is possible that the randomised oxygen combinations could have disorientated crayfish such that an avoidance response could not be made. This is pertinent as in nature *P. zealandicus* would be exposed to a different hypoxia regime (that of a progressive decline in oxygen levels).

It is most likely that *P. zealandicus* did detect low oxygen levels but the frequency of alterations prohibited a coherent behaviour to be initiated. In addition, as seen from previous studies (Chapters 3 and 4) *P. zealandicus* has extensive physiological and biochemical adaptations that allow them to endure oxygen levels as low as 10 mmHg for six hours. Thus, even though other species may initiate behavioural responses under similar conditions in attempt to maintain aerobic capacity, this study suggests the aerobic capacity of *P. zealaniducs* was not sufficiently impaired to elicit a response.

5.4.2. Emergence

While avoidance of hypoxia was not shown in the current study, emersion behaviour was. In the avoidance experiments the refuge PO_2 had no impact on the behavioural responses of freshwater crayfish. However, in emergence experiments crayfish did utilise the aerial refuge. In nature, high temperatures, low water flow and algal blooms can result in a rapid reduction in water oxygen levels (Taylor and Butler, 1973; Merrick, 1995). Under these conditions it would be advantageous to leave the hypoxic environment and attempt to uptake oxygen through aerial respiration. Although theoretically beneficial, for most aquatic animals their gills are not well adapted for aerial respiration and collapse when they are no

longer supported by water (deFur and McMahon, 1984). This is problematic as the decreased surface area for gas exchange diminishes oxygen uptake and anaerobic metabolism ensues. Thus, although more oxygen may be extracted from aerial respiration many animals initiate this emergence response only as a last resort. That *P. zealandicus* chooses to emerge, albeit at very low PO_2 values, suggests that it is better able to balance metabolism in air than in highly hypoxic waters, a finding that is consistent with the physiological and biochemical responses to severe hypoxia (i.e. poor oxyregulation, acidosis; Chapter 3 and 4). Emergence behaviour is often related to the critical oxygen tension at which MO_2 can no longer be maintained (i.e. P_{CRIT}) (Taylor *et al.,* 1973; Taylor and Wheatly, 1980). This has been shown in the crayfish *A. pallipes* which emerged at 40 mmHg and *O. rusticus* which partially emerges to ventilate its branchial chambers at 30 mmHg. These emersion PO_2 values all correlate with the respective animals P_{CRIT}. Titulaer (1995) showed this in the current study species *P. zealandicus* whereby emersion was initiated at 45 mmHg (its calculated P_{CRIT}). However, although emerging at P_{CRIT} is commonly observed, not all animals show this response. Rather, some animals have been shown to emerge prior to or after reaching P_{CRIT} (Martin, 1996). In the current study, *P. zealandicus* was shown to voluntarily leave hypoxic water when PO_2 fell below 5 mmHg- an oxygen level well below its P_{CRIT} of 45 mmHg.

The low emersion PO_2 of *P. zealandicus* reported in the current study could be due to the severity and exposure time of hypoxia. Titulaer (1995) exposed *P. zealandicus* to declining PO_2 over an 84-hour period. In the current study, water PO_2 levels decreased exponentially over a much shorter time period of 54 minutes. It could be suggested that water oxygen levels were reduced too rapidly for the crayfish to elicit a response. In the extreme and intermediate hypoxia experiments, *P. zealandicus* were placed into waters of PO_2 calculated from the initial progressive hypoxia emergence experiments (3.2 and 7.6 mmHg, respectively). Under both extreme and intermediate hypoxia they left the water significantly faster than they did under progressive hypoxia. In emergence experiments by Wheatly and Taylor (1979) and Taylor *et al*. (1973) oxygen levels were reduced over a period of 45 minutes which is the same as for the present experiments. Thus, it is unlikely that *P. zealandicus* emerged at such a low PO_2 because of the rate of oxygen decline.

