The effects of ocean acidification and temperature change on the West Coast rock lobster (*Jasus lalandii*)

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Notes

This thesis is presented in the format prescribed by the Department of Animal Sciences, Stellenbosch University. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by a summary chapter with the study objectives, followed by a general introduction chapter and culminating with a chapter for elaborating a general discussion and conclusions. Referencing format used is in accordance with the requirements of the Journal of Experimental Marine Biology and Ecology. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

Results from this dissertation that have been published in the following journals:

- Knapp, J.L., Bridges, C.R., Krohn, J., Hoffman, L.C., Auerswald, L., 2015. Acid–base balance and changes in haemolymph properties of the South African rock lobsters, *Jasus lalandii*, a palinurid decapod, during chronic hypercapnia. Biochem. Biophys. Res. Commun. 461, 475–480.
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Summary

The West Coast rock lobster (WCRL), *Jasus lalandii*, is a critical marine fisheries resource for South Africa and may in future be negatively affected by the changes in seawater parameters associated with the ongoing anthropogenic carbon dioxide (CO_2) emissions. These CO_2 emissions have been linked to a global decrease in ocean pH (termed "ocean acidification") and an increase in temperature. There are strong estimates that these changes are to worsen in coming centuries. This warranted research because of 1) the low current level of the resource (2.6% of pristine) and 2) the relatively unexplored physiological- and other biological responses of the WCRL to environmental stressors. This information is essential for the sustainable management of the resource by government scientists in times of global- and regional climate change.

In the short term, it was found that the WCRL was able to rapidly and reversibly respond to acute changes in seawater pH (pH 7.4), this was achieved primarily through the active up-regulation of bicarbonate levels in the haemolymph. Maintaining extracellular pH protects oxygen transport mechanisms, which are sensitive to pH changes due to the large Bohr effect that this study also revealed, in the respiratory protein, haemocyanin of adult WCRL.

The energy cost of actively maintaining extracellular pH, however, is expected to affect growth and potentially survival in the long term. This was tested on juvenile WCRL that were exposed to a reduced seawater pH of 7.3 (18.8 °C) over a period of 28 weeks. Results revealed that survival was not influenced and acid-base regulation in the hypercapnia-exposed lobsters was maintained throughout the duration of the trial, however, this led to a reduced growth rate. Subsequently, in order to replicate field conditions more closely, a combination of effects, namely seawater pCO_2 (pH 8 and 7.3) and different temperatures (15.6 and 19 °C) on the growth of juvenile WCRL were assessed over an exposure period of 48 weeks in a second chronic trial. In contrast to the initial trial (28 weeks), where hypercapnia was assessed separately, lobsters exposed to hypercapnia had a higher growth rate than those at the same temperature exposed to a "natural" (normocapnic) seawater pH. The difference was interpreted as an indication that food availability/quality may negatively affect stress response, as feeding in the first trial was later considered "sub-optimal" in comparison to that of the second trial. In the latter, although both hypercapnia and temperature affected growth rates, temperature was the largest contributor to differences observed between treatments. The order of growth rates for lobsters from different treatments was: hypercapnia/high temperature > normocapnia/high temperature > hypercapnia/low temperature > normocapnia/low temperature. In this trial too, irrespective of treatment, lobsters were able to maintain extracellular pH within a relatively narrow range over the extent of the trial and survival was not negatively affected by hypercapnia or high temperature.

In order to compare the sensitivity of juvenile WCRL to that of adults, with regards to the effect of changes in extracellular pH on oxygen transport, and to assess the impact of chronic hypercapnia,

haemocyanin from juveniles was studied in detail after the first growth trial. This revealed that juvenile WCRL have a similar Bohr effect to that of adults. In addition, the haemocyanin of hypercapnia-exposed juveniles showed an increased affinity to oxygen caused by an intrinsic change in its molecular structure. This was interpreted as an energy-saving mechanism, because at the same time, haemocyanin concentration in these animals was lower than in normocapnic lobsters.

At the termination of the second chronic trial, the immunological response to the combined stressors was assessed, namely total circulating haemocyte counts (THC) and the ability to clear/inactivate an introduced dose of a bacterium, *Vibrio anguillarum*. A pilot experiment on non-treated juveniles revealed a similar resting THC to that of other lobster species, and culturable *V. anguillarum* was rapidly cleared from their haemolymph. The effect of chronic exposure to a combination of effects, namely hypercapnia and different temperatures, was subsequently tested after termination of the second chronic trial. There were no differences between treatments in a) baseline THC (i.e. before bacterial challenge) and 2) the capability to clear culturable bacteria from haemolymph. The only difference was the circulating THCs post-bacterial challenge, as they were reduced in the hypercapnic-, high temperature treatment, compared with all other treatments. The reason is unknown, but it is speculated that it may have been linked to an increased metabolic demand in these lobsters.

Overall, these results demonstrate the great plasticity of the WCRL at the molecular-, biochemical and physiological level. They provide important initial information for government fisheries scientists to aid in predicting future development of, and potential threats to the WCRL resource, as well as providing a platform from which the direction of future studies can be determined.

Opsomming

Die Weskus-seekreef, *Jasus lalandii*, is ’n belangrike seevisseryhulpbron vir Suid-Afrika en kan in die toekoms negatief geraak word deur die veranderinge in seewaterparameters wat met voortgesette antropogeniese vrystellings van koolstofdioksied (CO_2) verband hou. Hierdie CO_2-vrystellings word met ’n wêreldwye daling in die pH van seewater (oftewel “oseaanversuring”) en ’n temperatuurstyging verbind. Alles dui daarop dat hierdie veranderinge in die volgende eeue sal vererger. Dít regverdig navorsing weens 1) die huidige skaarste aan dié hulpbron (2,6% van oorspronklike getalle), en 2) die betreklik onverkende fisiologiese en ander biologiese reaksies van die kreef op omgewingstressors. Hierdie inligting is noodsaaklik om staatswetenskaplikes in staat te stel om die hulpbron te midde van wêreldwye en streeksklimaatsverandering volhoubaar te bestuur.

Op kort termyn word daar bevind dat die Weskus-kreef vinnig en omkeerbaar op akute veranderinge in die pH van seewater reageer (pH 7,4). Dít is hoofsaaklik deur die aktiewe opwaartse regulering van bikarbonaatvlakke in die hemolimf vasgestel. Die handhawing van ekstrasellulêre pH beskerm die meganismes wat suurstof vervoer, wat gevoelig is vir pH-veranderinge weens die beduidende Bohr-effek in die respiratoriese proteïen, hemosianien, by die volwasse kreef – nóg ’n bevinding van hierdie studie.

Tog sal die energiekoste verbonde aan die handhawing van ekstrasellulêre pH na verwagting groei en moontlik ook oorlewing op lang termyn beïnvloed. Dít is getoets op jong Weskus-krewe wat oor ’n tydperk van 28 weke aan seewater met ’n verlaagde pH van 7,3 (18,8 °C) blootgestel is. Resultate dui daarop dat oorlewing nié geraak word nie, en dat suur-basis-regulering in die hiperkapnie-blootgestelde krewe vir die volle duur van die proef gehandhaaf is, hoewel dit tot ’n verlaagde groeitempo gelei het. Ten einde natuurlike omstandighede akkurater na te boots, is ’n kombinasie van uitwerkings, naamlik pCO_2 van seewater (pH 8 en 7,3) en verskillende temperature (15,6 en 19 °C), op die groei van jong krewe oor ’n blootstellingstydperk van 48 weke in ’n tweede chroniese proefneming beoordeel. In teenstelling met die aanvanklike proef (28 weke), is hiperkapnie afsonderlik beoordeel en het krewe wat aan hiperkapnie blootgestel is ’n hoër groeitempo getoon as dié by dieselfde temperatuur wat aan seewater met ’n ‘natuurlike’ (normokapniese) pH blootgestel is. Dié verskil is vertolk as ’n aanwyser dat voedselbeskikbaarheid/-gehalte ’n negatiewe uitwerking op stresreaksie kan hê, aangesien voeding in die eerste proefneming later as ‘suboptimaal’ beskou is vergeleke met dié van die tweede proef. In die tweede proef, hoewel hiperkapnie én temperatuur groeitempo’s beïnvloed het, was temperatuur die grootste bydraer tot die verskille wat tussen behandelings opgemerk is. Die orde van die kreefgroeitempo’s met die verskillende behandelings was: hiperkapnie/hoë temperatuur > normokapnie/hoë temperatuur > hiperkapnie/lae temperatuur > normokapnie/lae temperatuur. In die tweede proef kon die kreef ook, ongeag behandeling, ekstrasellulêre pH vir die volle duur van die proefneming binne ’n betreklik beperkte bestek handhaaf, en het nóg hiperkapnie nóg hoë temperatuur ’n negatiewe invloed op oorlewing gehad.

Om die gevoeligheid van jong Weskus-krewe met dié van volwasse krewe te vergelyk wat betref die uitwerking van veranderinge in ekstrasellulêre pH op suurstofvervoer, en om die impak van chroniese hiperkapnie te bepaal, is die hemosianien van jong krewe deeglik ná die eerste groeiproef bestudeer. Dít het aan die lig gebring dat die jong kreef ’n soortgelyke Bohr-effek as volwassenes toon. Daarbenewens toon die hemosianien van hiperkapnie-blootgestelde jong krewe ’n verhoogde affiniteit tot suurstof, wat deur ’n intrinsieke verandering in molekulêre struktuur veroorsaak word. Dít is as ’n energiebesparingsmeganisme vertolk, aangesien hemosianienkonsentrasie by hierdie diere terselfdertyd laer was as by normokapniese kreef.

Aan die einde van die tweede chroniese proefneming is die immunologiese reaksie op die gekombineerde stressors beoordeel, naamlik totale sirkulerende hemosiettellings (THC) en die vermoë om ’n toegediende dosis van die bakterie *Vibrio anguillarum* op te ruim/te deaktiveer. ’n Toetseksperiment met niebehandelde jong krewe dui op ’n soortgelyke rustende THC as dié van ander kreefspesies, en kweekbare *V. anguillarum* is vinnig uit die hemolimf opgeruim. Die effek van chroniese blootstelling aan ’n kombinasie van faktore, naamlik hiperkapnie en verskillende temperature, is vervolgens na afloop van die tweede chroniese proef getoets. Die verskillende behandelings lewer dieselfde a) THC op die basislyn (met ander woorde voor toediening van die bakterie), en 2) opruimingsvermoë van kweekbare bakterieë uit die hemolimf op. Die enigste verskil was die THC’s ná toediening van die bakterie, wat laer was met die hiperkapniese hoëtemperatuurbehandeling as met alle ander behandelings. Die rede hiervoor is onbekend, maar hou vermoedelik verband met ’n verhoogde metaboliese vereiste by hierdie krewe.

Oor die algemeen toon hierdie resultate die beduidende plastisiteit van die Weskus-seekreef op molekulêre, biochemiese en fisiologiese vlak. Dit bied belangrike aanvanklike inligting vir staatsvisserywetenskaplikes om die toekomstige ontwikkeling van én moontlike bedreigings vir die kreefhulpbron te voorspel, en voorsien boonop ’n platform van waar die rigting van toekomstige studies bepaal kan word.

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Chapter 1

Introduction

Marine living resources are invaluable to food security and the backbone of great industries worldwide. Crustaceans are one such resource, comprised of 248 lobster species (Chan, 2010). Of these, the most commercially important are the clawed-, spiny-, slipper lobsters and scampi (Briones-Fourzán and Lozano-Àlvarez, 2015). These species, however, are not only commercially valuable, but also play a vital role in ensuring diversity within the various marine ecosystems they inhabit (Barkai and Branch, 1988; Briones-Fourzán and Lozano- Àlvarez, 2015).

Marine resources, such as crustaceans, are under threat and have become a popular scientific topic, as their environments are changing at an alarming rate. The causal factor for this change is the ever-increasing anthropogenic emissions of carbon dioxide (CO_2) into the atmosphere (Ciais et al., 2013). This has led to both a decreased ocean pH (hypercapnia), termed "ocean acidification" (Rhein et al., 2013) and an increase in climate variability, with some regions along the South African coastline experiencing oceanic warming, while others -cooling (Jarre et al., 2015).

In South Africa, the West Coast rock lobster (*Jasus lalandii*), is a commercially important spiny lobster (Cockcroft et al., 2008). The commercially exploitable part of the population is located within the Benguela Current Large Marine Ecosystem (BCLME, Pollock, 1986), one of the largest Eastern boundary upwelling systems of the world (Summerhayes et al., 1995). The WCRL is exposed here to a host of environmental challenges. Water parameters are continually changing over the short term (Bailey and Chapman, 1991; Hutchings et al., 2009; Summerhayes et al., 1995) with upwelling being the main contributor. The seasonal upwelling leads to algal blooms which subsequently expire, leading to episodes of acute hypercapnic hypoxia (Pitcher and Probyn, 2010). These short-term changes however, may become more prolonged stressors as anthropogenic CO_2 concentrations continue to increase at rates far greater than what any geological system can counter (Blackford and Gilbert, 2007). The WCRL population may have responded already to (as yet unknown) environmental change, although at the behavioural level, by a southward shift of its main abundance (Cockcroft et al., 2008).

The WCRL resource is heavily relied upon by a number of fishing communities along the West Coast, as well as a large commercial fleet operating in the area. The industry is worth in excess of 250 million rand (Cockcroft et al., 2008), however, currently at a dangerously low level (2.6% of pristine; DAFF, 2014). *Jasus lalandii* is one of the best studied crustaceans in South Africa, but despite its economic and ecological importance, very little research has been conducted with regards to biological, physiological and biochemical response mechanisms to environmental change. It is difficult in this regard to use general models in order to predict future changes, since responses observed during

hypercapnic exposure differ between species (Whiteley, 2011). This highlights the need for species-specific studies on lobsters like the WCRL.

Some physiological responses to hypercapnia can be observed via monitoring changes that take place in the extracellular fluid (haemolymph) of crustaceans (Pörtner, 2008). In general, an external increase in pCO_2 would result in a higher internal pCO_2 (reduced pH), due to the high solubility of CO_2 in seawater and the fact that it is a freely diffusible gas (Henry and Wheatly, 1992). Compensation to reduced pH comes in the form of bicarbonate, mainly through ionic pumping from seawater via the gills (Cameron, 1978; Henry and Wheatly, 1992). The rate however, at which this compensation occurs, and ability to maintain this up-regulation of bicarbonate is species-specific (Metzger et al., 2007; Whiteley, 2011). Hypercapnia can also lead to a narrowing of the thermal window of species, with heat limits being reached sooner (Metzger et al., 2007). Investigation into various parameters, such as haemolymph pH, as well as molecular modulators (i.e. Ca^{2+}, Mg^{2+}) of haemocyanins' oxygen affinity, will provide valuable information with regards to the mechanisms utilized by the WCRL during these low pH events.

In the long-term, two physiological stressors related to a reduced seawater pH may contribute to a decreased somatic growth rate in the WCRL: the energy-costly up-regulation of bicarbonate for acid-base regulation (Pörtner et al., 2004) and the change in seawater carbonate chemistry with regards to calcification of exoskeleton (Kleypas and Langdon, 2000). The latter increases the energy cost to maintain the structural integrity and growth of the exoskeleton (Feely et al., 2008; Rhein et al., 2013).

Somatic growth rate and abundance are factors that feed into various inputs of the Operational Management Plan (OMP), which is used by the South African fisheries authority to manage the WCRL resource sustainably by setting annual Total Allowable Catches (TAC, Johnston et al., 2012). If growth rate and survival were to be negatively influenced by either ocean acidification and/or temperature change (all eventually feeding into abundance of WCRL), the resource will drop below the already low level, and the Total Allowable Catch (TAC) will be reduced further (currently ~1800 t / year).

In addition to acid-base balance and growth, other mechanisms may be influenced by the potential climatic changes. As mentioned, upwelling leads to both, a reduction in seawater pH and a reduced oxygen concentration, specifically towards the end of summer when large algae blooms collapse. Concentrations as low as 0.1 ml^{-1} have been recorded (Pitcher and Probyn, 2011). This, combined with temperatures lying on either side of the lobsters thermal range, may limit aerobic scope (Pörtner, 2010). It would therefore be of interest to determine how a reduced seawater pH would affect the transport of oxygen in the WCRL, specifically looking at the acute and chronic effects of pH on the respiratory pigment.

Climatic- and other environmental changes cause stress not only in crustaceans. The latter influences not only acid-base regulation but also the disease defence of the lobsters, i.e. immune functionality (Le Moullac and Haffner, 2000). Various studies have investigated the effects of temperature, hypercapnia,

hypoxia, and various combinations of these parameters on immune response in crustaceans (Le Moullac and Haffner, 2000). However, baseline data regarding the WCRL's immune mechanisms are still lacking. Investigation into this facet would therefore allow for estimation as to what extent the WCRL is resilient to diseases under conditions of climate change. Increased mortality due to viral or bacterial infections would, of course, eventually lead to lower abundance and therefore decline in the fishery.

The current state of knowledge on biological and physiological responses of the WCRL to environmental change is, as described above, suboptimal for predicting population- and resource development and, in turn, sustainable management. The present dissertation is a logic attempt to close some of the mentioned knowledge gaps and provide scientists and managers with vital information for future informed decisions.

The overall aims/objectives of the study were therefore:

- To investigate the acute physiological response of *J. lalandii* to a decreased seawater pH in order to provide a mechanistic basis from which further more complex studies could be designed. This involved assessing the short-term acid-base response, as well as the haemocyanin's (respiratory protein) sensitivity to a decreased pH.
- To investigate the effects of long-term exposure to a decreased seawater pH and, as a second step, in combination with increased temperature on somatic growth and survival of juvenile WCRL.
- To investigate the potential effect of long-term exposure to a decreased seawater pH on juvenile WCRL at an extracellular- and molecular level (i.e. haemocyanin), as well as to determine the sensitivity of the juvenile haemocyanin to changes in pH.
- To provide baseline immune data for the WCRL as well as investigate the effect of long-term hypercapnia at different temperatures on the WCRLs immune competency.
- To provide biological, physiological and biochemical insight into the WCRLs ability to deal with the predicted environmental changes. This is to consider relevant experimental results for incorporation into the OMP to ensure future sustainable management of the WCRL resource.

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Chapter 2

Literature review:

Sustainable management of a Southern African rock lobster resource in times of environmental change

Abstract

South Africa is a resource-rich country, with one of the most dynamic coastlines on the globe. A fishery of critical importance is the West Coast rock lobster, *Jasus lalandii*. The resource provides thousands of jobs through commercial fleets and artisanal fishermen. However, with the combination of predicted environmental stressors and a severely exploited resource, a once thriving lobster industry may be in jeopardy. The scope of this study was therefore to look at the West Coast rock lobster resource in terms of: 1) policy and management, 2) what is known about its biology, 3) environmental challenges it experiences, past and present, as well as how these are predicted to develop within the coming centuries, and 4) what is understood in terms of its physiological response to environmental perturbations. Briefly, the main findings of the review were as follows: 1) The resource (*J. lalandii*) is currently sustainably managed by means of an Operational Management Plan, however at a critically low level (2.6% of pristine). 2) Due to *J. lalandii's* economic importance, it is one of South Africa's best studied crustaceans, with various aspects of its biology known. 3) The majority of the lobster population resides in a highly changeable system - the Benguela Current Large Marine Ecosystem and are on occasion exposed to environmental extremes, such as low oxygen, temperature variability and decreased seawater pH over the short term, with these conditions expected to become more extreme in the future, and in the case of seawater pH, a decrease globally. It is also clear that there is a lack of accurate historical seawater data, specifically for pH, along the South African coast line. 4) There is an absence of studies investigating *J. lalandii's* ability to physiologically deal with the current and predicted environmental stressors. Various areas therefore warrant further scientific research, namely: How will *J. lalandii* react to perturbations such as hypercapnia alone, as well as in combination with temperature change over both the short- to long term, with regards to physiological responses, somatic growth rate, and vulnerability to disease. Understanding the effects of predicted environmental changes allows for incorporation into the current fisheries management tools and can contribute to the goal of sustainable management of the resource.

Keywords: Sustainability, Acid-base regulation, Physiology, Fisheries, Hypercapnia, Climate change, Upwelling.

1. Introduction

Lobsters are an important commodity globally, with capture fisheries for marine lobster amounting to 28 922 t in 2013 (FAO, 2013a). South Africa has in excess of 20 marine fishery resources, these accounted for 412 510 t with regards to total captures in 2013, including fish, crustaceans and molluscs (FAO, 2013b). In the latest report on South Africa's fisheries (DAFF, 2014), the state of 50% of them fell into the category "be concerned", and of these, 22% into the "heavily depleted" category. The West Coast rock lobster (WCRL, *Jasus lalandii*) falls into the latter. It is South Africa's most important rock lobster fishery, worth in excess of 250 million rand (US $19.5) per annum, and provides some 4200 jobs (DAFF, 2014). The WCRL occurs primarily along the West Coast of South Africa, in a system known as the Benguela Current Large Marine Ecosystem (BCLME), one of the largest upwelling systems in the world, and therefore one that shows great variability in the short- to medium term.

In the current literature, a "hot" topic is that of Ocean Acidification (OA), this is the decrease in seawater pH due to the absorption of anthropogenic carbon dioxide (CO_2) by the oceans (Rhein et al., 2013). This leads to a condition called hypercapnia, which is essentially an elevated pCO_2 that causes a decrease in seawater pH (Pörtner et al., 2004). Almost half of the CO_2 emitted into the atmosphere has been absorbed by the oceans (Sabine et al., 2004). Since pre-industrial times, atmospheric CO_2 has been on the rise, and in future this trend is expected to continue (Caldeira and Wickett, 2003). Ocean acidification is, however, considered to be the "other" CO_2 problem (Doney et al., 2009), as there is another, which has in the past led to much controversy, namely; "Global Warming". This warming is understood to be induced by the amount of green-house gasses (i.e. CO_2), and non-CO_2 greenhouse gasses (i.e. methane, CH_4) that are released into the atmosphere (Hansen et al., 2000; Rhein et al., 2013). These stressors combined, will lead to a host of environmental changes, from below the organismic level up to entire ecosystem function.

In literature, a variety of responses are observed when marine biota are experimentally exposed to OA conditions or warming separately (Branch et al., 2013; Byrne, 2011; Fabry et al., 2008; Hofmann and Todgham, 2010; Hofmann et al., 2010; Kelly and Hofmann, 2013; Pörtner, 2008; Pörtner et al., 2004; Somero, 2010; Whiteley, 2011). Subsequently, when exposed in combination, which is more likely to occur in the field, the effects seem to be compounded (Whiteley, 2011). In crabs and lobsters, on exposure to these environmental stressors in the short term, an acid-base response occurs, in the medium term, intrinsic changes to internal mechanisms, and lastly, in the long term, a decrease or increase in somatic growth rate (Green et al., 2014; Whiteley, 2011).

The resource of *J. lalandii* is currently at 2.6% of its pristine level (DAFF, 2014). With the predicted dramatic changes in the physiochemical parameters associated with its environment, it is not known whether this resource will be able to deal with both, the environmental and commercial strains that are placed on it. Therefore, it is essential to determine the underlying mechanisms at the physiological level

to explain and estimate as to how the resource may react to a change in its environment in terms of OA and warming. Despite these challenges, South African fishery scientists are confident that, in the OMP, they have developed a tool that allows for sustainable management and even partial restoration of the WCRL resource. In the process of continuous updating of the OMP, knowledge about biological response mechanisms can be incorporated to aid future management decisions. This chapter describes details of as to what knowledge has to be considered and how it can enter the OMP process.

2. Sustainable WCRL resource management

Management of the South African WCRL resources was initially unregulated. Later, in order to achieve sustainable management of the fishery, regulatory mechanisms were introduced. Environmental considerations were only incorporated into management towards the end of the last century. This process is described in more detail below.

2.1. Historic development towards a sustainable management

The development of the WCRL industry has been well reviewed (Cockcroft et al., 2008; Melville-Smith and van Sittert, 2005; Schoeman et al., 2002) and the most important aspects will be described here (Table 1). The WCRL became a commercially exploited resource in the late 1900s (Cockcroft and Payne, 1999) and the industry peaked in the early 1950s, with a record annual catch of close to 18 000 t of lobster (Johnston and Butterworth, 2005). Since this inflection point, there have been a number of set states in annual catches, with a levelling off at around 10 000 t between the end of the 1950s and 1960s (Cockcroft and Payne, 1999). In the mid-1980s, the resource was considered supportive of a sustainable supply to the commercial industry of between 3500 - 4000 t annually (Johnston and Butterworth, 2005). However, in the early 1990s, the stability was interrupted, somatic growth rates decreased sharply, leading to decreased recruitment of legally-sized lobsters (Cockcroft, 1997). Assessments estimated that the harvestable component (> 75 mm carapace length, *CL*) of the resource was at 5% and spawning biomass (females > 65 mm *CL*) at 20% of pre - exploited (pristine) levels in 1999 (Cockcroft and Payne, 1999). The harvestable component subsequently decreased to reach approximately 2.6% currently (DAFF, 2014). As lobster densities decrease, the catch per unit effort (CPUE) decreases, and with this, profit margins will be reduced, ultimately leading to job losses.

There have been two major events associated with the WCRL resource, namely a decrease in somatic growth rates and a southward “shift” (Cockcroft, 1997; Cockcroft et al., 2008). The cause however, is unknown, some have speculated that the decreased growth rate, a drastic 50% reduction at sexual maturity is due to food availability and quality, low oxygen, competition or a combination of these factors (Blamey et al., 2015; Pollock and Shannon, 1987; Pollock et al., 1997; Shannon et al., 1992). The southward “shift” of the lobster population is suggested in Blamey (2015) to be due to an sudden

recruitment of adults (Cockcroft et al., 2008; Jarre et al., 2015; Tarr et al., 1996), and an expansion of lobsters from a population close by (Cockcroft et al., 2008), the reason however, is still not known.

Over the years, the primary method of harvesting has changed from the use of hand-hauled baited hoop-nets to that of traps deployed by large vessels, the latter now account for about 75% of annual catches (Cockcroft and Payne, 1999). In order to ensure sustainable management of the WCRL fishery, management tools have been implemented successively. The chronological order of important events and measures taken to finally reach a sustainable management of the WCRL resource are summarised in Table 1. The most important measures implemented with regards to sustainability of the resource were the initial implementation of a size limit (by *CL*) in 1933, implementation of a Total Allowable Catch (TAC) in 1979 and the implementation of the first Operational Management Plan (OMP) in 1997. The latter was a very important step since it indirectly took, for the first time, the impact of potential environmental changes into account. The OMP is still successfully used and regularly reviewed and adjusted. According to this OMP, the TAC is currently recommended by structures within the Branch: Fisheries of the Department of Agriculture, Forestry and Fisheries (DAFF) of South Africa.

2.2. Current and future management

The overall TAC, which is set annually, is divided amongst several commercial and non-commercial sectors, namely offshore-, nearshore-, "interim relief-" (subsistence fishermen) and recreational sectors. The area in which *J. lalandii* is harvested is divided into five super areas (Cockcroft et al., 2008) in each of which a respective TAC is set for each of the sectors (Figure 1).

On an annual basis, lobster population, fishery- and ecosystem indicators are reviewed by the WCRL Scientific Working Group (SWG), consisting of DAFF scientists, academics and stake holders (representatives of the various sectors and industries) and a TAC is proposed accordingly. There are two routes via which the impact of environmental changes are accommodated: 1) stock assessment data feeding into TAC calculations (Depiction. 1, Equation I) where they influence the harvestable portion of the resource and 2) implementation of a metarule process, in the case of a drastic event or in preparation of one ("exceptional circumstances", see below). The two key inputs in calculating the TAC, namely the abundance index (A, Equation II) and secondly the somatic growth index (IV) provide indirectly for potential environmental impacts on the WCRL resource. Equation (III) accounts for high mortalities and movement of lobsters from an area.

Table 1. Chronological implementation of management tools to regulate the WCRL resource.

Date	Management tools implemented	Reason
1933	Introduction of a minimum carapace length (*CL*) of 89 mm (not throughout zones)	To protect slower growing females
1970	The production quota was cut, to a tail mass equivalent of 5513 tons and a minimum size limit of 89 mm *CL* was implemented throughout zones	To address over-exploitation, specifically in northern areas where the *CL* size limit was set at 76 mm after 1959
1979	Tail mass quota was replaced by a whole lobster quota and managed by means of a total allowable catch (TAC) limit	Alleviate pressure on resource
1989	Due to sharp decline in somatic growth rates of the previous two decades, there was a decrease in the recruitment of lobsters greater than that of the minimum size limit (89 mm)	Sharp decline in somatic growth rate in previous two decades (reason unknown), translated to decreased recruitment
1991/92	Minimum size limit was reduced to 75 mm (*CL*)	Poor catches and increased handling of undersized lobsters
1997	Implementation of the first operational management plan (OMP), a recovery rate of 20% above the 1996 level was set	To resolve difficulty associated with reaching consensus in SWG, due to projections and advice relying hugely on assumptions with regards to predicted somatic growth
2011/2012	OMP revised, Global TAC set at 2 426 tons	Set to ensure 35% biomass recovery of resource by 2021 compared with 2006 (biomass will then be considered at 4.8% of pristine)
2012/2013	Two new protective provisions incorporated into OMP model	Allow for 1) a larger reduction in TAC should it be needed, 2) closure of a super-area, if underperforming to a certain degree
2013/2014	Dassen Island, super zone closed to fishing, with exception of experimental fishing (80 tons) between December 2013 and March 2014. Global TAC set at 2 160 tons	Stock assessment data showed almost no biomass of lobsters greater than 75 mm (*CL*)
2014/2015	OMP revision was delayed till 2015, TAC set at 1 801 tons	Possible consequences of small-scale fisheries policy on the resource. TAC reduced due to underperforming of zone supplying bulk of global rock lobster catch (zone 8)

References: Bergh (2014); Cockcroft and Payne (1999); Cockcroft (1997); Cockcroft et al. (2008); DAFF, (2014); Hutchings et al. (2009); Johnston and Butterworth (2005); Johnston and Butterworth (2012).
Note: Management "decisions" important for response to the impact of environmental changes to the resource are highlighted.

The framework for the present annual setting of the TAC is provided by an Operational Management Plan (OMP). The OMP is revised every four years, key inputs each year are: commercial Catch Per Unit Effort (CPUE) of both hoop net (row boats and deck boats) and trap fisheries, Fisheries Independent Monitoring Survey (FIMS), and recent somatic growth rates observed by area-specific tagging of male sized lobsters.

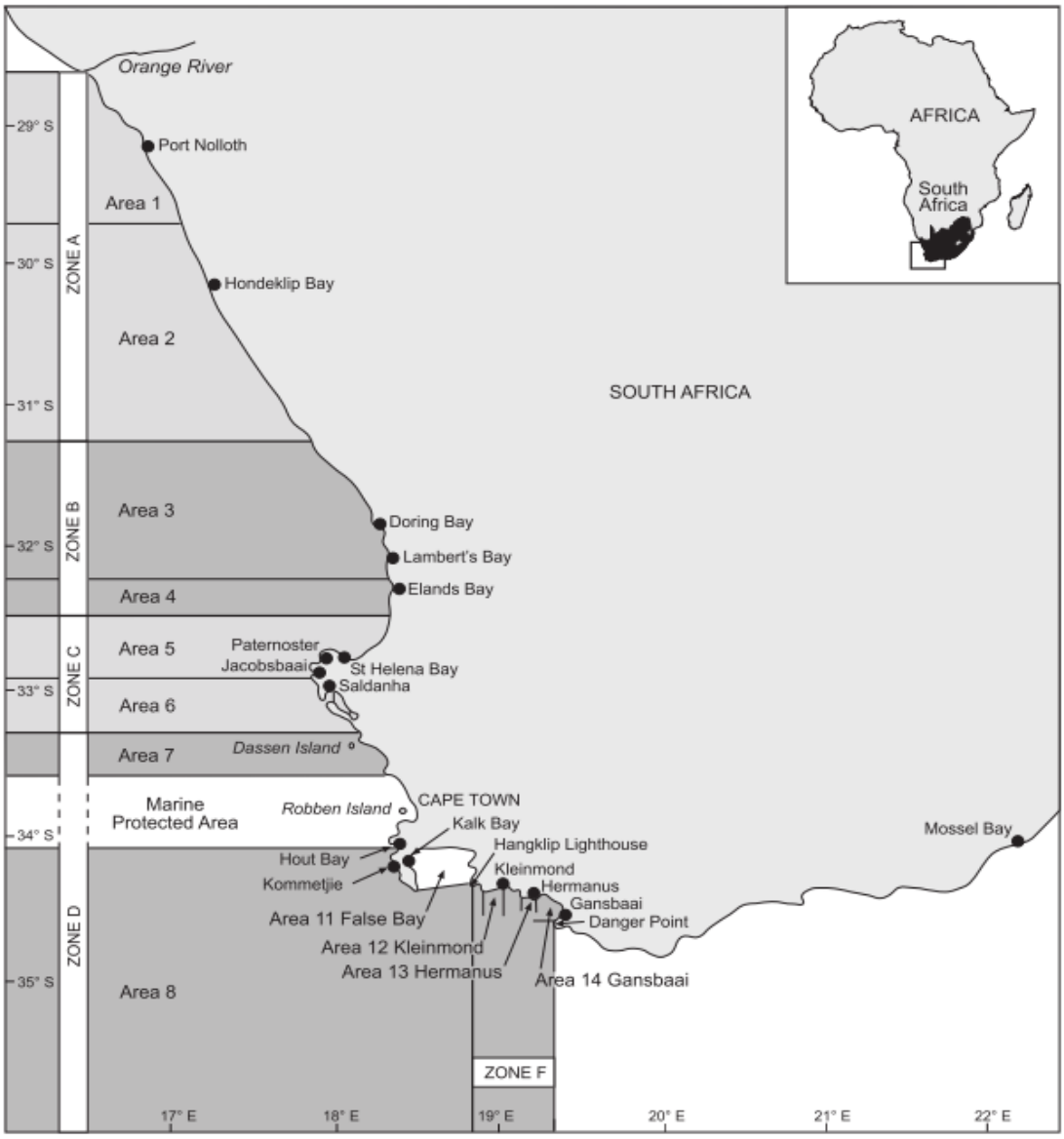


Figure 1. Divisions of the WCRL resource into respective fishing zones (reproduced with permission from A.C. Cockcroft (DAFF).

Depiction 1. Important equations used in setting of the annual TAC, also included are the relevant equations in which environmental impacts would be reflected during the stock assessment process.

TAC set according to:

$$\text{I.} \quad \boldsymbol{TAC_y^{G,2} = TAC_y^{G,1} + Z}$$

Key inputs:

A) Abundance Index

$$\text{II.} \quad \boldsymbol{TAC_y^{G,1}} = \alpha(\bar{J}_y - J_{min})$$

- α & J_{min} : Two tuning parameters.
- $\bar{J}_y$: Combined abundance index (both super-areas and gear-types), calculated via:

$$\text{III.} \quad \bar{J}_y = \sum_{gear=1}^{3} W^{gear} J_y^{gear}$$

- W^{gear}: Relative weight given to specific gear type (selected by SWG).
- J_y^{gear} : Relative measure of the immediate past level in the abundance index "gear", for gear type, trap, hoop (CPUE) or FIMS (CPUE), determined by calculating a weighted biomass above a set *CL* for year *y*.

B) Somatic Growth Index

$$\text{IV.} \quad \boldsymbol{Z} = \bar{x}\frac{SG_{y-1,y-2,y-3} - SG_{low}}{SG_{med} - SG_{low}}$$

- $SG_{y-1,y-2,y-3}$: Geometric mean of the combined somatic growth index for the three most recent seasons.
- $\bar{x}$: Calculated by comparing the tonnage differentials between the low and medium somatic growth rates that would result in the same male biomass level for the resource as a whole after several years.
- Growth is measured by means of tagging male lobsters.

Key:

- SWG: Scientific working group – Carries out all the work involved in recommending the TAC to the minister in accordance with the OMP.
- $FIMS$: Fisheries Independent Monitoring Survey.
- CL: Carapace length.
- SG: Somatic growth.

Furthermore, specified fishing times (08:00 - 16:00) during the fishing season (currently 21 days annually) and maximum number allowed per day (4 lobsters, CL >75 mm) for the recreational sector to promote sustainable harvesting. The commercial sector is further regulated by specified fishing zones and protection of berried or soft-shelled lobsters (Cockcroft, 2001; Hutchings et al., 2009; Johnston and Butterworth, 2005).

As mentioned above, in the case of "exceptional circumstances", a metarule process is applied, which is provided for in the OMP. Three potential inputs that would lead to an metarule being initiated are: 1) research data shows a drastic decline of the resource, 2) population-, fishery- and ecosystem indicators do not fall within the bounds projected in OMP testing (done annually), i.e. when industry thresholds are crossed downwards, based on a 3-year average or 3) in-depth stock assessment and indicators review return results that show evidence for exceptional circumstances (done on a two year basis, Johnston et al., 2012).

If this is the case, a series of actions is initiated and coordinated by the SWG. Once the severity of this "event" has been assessed by the SWG, the Chief Director: Research of Branch: Fisheries of DAFF approves the SWG's recommendation and, ultimately, the Deputy Director General (head) of Branch: Fisheries decides on the implementation.

If allocation of the resource is changed in the subsistence or commercial sectors (near- and offshore), the quota of each right holder will be adjusted according to that of the whole sector, while the recreational sector is changed by adjusting the duration of the fishing season. The latest (2014/2015) TAC was set at approximately 16.8% below that of the previous year.

Because the WCRL resource is currently at only 2.6% of pristine levels (DAFF, 2014), other parts of the society have stepped in to protect the stock. The Southern African Sustainable Seafood Initiative (SASSI), together with other stakeholders, for example, has assigned the species an orange status (on a scale ranging from green to red, Figure 2) by indicating to customers in shops and restaurants that the species' stock is depleted as a result of overfishing and cannot sustain current fishing pressure.

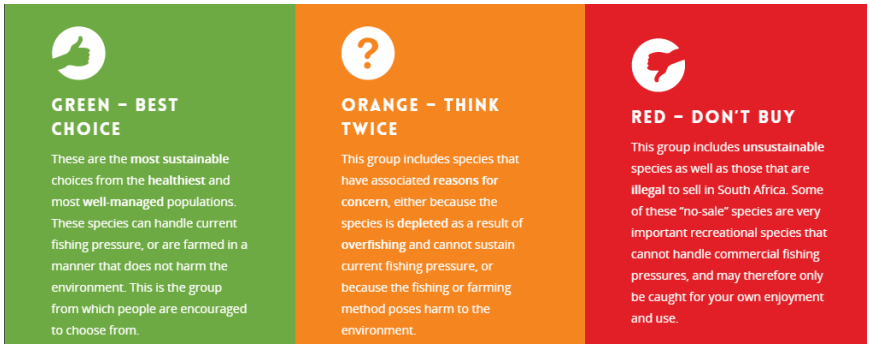


Figure 2. The SASSI scale, including a description for each colour code (WWF SASSI, 2015).

In this way, the label encourages the consumers to consume WCRL with caution or not at all. Moreover, Judy Sole, a Green Party founder recently publically proposed a total ban on fishing of the WCRL on the base of its low abundance compared with pristine levels (Independent online, 2015).

The actions of these two groupings show that the public is aware that the WCRL stock is threatened. In the public opinion, however, the threat is almost exclusively attributed to the perceived or real over-exploitation. The episodic occurrences of "walkouts", however, clearly show that environmental factors already have an impact on the resource. Despite this, the assumption of DAFF- and other scientists is, that the resource can be managed sustainably with the current (and regularly updated) OMP at hand. The current OMP, for example, targets a build-up of the WCRL resource by 2021 by 35% above the 2006 level (DAFF, 2014).

3. Biology of the West Coast rock lobster (*Jasus lalandii*)

The palinurid decapod *J. lalandii* is one of the best studied crustaceans in South Africa. Various facets of its life have already been studied, such as distribution (Atkinson et al., 2005; Cockcroft et al., 2008; Groeneveld et al., 2010; Pollock and Beyers, 1981), growth (Goosen and Cockcroft, 1995; Hazell et al., 2001a; Mayfield et al., 2000; Pollock and Beyers, 1981; Pollock et al., 1997) and sexual maturation (Beyers and Goosen, 1987; Cockcroft and Goosen, 1995).