Many animals differ in their emergence response and respiratory behaviour on emersion (Wheatly and Taylor, 1981). *O. rusticus* and *C. maenas* partially surface and ventilate their branchial cavities with air (Taylor and Butler, 1973). Conversely, *A. pallipes*

completely emerges out of the water (Wheatly and Taylor, 1981). Variations in emergence response are thought to have different advantages with respect to the maintenance of acid-base balance (Wheatly and Taylor, 1979). Although oxygen extraction may increase in aerial respiration, because CO_2 is more soluble in water than in air, CO_2 accumulation is common in emerged animals (DeJours, 1981; Truchot, 1990). This increase in CO_2 can decrease pH and lead to acidosis. Therefore, some animals emerge to better extract oxygen but still remain in water to excrete CO_2. Therefore, how a researcher defines emergence, will influence the exact PO_2 reported for the onset of this phenomenon. In the present study, the emergence response was defined as the crayfish completely leaving the water and moving onto the ramp for more than 4 minutes. However, before full emergence occurred, *P. zealandicus* was often seen partially raising its head above the water at the sides of the chamber, or temporarily emerging for a few seconds at a time. This definition of emergence, therefore represents a point at which partial emersion was no longer sustainable and so may represent a more extreme endpoint than that reported by other authors.

The methodological approach may be responsible for the low emersion PO_2. The temperature reduction (caused by gassing chambers at high rates with nitrogen) observed in the current emergence experiments could partially explain the low PO_2 at which *P. zealandicus* emerged. The impacts of temperature on behavioural responses to hypoxia have been seen in other studies. For example, in *C. maenas* emersion took place at 18, 21, and 59 mmHg at 6, 12 and 17 °C, respectively (Taylor *et al*., 1973). Physiological processes such as respiratory frequency and heart rate increase as temperature increases, and as a consequence temperature induces a greater oxygen demand. This is known as the Q_{10} effect where there are greater oxygen demands at higher temperatures (Section 1.1.2.). Consequently a reduced water temperature might subdue metabolism, and thereby lead to a better ability to maintain sustainable oxygen uptake, and/or extend the duration of tolerance to anaerobic waste products. This may partially explain why *P. zealandicus* emerged at a PO_2 significantly lower than its calculated P_{CRIT} (Taylor *et al*., 1973).

Perceived risk of predation due to observer disturbance has previously been shown to delay aerial emergence in the whitebait fish species inanga (Dean and Richardson, 1999) and the grey mullet *Mugil cephalus* (Shingles *et al*., 2005). In the present study, chambers where surrounded by black plastic and panels placed around them to block visual cues. However,

activity was occurring in the lab that may have triggered vibrations or noise that could have influenced emergence and explain why crayfish took so long to emerge.

5.5. Summary

P. zealandicus did not show an obvious avoidance pattern when subjected to hypoxic waters. However, when offered an aerial refuge *P. zealandicus* initiated an emergence response, at a PO_2 much lower than P_{CRIT}, and lower than that observed previously for this species (Titulaer, 1995). This suggests *P. zealandicus* can tolerate very low oxygen levels before eliciting behavioural responses. In addition, the low emersion PO_2 observed in the current study suggests other factors such as temperature and perceived risk of predation may delay the onset of this response.

Future behavioural studies would benefit from simultaneous measurement of physiological and biochemical responses that would allow an assessment of whether there is a biochemical/physiological driver for the behaviours observed. If the emergence responses observed in the current study do reflect the scenario in the natural environment then it suggests a strong tolerance to hypoxia, and hints at an ability to utilise aerial respiration. Such a response will undoubtedly contribute to the survival of crayfish species during hypoxic episodes. Responding quickly to hypoxia is advantageous to allow escape before oxygen levels decline and the environment becomes too stressful (Renaud, 1986). However, behavioural responses may not be required if an animal has a wide tolerance and physiological and biochemical responses to compensate.