The species is found primarily on rocky reefs, but occasionally also occurs on light foul ground ranging from intertidal depths down to 200 meters (Groeneveld et al., 2010) in an area that spans from Walvis Bay (Namibia) in the northern Benguela Current sub-system to East London on the east coast of South Africa (Groeneveld et al., 2010). *J. lalandii* is a keystone benthic predator (Barkai and Branch, 1988) and is known to feed primarily on sea urchins and various species of mussels (Barkai et al., 1996; Booth, 2006). However, as with most feeding habits, species found in different locations will have different feeding preferences due to abundance and availability of prey; differences can also be found in the preferred prey of different size classes of the lobster (Griffiths and Seiderer, 1980).

Mating and spawning occurs in the southern population during autumn/winter between June and July (Figure 3, Western Cape population), with external fertilization of eggs (Booth, 2006). The brood period is approximately three months, the larvae therefore hatch in spring to summer (Silberbauer, 1971).

Like other palinurid crustaceans, *J. lalandii* has a complex life cycle (Table 2; Booth, 2006). Briefly, males mate with females shortly after females moult while the exoskeleton is still soft, after which oviposition takes place (Berry and Heydorn, 1970) and females attach berry (eggs) onto their

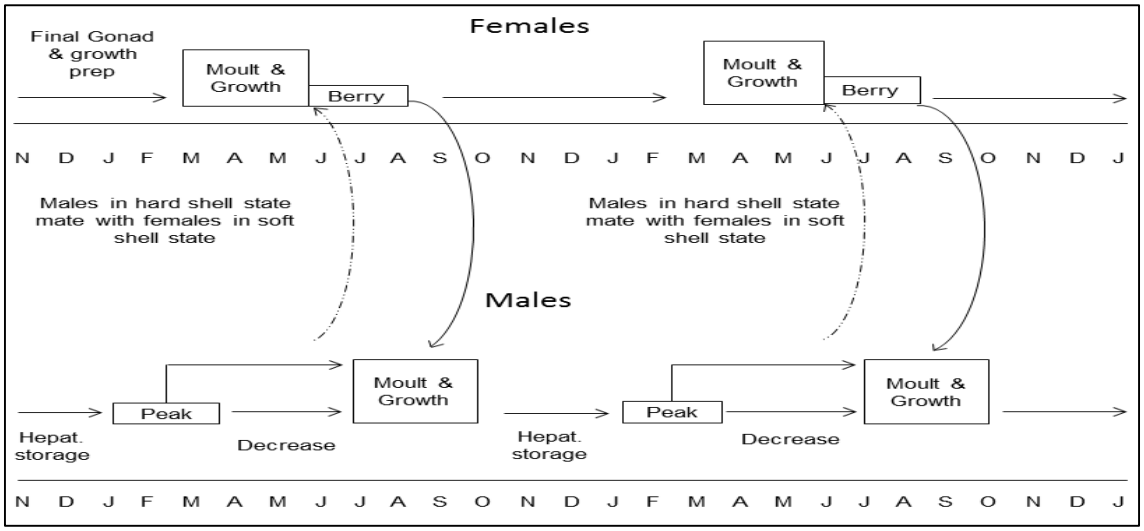
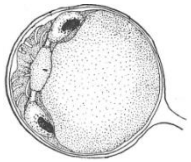
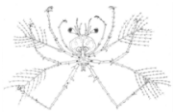
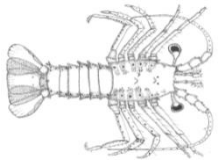
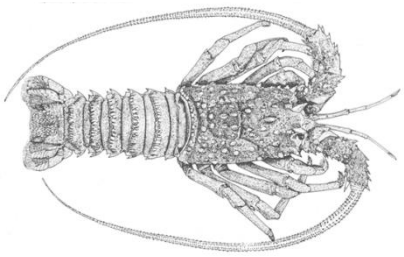


Figure 3. Synchronisation between growth- and reproductive cycles of male and female *J. lalandii* (Western Cape population). Reproduced with permission of A.C. Cockcroft (DAFF).

pleopodal setae (Dubber et al., 2004). Females carry the eggs for three months during which the embryo develops in several, well visible stages (Silberbauer, 1971). After the brood period, a naupliosoma larva hatches (Booth, 2006). The naupliosoma stage is short-lived (hours) and followed by a planktonic larval phase (MacDiarmid, 1985). This so-called phyllosoma phase comprises of 11 stages of development and takes up to 7 - 8 months (Booth, 1997, 2006; Dubber et al., 2004; Kittaka, 1988). A post-larval, non-feeding stage termed a puerulus links the larval- with the post-larval, juvenile phase. The puerulus is a strong horizontal swimmer and returns inshore (Booth, 2006) and, after 10 days of pigmentation, moults into a juvenile (Dubber et al., 2004). Once the carapace of the post-puerulus has hardened due to calcification (15 days), the solitary juvenile lobster will moult and grow fast (Dubber et al., 2004; Hazell et al., 2001a). Puerulus settlement is currently not well known, but studies revealed that high puerulus numbers occur in a North - South trend, starting in Lüderitz in winter progressing into summer in Table Bay (Groeneveld et al., 2010; Keulder, 2005; Pollock, 1973), although settlement times may be influenced by several environmental factors (Groeneveld et al., 2010).

As the WCRLs grow, which is achieved via shedding of the old restrictive exoskeleton, they become more communal and they start aggregating in retreats. During mating, however, they tend to be more solitary as the males become aggressive (Booth, 2006). It takes the female around 3 - 7 years (carapace length of 56 – 120 mm) before reaching sexual maturity (Beyers and Goosen, 1987; Booth, 2006), while males found in the same area reach sexual maturity at a similar or smaller size than females *(J. edwardsii*, MacDiarmid, 1989; Turner et al., 2002). This large time discrepancy may be due to distribution effects, such as temperature and food availability (Beyers and Goosen, 1987).

Table 2. Life stages of *J. lalandii*.

Stage	Description	Depiction (egg - puerulus[12]; adult[5])
Egg	Duration : ~3 months[12] Processes: hatch overnight due to osmotic pressure, water content increases from 27 - 41.5% (3 - 5 days)[12] Egg size : 0.8 - 1 mm[12]	  Stage of development: 30 - 60 days
Nauplisoma	Duration : 10 - 20min[7] - ½ day (3 hrs after hatching assumes mature form)[12] No's. peak: Lüderitz in Aug – Sept[6] Saldanha Bay in Nov – Jan[3] Table Bay in Dec – April[9]	
Phyllosoma	Duration : 231[12] – 306[7] days Processes: Consists of 11 stages[12, 7], 15 moults[5] Size : 1.6 - 37.5 mm[12]	 Stage 1  Stage 11
Puerulus	Duration : 25[12] – 31[7] days Processes: Consists of two stages, puerulus and post-puerulus[12] Size : 20-33 mm[12] *CL* = 10 – 20[7], 7.3 - 10.4 mm[3]	
Juvenile	Duration : - Processes: Moults several times annually[9] Size : > 8.5 mm, average 9 - 10.4 mm[3]	
Adult	Duration : Sexually mature at: Female = 3 - 7 yrs (*CL* = 56 - 120mm)[1,2], Male < or equal in size[10, 8, 13] Processes: Undergo anecdysis (annual moult)[4] Size : Maximum recorded *CL*: M = 190, F = 140 mm[11]	

Source presented by superscript letter: [1] Beyers and Goosen (1987); [2] Booth (2006); [3] Groeneveld et al. (2010); [4] Hazell et al. (2001); [5] Holthius (1991); [6] Keulder (2005); [7] Kittaka (1988); [8] MacDiarmid (1989, *J. edwardsii*); [9] Pollock (1973); [10] Pollock (1986); [11] Pollock (1991); [12] Silberbauer (1971); [13] Turner et al. (2002, *J. edwardsii*).

Size at sexual maturation also decreases from the South African waters northwards to the Namibian coast which may be attributed to food supply, oxygen concentration and/or temperature (Pollock and Beyers, 1981). Juvenile males and females have similar growth rates until they reach maturity, after which the females' moult increment is smaller as more energy is diverted for gonad production rather than growth. An age-specific-, rather than size-specific relationship exists with regard to the onset of sexual maturity in the female (Beyers and Goosen, 1987).

Various aspects of the biology and physiology of the WCRL are not fully understood. This is especially true for the larval phase, which is considered the most vulnerable part of the life cycle of benthic calcifying organisms (Kurihara et al., 2007). The effects of permanent and more prolonged change, such as climate change with all its facets, are less apparent and difficult to detect in the field. They are nonetheless likely to affect the resource in the future. These changes require energy-costly adjustments to maintain internal equilibria. As a consequence, growth and several other parameters may be negatively affected, eventually impacting the fishery of the WCRL.

4. Environmental challenges

Globally, several changes are anticipated to occur with regards to the physicochemical parameters of the oceans, namely: warming/cooling of surface waters, "acidification", intensified wind stress, greater stratification and an increased occurance of low oxygen water (LOW, Moloney et al., 2013).

The Benguela Current Large Marine Ecosystem (BCLME) is one of the largest Eastern boundary upwelling systems (Summerhayes et al., 1995) with water parameters continually changing over the short term (Blamey et al., 2015; Hutchings et al., 2009; Pitcher and Probyn, 2010; Pitcher et al., 2014; Summerhayes et al., 1995). Within this system, the WCRL resource and its sustainable management are expected to face the following environmental challenges:

1) Increased Upwelling
2) Low-oxygen events and
3) Ocean Acicidification (OA) and temperature change

Below, the short-term variability, as well as predicted long term changes, will be discussed in more detail.

4.1. Upwelling

The BCLME, located off the West Coast of Southern Africa is highly dynamic in nature (van der Lingen et al., 2006). It can be divided into two sections, namely a northern and a southern sub-system (Cury and Shannon, 2004). The latter is were the majority of South Africa's commercial fisheries can be found, including that of the WCRL (Blamey et al., 2015). Here, the local alongshore winds

lead to a phenomenon known as "Ekman transport" (Pitcher et al., 2010), essentially the net movemement of surface waters offshore due to the combination of wind stress and the Coriolis force (Price et al., 1987). Subsequently, upwelling ensues, which occurs along the extent of the West Coast of Southern Africa (Nelson and Hutchings, 1983). The upwelling events occur in 3-10 day cycles (Hill et al., 1998) whereby cold, nutrient-rich water moves into the euphotic zone (Pitcher et al., 2010) and in some cases this upwelled water is also low in pH (Feely et al., 2008; Gregor and Monteiro, 2013). Generally, these upwelling cycles reach a maximum during spring and summer when the winds that drive them are most prominent (Pitcher et al., 2010).

Upwelling in the past has gone through various cycles (Jarre et al., 2015). Since the 1990s, however, an increase in upwelling has occurred from Cape Columbine in the southern sub-system southwards, with a slight deterioration in the early 2000s (Jarre et al., 2015). The predicted global increase in wind stress due to the faster rise in land- to sea temperatures (Bakun et al., 2010; Sydeman et al., 2014) would naturally suggest that upwelling will increase in intensity, as is predicted for other systems (Bakun, 1990; Vargas et al., 2007). It is currently not possible, however, to say that the BCLME will react in the same manner (Bakun et al., 2010).

In late summer, southerly winds, upwelling and calm seas allow for oxygen deficient bottom waters - which are well documented along the South African coast (Bailey et al., 1985; Blamey et al., 2012; Jarre et al., 2015; Pitcher and Probyn, 2011; Pollock and Shannon, 1987) - to be transported closer inshore (Newman and Pollock 1971).

4.2. Low oxygen

Upwelling in the late summer/autumn period is often lagged by the occurance of harmful algal blooms (HABs), due to a decline in wind stress and rise in solar irradiance (Pitcher et al., 2010). These blooms can lead to extremely low dissolved oxygen in the water column, with concentrations as low as 0.1 ml l^{-1} being recorded for an entire water column (4 m) at Dwarskersbos during a phytoplankton bloom (just south of Elands Bay, Pitcher and Probyn, 2011).

The occurrence of phytoplankton blooms have increased globally (Glibert and Burkholder, 2002; Glibert et al., 2005). The oxygen deficient situations associated with these blooms are becoming more prevelant with the additional stress of eutrophication (Diaz and Rosenberg, 2008; Pitcher and Probyn, 2011). These hypoxic (oxygen < 2 ml l^{-1})/dead zones are found in 400 systems globally, affecting a vast area (Diaz and Rosenberg, 2008).

With a strong positive correlation found between a decrease in oxygen concentration and pH (Cai et al., 2011), particularly in upwelling systems of this nature (Frieder et al., 2012; Paulmier et al., 2011), low pHs are expected to exist in the water column during these upwelling events. Extreme pHs of 6.6 have been recorded for the nearshore area on the South African West Coast during a phytoplankton bloom (Pitcher and Probyn, 2010).

These LOWs lead to a mass movement of lobsters to the shallows as they try to locate oxygen-rich water created by wave action. Migrations like this during hypoxic conditions have been recorded for both fish and crustaceans (Pihl et al., 1991). As the lobsters move into the shallows, oxygen decreases and overcrowding occurs, leading to aggression and juvenile mortalities (Bailey et al., 1985). Once the tide begins to recede, especially during spring tide, the lobsters are stranded (Figure 4), female lobsters tend to contribute to the bulk of the catch in the initial phase of the "walkout", followed by larger males later on (Newman and Pollock, 1971; Pihl et al., 1991), possibly due to the fact that the larger male lobsters are in deeper water initially (Cockcroft, 2001). Mass mortalities caused by oxygen deficiency events of marine crustaceans have been reported elsewhere too (Baden et al., 1990; Feldmann et al., 1999).

Figure 4. Stranded lobsters litter the beach during a "walkout" at Elands Bay. With permission of D. van Zyl.

In Table 3, estimates of the total number of lobsters that were involved in the "walkouts" between 1993 and 2015 are given. Within an 80 km stretch of coastline (Lamberts Bay to Elands Bay), 94% of the total lobster mortalities where at Elands Bay in the 1990s (Cockcroft, 2001). The highest loss of lobster biomass was recorded in 1997 where the event extended for a period of 67 days and lead to the walkout of 1 955 t. For comparison, the TAC for this period was 2 040 t (Johnston and Butterworth, 2005). Only 308 t of these lobsters were returned, although the survival rate of such returned lobsters is still unknown to date.

The effects of low oxygen on other species of lobster have been recorded in several publications (Baden et al., 1990; Eriksson and Baden, 1997; Hagerman and Baden, 1988; Hagerman and Uglow, 1985; McMahon and Wilkes, 1983). The negative impact on the WCRL may in future be aggravated

by the continuous addition of anthropogenic CO_2 to oceans, termed "ocean acidification" (Feely et al., 2008).

Table 3. Recorded low oxygen events along the South African coast line.

Locality	Fishing zone	Date	Approximate event duration (days)	Amount (t)	Sex ratio M-F	Mean *CL* (mm)	Amount returned
Lamberts Bay, northwards	B	Feb-93	14	10	-	-	-
Elands Bay	B	Feb-94	38	5	-	-	-
Dwarskersbos	C	Mar-94	-	3	-	-	-
St Helena Bay	C	Mar-94	-	60	-	-	-
Elands Bay	B	Mar-97	67	225	20:80	67.7	40
Elands Bay	B	Apr-97	-	1 700	37:63	67.9	250
Dwarskersbos	C	May-97	-	30	63:37	70.2	18
Dwarskersbos	C	Apr/May 98	14	30	54:46	57	15
Elands Bay	B	Apr-99	25	200	25:75	65.2	72
Elands Bay	B	Mar-00	1	1	-	-	1
Elands Bay	B	Mar-06	1	5	-	-	2
Elands Bay	B	Mar-06	3	0.5	-	-	-
St. Helena Bay	C	Mar-06	7	15-20	-	-	15
Elands Bay	B	Apr-06	3	20	-	65	1
Elands Bay	B	Mar-09	1	5	-	-	Minor stranding, lobsters remained in shallows
Dwarkersbos	C	May-09	2	50	-	-	3
Elands Bay	B	Mar-12	3	3.5	-	-	-
Dwarskersbos	C	May-12	2	10	-	-	3
Elands Bay	B	Feb-15	-	307	68:32	50.9	<4
Elands Bay	B	Mar-15	-	127.3	41:59	60.3	-

Modified and updated from Cockcroft (2001).

4.3. Ocean acidification and temperature change

The rise of atmospheric pCO_2 has resulted in more CO_2 being dissolved in the upper thermocline of the oceans (Sabine et al., 2004), thus following Henry's law (Pörtner et al., 2004). The oceans have the ability to buffer the effect of absorbed CO_2 via the so-called "carbonate system" (Turley et al., 2005) whereby dissolved atmospheric CO_2 forms carbonic acid and bicarbonate:

$$CO_{2\,(atmosphere)} \leftrightarrow CO_{2\,(aqua)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

Due to their large volume and the ability of seawater to buffer CO_2, the oceans have absorbed a large amount of anthropogenic CO_2 from the atmosphere (Brierley and Kingsford, 2009; Pörtner, 2008). This amounts to an estimated one third of anthropogenic emissions since the beginning of the industrial revolution (Feely et al., 2008; Sabine and feely, 2007). Due to the exceptional rate of increase of CO_2 in the atmosphere, geological feedbacks that would normally counter the declining pH, are too slow to have a serious effect (Blackford and Gilbert, 2007, Figure 5). One can find several instances throughout the literature where the magnitude of the current CO_2 atmospheric level is conveyed (Caldeira and Wickett, 2003; Feely et al., 2008; Floch et al., 2008; Petit et al., 1999). According to Kleypas and Langdon (2000), even if fossil fuel emissions steadied at present levels, the atmospheric CO_2 value would surpass double pre-industrial levels by the turn of the century and, unlike that of climate forecasts, future changes in ocean chemistry can be predicted (Doney et al., 2009).

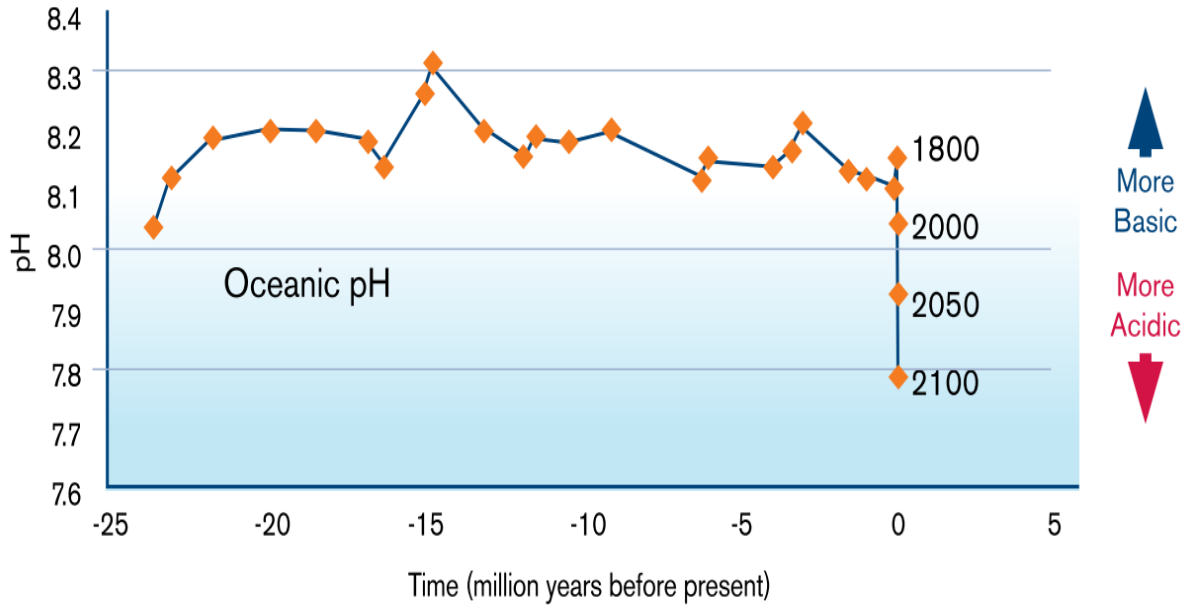
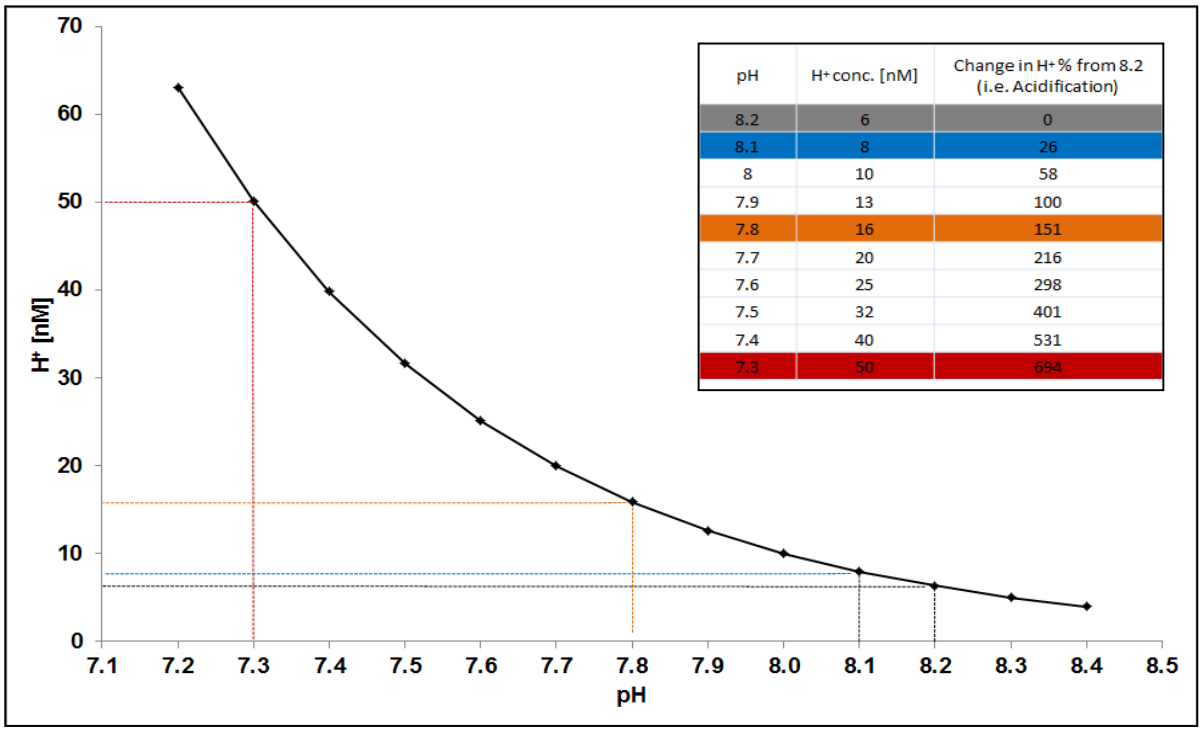


Figure 5. Rapid seawater pH change since the industrial revolution (Huelsenbeck, 2012- modified from Turley et al. (2006).

This predicted long-term decrease in pH, termed "OA", has recently become a priority in various publications, articles and media, with the average ocean pH having decreased by 0.1 units since the beginning of the industrial era, moving globally from 8.21 to 8.10 (Raven et al., 2005; Rhein et al., 2013). This 0.1 decrease in pH lead to a change in the concentration of H^+ ions in the surface water, i.e. acidification, by approximately 26% (Rhein et al., 2013). Figure 6 was constructed according to anti-logged values for the logarithmic pH scale to make this more understandable. Currently, pH levels around the world are decreasing at rates between 0.0014 and 0.0024 units yr^{-1} (Rhein et al., 2013).



pH	H⁺ conc. [nM]	Change in H⁺ % from 8.2 (i.e. Acidification)
8.2	6	0
8.1	8	26
8	10	58
7.9	13	100
7.8	16	151
7.7	20	216
7.6	25	298
7.5	32	401
7.4	40	531
7.3	50	694

Figure 6. Change in acidity (concentration of H^+ ions) as pH changes (constructed from pH and antilog pH). Dotted lines illustrate association between pH and Hydrogen ion concentration at certain points. Colours represent specific pH values, H^+ concentration and change in H^+ ion concentration relative to pre - industrial pH levels

Furthermore, the oceans' pH is expected to continue to decline within the coming centuries, with a predicted decrease of ~0.3 units for 2100 (Figure 5), and ~0.7 for 2300 (Caldeira and Wickett, 2003, Figure 7). Although the trend for the decrease in oceanic pH is widely accepted, there are more speculations and uncertainties about that of the "global warming" phenomena.

Whereas some authors have confidence that non-CO_2 greenhouse gases (GHGs) are responsible (Hansen et al., 2000), others show an association between global temperature change and the amount of CO_2 in the atmosphere (Doney et al., 2014). Some records show a 0.5 °C increase in global surface

temperature since the 1970s (Hansen et al., 1999; Jones et al., 1999), others a 0.1 °C increase per decade from 1971 to 2010 in the upper 700 meters (Rhein et al., 2013).

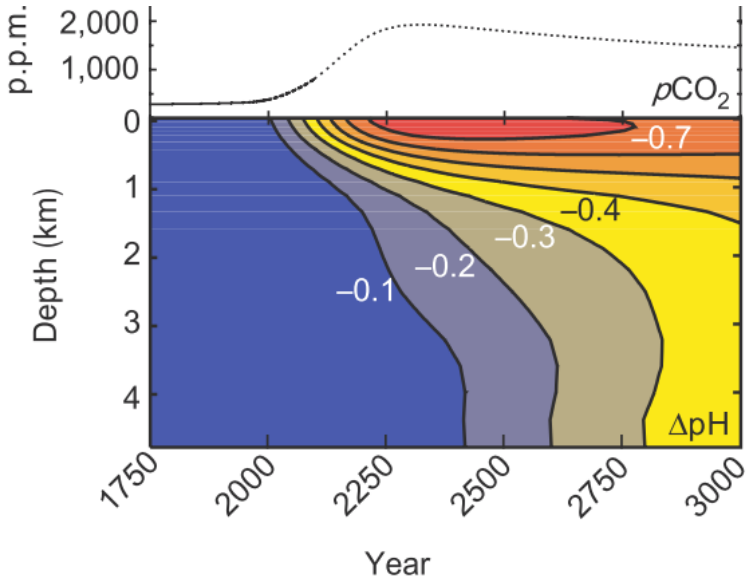


Figure 7. Anthropogenic CO_2 emmissions and resultant global pH, of past-, present- and predicted CO_2 levels (Caldeira and Wickett, 2003).

Currently, atmospheric CO_2 concentration is at 395 ppm (Ciais et al., 2013) and if ignoring the particularly episodic states of the climate change systems (Lenton et al., 2008), and the current rate of CO_2 emissions is used to predict future scenarios, a "dangerous" threshold of 450 ppm (Hansen et al., 2007a) will be surpassed by 2040 (Brierley and Kingsford, 2009). This would lead to a global 2 °C increase in surface temperature relative to pre-industrial levels (Brierley and Kingsford, 2009) and could have disastrous and irreparable consequences (Hansen et al., 2007b). If emissions continue as present, very high atmospheric CO_2 concentrations are probable in the future and will lead to a land surface temperature increase in excess of 5 °C by 2100 (Romm, 2008). Predicting temperature change for a specific system, such as the BCLME or even its sub - systems, is even more difficult due to the contributing multitude of global and local factors.

The West- and South Coasts of South Africa show variability in terms of medium-term ocean temperature, there are conflicting assumptions with regards to whether it is warming or cooling. The area off the Cape Peninsula, according to Blamey et al. (2012), is warming throughout the year, whereas Rouault et al. (2010) and Lima and Wethey (2012) show a different scenario. The northern Benguela sub-system (Namibia and Angola) and parts of the southern Benguela sub-system (Orange river - Hondeklip Bay), however, are seemingly warming (Blamey et al., 2012; Jarre et al., 2015). There are two occurrences, amongst others, described that lead to the variability in average temperatures seen in the BCLME: 1) El Niño, which takes place every several years and leads to warming in the southern

and cooling of the northern Benguela, with La Niña creating the opposite effect (Dufois and Rouault, 2012; Rouault et al., 2010) and 2) the relationship between the seasonal South Easterly (SE) winds and cooling of the adjacent ocean due to upwelling (Blamey et al., 2012). If the trend of the land/sea warming ratio persists (Sutton et al., 2007), an increase in frequency and strength of the SE winds and, in turn, frequency and intensity of upwelling events in spring and summer can be expected (Bakun, 1990; Wang et al., 2015).

Interestingly, not only the degree by which the oceans are going to warm, but also the rate of warming seems to affect biota (Peck et al., 2009). Unfortunately, even if emissions were to stop today, warming would continue due to thermal inertia (Rhein et al., 2013). Climate change is significantly affecting physiological and biological systems universally (Rosenzweig et al., 2008), the question no longer being asked is, "if" extinctions will occur due to temperature change, but rather how soon (Hughes, 2000).

As discussed, the habitat of the WCRL shows a large degree of variation. Therefore, the lobsters experience a host of physicochemical changes in water parameters in the short- to medium term. In addition, long-term predictions could mean even more change for the species, and so strain the resource further. The responses of the WCRL in particular to these changes is yet unknown, but in general, it is known that crustaceans often exhibit some physiological plasticity due to the variability of their environment (Bridges, 2001). Species differ, however, with regards to responses (Whiteley, 2011).

5. Biological responses to environmental challenges

Environmental change will most likely impact *J. lalandii* from the molecular - via the organismic - to the population level. In the following section, some of the broader areas of impact have been reviewed, mainly looking at the temperature and acidification (hypercapnia) aspects of climate change. With regards to the affected mechanisms, the review focusses on the organismic level of impact and below, such as acid-base regulation and respiration, calcification, growth, and disease. Reproduction and ontogenetic development will, of course, also be affected but were excluded here because they do not play a role in the present dissertation.

5.1. Acid-base regulation and respiration

Acid-base regulation is central to all living processes (Henry and Wheatly, 1992). Mitochondria in the respiring tissues of crustaceans, as in all animals, produce carbon dioxide (CO_2) which then moves into the cytoplasm and extracellular tissue, where it reacts with water to become carbonic acid (H_2CO_3). The latter then dissociates to a bicarbonate (HCO_3^-) ion and an hydrogen (H^+) ion, as follows (Henry and Wheatly, 1992):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

Metabolically produced CO_2 is the major source of acid, with the CO_2/HCO_3^- system acting as the principle buffer mechanism (Henry and Wheatly, 1992). In order to maintain a certain acid-base balance, respiratory gas exchange-, intermediary metabolism- and ion transport regulation are involved (Henry and Wheatly, 1992). Acid-base regulation and ion transport are linked molecularly by carbonic anhydrase, the enzyme which catalyses the hydration of CO_2 to H^+ and HCO_3^-, thereby providing the Na^+ and Cl^- transport mechanism with counter ions (Henry and Cameron, 1983). A suggested fourth potential mechanism used for acid-base regulation is the dissolution of calcium carbonate ($CaCO_3$) in the shells and liberation of HCO_3^- (Spicer et al., 1988). It is, however, ion transport that is the primary mechanism used, and not respiration as water is a poor O_2 medium and, therefore, respiration is controlled by the necessity to take oxygen up from the water (Henry and Wheatly, 1992).

The acid-base steady state of crustaceans' haemolymph falls within a narrow range (Henry and Wheatly, 1992) and any change in seawater constituents will affect the haemolymph first, followed by an acid-base response (Pörtner, 2008). Environmental hypercapnia leads to an acute respiratory acidosis whereby HCO_3^- levels are increased and compensate for any intracellular/extracellular acidosis experienced via transmembrane exchange (Pörtner, 2008). During hypoxia, which can be experienced during low-oxygen situations and emersion, hyperventilation occurs to ensure CO_2 elimination and a respiratory alkalosis is observed (Burnett and Johansen, 1981; Henry and Wheatly, 1992). In addition, temperature change and extracellular pH are inversely related (Truchot, 1978) and, therefore, a temperature change will lead to a change in total dissolved CO_2, primarily in the form of HCO_3^-. Cameron and Iwama (1987) deduced that some sort of a compromise is reached between pH and ion balance in the haemolymph, with compensation for acidosis not always being complete.

Several spiny lobster species have been studied with regards to regulation in response to changes in environmental parameters (Hammer, 2012; Lorenzon et al., 2007; Ridgway et al., 2006; Schmitt and Uglow, 1997; Taylor and Waldron, 1997; Vermeer, 1987). Literature on *J. lalandii*, however, is scarce. Haemocyanin is a respiratory pigment that is found in solution in the haemolymph of crustaceans and other invertebrates (Lockwood, 1968a). Its primary function is to increase the ability of blood to carry oxygen (Bridges, 2001). At molecular level, haemocyanin oxygen affinity is affected via changes in molecular modulators (L-lactate, Ca^{2+}, Mg^{2+} etc.), as well as changes in several physicochemical water parameters (Bridges, 2001). As described in section 3, several seawater parameters are known to change in the short term, where the lobsters will be exposed to a variety of conditions that will negatively affect the affinity of haemocyanin for oxygen, namely hypercapnia, hypoxia and increased water temperature. Any alteration in the affinity is conveyed as a change in the half-saturation or P_{50}, known as the "Bohr effect" and is dependent on the organism's physiological range (Bridges, 2001). Changes in the P_{50} counteract for, among others, acid-base and/or energy requirements. For instance, under hypoxic conditions, hyperventilation leads to an alkalosis, allowing for an increase in affinity of haemocyanin to carry oxygen (Henry and Wheatly, 1992), subsequently increasing branchial uptake of oxygen. Under

decreased temperature, a decreased metabolism in the blue crab (*C. sapidus*) led to an increased affinity for oxygen (Mauro and Magnum, 1982).

In the medium- to long term, some crustaceans can intrinsically modify the haemocyanin's affinity for oxygen by changing the structural (molecular) make-up of the pigment, possibly in order to conserve energy (Bridges, 2001; Truchot, 1992). *J. lalandii* and other spiny lobsters have developed mechanisms to deal with changes in their environment, such as emersion from seawater. This can naturally occur during so-called lobster "walkouts" (Cockcroft, 2001) and, not naturally, handling during fishing of the lobsters, as well as during scientific experiments, as emersion cannot always be avoided.

Emersion leads to a drastic alteration in acid-base status in *J. lalandii* on a seasonal basis during the period associated with phytoplankton blooms. Generally, complete emergence of crustaceans into air leads to a haemolymph acidosis initially (deFur and McMahon, 1984; Harris and Andrews, 2005; Ridgway et al., 2006; Truchot, 1975; Vermeer, 1987) due to collapsed gills (deFur et al., 1988), inhibiting the lobster's ability to remove CO_2 (Morris and Oliver, 1999; Taylor and Waldron, 1997). This, in turn, leads to excess CO_2 in the haemolymph which then binds with water to form H_2CO_3 and contributes to the pH decrease along with lactic acid build-up (Burnett, 1992; Vermeer, 1987). In Haupt et al. (2006), this process of events was described for *J. lalandii* after air exposure for 5 h. This caused a decrease of pH by 0.6 units and a 17-fold increase in lactate concentration. Such a magnitude of change has consequences for the lobsters, resulting in high mortality within the observed 5 h.

5.2. Calcification

Change to the physicochemical make-up of water not only affects the lobster in terms of acid-base balance but may negatively influence other processes, too. Calcification of hard structures, a mechanism that is not fully understood (Whiteley, 2011), is such a process.

Calcifying organisms are thought to be the most vulnerable to changes in the carbonate chemistry of the surrounding environment (Findlay et al., 2009; Orr et al., 2005). With an increase in seawater pCO_2, there is a concomitant decrease in the calcium carbonate saturation state (Ω), i.e. availability for shell formation, due to the increase in H^+ ions, which lowers pH and reduces available carbonate ions (Feely et al., 2008). Shell formation is favoured if Ω (calcite or aragonite) saturation is greater than one (Feely et al., 2008), this horizon in the oceans is however, moving closer to the surface as pCO_2 increases (Feely et al., 2004; Orr et al., 2005). The solubility of $CaCO_3$ is also known to change with seawater temperature and depth (Watson et al., 2012). A large change in carbonate saturation is predicted to occur by 2100, a change that will likely have significant effects on marine ecosystems, as well as the various economies that utilize these ecosystems (Feely et al., 2008).

Due to the fact that lobsters shed their old exoskeleton, they need to be efficient at moving calcium and other ions across epithelia and gills (Neufeld and Cameron, 1993; Robertson, 1960). Calcifying organisms are predicted to be negatively affected by decreased carbonate ion availability (Findlay et

al., 2009). Three mechanisms are present which could lead to the evasion of this: 1) Carbonate is rarely transported across membranes in calcareous organisms (Findlay et al., 2009), 2) the calcification process is secluded from that of environmental changes (Whiteley, 2011) and 3) seawater bicarbonate-, rather than carbonate ions, are used for calcification (Cameron,1985). Hence, calcification rates may not be negatively affected in crustaceans. Rather, as described by Whiteley (2011), calcification will possibly be indirectly affected through the effects of increased environmental H^+ ions, which negatively influence uptake of HCO_3^- by gills (Cameron and Wood, 1985).The latter, along with Ca^{2+}, is taken up post-moult to ensure hardening of the exoskeleton (Neufeld and Cameron, 1992; Wheatly, 1997).

In future, net carbonate production in the exoskeleton will be a compromise between calcification and dissolution (Findlay et al., 2009). Dramatic size reduction of organisms has been observed in historical records in the presence of increased environmental CO_2 (Hautmann, 2006), as well as changes in shell mineralogy to a polymorph (aragonite *vs*. calcite) of $CaCO_3$ which is less prone to dissolution (Hautmann, 2006). A delay in the calcification process could potentially extend the post-moult period for crustaceans, such as *J. lalandii*, when the exoskeleton is softer and make them more vulnerable to predation (Whiteley, 2011). If the net calcification rate is negatively influenced, growth will be directly affected due to possible redirection of energy to maintaining the integrity of the exoskeleton, as suggested in the barnacle *Semibalanus balanoides* (Findlay et al., 2010). Since the mechanical strength of claws and mandibles may also be affected, the size and type of species that *J. lalandii* will be able to predate on may be more limited than currently.

5.3. Growth

In crustaceans, growth is achieved via ingestion and absorption of water after shedding the old restricting exoskeleton (Dall and Smith, 1978). Growth only occurs after exuviation, and the so-called moult cycle (physiological and morphological events from moult to moult) is divided into several moult stages, with differences found between species regarding the relative duration of stages and associated physiological changes such as water/muscle ratio, feeding/non-feeding and ion concentrations. In *J. lalandii*, moult stages range from AB, C, through to D_4 (Marco, 2012), determined by microscopic analysis of the pleopods (five pairs, females having more) on the abdomen of the lobster. In juvenile crustaceans, the length of each moult stage is compressed compared to that of adults due to the shorter Intermoult Period (IMP = duration between two moults; Lockwood, 1968b). Intermoult (stage C) is considered to be the longest phase in lobsters such as *J. lalandii* that undergo an anecdysis moult cycle (seasonal, annual basis) as adults (Hazell et al., 2001b). This type of moult cycle is associated with a window of feeding, primarily in intermoult (stage C) and in early pre-moult (stage D), while lobsters in a diecdysis cycle (throughout the year), tend to begin feeding sooner and more continuously with a lesser dependence on reserve build-up (Lockwood, 1968b). This makes studying growth in crustaceans a complex challenge, as it is not continuous, periods of fasting exist, and meaningful growth measurements can only be obtained after moulting.

In pueruli (post-larvae of palinurids) and juvenile lobsters, a decrease in growth leads first to a delay in moult frequency and only later to a reduced moult increment (Chittleborough, 1975; Serfling and Ford, 1975). In contrast, exposure to unfavourable environmental conditions in adult *J. lalandii* were hypothesised to firstly lead to a reduction in moult increment, rather than a delay in moult, due to the tight coupling of moult with reproductive cycles in *J. lalandii* (Cockcroft and Goosen, 1995). Field data have shown that unfavourable environmental conditions can cause shrinkage at moult in *J. lalandii* (Cockcroft and Goosen, 1995). A Similar response has been observed in another spiny lobster, *P. interruptus* (Lindberg, 1955). Cockcroft and Goosen (1995) attributed this response in *J. lalandii* to an insufficient reserve build-up, as an alternative to a physiological response used to conserve energy, as suggested for other crustaceans (Ikeda and Dixon, 1982).