Chapter Six

6. General discussion

6.1. Aims and background

The aim of this study was to investigate the physiological, biochemical and behavioural responses of freshwater crayfish, *Paranephrops zealandicus*, to both low oxygen levels and high nutrient levels. Due to its frequency, impact on biota and geographical spread, hypoxia caused by eutrophication is considered one of the foremost anthropogenic impacts in aquatic ecosystems (Diaz and Rosenberg, 2008; Turner *et al*., 2005). The intensification of agricultural and industrial activities in New Zealand not only threatens the health of the streams that flow through these farmlands but also the organisms that inhabit them. Both globally and in New Zealand, there has been correlation between hypoxia caused eutrophic waters and the loss of stream biodiversity and species richness (Levin *et al*., 2009; Franklin, 2014). In response to eutrophication, freshwater organisms have developed a variety of responses to reduce the impacts of environmental change (Reiber, 1995; McMahon, 2001). The current study showed that *P. zealandicus* initially responded to both acute and prolonged hypoxia in the absence and the presence of increased nutrients with a series of coordinated physiological (reduced oxygen consumption) and biochemical (activation of anaerobic metabolism) responses. In addition, *P. zealandicus* relied on behavioural adaptations (emersion out of hypoxic waters) as a last resort to deal with extreme aquatic hypoxia. Overall, these results indicated that, at least in the short-term, *P. zealandicus* is tolerant to moderate hypoxia.

6.2. Physiological responses to hypoxia

6.2.1. Oxygen consumption responses to hypoxia

The average response of *P. zealandicus* in progressive hypoxia was to maintain MO_2 until a critical concentration (P_{CRIT}) was reached, at which point regulation failed and the animals reduced MO_2 as PO_2 dropped. This is similar to other crustaceans (Taylor and

Wheatly, 1980; Massabuau and Burtin, 1984; Morris and Callaghan, 1998) and is a mechanism that limits the need to activate anaerobic metabolism, and its potentially toxic consequences. The exact PO_2 of P_{CRIT} differed (20 mmHg *versus* 45 mmHg) depending on how it was calculated, most likely due to the intraspecific variability of oxygen consumption patterns. The minimal lactate accumulation in crayfish exposed to 30 mmHg suggested animals were not hypoxia-stressed under these conditions. This supports the P_{CRIT} value of 20 mmHg. The lack of bradycardia under both progressive and prolonged hypoxia also corroborates this value. This variability in regulation of oxygen consumption could be beneficial in the natural environment where conditions are very dynamic. If an animal is able to alter its metabolic rate under hypoxia depending on factors such as nutritional state, temperature, moulting cycle or developmental stage, then it will be more adaptable to change (Thompson and Pritchard, 1969; Pörtner and Grieshaber, 1993).

6.2.2. Heart rate responses to hypoxia

P. zealandicus largely maintained its heart rate under severe hypoxia (10 mmHg). This pattern, while observed in crustaceans before (Morris *et al*., 1996), runs contrary to the usual bradycardia response (Wheatly and Taylor, 1980; Ahern and Morris, 1999; Reiber *et al*., 1992). This suggests a tolerance of *P. zealandicus* to hypoxia and/or suggests that other compensatory functions such as increases in cardiac output or stroke volume could be occurring. The ability of *P. zealandicus* to maintain MO_2 and heart rate under moderate hypoxia suggests that it possesses a great capacity for oxygen extraction and therefore has the predisposition to exploit and tolerate more oxygen variable environments. Having such a low P_{CRIT} also limits the need to activate anaerobic metabolism which prolongs the time that the limited quantity of glucose and glycogen can support metabolism, and therefore will extend survival (Richards, 2009).

6.3. Biochemical responses to hypoxia

6.3.1. Haemolymph responses to hypoxia

The physiological responses of oxygen consumption and heart rate were underpinned by changes at the biochemical level. Haemolymph acidosis is the reduction in haemolymph pH, usually from an accumulation of CO_2 and/or lactate, and is an indicator of stress. The reduction in pH in crayfish exposed to severe hypoxia is most likely due to the accumulation of lactate observed in the current study. The lack of CO_2 accumulation and minimal increase in lactate in crayfish exposed to moderate hypoxia explained the absence of acidosis in this treatment. These results further support the assumption that moderate hypoxia is not stressful for *P. zealandicus* and that its P_{CRIT} is lower than 30 mmHg. The ability to avoid acidosis at such low oxygen levels will have important implications on acid-base status, enzymatic functioning, and ability to survive hypoxia (Campbell, 1973; Henry and Wheatly, 1992; Pavasovic *et al*., 2004).