A change in the lobsters' immediate environment, such as a change in pH, temperature or oxygen concentration, especially in a combination, will more than likely have an influence on growth. As discussed in section 4.1, hypercapnia would lead to a rapid response of acid-base regulation with growth unlikely being affected over the short-term. However, if exposed chronically to a decreased pH, energy-costly proton pumping will need to be maintained (Pörtner et al., 2004) and may hence lead to diversion of energy away from energy-consuming processes involved in growth (Keppel et al., 2012). Negative effects of hypercapnia on the growth of clawed lobsters have been observed (Arnold et al., 2009; Keppel et al., 2012). The influence of hypercapnia on *J. lalandii* needs further investigation as the effects on growth are currently unknown, while the influence of temperature is far better studied.

Lobsters are poikilothermic, meaning that temperature is one of the primary factors influencing growth (Green et al., 2014). Various studies carried out on spiny lobsters have shown that, with an increase in temperature, there is an associated increase in growth rate, implying that moult increment is increased and/or intermoult period reduced (Hazell et al., 2001a; Lellis and Russell, 1990; Phillips et al., 1977; Pollock and Beyers, 1981). Increased temperature also leads to both, an increased oxygen consumption and metabolic activity, however only up to a certain limit (Chittleborough, 1975; Thomas et al., 2000). In the edible crab *C. pagurus* hypercapnia led to a decrease in the thermal tolerance limit (Metzger et al., 2007) and the same response may be observed in *J. lalandii*. The association between temperature and growth is relatively well studied in *J. lalandii* which grows fastest between 18 - 20 °C, above (and below) which growth rate decreases (Beyers et al., 1994; Dubber et al., 2004). Naturally, higher oxygen consumption in *J. lalandii* with increasing temperature is associated with an elevated energy demand (Beyers et al., 1994). The combination of low oxygen and increased temperature, as observed during phytoplankton blooms, can therefore be detrimental to growth and ingestion (Beyers et al., 1994; Crear and Forteath, 2000).

5.4. Disease

Environmental change has the potential to negatively impact the immune system of lobsters and, in turn, could lead to an increased incidence of diseases. Marine lobsters, primarily clawed-, spiny-, and slipper lobsters, as well as scampi (Briones-Fourzán and Lozano-Àlvarez, 2015) are important sources of seafood worldwide, with aquaculture producing 2 035 t in 2012 (FAO, 2013c) and capture fisheries providing some 28 922 t in 2013 (FAO, 2013a), yet studies describing diseases and immune response are scarce (Le Moullac and Haffner, 2000; Shields, 2011). This is unfortunate, since pathogens could have devastating consequences for fisheries and aquaculture (Shields, 2011). In aquaculture, for example, poor water quality can be detrimental to the health of lobsters and is known to cause, amongst others, accumulation of external growth, fungal disease, infection of wounds (*Vibrio* species) and bacterial shell disease (Evans et al., 2000).

The lobsters' first defence against pathogens is the exoskeleton, if damaged, a rapid wound sealing system in the haemolymph is initiated (Evans et al., 2000), involving coagulation and haemocyte accumulation, followed by melanisation (Evans et al., 2000). The main cell type associated with immune response are haemocytes (Johnson, 1987; van de Braak et al., 2002). There are three primary types, namely hyaline-, semi-granular- and granular haemocytes (Bauchau, 1980), each having a particular response mechanism (Johansson et al., 2000). Total haemocyte counts (THC) vary with regards to physicochemical water- and biological parameters (Cheng and Chen, 2001; Cheng et al., 2003; Hernroth et al., 2012; Lin et al., 2012; Ridgway et al., 2006; Verghese et al., 2007, 2008), with low circulating haemocyte numbers being strongly correlated to a greater sensitivity to pathogens (Le Moullac et al., 1998; Persson et al., 1987).

After exposure to a pathogen, specifically bacteria, an immune response is initiated in the following sequence: 1) Bacteria adhere to the body cavity, 2) haemocytes attach to the bacteria and 3) bacteria are subsequently broken down (Martin et al., 1996). Those that move into circulation are trapped in tissues by fine capillary networks, i.e. the gills and hepatopancreas. Entrapment occurs as a result of large nodule formation as haemocytes aggregate on the bacterium (Martin et al., 1998, 1993; Smith and Ratcliffe, 1980). Crustaceans can be very proficient in inactivating bacteria, as observed in the Penaeid shrimp (*Sicyonia ingentis*), with more than 90% of bacteria cleared within the first 10 min after exposure (Martin et al., 1993).

Changes in environmental parameters, such as hypercapnia, not only affect THC but also haemocyte functionality (Bibby et al., 2008; Hernroth et al., 2011), bacteriolytic- and antibacterial activity, and phenoloxidase activity (Lu-Qing et al., 2005). In a recent study, hypercapnia combined with temperature increase led to a pronounced negative effect in the Norway lobster (*Nephrops norvegicus*) in terms of THC (Hernroth et al., 2012). Due to the negative effect that environmental stressors, such as hypercapnia and increased temperature, commonly have (Hernroth et al., 2012; Le Moullac and

Haffner, 2000; Oweson and Hernroth, 2009), and the predicted increase in disease incidence (Stentiford et al., 2012), a weakened immune response and subsequent higher prevalence of diseases may be observed in *J. lalandii*. This could potentially impact growth and mortality as observed in aquaculture (Stentiford et al., 2012).

6. Conclusion

It is clear that the environment in which *J. lalandii* resides is particularly variable. It is exposed to a host of natural and non-natural stressors which will lead to a number of physiological responses. The resource is invaluable to South Africa, yet at a critically low level, and it is fairly certain that the environment that it inhabits is going to be changing over the long-term. Although there is a body of literature describing the biology of *J. lalandii*, the underlying mechanisms of physiological processes involved are absent. Therefore, a knowledge void exists, that needs urgent addressing. By studying the ability of *J. lalandii* to deal with the predicted environmental changes, a better understanding of fragility of the resource can be gauged. These studies will not only provide valuable general knowledge, but also direction for future studies on the species. The results obtained will offer potential inputs for the OMP and, subsequently, ensure better management of the resource in times of a changing environment.

7. References

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Chapter 3

The effects of hypercapnia on the West Coast rock lobster (*Jasus lalandii*) through acute exposure to decreased seawater pH - Physiological and biochemical responses*

Abstract

The cold water palinurid *Jasus lalandii* ("West Coast rock lobster") is a commercially important crustacean in South Africa and Namibia and inhabits the Beguela Current Large Marine Ecosystem (BCLME). This habitat is characterised by strong upwelling events in summer and algal blooms with their subsequent decay in autumn. Upwelling can lead to acute hypercapnia whereas the algal decay is associated with acute hypercapnic hypoxia. Both types of hypercapnic events could become more frequent and severe in future due to ongoing climate change. The aim of the present study was, however, to study the capability and mechanisms of response in *J. lalandii* to hypercapnia exclusively. Accordingly the following research questions were formulated: 1) To what extent is heamocyanin oxygen-binding affinity of adult *J. lalandii* pH-sensitive? 2) Can adult male WCRL respond swiftly to drastic changes in pH? 3) What physiological mechanisms facilitate a potential response to a drastically declining pH, i.e. acute hypercapnia? These questions were answered by analysing 1) the pH sensitivity of the haemocyanin's oxygen binding properties and 2) *in vivo* changes in the acid–base balance of adult *J. lalandii* during acute exposure to hypercapnia (pH 7.4). Results showed the following: 1) Haemocyanin displays a strong Bohr shift (whole blood: $\Delta\log P_{50}/\Delta pH$ = -1.17; dialysed blood: $\Delta\log P_{50}/\Delta pH$ = -0.84) in response to lowering of pH. 2) Acute hypercapnia leads to strong transitional, un-compensated acidosis of 0.3 pH units within 100 min of exposure. 3) After 1.5 h, active compensation becomes apparent as the bicarbonate levels start to increase, with complete compensation reached after 5 h of exposure (+ 2.3 mmol l^{-1}; + 48%). 4) This bicarbonate increase is reversed when returning lobsters to normocapnia (pH 7.9). 5) Haemocyanin concentration and molecular modulators of haemocyanin oxygen affinity (Ca^{2+}, Mg^{2+} and L-lactate) do not change during acute exposure to hypercapnia. The West Coast rock lobster is therefore well equipped for its habitat where these hypercapnic events are known to occur frequently.

Keywords: Upwelling, Ocean acidification, Haemocyanin, Bohr shift, Acute hypercapnia, Acid-base balance

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1. Introduction

The West Coast rock lobster (WCRL), *Jasus lalandii*, is a palinurid decapod that supports one of South Africa's oldest (Melville-Smith and van Sittert, 2005) and most important fisheries, valued in excess of 19.3 million US dollars annually and provides work for some 4200 individuals (Cockcroft et al., 2008). The species occurs in a depth range from the sub-tidal down to 200 meters (Groeneveld et al., 2010). The WCRL occurs in an area that spans from Walvis Bay (Namibia) in the northern Benguela Current sub-system to East London on the east coast of South Africa. Commercial operations are restricted to the Southern Benguela Current sub-system, i.e. just north of Lüderitz in Namibia (25 ˚S) on the West Coast to just east of the Cape Town in South Africa, where sufficient densities of *J. lalandii* are present (Beyers et al., 1994; Hutchings et al., 2009).

The Benguela Current Large Marine Ecosystem (BCLME) is one of the largest upwelling systems in the earth's oceans (Summerhayes et al., 1995) and is highly changeable in the short term. For example, temperatures in the sub-tidal range from 8 to 20 °C (recordings at the Marine Research Aquarium of the Department of Agriculture, Forestry and Fisheries from 1999 to 2015). Upwelling occurs primarily during spring and summer periods in the BCLME, in 3 - 10 day cycles (Hill et al., 1998) moving cold (~10 ºC), nutrient rich, low pH (pH 7.4 - 7.6) water into the euphotic zone (Pitcher and Probyn, 2010; Pitcher et al., 2010, 2014). The upwelling season is often followed by the collapse of a phytoplankton bloom in late summer/autumn (Probyn et al., 2000). Although pH data for the South African coastline is extremely scarce, there is substantial literature on oxygen concentrations observed in the southern Benguela (Pitcher and Probyn, 2011; Pitcher et al., 2014). One could assume with low O_2 concentrations, a high CO_2 concentration would be present as changes in oxygen and pH are strongly positively correlated in systems of this nature (Frieder et al., 2012; Paulmier et al., 2011) so that it is likely organisms would be exposed to decreased pH values. During such periods, pH levels as low as 6.6 – 7.4 have been recorded for the nearshore area for an entire water column (from surface to 8 m deep), specifically an area known as Dwarskersbos (Pitcher and Probyn, 2010). The habitat of *J. lalandii* is located in the zone where these dramatic pH changes occur (see above).

There are two trends that will potentially aggravate the situation in the BCLME with regards to hypercapnic situations: 1) The continuous absorption of atmospheric CO_2 from anthropogenic sources to the oceans feeds the on-going "ocean acidification" processes (Caldeira and Wickett, 2003; Fabry et al., 2008) leading to decreased global pH levels. The upwelled water is predicted to become more hypercapnic and, in addition, upwelling events were predicted to, and have become more frequent and severe in the Eastern Boundary Current Systems (EBCSs, Bakun, 1990; Sydeman et al., 2014), with much variability observed in the BCLME (Jarre et al., 2015). 2) Globally, eutrophication has led to an increase in the frequency, severity and duration of oxygen deficient events (Diaz and Rosenberg, 2008; Pitcher and Probyn, 2011) associated with increased phytoplankton blooms in recent decades (Glibert and Burkholder, 2002; Glibert et al., 2005). This also applies to the BCLME, where above-mentioned

nearshore hypercapnic events could become more common after algal blooms. A decrease in seawater pH affects organisms in two ways: through reduced pH and increased CO_2 (hypercapnia; Wood et al., 2008). There have been a number of studies investigating the acute effects of decreased seawater pH on crustaceans (Whiteley, 2011), but it is difficult to configure general predictions as there have been a great diversity of experimental conditions and species' responses.

Environmental hypercapnia is a potential threat to oxygen transport since many crustaceans display a substantial Bohr shift, i.e. a reduced oxygen affinity of haemocyanin with decreasing pH (Bridges et al., 2001). Acid-base adjustment by proton buffering of the extracellular fluid is therefore required. Such proton buffering is a general guarding response to CO_2-induced acidification and is achieved by a bicarbonate- and non-bicarbonate component of body fluids in all animals (Melzner et al., 2009). In crustaceans, a general response, when experiencing an acute respiratory or extracellular acidosis, is an increase in the haemolymph HCO_3^- concentration (Cameron, 1978; Henry et al., 1981; Pane and Barry, 2007; Spicer et al., 2007). This increase leads to a stabilization of the extracellular pH to resting levels (Spicer et al., 2007) or close to the original value (Cameron, 1978; Henry et al., 1981; Pane and Barry, 2007).

Like other palinurids (Crear and Forteath, 2000; Winget, 1969), *J. lalandii* is assumed to be an oxygen regulator (Zoutendyk, 1989) and would thus be able to maintain its metabolic rate by increasing the efficiency of the respiratory system through changes in circulation and ventilation at the physiological level (Burnett, 1992; Winget, 1969). At the molecular level, many crustaceans regulate oxygen transport capability of the haemolymph by modulators (lactate, magnesium and calcium ions) as these effect haemocyanins (respiratory protein) oxygen affinity, which is quantified as the change (increase/decrease) in partial pressure of O_2 required to half saturate the protein (P_{50}, Bridges, 2001). The occurrence of protons (H^+) during acidosis can have an adverse effect on haemocyanins oxygen affinity and the extent of this effect (Bohr coefficient) is dependent on the species physiological pH range (Bridges, 2001) and a reduction in haemolymph pH, if not compensated for, can lead to a decreased affinity of haemocyanin for oxygen (Morris and Oliver, 1999, Vermeer, 1987).

Due to its economic importance, there is a large body of literature on *J. lalandii*. There is little information, however, on its physiology. Known molecular and physiological aspects that are relevant to the present study are limited to the molecular weight (Joubert, 1954) and polymerization behaviour (Moore et al., 1968) of haemocyanin, the response to low oxygen (Beyers et al., 1994) and the effect of emersion on escape behaviour and haemolymph chemistry (Haupt et al., 2006). However, there is no data available with regards to the capability of adult *J. lalandii* to adjust acid-base regulation and respiration during hypercapnic exposure.

The aim of the present study was to analyse the adult WCRL's sensitivity to hypercapnia and its response mechanisms to mitigate exposure to such conditions. Accordingly, we formulated the

following research questions: 1) To what extent is haemocyanin oxygen-binding affinity of adult *J. lalandii* pH-sensitive? 2) Can adult male WCRL respond swiftly to drastic changes in pH? 3) What physiological mechanisms facilitate a potential response to a drastically declining pH, i.e. acute hypercapnia? To answer these questions, we measured a) the pH-sensitivity of the oxygen-binding properties of haemocyanin of resting lobsters and concentrations of molecular modulators during acute hypercapnia and b) the adjustment of the acid-base balance during acute hypercapnia and subsequent recovery.

Although hypercapnia occurs sometimes together with hypoxia in some parts of the WCRL's environment (see above), the present study was designed as a single-factor investigation. This is an initial step in investigating the acute physiological response of *J. lalandii* in order to provide a mechanistic basis from which further more complex multifactorial studies can be designed, imitating more closely what occurs in their natural environment.

2. Materials and methods

2.1. Experimental animals

Adult *Jasus lalandii* of both sexes were collected by use of baited traps from the research vessel "Ellen Khuzwayo" of the Department of Agriculture, Forestry and Fisheries (DAFF) of South Africa, during a routine *J. lalandii* tagging and sampling trip in the South-Eastern Atlantic in the vicinity of Cape Town (34.07 °S, 18.33 °E). These lobsters were placed into a flow through system aboard the ship for two days and, upon arrival, were transported to the Sea Point Research Aquarium of DAFF in 29 l polystyrene containers within 30 min. There, lobsters were transferred into a flow-through tank (1500 l) with a flow rate of approximately 430 l h^{-1} where they remained in normocapnic conditions for three months prior to experimentation (T_A ranged from 9 to 17 °C, salinity 34.5 - 35.0 ‰). Lobsters were fed *ad libitum* with a mixed diet of mussel (*Choromytilus meridionalis* and *Mytilus galloprovincialis*), sardines (*Sardinops sagax*) and Horse mackerel (*Trachurus trachurus)* once to twice a week in the afternoon. Four days prior to experimentation all lobsters were fed *ad libitum* with sardines, excess subsequently removed and feeding discontinued.

2.2. Haemocyanin oxygen affinity

For analysis of haemocyanin properties, 30 male and female lobsters (*CL*: 69.6 ± 0.9 mm; *w*: 186 ± 5 g) were selected from the holding tank and 1.8 ml haemolymph immediately withdrawn from each as described below and frozen in microcentrifuge (Eppendorf) tubes at -80 °C. Subsequently, the moult stage of each lobster was determined microscopically from setagenic stages of pleopods (Marco, 2012), to confirm that they were all in inter-moult stage, as assumed from time of the season. The haemolymph samples were then stored at -80 °C until transport on ice to Düsseldorf, Germany, where haemocyanin

oxygen binding properties were analysed as described previously (Knapp et al., 2015, Chapter 5). Briefly, an aliquot (1.5 ml) was dialysed (1:1000 ratio haemolymph vs. Ringer) at 4 °C for 24 h against a Ringer (0.017 M NaCl, 0.006 M $CaSO_4$, 0.003 M $MgSO_4$, 0.013 M KCl, and 0.012 M $NaHCO_3$, pH 7.8) that was based on the measured *in vivo* haemolymph composition of the same lobsters. Thereafter, haemocyanin concentration was doubled in both, the whole and dialysed haemolymph aliquot. From these preparations, oxygen affinity curves were established and analysed for whole and dialysed haemolymph by adjusting the CO_2 in the gas mix between to 0.1 and 0.5% (~0.75 - 3.7 Torr).

2.3. Acute exposure

For the acute trial, 14 male lobsters of similar size were selected from the holding tanks and transferred to an acclimation tank (1000 l), where they were kept for 24 h prior to experimentation at water conditions given in Table 1. Following acclimation, a haemolymph sample was taken from each lobster (see below), and five of the lobsters (*CL*: 77.6 ± 0.7 mm, *w*: 251 ± 6 g) transferred into individual compartments inside a glass tank (141 l) with normocapnic conditions whereas five (*CL*: 77.6 ± 0.2 mm, *w*: 237 ± 4 g) and four individuals (*CL*: 76.8 ± 0.3 mm, *w*: 236 ± 5 g), respectively, were placed in same size tanks with hypercapnic conditions. The normocapnic pH represents that of the incoming seawater on this day and is close to the level during non-upwelling periods in the sub-tidal zone. The level of hypercapnia was selected to be within the range of a typical hypercapnic situation in the habitat of the WCRL (see Introduction). In order to minimize the time difference in exposure to the set seawater parameters of the respective treatments, consecutive lobsters were placed in alternating treatments after the initial haemolymph sample was withdrawn (i.e. first normocapnia, second hypercapnia). At the given time points (Table 2), pre-branchial haemolymph (max. 1 ml) was withdrawn from the arthrodial membrane at the base of the fifth pair of pereiopods by syringe with hypodermic needle (Neomedic 1 ml, 29 G); avoiding tail flips (i.e. accumulation of L-lactate) by securing the abdomen with a firm grip. After the 24 h exposure period, all lobsters from the normocapnic- and the five lobsters from one hypercapnic group were transferred into same size recovery tanks. In order to investigate the response in the initial 90 min not covered in the above hypercapnic group, haemolymph samples were taken at short intervals for the first 100 min of exposure from four lobsters. All water conditions are given in Table 1. Circulation in the tanks was achieved by JVP-202 12000 l h^{-1} propellers (JVP, China) and air was provided from the aquariums compressed air system. The pH of the hypercapnic tanks was set as described previously (Knapp et al., 2015, Chapter 5). T_A, pH, A_T and salinity were measured and pCO_2, HCO_3^- and CO_3^{2-} were calculated as outlined in (Knapp et al., 2015, Chapter 5) using the appropriate constants. Oxygen concentration was determined using a Multi 350i meter set (WTW, Germany). Water quality was monitored by measuring NH_4^+ concentration (Ammonia test kit, Sera, Germany) and never exceeded 0.4 mg l^{-1}. Moult stage was determined for each lobster after the trial (see above).

Table 1. Physicochemical seawater conditions recorded during acclimation and subsequent acute exposure of adult *J. lalandii* to normocapnic and hypercapnic conditions.

Treatment	T_A °C	pH	AT (µmol kg $^{-1}$)	O_2 (%)	Salinity (‰)	Ca^{2+} (mmol l^{-1})	Mg^{2+} (mmol l^{-1})	pCO_2 (Torr)	HCO_3^- (mmol l^{-1})	CO_3^{2-} (mmol l^{-1})
Acclimation	18.8 ± 0.0	7.82 ± 0.06	2028 ± 24	96	34.5 ± 0.1	8.4 ± 0.9	57.5 ± 9.5	0.5 ± 0.1	1.8 ± 0.0	0.1 ± 0.0
Normocapnia	18.7 ± 0.1	7.85 ± 0.03	2011 ± 25	90	34.5 ± 0.0	10.3 ± 1.0	51.5 ± 3.7	0.4 ± 0.0	1.8 ± 0.0	0.1 ± 0.0
Hypercapnia	18.3 ± 0.3	7.39 ± 0.08	2011 ± 02	96	34.5 ± 0.0	9.6 ± 1.7	54.5 ± 2.0	1.4 ± 0.3	2.0 ± 0.0	0.0 ± 0.0
Recovery	18.8 ± 0.1	7.89 ± 0.07	2018 ± 32	93	34.8 ± 0.3	10.2 ± 1.1	46.0 ± 1.9	0.4 ± 0.1	1.8 ± 0.0	0.1 ± 0.0

Values are mean ± S.E.

Measurement of total CO_2 (cCO_2) and pH from 100 µl of haemolymph and subsequent calculation of pCO_2 and bicarbonate concentration as well as determination of the concentrations of Ca^{2+}, Mg^{2+}, L-lactate and haemocyanin were done as described in Knapp et al. (2015, Chapter 5).

2.4. Statistical analysis of data

Data analysis was carried out using SAS software (Statistical Analysis System, Version 9.3, SAS institute Inc., Cary, NC, USA). A repeated measures ANOVA (PROC MIXED) was used to test for differences between treatments per time interval as well as for differences between time points per treatment. Residuals were assessed for normality and homoscedasticity by means of Shapiro-Wilk and Bartlett tests, respectively, and where necessary, values where log-transformed to meet underlying assumptions of models. Bonferroni adjustment of P-values was employed to assess differences within and between treatment groups where F-tests were significant.

3. Results

3.1. Respiratory capacity of haemolymph

At a CO_2 tension of 0.1% (~0.75 Torr) whole haemolymph (P_{50} = 14.1 Torr, pH 7.84) and dialysed (to reduce influence of some molecular modulators of oxygen affinity) haemolymph (P_{50} = 15 Torr, pH 7.87) had a similar oxygen affinity. An increased pCO_2 led to a decreased oxygen affinity (i.e. increased P_{50}) of haemocyanin: A change of 5.9 Torr occurred in whole haemolymph by increasing pCO_2 from 0.1 - 0.5%. A similar response was observed in the dialysed haemolymph, with a change of 3.8 Torr. In order to determine the influence of molecular modulators (Ca^{2+}, Mg^{2+} and L-lactate) on the oxygen binding affinity of the haemocyanin, the relationship between O_2 affinity and pH of whole and dialysed haemolymph was investigated, it can be described by the Bohr coefficient (attained by plotting P_{50} against pH), which were -1.17 (r^2 = 0.88) for whole haemolymph and -0.84 (r^2 = 0.85) for dialysed haemolymph.

Some differences in haemocyanin concentration occurred within treatments over the time course of the 32 h acute exposure experiment, with the highest concentration recorded in each treatment at the 3 h sampling and the lowest after recovery (Table 2). However, these changes were small compared with the variation of individual values (i.e. S.E.s). There were no differences between treatments. In the 100 min, no change occurred.

Potential molecular modulators (Ca^{2+}, Mg^{2+}, L-lactate) showed only small, but in some cases significant differences within treatments over their respective time intervals (Table 2). There were no differences, however, between the 32 h treatments.

3.2. Acid-base regulation

The acute exposure experiment showed a marked difference between the responses to normocapnic and hypercapnic conditions, respectively.

In the normocapnic control group, pH increased by 0.08 units during the first 1.5 h from an initial pH of 7.64 but, within another 1.5 h, had returned to values close to that of initial levels were it remained for the rest of the trial. Extracellular total CO_2 levels decreased by 49% within 8 h from the initial level measured, thereafter increased to a value 21% below the initial level at the 32 h time point (i.e. after 8 h recovery). Levels of pCO_2 decreased from the initial value until the 8 h mark, thereafter returned closer to the initial value. The Henderson-Hasselbalch diagram of the normocapnic treatment showed very little movement in [HCO_3^- + CO_3^{2-}], pCO_2 and pH compared with that of hypercapnic exposure (Figure 2A, B; Table 2).

The lobsters in the hypercapnic treatment, due to the increase in seawater pCO_2 and subsequently haemolymph pCO_2 (Table 2), showed an extracellular pH decrease of 0.07 units from an initial value of 7.63 within 1.5 h after sudden exposure to hypercapnia (Figure 1A, Table 1 and 2). While this suggests a moderate increase in acidity of about 16%, a sub-set of data, that covered the first 100 min of exposure in more detail, revealed that the decline in extracellular pH is more drastic by approximately 0.3 units (insert in Figure 1A) and that, at the 1.5 h mark, pH was already actively recovering. The decline in pH measured during the initial 100 min, represents an acidification of approximately 110% (to ~53 nM H+) from the start to 60 - 75 min of exposure. Subsequently, in the 32 h trial haemolymph pH increased steadily from the 1.5 h interval until reaching a plateau at approximately pH 7.7 after 8 h, where it remained for the last 16 h of exposure. This is an over-compensation of pH by approximately 0.06 pH units or reduction in [H+] by 3 nM (13%) compared with initial levels. Thereafter, recovery in normocapnic seawater did not change the haemolymph pH. During 24 h of hypercapnic exposure, extracellular total CO_2 (cCO_2) levels rose by 53% from an initial 4.9 $mmol \cdot l^{-1}$ (Table 2). Recovery in normocapnic seawater subsequent to 24 h hypercapnic exposure caused a sharp decline to 39% below the initial extracellular cCO_2 level. Values for [HCO_3^- + CO_3^{2-}] followed a similar trend (Table 2, Figure 1B). The partial pressure of CO_2 increased from its initial level peaking at the 3 h time interval after which it decreased marginally but remained raised above the initial value. Data was used to construct Henderson-Hasselbalch diagrams, depicting the extracellular pH, calculated [HCO_3^- + CO_3^{2-}] and pCO_2 values (Figures 2B, C). A respiratory acidosis is apparent under exposure to hypercapnia: As the pH decreases, there is an increase in [HCO_3^- + CO_3^{2-}], buffering the extracellular acidosis after 5 h of hypercapnia. This is indicated by a shift to the right, i.e. leading to an alkalosis when compared to the initial pH measured. This alkalosis is carried over to the 32 h (recovery) value. Between treatments, haemolymph pHs only differed at the 1.5 h time interval, after which they remained at similar, stable levels, including after 8 h of recovery (Table 2, Figure 1A).

Table 2. Time course of *in vivo* haemolymph parameters of adult *J. lalandii* during acute exposure to normocapnic (pH 7.9) and hypercapnic (pH 7.4) conditions.

Exposure time	pH	cCO_2 ($mmol \cdot l^{-1}$)	pCO_2 (Torr)	$[HCO_3^- + CO_3^{2-}]$ ($mmol \cdot l^{-1}$)	Ca^{2+} ($mmol \cdot l^{-1}$)	Mg^{2+} ($mmol \cdot l^{-1}$)	L-Lactate ($mmol\ l^{-1}$)	Haemocyanin ($mg\ ml^{-1}$)
Normocapnia (h)								
0	$7.64^{a} \pm 0.01$	$4.7^{a} \pm 0.1$	$2.4^{a} \pm 0.1$	$4.6^{a} \pm 0.1$	$11.5^{a} \pm 0.9$	$8.9^{a} \pm 0.5$	$0.0^{a} \pm 0.0$	$65.2^{a,b} \pm 9.7$
1.5	$7.72^{a} \pm 0.03$	$3.6^{a,b} \pm 0.3$	$1.5^{b} \pm 0.1$	$3.5^{a,b} \pm 0.3$	$13.3^{a,b} \pm 0.6$	$8.9^{a} \pm 0.4$	$0.0^{a} \pm 0.0$	$65.8^{a,b} \pm 8.5$
3	$7.60^{a} \pm 0.05$	$2.9^{b,c} \pm 0.2$	$1.6^{a,b} \pm 0.2$	$2.8^{b,c} \pm 0.2$	$14.3^{a,b} \pm 0.5$	$9.1^{a} \pm 0.4$	$0.1^{a} \pm 0.1$	$83.0^{a} \pm 9.2$
5	$7.64^{a} \pm 0.01$	$2.6^{b,c} \pm 0.1$	$1.3^{b} \pm 0.1$	$2.6^{b,c} \pm 0.1$	$15.4^{b} \pm 0.5$	$8.8^{a} \pm 1.2$	$0.1^{a} \pm 0.1$	$62.6^{a,b} \pm 7.3$
8	$7.67^{a} \pm 0.01$	$2.4^{c} \pm 0.1$	$1.1^{b} \pm 0.0$	$2.3^{c} \pm 0.1$	$15.4^{b} \pm 0.6$	$10.3^{a} \pm 0.7$	$0.1^{a} \pm 0.1$	$73.9^{a,b} \pm 8.1$
24	$7.65^{a} \pm 0.03$	$3.4^{b,c} \pm 0.3$	$1.7^{a,b} \pm 0.2$	$3.3^{b,c} \pm 0.3$	$12.8^{a,b} \pm 0.4$	$9.6^{a} \pm 0.3$	$0.0^{a} \pm 0.0$	$64.5^{a,b} \pm 7.0$
32 (Recovery)	$7.65^{a} \pm 0.03$	$3.7^{a,b} \pm 0.3$	$1.9^{a,b} \pm 0.2$	$3.6^{a,b} \pm 0.3$	$14.0^{a,b} \pm 0.5$	$9.0^{a} \pm 0.4$	$0.0^{a} \pm 0.0$	$57.5^{b} \pm 6.7$
Hypercapnia (h)								
0	$7.63^{a} \pm 0.03$	$4.9^{a} \pm 0.4$	$2.6^{a} \pm 0.2$	$4.8^{a} \pm 0.4$	$13.6^{a} \pm 0.5$	$6.9^{a} \pm 1.5$	$0.0^{a} \pm 0.0$	$74.0^{a} \pm 13.4$
1.5	$7.56^{a*} \pm 0.02$	$5.8^{a,b*} \pm 0.2$	$3.5^{b*} \pm 0.2$	$5.7^{a,b*} \pm 0.2$	$15.2^{a} \pm 0.4$	$8.3^{a} \pm 1.1$	$0.1^{a} \pm 0.0$	$59.9^{a,b} \pm 5.9$
3	$7.58^{a} \pm 0.05$	$6.7^{b,c*} \pm 0.4$	$4.0^{b*} \pm 0.4$	$6.5^{b,c*} \pm 0.4$	$15.0^{a} \pm 0.7$	$10.3^{a} \pm 0.4$	$0.1^{a} \pm 0.1$	$73.5^{a} \pm 5.6$
5	$7.67^{a} \pm 0.03$	$7.2^{c*} \pm 0.2$	$3.4^{ab*} \pm 0.2$	$7.1^{c*} \pm 0.2$	$16.2^{a} \pm 0.9$	$10.7^{a} \pm 0.3$	$0.2^{a} \pm 0.1$	$61.6^{a,b} \pm 7.4$
8	$7.69^{a} \pm 0.01$	$7.2^{c*} \pm 0.1$	$3.3^{ab*} \pm 0.1$	$7.0^{c*} \pm 0.1$	$14.7^{a} \pm 0.5$	$10.6^{a} \pm 0.1$	$0.1^{a} \pm 0.0$	$63.2^{a,b} \pm 5.8$
24	$7.69^{a} \pm 0.01$	$7.5^{c*} \pm 0.2$	$3.4^{ab*} \pm 0.2$	$7.4^{c*} \pm 0.2$	$13.3^{a} \pm 0.7$	$7.9^{a} \pm 0.9$	$0.0^{a} \pm 0.0$	$51.7^{b} \pm 5.6$
32 (Recovery)	$7.69^{a} \pm 0.03$	$3.0^{d} \pm 0.2$	$1.4^{c} \pm 0.0$	$3.0^{d} \pm 0.2$	$13.9^{a} \pm 1.2$	$8.1^{a} \pm 1.0$	$0.3^{a} \pm 0.2$	$50.9^{b} \pm 4.3$
Hypercapnia (min)								
0	$7.59^{a} \pm 0.05$	$5.3^{a} \pm 0.1$	$3.0^{a} \pm 0.6$	$5.2^{a} \pm 0.2$	$15.2^{a} \pm 0.6$	$8.7^{a} \pm 0.4$	$0.1^{a} \pm 0.0$	$56.2^{a} \pm 5.4$
25	$7.37^{b} \pm 0.09$	$5.7^{a} \pm 0.4$	$5.3^{b} \pm 1.3$	$5.4^{a} \pm 0.8$	$17.1^{a} \pm 1.1$	$8.8^{a} \pm 0.2$	$0.2^{a,c} \pm 0.1$	$56.8^{a} \pm 2.9$
50	$7.32^{b} \pm 0.08$	$5.7^{a} \pm 0.6$	$5.8^{b} \pm 0.9$	$5.4^{a} \pm 1.3$	$15.1^{a} \pm 1.5$	$8.4^{a} \pm 0.7$	$0.3^{a,b,c} \pm 0.1$	$45.3^{a} \pm 4.0$
60	$7.27^{b} \pm 0.07$	$5.3^{a} \pm 0.6$	$6.0^{b} \pm 0.6$	$5.1^{a} \pm 1.3$	$15.6^{a} \pm 0.9$	$8.0^{a} \pm 0.2$	$0.6^{b,c} \pm 0.1$	$58.7^{a} \pm 8.1$
75	$7.28^{b} \pm 0.08$	$5.4^{a} \pm 0.5$	$6.0^{b} \pm 0.9$	$5.1^{a} \pm 1.1$	$18.3^{a} \pm 0.8$	$9.2^{a} \pm 0.2$	$0.7^{b} \pm 0.2$	$54.2^{a} \pm 4.7$
100	$7.30^{b} \pm 0.08$	$5.6^{a} \pm 0.7$	$6.0^{b} \pm 0.5$	$5.3^{a} \pm 1.5$	$17.5^{a} \pm 1.1$	$8.3^{a} \pm 0.5$	$0.7^{b} \pm 0.2$	$54.2^{a} \pm 3.9$

Values are means ± S.E. (n = 4 – 5).

^{a,b,c}Values within a column sharing same superscript do not differ significantly within the respective treatment ($p < 0.05$).

*Significantly different from the respective sampling time of the normocapnic group (32 h).

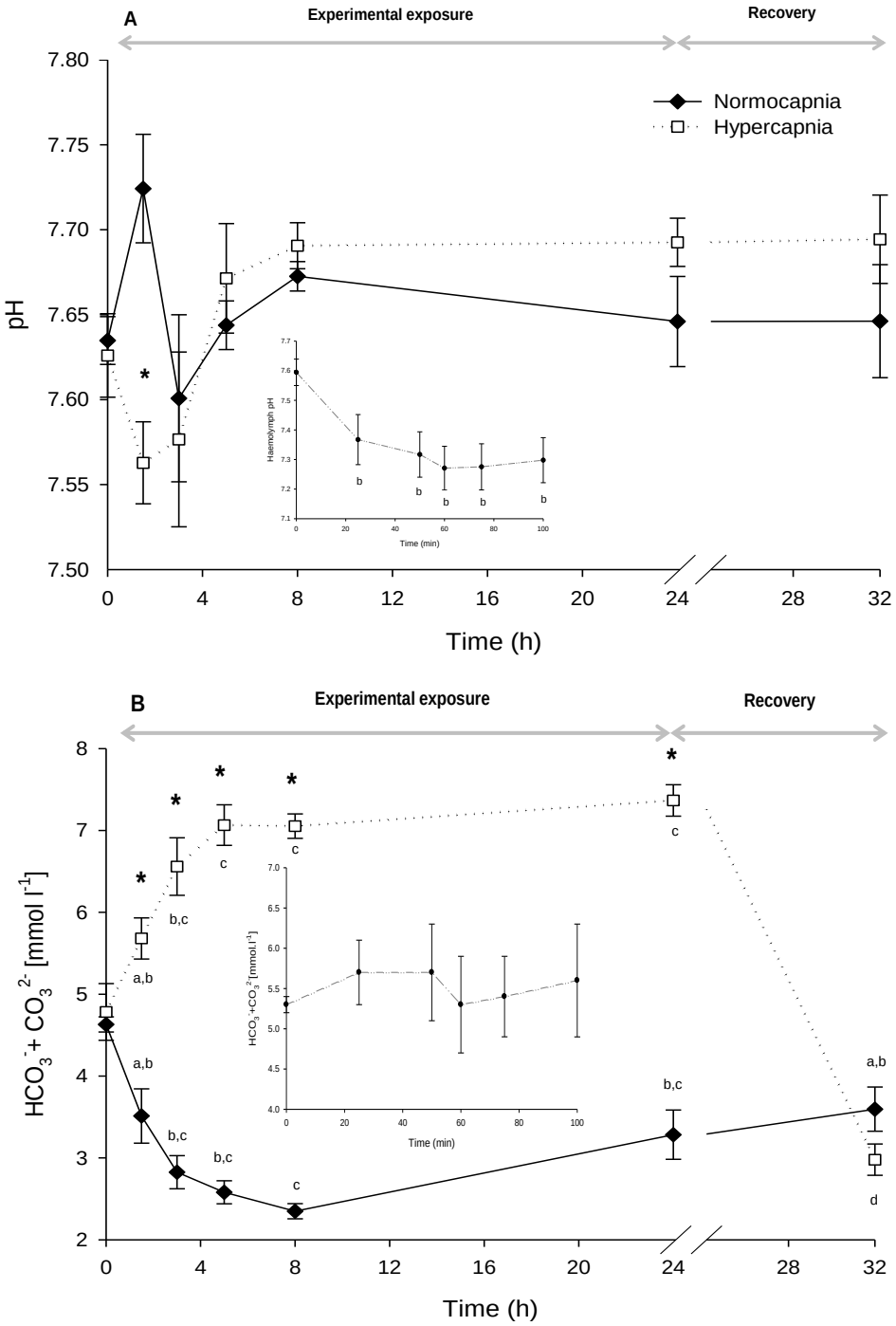

Figure 1. Time course of *J. lalandii* haemolymph (A) measured pH and (B) calculated [HCO_3^- + CO_3^{2-}] during acute exposure to normocapnic seawater (solid line, n = 5) and hypercapnia (dashed line, n = 5) for 24 h followed by 8 h recovery in normocapnic seawater. Inserts depict haemolymph pH and [HCO_3^- + CO_3^{2-}] for a group of *J. lalandii* (n = 4) that was frequently sampled during a short exposure to hypercapnia for 100 min. Values are mean ± S.E. *Significantly different from the respective sampling time of the normocapnic group ($p < 0.05$). Different lower case letters indicate significant differences within treatments over time ($p > 0.05$).