6.3.2. Lactate responses to hypoxia

The activation of anaerobic metabolism is a common response when animals are exposed to oxygen levels below their P_{CRIT}. In the present study exposing crayfish to moderate hypoxia resulted in a five-fold increase in haemolymph lactate levels (from 0.006 to 0.03 mg mL^{-1}). However, this is minimal compared to the 50-fold increase (from 0.006 to 0.29 mg mL^{-1}) observe in crayfish exposed to severe hypoxia. Thus, *P. zealandicus* were more hypoxia-stressed under severe hypoxia than moderate hypoxia. Yet this accumulation, and therefore hypoxia stress, is much lower compared to other species in similar conditions. The reduced accumulation of lactate could be explained by diffusive loss of lactate into the water, a limitation in terms of energy substrates, or a low limit of tolerance to circulating lactate and/or its acid-base consequences.

6.3.3. Glucose responses to hypoxia

In most hypoxia-stressed animals, a rise in lactate under low oxygen levels occurs concurrently with an increase in glucose as glycogen is broken down to supply substrate for anaerobic glycolysis (Oliveira *et al*., 2001; da Silva-Castiglioni *et al*., 2010, 2011; Marqueze *et al*., 2011). In the present study, the decline in haemolymph glucose at both moderate, but especially in severe, hypoxia could suggest that the use of glucose may have been greater than the breakdown of glycogen. Measurements of glycogen concentrations would be required to confirm this. The surprisingly small increase in lactate and decrease in glucose of animals exposed to moderate hypoxia (30 mmHg) compared with control animals suggests a tolerance to dissolved oxygen levels lower than its P_{CRIT}. This may prove beneficial to freshwater crayfish inhabiting hypoxic areas.

6.3.4. Responses to nutrients

Eutrophic waters are both low in dissolved oxygen levels and high in nutrient levels. Knowing how crayfish, which are found in eutrophic lakes, physiologically copes with a combination of stressors will give a better insight into their adaptive abilities. The few studies that have investigated the combined exposure of hypoxia and enriched nutrients show an exacerbation of effects over exposure to single stressors. In the present study, the combination of high ammonia and nitrite with severe hypoxia did not alter the physiological and biochemical responses of *P. zealandicus*. Although levels tested were above those found naturally in a healthy environment, with pastoral land use increasing and temperature rising, algal blooms and run-offs are likely to become more common. Thus, the fact that high levels of aquatic nutrients do not exacerbate the effects of hypoxia in *P. zealandicus* has important management implications.

6.4. Behavioural responses

6.4.1. Avoidance

The tolerance of *P. zealandicus* to low oxygen levels was also shown in the behavioural responses. The avoidance of hypoxic waters by migrating away from deleterious conditions and toward more oxygenated waters is seen in many species. This was not, however, observed in *P. zealandicus*. Although the conclusions reached were inevitably shaped somewhat by the choice of experimental apparatus (see Section 5.2), the more probable reason for the absence of avoidance was that the oxygen levels tested may not have been low enough to elicit a behavioural response.

6.4.2. Emergence

Emergence is a type of avoidance where animals migrate out of the water in an attempt to escape the deleterious effects of hypoxia. The low emergence PO_2 (4.2 mmHg) observed in the present study supports the lack of avoidance in the choice chamber (which had a minimal PO_2 of 30 mmHg). Emerging out of the water increases both risk of desiccation and predation and is therefore often only used as a last resort when water conditions become unbearable. Because *P. zealandicus* emerged at a much lower PO_2 than other freshwater crustaceans, this suggests a comprehensive compensatory ability of the physiological and biochemical responses that allow the animal to remain in such hypoxic waters. The ability of organisms to tolerate low oxygen levels is of great environmental importance. When hypoxia persists and oxygen supply is insufficient to meet the minimal energy demands of essential functions (e.g. predator avoidance, and feeding), death is inevitable (Franklin, 2014). Therefore, animals that are able to function at lower oxygen levels may have higher survival rates. The presence of hypoxia, coupled with a lack of tolerance to such conditions, can decrease species diversity/richness and alter community structure in crustacean and freshwater communities (Kilgore and Hoover, 2001). The low sensitivity of *P. zealandicus* found in the present study suggests this species is more robust than other species.