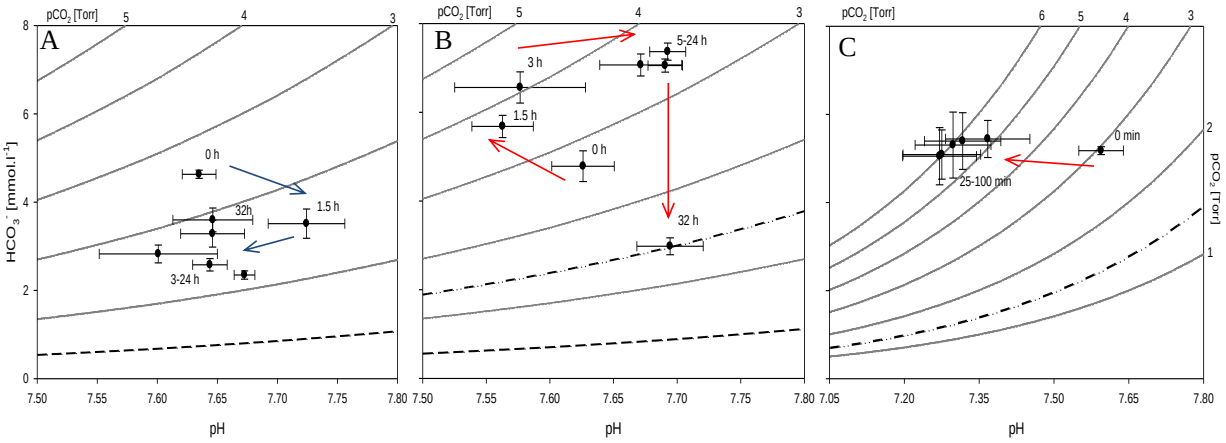

Figure 2. Henderson-Hasselbalch (pH-bicarbonate) diagrams for haemolymph of adult *J. lalandii* constructed from the time course of values during acute exposure presented in Table 2. (A) During 24 h normocapnia and subsequent 8 h recovery (n = 5), (B) during 24 h hypercania followed by 8 h recovery (n = 5) and (C) during 100 min of hypercania (n = 4). The pCO_2 isopleths (grey lines) were derived from the Henderson-Hasselbalch equation. Appropriate values for the first dissociation constant (pK'_1) and solubility coefficient (α) were derived from (Truchot, 1976). Dashed line = normocapnic/recovery seawater isopleth, dash dot dot line = hypercapnic seawater isopleth. Values are mean ± S.E.

Total CO_2, calculated pCO_2 and [HCO_3^- + CO_3^{2-}], however differed already at 1.5 h time point (Table 2, Figure 1B). Thereafter, the difference increased steadily until the 8 h sampling, from which it remained stable throughout the rest of the 24 h. During the recovery period, all three parameters declined sharply to reach the low level of the normocapnic group. Levels of molecular modulators did not differ between the 32 h treatments throughout the experiment, Lactate levels, however were raised in the 100 min (Table 2).

4. Discussion

The habitat of *J. lalandii* is characterised by periodic episodes of hypercapnia resulting from upwelling events in summer and low oxygen events from algal decay in autumn. The two major findings of the present study, that have relevance to those conditions, are:

1. Haemocyanin displays a large Bohr shift, making the WCRL vulnerable to low extracellular pH.
2. A rapid adjustment of the acid-base balance by increased bicarbonate levels keeps pH within physiological range, protects oxygen affinity of haemocyanin and ensures an outward CO_2 gradient.

4.1. pH sensitivity of oxygen carrying capacity of haemolymph

The haemocyanin oxygen affinity of *J. lalandii* (P_{50} = 14.1 Torr at 15 ºC) is within the range of the closely related Australian species *Jasus edwardsii* (P_{50} = 25.8 Torr at 20 °C), when considering the higher experimental temperature (Morris and Oliver, 1999). Morris (1991) suggested that haemocyanin oxygen affinity is an adaptation to environmental oxygen levels and that there is potential correlation between low environmental O_2 and haemocyanin affinity. The slightly higher affinity in adult *J. lalandii* is therefore a possible result of consistent exposure to low oxygen events in the Benguela Current system whereas there is a low likelihood of exposure to such conditions for *J. edwardsii* (Morris and Oliver, 1999). Interestingly, although frequent exposure to hypoxic conditions is known for adult lobsters, their oxygen affinity is similar to juveniles (Knapp et al., 2015, Chapter 5). Thus, the overall oxygen carrying capacity of adults should be several times higher than in juveniles when taking the much lower haemocyanin concentration in juveniles (Knapp et al., 2015, Chapter 5) into account and this may play a vital role in enabling them to survive moderate hypoxic environments as experienced frequently along the West Coast of Southern Africa. However, haemocyanin concentration is known to vary, depending on moult cycle and size (Chen and Cheng, 1993). Nonetheless, the lobsters used in the acute trial were all males and were in a similar moult stage. Interestingly in both treatments, haemocyanin concentration peaked at the 3 h time interval and were lowest during recovery. The small extent of these changes, however, should be of low biological significance.

Molecular modulators of haemocyanin oxygen affinity are unlikely to play a role under acute hypercapnic conditions: Firstly, although removal of modulators by dialysis marginally reduced oxygen affinity of haemocyanin (reduced P_{50}), this was outside the physiological pH range measured during the trial. Closer to the *in vivo* pH range, oxygen affinities of whole and dialysed haemolymph were almost identical (a further, more in-depth investigation is needed here). Secondly, in our 32 h acute experiment, measured concentrations of potential molecular modulators (Ca^{2+}, Mg^{2+}, L-lactate) did not change. In the 100 min L-lactate increased significantly, however this is more than likely due to the short periods between sampling and thus the stress associated with this. The findings in the 32 h experiment resemble the situation in juvenile *J. lalandii* after prolonged hypercapnia, where molecular modulators (Knapp et al., 2015).

The oxygen affinity of haemocyanin from adult *J. lalandii* decreased strongly with increasing pCO_2 (i.e. displaying a strong Bohr shift) in the range that the lobsters would experience naturally as well as that observed during the acute trial. This indicates that haemocyanin of adults is more sensitive than that of juveniles which have a lower Bohr coefficient (-0.57, pH 8, 15 °C, Knapp et al., 2015). For whole haemolymph of adult *J. lalandii*, a coefficient of -1.17 was calculated, the same as the -1.17 (pH 7.9, 15 °C) in *Homarus vulgaris* (Bouchet and Truchot, 1985), but greater than the -0.83 (pH 7.8, 15 °C) reported for *C. maenas* (Lallier and Truchot, 1989).

4.2. Regulation of acid-base balance

Oxygen affinity needs to be protected in the presence of a pronounced Bohr shift (see above) to guarantee loading of haemocyanin with oxygen at the gills and offloading at the tissues. In addition, extracellular pCO_2 must be maintained above environmental pCO_2 to ensure an outward gradient for CO_2 removal. Both are rapidly achieved in *J. lalandii* by elevation of [HCO_3^-] by a net 2.6 mmol·l^{-1} (+ 54%) after 24 h. Such increase in bicarbonate levels is typical in crustaceans in response to respiratory or extracellular acidosis (Cameron, 1978; Henry et al., 1981; Pane and Barry, 2007; Spicer et al., 2007). This increase in HCO_3^- is also needed to compensate for disturbance of acid-base balance to achieve resting pH levels (Spicer et al., 2007) or close to original values (Cameron, 1978; Henry et al., 1981; Pane and Barry, 2007).

Such compensatory capacity is present in *J. lalandii*: When exposed to an ambient pH of 7.4, they fully compensated for the decreased environmental pH within 5 hours post exposure, with a net increase of 0.066 pH units after 24 h (Figure 1A, Table 2). This is rapid compared with other crustacean species and some species seem unable to fully compensate. The Grooved Tanner crab (*Chionoectes tanneri*), for example, when exposed to a pH of 7.1, experienced a net reduction of 0.32 pH units (HCO_3^- - 3mM) over 24 h and the Dungeness crab (*Cancer magister*) showed an incomplete extracellular compensation after 24 h, with a net reduction of 0.08 pH units (HCO_3^- - 12mM, Pane and Barry, 2007).

The pre-branchial initial extracellular pH for *J. lalandii* throughout experiments was approximately 7.6 (T_A 18 - 19 °C, Table 2). During exposure to a pH of 7.4, lobsters reached a minimum extracellular pH of 7.56 after 1.5 h, only being 0.07 pH units lower than the initial value (Table 2). This is misleading, however, as apparent in the group that was more frequently sampled in the first 100 min of hypercapnia: An uncompensated acidosis occurred within the first 60 – 75 min (Insert in Figure 1A, Table 2) after which extracellular pH slowly increased. It did not recover to the extent of the 1.5 h sampling in the other hypercapnic group, possibly because of the observed L-lactate built-up due to frequent handling (Table 2), this L-lactate may also very well have aided in the reduced pH observed. A short fall here, is that no control treatment was used and so the extent that the L-lactate had on the pH cannot be assessed. In the other treatments, no such built-up of L-lactate was measured because lobsters had sufficient time to recover until the next sampling.

The above-mentioned acidosis was not detected at the 1.5 h sampling point in the other hypercapnic group, possibly due to the strong and rapid response to decreased seawater pH which became apparent at this time point. Additionally, it may have been masked by hyperventilation (Burnett and Johansen, 1981) as can be assumed from the observations in the normocapnic group (18% alkalosis compared with resting) at the 1.5 h mark. Other authors noted that they may have missed physiological changes in this early period. For example, an acidosis was not detected in *N. puber* during exposure to a pH of 7.3 when the first haemolymph sampling was done only 48 h post exposure (Spicer et al., 2007). In *J.*

lalandii, that had been exposed to hypercapnia, a new resting steady state was reached eight hours after returning the lobsters to normocapnic seawater for recovery, indicated by a return of bicarbonate levels to initial values and an unchanged pH.

The Henderson-Hasselbalch diagrams provided an illustration of the interaction of extracellular pH, calculated haemolymph bicarbonate and pCO_2: In the group of lobsters exposed to normocapnic conditions, there is very little movement compared with hypercapnic exposure (Figures 2A, B). The small changes observed, however, were caused by the changes in both extracellular pH and HCO_3^- (Table 2, Figure 2A). This may indicate hyperventilation (response to handling stress) during sampling. This was not obvious in the hypercapnic groups where it may have been masked by the exposure to a reduced seawater pH and the subsequent decrease in haemolymph pH, which would counteract the increase in haemolymph pH associated with hyperventilation.

After exposure to hypercapnic conditions, there is a strong transitional acidosis (Table 2, Figure 1A, 2B, C) within 1.5 h after which compensation by bicarbonate becomes effective (Figure 1B, 2B). Acidosis is more drastic and uncompensated when looking at the hypercapnic group that was sampled more frequently during the period (first 100 min of exposure) not covered in the other hypercapnic group (Figure 2C). After 3 h of exposure, compensation by increased bicarbonate levels (upward move) becomes apparent and the pH starts to increase (movement to the right). Subsequently, further increasing bicarbonate levels caused a move into respiratory alkalosis and over-compensation (Figure 2B). When lobsters were moved from hypercapnic to normocapnic conditions after 24 h, alkalosis remained despite a substantial and fast decline in bicarbonate concentration. Adjustment of pH to initial levels takes probably longer to allow cellular processes to adjust.

5. Conclusion

The experimental findings reveal that *J. lalandii* is able to rapidly and fully compensate for hypercapnic induced extracellular acidosis over the short term period of 24 hours. Haemolymph pH declines drastically shortly after onset, but this is quickly compensated by a, slightly lagging, and reversible increase in extracellular bicarbonate. This adjustment protects the oxygen carrying capacity of haemocyanin under hypercapnic conditions, therefore equipping the species to deal with hypercapnic events that occur frequently in its habitat.

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Chapter 4

Synergistic effects of elevated seawater pCO_2 and temperature on growth and survival of a South African cold water palinurid, *Jasus lalandii*

Abstract

Somatic growth rate and abundance are two factors that feed into various inputs of the Operational Management Plan (OMP), which is used to ensure sustainable management of the South African West Coast rock lobster (WCRL) resource. With the predicted global changes, however, associated with anthropogenic CO_2 emissions, such as, ocean acidification (decreased seawater pH) and temperature increase, somatic growth rate may be influenced. In the present study, two separate long-term trials were conducted, whereby the effects of a single stressor was investigated first, namely increased seawater pCO_2 (pH 8 and 7.3, 18.8 °C, ~28 weeks). Lobsters from the normocapnic treatment grew faster (24% *CL*, 34% *w*) than those from hypercapnic. Secondly, four combinations of two stressors: seawater pCO_2 (pH 8 and 7.3) and temperature (15.6 and 19 °C, ~48 weeks). In spite of the extended period of hypercapnia, juvenile lobsters were able to actively maintain acid-base balance throughout the duration of the trials. Growth rate was highest in lobsters exposed to a combination of hypercapnia and high temperature, while slowest growth- and highest mortality rates were observed in lobsters exposed to normocapnia and low temperature conditions. Overall, effects on average daily gain (ADG) occurred in the following order: high temperature (31% carapace length (*CL*), 49% weight (*w*) higher than low temperature) > hypercapnia (20% *CL*, 34% *w*) > normocapnia (11% *CL*, 14% *w*) > low temperature. The two trials showed contrasting impacts of hypercapnia, the differences are discussed. The results show that juvenile WCRL will cope well under future global climate and ocean scenarios, if food availability does not become limiting.

Keywords: *Jasus lalandii*, Ocean acidification, Climate change, Long-term exposure, Growth rate, Crustaceans.

1. Introduction

The increased variability associated with upwelling and global temperature change in South African coastal waters (Jarre et al., 2015) and long-term changes predicted for our oceans over the coming centuries in general (Caldeira and Wickett, 2003; Rhein et al., 2013), will affect various marine biota (Hofmann et al., 2010; Whiteley, 2011). The organisms most likely to be affected by the associated changes in the physicochemical properties of seawater are those that rely on the process of calcification to build exoskeletons (Fabry et al., 2008; Findlay et al., 2009; Ries et al., 2009). With the increasing amount of anthropogenic CO_2 absorbed by the oceans, seawater becomes more corrosive due to a decreased pH (Raven et al., 2005; Rhein et al., 2013), and under-saturated with regards to carbonate species involved in shell formation (Feely et al., 2008). In order, therefore, to maintain shell integrity, an increased energy demand is placed on powering proton pumps which may divert energy away from important processes, such as growth (Keppel et al., 2012; Pörtner et al., 2004). Hypercapnia may not only affect growth negatively, but also lead to a reduced thermal tolerance (Metzger et al., 2007). In the field, the combination of hypercapnia and increased temperature may translate to a greater energy deficit, as well as increased mortality.

Crustaceans investigated thus far, however, have shown to be relatively unperturbed by the effects of hypercapnia in the short- to medium term. In the short term a strong acid-base response protects underlying mechanisms (e.g. oxygen transport, Bridges, 2001) and allows for their proper functioning, in spite of the environment. While, over the medium term, literature thus far shows no negative affect on calcification, in fact, calcification rate is either unchanged or increased (Ries et al., 2009). The limited literature currently available on the impact of hypercapnia on growth rates of lobsters showed contrasting results: The American lobster (*Homarus americanus*) was negatively affected (Keppel et al., 2012), whereas the European lobster was not (*Homarus gammarus*, Arnold et al., 2009). The body of literature describing the effect of temperature on growth, however, is far greater. As temperature increases, growth and metabolic rate increase, up until a species-specific thermal tolerance limit is reached (Chittleborough, 1975; Thomas et al., 2000). At, and above this tolerance limit, growth is negatively affected. In lobsters, if growth is stunted, it is conveyed in either 1) an increased intermoult period (IMP) and/or 2) a decreased moult increment (i.e. decrease in weight and/or carapace length), while an increase in growth has the opposite effect (Cockcroft and Goosen, 1995; Dubber et al., 2004; Hazell et al., 2001; Lockwood, 1968; Pollock and Beyers, 1981). Developmental stage however, will more than likely play a role in determining which of the two occurs first, as adult lobsters are dependent on seasonal moulting for synchronization of the reproductive cycle (Cockcroft and Goosen, 1995), while juveniles are not.

Studying growth in crustaceans is somewhat challenging, as it is not a continuous process, being interrupted by shedding of the old exoskeleton in order to increase in size. This process is associated with a period of fasting, major internal ion concentration adjustments, cannibalism and mortality

(Barkai and Branch, 1988; Cameron and Wood, 1985; Lockwood, 1968; Neufeld and Cameron, 1992; Robertson, 1960; Sakamoto et al., 2009). Currently, to the best of my knowledge there is not a single study on the combined effects of hypercapnia and different temperatures on somatic growth of spiny lobsters. The West Coast rock lobster (WCRL), *Jasus lalandii*, is a cold water palinurid that forms the backbone of a large industry (> 250 million rand, Cockcroft et al., 2008) in South Africa. It is found in the Benguela Current Large Marine Ecosystem (BCLME); one which shows substantial variation over the medium term in relation to temperature change (Blamey et al., 2012; Jarre et al., 2015; Rouault et al., 2010). One process responsible for this variability, is the occurrence of upwelling, associated with cold, nutrient rich, corrosive (low pH), low oxygen water (Gregor and Monteiro, 2013; Pitcher et al., 2010; Pitcher and Probyn, 2011). The effects of temperature and oxygen concentration on the somatic growth rate in *J. lalandii* have been studied previously. Both had an effect on growth rate, the latter however, only at an oxygen concentration of less than 35% (Beyers et al., 1994; Dubber et al., 2004).

The South African WCRL resource is currently at a critical level of 2.6% of pristine (DAFF, 2014) and any further deterioration could lead to difficulties for the Department of Agriculture, Forestry and Fisheries (DAFF) to manage the resource. This would have severe impacts on the industry and, in turn, on job security and income. So far, the resource is managed sustainably, however, by DAFF according to an Operational Management Plan (OMP) that provides for the indirect input of the impacts of environmental change. Somatic growth and abundance are two such parameters that provide for the entry of climate change-related effects (Johnston et al., 2012). These experiments were conducted to determine the effects of long-term exposure to 1) a much reduced seawater pH on the WCRL in terms of various physiological and biological parameters and 2) more closely mimicking what would be found in the field, the effects of a combination of hypercapnia and temperature change over the long term with regards to similar parameters to those assessed in 1. These results would have the potential to provide key inputs into the current OMP as well as insight into the future development of the resource.

2. Methods and materials

2.1. Trial 1

2.2.1. Experimental animals

Juvenile lobsters were collected in Saldanha Bay, which is a large semi-enclosed bay on the West Coast of South Africa (Groeneveld et al., 2010). Lobsters were obtained from oyster stacks at an offshore oyster farm (Saldanha Bay Oyster Company) situated opposite the mouth of the bay (32°59′–33°05′ S, 17°56′– 18°02′ E). Oyster stacks were sampled during routine maintenance operations between September and December 2012. All specimens (n = 43) were placed in plastic bags filled to 50% with 4.5 l seawater. These were bubbled with oxygen before sealing and the bags then placed into a polystyrene container with ice bricks to ensure T_A < 20 °C. Traction for the lobsters in the bags was

provided by mesh cloth; this minimised stress during transport. These lobsters were subsequently transported to the Marine Research Aquarium of DAFF in Sea Point, Cape Town. Immediately upon arrival, the juveniles were placed into glass tanks *(L × W × H: 100 × 48 × 35 cm).* Seawater flowed through at a rate of ~240 $l \cdot h^{-1}$.The tanks were constantly aerated and photoperiod was maintained at a 12 hr day/night cycle.

The lobsters were held in these tanks for ± 4 months, prior to experimentation. While in the tanks, lobsters were fed a mixed diet of: mussels (*Choromytilus meridionalis* and *Mytilus galloprovincialis*), sardine (*Sardinops sagax*) and Horse mackerel (*Trachurus trachurus)*, once to twice a week in the afternoon *ad libitum*. Excess food was removed. Temperature and pH within the tanks fluctuated according to the environment over the holding period in 2012/2013 (pH ranged from 7.9 to 8.1; T_A from 8.4 to 16.8 °C). Four days prior to placement into the chronic experiments, feeding was discontinued.

2.2.2. Hypercapnic exposure

Juveniles were removed from the holding tanks, each individual was weighed to 1 mg (*w*) using a M-power balance (Sartorius, Germany) and carapace length (*CL*, tip of rostrum to mid-caudo-dorsal margin of carapace) measured to the nearest 0.1 mm with a digital calliper (Mitutoyo Corp, Japan) and any deformities were noted. Subsequently, each lobster was placed into an individual container (*L × W × H: 10 × 10 × 7½ cm*), labelled with a unique number. This was done, as it allowed for precise growth monitoring of each individual, prevented cannibalism and competition for food. The containers had been perforated with forty-four 3 mm holes on each side to ensure sufficient water exchange with the surrounding environment. Perforation also provided the lobsters with better traction on the container to prevent exhaustion and to assist with moulting. All individuals were then placed into conditions which replicated those of the acclimation tank during biomass calculations, ensuring an equal size distribution among treatments. Once this had been accomplished (1 day) 22 individuals were placed into a tank (*L × W × H: 148 × 100 × 90 cm*) for normocapnic treatment and 21 individuals into a second tank (*L × W × H: 148 × 100 × 90 cm*) for hypercapnic treatment (Table 1). An analysis of variance revealed no difference in *w* between the two groups. Tanks were well mixed and aerated. Lobsters were acclimatized for a week to around 18 °C after which the pH in one tank was lowered in two steps in five days from approximately 8.1 to 7.3 using a pH controller (7074/2, TUNZE, Germany) containing a solenoid valve (7074.111) and a pH electrode (7070.110) attached to a 9 kg CO_2 bottle (technical). Once these parameters had stabilised, the chronic trial began and continued for 195 days (~28 weeks). Light conditions were kept on a 12 hour regime using a Major Tech, MTD 9 digital multi-timer and 75 W globes. Each tank had a light placed directly above it to ensure equal light exposure. Approximately 80% of the tanks' volume was exchanged with pre-conditioned seawater twice a week. Seawater pH and T_A were measured five times a week, A_T and salinity twice weekly. From these carbonate species concentrations were calculated (described below) and summarised in Table 2.