Not only does this adaptive strategy of emerging out of hypoxic waters enable crustaceans to avoid anaerobiosis and the concomitant accumulation of metabolites, it could also allow for the migration over land to more favourable habitats. The observed emersion of *P. zealandicus* in the current study along with anecdotal evidence presented in Titulaer (1995), would suggest that migration out of hypoxic waters to more favourable conditions is a possibility for *P. zealandicus*. This behaviour could prove advantageous in fragmented waterways such as those found in forest streams. With this information the repopulation and conservation of this culturally, ecologically and economically import species can be improved.

6.5. Methodological considerations and issues highlighted for further study

The present research suggests that adult *P. zealandicus* are able to maintain their MO_2 down to low oxygen levels in 15 °C waters. Freshwater crayfish are poikilotherms, therefore environmental temperatures affect the rate of metabolism (Farkas and Nevenzel, 1981). Consequently, a change in temperature may alter the rate of MO_2 and, thus, the critical oxygen tension at which anaerobic metabolism ensues. This would in turn impact how tolerant crayfish are to hypoxia. The results of the behavioural study (Chapter 5) suggested that a decline in temperature lowered the PO_2 at which emersion was initiated. Therefore, it can be assumed that an increase in temperature would do the opposite. With many New Zealand freshwater streams already exceeding 15 °C (Wilcock, 1996; Collier and Smith, 2000) and global temperatures rising (Mann *et al.,* 1998) investigating the responses of freshwater crayfish to hypoxia at higher temperatures needs to be conducted.

The results of the current study indicate an absence of hypoxia-induced bradycardia under progressive hypoxia and minimal bradycardia under prolonged hypoxia. Bradycardia is seen in the majority of crustaceans and is energetically advantageous. One possibility for the lack of bradycardia is that exposure was not severe enough or long enough to elicit such a response. However, considering the observed reduction in MO_2, compensatory adjustments of other cardiac functions seems more plausible. Therefore, more extensive studies are required to measure stroke volume and cardiac output to determine whether the observed

bradycardia is due to an unexpected tolerance or if other responses were instead compensating (Farrell, 1993; Reiber, 1995; McMahon 2001).

The results of the physiological and biochemical experiments (Chapter 3 and 4) suggest *P. zealandicus* is not impacted by ammonia and nitrite. However, nutrient exposures were never conducted in isolation from severe hypoxia. In part this was owing to the specific goal of replicating eutrophied waters where hypoxia is always accompanied by enhanced nutrient levels. Exposing crayfish to ammonia and nitrite singularly would however be useful to show whether nutrients truly had no impact because the responses would be comparable to the combined treatments.

6.5.1. Variability in responses

The intraspecific variability within many of the experiments requires further attention. The variability in oxygen consumption under hypoxia was interesting and although it has been seen in many other species, it had not previously been shown in *P. zealandicus*. Larger sample numbers and more replicates are required to evaluate the cause of this intraspecific variability in responses. Within the current study some variability may have been reduced had different intervals of measurements been adopted. For example, MO_2 was measured at 20 minute intervals. Measuring the reduction in dissolved O_2 levels, and therefore MO_2, continuously will give more data points in real time. This could help better determine this species MO_2 pattern and therefore its oxyregulation response.

Variability was not restricted to hypoxia exposures but was also seen in concert with elevated nutrients. One key factor that may control variability of response to nitrite is chloride uptake rates. Intraspecific variation in branchial chloride uptake rates has been shown in rainbow trout (Aggergaard and Jensen, 2001) and eel (Hyde and Perry, 1989). Chloride uptake rates effect nitrite uptake and as such is proportional to toxicity and therefore tolerance to hypoxia (Aggergaard and Jensen, 2001). Whether *P. zealandicus* also exhibit variability in chloride uptake rates is not known, but if they did nitrite would be more toxic to those individuals with higher chloride uptake rates. If this is the case, this could explain the variability for heart rates in the nitrite-exposed crayfish (Williams and Eddie, 1986; Aggergaard and Jensen, 2001). Thus, future work should investigate the branchial chloride uptake rates in *P. zealandicus.* This could be achieved by branchial cannulation

studies, which have been previously used to determine chloride uptake in crustaceans (Morris *et al.,* 2000).

Glucose, lactate and glycogen levels are highly fluid during the course of a hypoxia exposure, varying as the animal employs different strategies to manage the stress. The current study only tested the lactate and glucose levels at the end of six hours, providing only a snapshot of the anaerobic state of the crayfish. To understand how *P. zealandicus* initiates anaerobic metabolism over time, sampling at smaller intervals is required. Fluctuations in lactate over time have been seen in other studies (da Silva-Castiglioni *et al.,* 2010). Similarly, the acid-base balance of animals exposed to hypoxia in the current study suggests a fluctuation in response depending on the severity of hypoxia. For example, under moderate hypoxia the haemolymph results suggest *P. zealandicus* was experiencing alkalosis, although this was not a significant effect. Conversely, under severe hypoxia a significant acidosis occurred. Taking haemolymph samples at more frequent intervals under prolonged hypoxia would better elucidate the pattern of acid-base balance. On initial exposure to hypoxia many species hyperventilate in order to gain more oxygen from the water (a factor underlying the putative alkalosis). The oxygen tension at which this ventilation ceases can be used as in indicator of stress, and would therefore be a useful complement to measurements of haemolymph acid-base status of stressed animals.

6.5.2. Behavioural methodologies

The lack of avoidance shown in the current study could be due to the fact that the experimental design influenced the experimental outcomes. The design was based on that of Cook *et al*. (2013) and Herbert and Cook (2012) with a few alterations. For example, water flowed through the chamber and was not recycled to avoid chemical signals altering responses. This meant that substantial gassing, by manually altering N_2 flow rates, was required to modify water PO_2 levels especially at 30 mmHg. In the methods reported by Cook *et al*. (2013) Oxyreg units (Loligo Systems, Tjele, Denmark) were coupled to computer software which allowed the continuous tracking and automatic alteration of oxygen levels. This equipment would offer a better control of oxygen levels throughout experimentation

The percent time in each PO_2 was the only avoidance-related behaviour recorded in the current study (see Chapter 5). In previous studies other behavioural responses such as the frequency of movements between the oxygen flows have been reported (Cook *et al.,* 2013). Depending on the species, behavioural responses to hypoxia can consist of either an increase or a decrease in activity (Skjæraasen*et al*., 2008; Herbert *et al*., 2011). Sedentary species that encounter hypoxia often tend to reduce their activity in hypoxia (Chapman and McKenzie, 2009). Conversely, more active species generally increase activity in an attempt to enhance their probability of encountering higher oxygenated waters (Skjæraasen*et al*., 2008; Chapman and McKenzie, 2009). The activity levels of *P. zealandicus* exposed to hypoxia have never been studied, but this would be a useful addition to future work.

Interestingly, the PO_2 at which emergence was initiated in *P. zealandicus* was extremely low compared with other species. It is possible that this low value was partially due to intermittent temperature drop below 15 °C during the experiment which was caused by the high flow of nitrogen from the tank. A reduction in temperature would reduce metabolic rate and thus the amount of oxygen consumed (Taylor and Wheatly 1979; Wheatly and Taylor 1979). Reducing the amount of gas flow required to get PO_2 down to such low levels would correct this. The use of a Styrofoam cover or beads to restrict the air-water interface and better facilitate water deoxygenation has been shown in other studies (Morris and Callaghan, 1998). This would, however, impede visual recording. The strict definition of emergence employed in the current study (complete emergence for 4 minutes) could also explain the low emergence PO_2. Some studies have placed infra-red detectors at intervals above the air/water interface to record when the animal first emerges out of the water and the duration of this emergence (Morris and Callaghan, 1998). This would allow for all types of emersion to be recorded and may therefore give a more detailed indication of the behavioural response patterns of *P. zealandicus* to hypoxia.

6.5.3. Future studies

Many animals that emerge do so because they have the ability to maintain a similar MO_2 and heart rate in air as in water (Wright and Raymond, 1978; Yoshiyama and Cech, 1994; Martin, 1996; Sloman *et al*., 2008). Although emergence was shown as a viable behavioural response to severe hypoxia in the current study it is not known whether

physiological responses were maintained. Maintenance of MO_2 would suggest bimodal respiration and a tolerance to hypoxia, as seen in tidepool sculpins after 72 hours of emersion (Sloman *et al*., 2008). In addition, the measurement of haemolymph lactate and glucose levels on initial emergence would also determine if the activation of anaerobic pathways explains the emergence. This was seen in *C. maenas* which showed no lactate build-up even after three hours of emersion (Taylor and Butler, 1978). Lactate and glucose levels would also allow assessment as to whether the low emergence PO_2 observed in the current study truly reflects a tolerance to hypoxia or just an unwillingness to emerge. If the low emersion PO_2 seen in *P. zealandicus* is due to a tolerance to hypoxia it should have an uncompromised aerobic capacity as represented by a minimal lactate accumulation and maintenance of MO_2 on emersion.

Responses to short-term hypoxia can be vastly different to those induced by chronic hypoxia (Dalla Via *et al*., 1994). In the current study the responses from the six hour exposures were similar to the short-term exposures. To better evaluate the tolerance of *P. zealandicus* to hypoxia and high nutrients exposures longer than six hours need to be tested. Chronic exposure could also be used to investigate the biological consequence of hypoxia exposure. The physiological and biochemical responses will come at a cost to the animal, and as such they could impact other energy-demanding processes such as growth and reproduction (Diaz and Rosenberg, 1995).

All the data in the current study were obtained from male crayfish. Juvenile and female crayfish often have a lower body mass and thus greater oxygen consumption demands (Thompson and Pritchard, 1969; Pörtner and Grieshaber, 1993). As such they may be more susceptible to hypoxia. Furthermore, the current study examined farmed crayfish. The responses of farmed crayfish may differ markedly to animals that have inhabited the natural environment (Martin, 2014). This is because farmed animals are often bred over generations and artificially selected for particular traits which reduces their genetic variability and eventual evolutionary divergence from their wild caught counterparts (Cross and Challanain, 1991; Fleming *et al.,* 1996). Farmed animals are also exposed to different factors compared with wild animals. For example, farmed *Procambarus clarkii* have different diets, are handled more and often have a reduced health (Martin, 2014). Caution should also be taken when extrapolating laboratory data to the natural environment, as crayfish in the wild would be met with greater energetic demands owing to feeding, exercise and other

confounding factors. Thus, future studies examining the impacts of *in situ* exposures are required to add further insight into the responses of *P. zealandicus* to hypoxia.

The present study along with these future studies will give a better insight into the physiological, biochemical and behavioural responses of *P. zealandicus* to both current and future environmental perturbations. An insight of the found tolerances that may be used to a slow the decline of this ecologically important species.

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Appendix

A

Underline sentences confer what was completed and indented sentences indicated the code ran to achieve this. **X**= PO_2, **Y**= MO_2.

Attach data

```
data.name<-read.table(file="brokenstick.csv",header=T,row.names=NULL,sep=",")

attach(data.name)
```

Load the R package SiZer

```
library(SiZer)
```

Fit a piecewise linear model to your data

```
model.name<-
piecewise.linear(X,Y,middle=1,CI=FALSE,bootstrap.samples=1000,sig.level=0.05)
```

Show results

```
print(model.name)
```

Plot results

```
plot(X,Y)

lines(model.name,lty=2)
```