Lobsters were fed *ad libitum* five days a week, 3 days with pelleted feed (Nutrafin Max, A6792U) and fresh mussel (*Choromytilus meridionalis* and *Mytilus galloprovincialis*) on the other two days. Individuals were monitored daily for moults, if a moult was discovered it was removed and frozen at -20 °C. Individuals were weighed and measured several days after moults once the carapace had hardened sufficiently. Excess food and moults were removed and water quality monitored by measuring NH_4^+ concentration (Ammonia test kit, Sera, Germany). The latter never exceeded 0.4 $mg \cdot l^{-1}$.

2.2. Trial 2

2.2.1. Lobsters

Collection site and methods were identical to those described for trial 1. Lobsters were collected over the same months, however in the year of 2013. The temperature and pH within the holding tanks fluctuated according to the environment over the holding period in 2013/2014 (pH ranged from 7.9 to 8.1; T_A from 11 to 17 °C).

2.2.2. Exposure to different pH- and temperature levels

Methods for holding conditions replicated those of trial 1, with the following exceptions: Due to a higher availability of juvenile lobsters, 30 individuals were placed into each of the treatment tanks. Analysis of variance revealed no significant differences between the biomass of treatments (Table 1). The pH of the hypercapnic tanks was set as described previously, and to ensure sufficient mixing a submersible pump (BOYU, SP 103-2400) was placed in each tank. Temperature was maintained via a universal STC-1000 Digital Temperature Controller (DTC, AGPtek, USA), connected to two 300 W aquarium heaters (Eheim Jäger, Germany) for treatments where high temperature was set as an environmental parameter, and in treatments where low temperature was set, a combination of heaters (as above) and a chiller (Hailea, HS 66-A, China) were used to ensure a stable temperature throughout the trial. The latter variables were also controlled via a DTC. Each tank functioned as its own unit, set up as a semi recirculation/semi-flow through system. Water exchange rates were set for approximately a full tank exchange (~1000 l) every two days. Water quality was monitored by measuring NH_4^+ concentration (Ammonia test kit, Sera, Germany).

After assignment of juvenile lobsters to respective treatments, water conditions were adjusted to the various experimental parameters (combinations) over a period of approximately two weeks. Initially seawater parameters remained unchanged for two days (pH ranged from 8.0 to 8.1; T_A ranged from 17 to 19 °C), in order to allow lobsters to settle. Subsequently, pH was dropped in two steps from 8.0 to 7.3 over five days in treatments where the effect of hypercapnia was one of the variables to be assessed. Thereafter, temperature was decreased from 18° C, at a rate of approximately 1 °C a day, to a temperature of 15.5 °C in the treatments where low temperature was to be one of the variables assessed. Once these parameters had stabilised, the chronic trial began and continued for 334 days. Juveniles were fed *ad libitum* twice a week, with fresh or frozen mussel (*Choromytilus meridionalis* and *Mytilus*

galloprovincialis), depending on availability. Every evening of the day after feeding, all excess feed was removed. As the lobsters grew, feed rations were increased accordingly. Water quality was monitored by measuring NH_4^+ concentration (Ammonia test kit, Sera, Germany). The latter never exceeded 0.5 $mg \cdot l^{-1}$. Due to the rapid, and unexpected growth rate observed, when lobsters reached a size of 27 mm carapace length, they were transferred into a larger container *(∅ × H: 11 × 16.3 cm)*.

In an effort to account for potential "tank effects", approximately mid-way through the trial (day 146 of exposure), treatments along with their respective water parameters were switched between tanks, i.e. lobsters environmental conditions did not change, however, their tanks did.

2.3. Shared methodology

The methods described below were used for analysis in both growth trials.

2.3.1. Seawater parameters

Oxygen concentration was determined using a Multi 350i meter set (WTW, Germany). For determination of total alkalinity (A_T), 0.05% saturated mercury chloride ($HgCl_2$) was added to a water sample to inhibit biological activity. A_T was later determined by a colorimetric method (Sarazin et al., 1999). Seawater pCO_2, HCO_3^- and CO_3^{2-} were calculated using measured pH, T_A, salinity and A_T as constants in CO2SYS software (Pierrot et al., 2006), using dissociation constants refitted by Dickson and Millero (1987) from Mehrbach et al. (1973) and KSO_4 from Dickson (1990, Table 2). Concentration of divalent ions (Ca^{2+} and Mg^{2+}) were quantified using test kits as described below for haemolymph. Ca^{2+} was diluted with distilled water at a ratio of 1:3 and Mg^{2+} 1:100.

2.3.2. Haemolymph sampling

On termination of the respective trials, the maximum volume of haemolymph possible was extracted from all lobsters. Pre-branchial haemolymph was extracted from the arthrodial membrane at the base of the fifth pair of pereiopods by syringe with a hypodermic needle (Neomedic 1 ml, 29 G, Figure 1). Tail flips were avoided by securing the abdomen with a firm grip. The samples were then placed in a microcentrifuge (Eppendorf) tube for immediate acid-base analysis and subsequently frozen at -80 °C for further analysis.

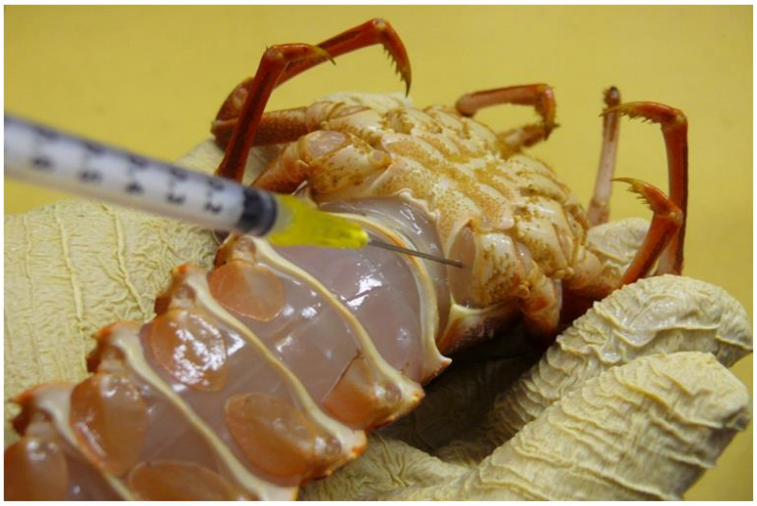

Figure 1. Pre-branchial haemolymph was extracted from the arthrodial membrane at the base of the fifth pair of pereiopods.

2.3.3. Haemolymph acid-base balance

pH was measured within 20 s after sampling using an Orion 3 star pH meter equipped with an Orion 8220 BNWP micro pH electrode (Thermo Scientific, USA). Calibration was performed with NBS precision buffers (Applichem, Germany). A haemolymph subsample (50 µl) was immediately injected into a de-gassing (magnetic stirrer) chamber containing 200 µl of 100 mM H_2SO_4 and liberated total CO_2 (cCO_2) determined by a CO_2 analyser (SBA4, PP Systems, USA) using CO_2-free N_2 (technical) as carrier gas (50 ml·min^{-1}) calibrated against freshly made Na_2HCO_3 standards (0.78 – 25 mM, Figure 2).

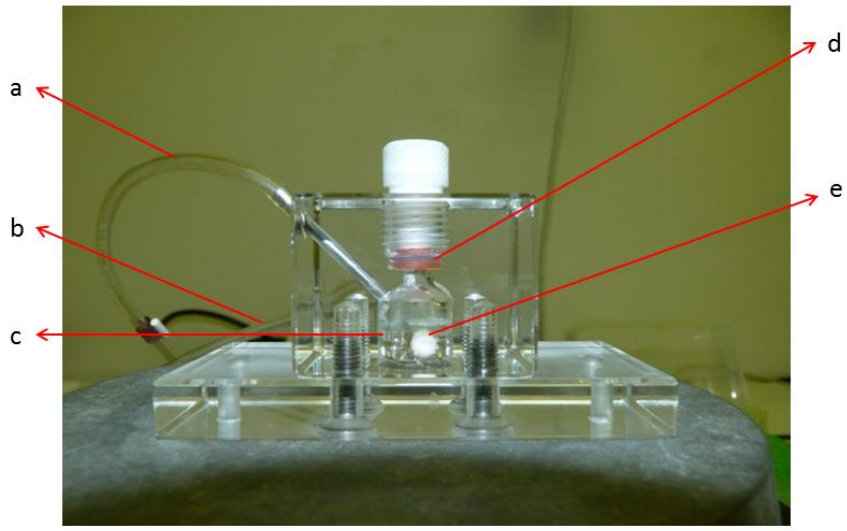

Figure 2. Sample chamber used in the determination of cCO_2. a) Tubing and chamber outlet, b) tubing and chamber inlet, c) chamber, d) seal, e) stirring bead.

Table 1. Initial carapace length and weight of juvenile West Coast rock lobster in each of the respective treatment.

Measured parameter	Trial 1		Trial 2			
	Normocapnia (18.8 °C)	Hypercapnia (18.8 °C)	Hypercapnia/ Low temperature	Normocapnia/ High temperature	Normocapnia/ Low temperature	Hypercapnia/ High temperature
Initial *CL* (mm)	$15.0^{a} \pm 1.9$	$14.3^{a} \pm 0.6$	$13.5^{a} \pm 0.3$	$13.2^{a} \pm 0.3$	$13.3^{a} \pm 0.3$	$13.3^{a} \pm 0.3$
Initial *w* (g)	$1.94^{a} \pm 0.25$	$1.70^{a} \pm 0.22$	$1.31^{a} \pm 0.10$	$1.29^{a} \pm 0.10$	$1.29^{a} \pm 0.10$	$1.30^{a} \pm 0.10$

Seawater parameters: Normocapnia – pH 8; hypercapnia – pH 7.3; high temperature – 19 °C; low temperature – 15.6 °C.
^{a,b}Values within a row per trial sharing same superscript do not differ significantly ($p < 0.05$).
Values are means ± S.E.

Table 2. Physicochemical seawater parameters recorded and calculated for the duration of experimental exposure for trials 1 and 2.

Treatment	Trial 1 (28 weeks)		Trial 2 (48 weeks)			
	Normocapnia	Hypercapnia	Hypercapnia/ Low temperature	Normocapnia/ High temperature	Normocapnia/ Low temperature	Hypercapnia/ High temperature
Salinity (‰)	34.4 ± 0.1	34.4 ± 0.0	35.1 ± 0.1	35.0 ± 0.0	35.0 ± 0.1	35.0 ± 0.1
T_A(°C)	18.8 ± 0.1	18.8 ± 0.1	15.6 ± 0.1	18.9 ± 0.1	15.6 ± 0.1	19.0 ± 0.1
A_T (µmol kg^{-1})	1903 ± 8	1925 ± 7	2063 ± 12	2051 ± 11	2065 ± 13	2037 ± 23
pH	8.02 ± 0.02	7.32 ± 0.01	7.34 ±0.01	8.00 ± 0.01	8.02 ± 0.01	7.35 ± 0.01
pCO_2 (Torr)	0.27 ± 0.01	1.57 ± 0.03	1.60 ±0.03	0.30 ± 0.01	0.29 ± 0.01	1.55 ± 0.03
HCO_3^- (mmol l^{-1})	1.58 ± 0.02	1.88 ± 0.01	2.03 ±0.01	1.71 ± 0.01	1.75 ± 0.02	1.99 ± 0.02
CO_3^{2-} (mmol l^{-1})	0.14 ± 0.01	0.04 ± 0.00	0.03 ±0.00	0.15 ± 0.00	0.14 ± 0.00	0.04 ± 0.00
Ω_{Ca}	3.40 ± 0.137	0.81 ± 0.018	0.80 ±0.02	3.60 ±0.08	3.35 ± 0.08	0.93 ± 0.02
Ω_{Arag}	2.20 ± 0.089	0.53 ± 0.011	0.52 ±0.01	2.33 ± 0.05	2.15 ± 0.05	0.60 ± 0.01

Values are means ± S.E.

From measured pH and cCO_2 values, pCO_2, and [HCO_3^-] were calculated using derivatives of the Henderson Hasselbalch equation (I and II). The appropriate solubility coefficient αCO_2 and dissociation constant pK'_1 of carbonic acid were adopted as described previously for *Carcinus maenas* (Truchot, 1976).

$$\text{I.} \quad pCO_2 = \frac{cCO_2}{10^{pH-pK^1} \times \alpha CO_2 + \alpha CO_2}$$

$$\text{II.} \quad HCO_3^- = cCO_2 - \alpha CO_2 \times pCO_2$$

2.3.4. Calculated indices

After haemolymph sampling, lobsters were sacrificed by dipping the head into liquid N_2. Thereafter, each lobster was dissected carefully and their hepatopancreas and tail removed and weighed. Care was taken to ensure that each tail was dissected cleanly at the same place from the body to ensure comparability within- and amongst treatments. The hepatosomatic- (HSI) and muscle-somatic (MSI) indices for each individual were then calculated using equations III and IV (below), respectively. For determination of moisture content of muscle, the third segment of the abdomen (*w:* 0.060 - 0.198g) was dissected from each lobster and the muscle tissue removed. Samples were then placed into pre-weighted microcentrifuge (Eppendorf) tubes and dried for 24 hours at 90 °C, after which the tubes were re-weighed and the % moisture content (MC) calculated. Biological indices (NTI = nutritional index) for each lobster were calculated as follows:

$$\textit{III.} \quad HSI = \frac{Wet\ weight\ of\ hepatopancreas}{Total\ body\ weight} \times 100\%$$

$$\textit{IV.} \quad MSI = \frac{Tail\ weight}{Total\ body\ weight} \times 100\%$$

$$\textit{V.} \quad MC = 100\% - \left(\frac{Wet\ weight}{Dry\ weight} \times 100\%\right)$$

$$\textit{VI.} \quad NTI = \frac{Carapace\ length}{Total\ body\ weight} \times 100\%$$

2.3.5. Moult stage determination

The stage in the moult cycle of each lobster was determined via microscopic analysis of the setagenic stages based on a moulting stage range of AB, C, D_0 through to D_4 (Marco, 2012). Briefly, the distal section of the first pleopod (as morphological discrepancies have been found within the pleopods of a single lobster) was removed from each lobster and stored at -20 °C until analysis was possible. To assess moult stage, the pleopods were floated on a cavity slide, covered with a cover slide and examined with a compound eclipse Ni microscope (Nikon, Japan).

2.3.6. Haemolymph sample preparation

Samples were removed from the storage freezer and allowed to defrost. They were then homogenized with a pestle (Scienceware, A Bel-Art product) and placed in a PrismR cooling centrifuge for 15 min at 17 136 g and 4 °C (Labnet, USA). The pellet was removed and discarded (pellet not used as it leads to rapid coagulation of sample) and the supernatant used to determine haemolymph parameters, namely Ca^{2+}, Mg^{2+} and haemocyanin concentrations.

2.3.7. Measurement of the concentrations of divalent ions and haemocyanin in the haemolymph

Ca^{2+} and Mg^{2+} concentrations were determined by use of commercial test kits (Diaglobal GmbH, Germany). Haemolymph supernatant (see above) was mixed using a vortex mixer and subsequently diluted at a ratio of 1:10 with distilled water due to the high expected concentration. The colorimetric reaction of the ions was analysed in a micro plate reader (SIRO, SEAC, country) at 540 nm. For haemocyanin determination, supernatant was diluted (1:50) with *Jasus lalandii* Ringer solution (0.52 M NaCl, 0.015 M $MgSO_4$, 0.013 M $CaSO_4$, 0.005 M KCl, 0.005 M $NaHCO_3$, pH 7.8) and absorbance measured at 335 nm in a Libra S12 spectrophotometer (Biochrom, UK). Concentration was calculated using an extinction coefficient of $\varepsilon_{335} = 0.233$ ΔE units $\cdot mg^{-1} \cdot ml^{-1}$(Nickerson and Van Holde, 1971).

2.4. Statistical analysis of data

Data analysis was generated using SAS software (Statistical Analysis System, Version 9.3, SAS institute Inc., Cary, NC, USA). If assumptions were met, namely normality and equal variance, parametric tests were carried out as follows (Bonferoni post hoc-test, used in all cases):

The first moult period was excluded from analysis in both trials, to account for any effect of acclimation conditions on the somatic growth rate and allow for better estimation of average daily gain (ADG) between treatments. In order, however, to ensure that a sufficient sample size was present throughout the growth period, moults 1 to 4 were used in the first trial and moults 1 to 6 in the second trial. This was necessary due to the unsynchronized moult cycles, differing growth rates and length of experimental exposure.

2.4.1. Trial 1

Somatic growth rate: Total weight (*w*) gain and carapace length (*CL*) gain over the duration of the exposure period were investigated as follows: 1) Individuals which had less than three measured data points (for either *w* or *CL*) were excluded. 2) A regression line was then fitted for each individual, for *w* and *CL* over both time (days) and moult stages recorded. All individuals showed strong positive linear association for both *w* and *CL* gain. 3) Individual lines for average daily gain (ADG) and average moult gain (AMG) were compared with ANCOVA, using their intercept as a covariate in order to determine differences between treatments.

Endpoint data, i.e. from sacrificed lobsters, was investigated by means of a one-way ANOVA.

2.4.2. Trail 2

Somatic growth rate: This was analysed as above, however, ANCOVA analysis was subsequently applied to investigate firstly an interaction of the main effects (pH × T_A). If not significant, the main effects could be further analysed. The interaction between the main effects of pH and temperature was also included in the model.

Endpoint data was investigated by means of a two-way ANOVA, if the interaction was not significant, then main effects were investigated (as above) further.

In both trials, where MC, NTI, HSI and MSI were investigated, an ANCOVA was used, with the covariate being moult stage.

3. Results

3.1. Trial 1

Measured body parameters throughout trail 1 are depicted in Figure 3, whereas Table 3 summarises final length and weight data. Averaged data per respective moult, spanning four moults (see Material and Methods for explanation), revealed no differences ($p > 0.05$) between experimental treatments with regards to *CL*, *w* and IMP (intermoult period), although IMP was consistently shorter in normocapnic treatment (Figures 4A, B, C). It is noteworthy that the same IMP differed in time points by several days between individual lobsters. Calculation of average daily gain (ADG, over moults 1 - 4) was therefore performed and showed that lobsters from the normocapnic treatment grew faster ($p < 0.05$, 24% *CL*, 34% *w*) than those from hypercapnic exposure (Table 4). A similar trend was found when average moult gain (AMG, over moults 1 - 4) was calculated: Here, lobsters from the normocapnic group also grew faster ($p > 0.05$, 12% *CL*, 25% *w*, Table 4). When *CL* and *w* data, collected throughout the experiment, were plotted, the exponential growth of *w* in relation to *CL* in both treatments became apparent (Figure 4D). Mortalities began between day 19 and 34 in both treatments and were slightly lower under normocapnic- (18%) than under hypercapnic (29%) conditions (Figure 4E).

Indices for trial 1, which were collected on the day of termination (after sacrificing the lobsters) are depicted in Figure 5. No differences ($p > 0.05$) were found in HSI, MSI, and NTI, whereas MC was lower (4%, $p < 0.05$) in lobsters exposed to normocapnic seawater conditions (Table 3, Figure 5B). Each experimental group consisted of a cohort of lobsters that were in a similar range of moult stages. In the normocapnic group, three lobsters were at stage AB, five at C, four at D_0, five at D_1 and one at D_4. In the hypercapnic group, three lobsters were at stage AB, five at C, two at D_0, four at D_1 and one at D_2. Acid-base parameters (Table 5) for this trial are discussed in Chapter 5.

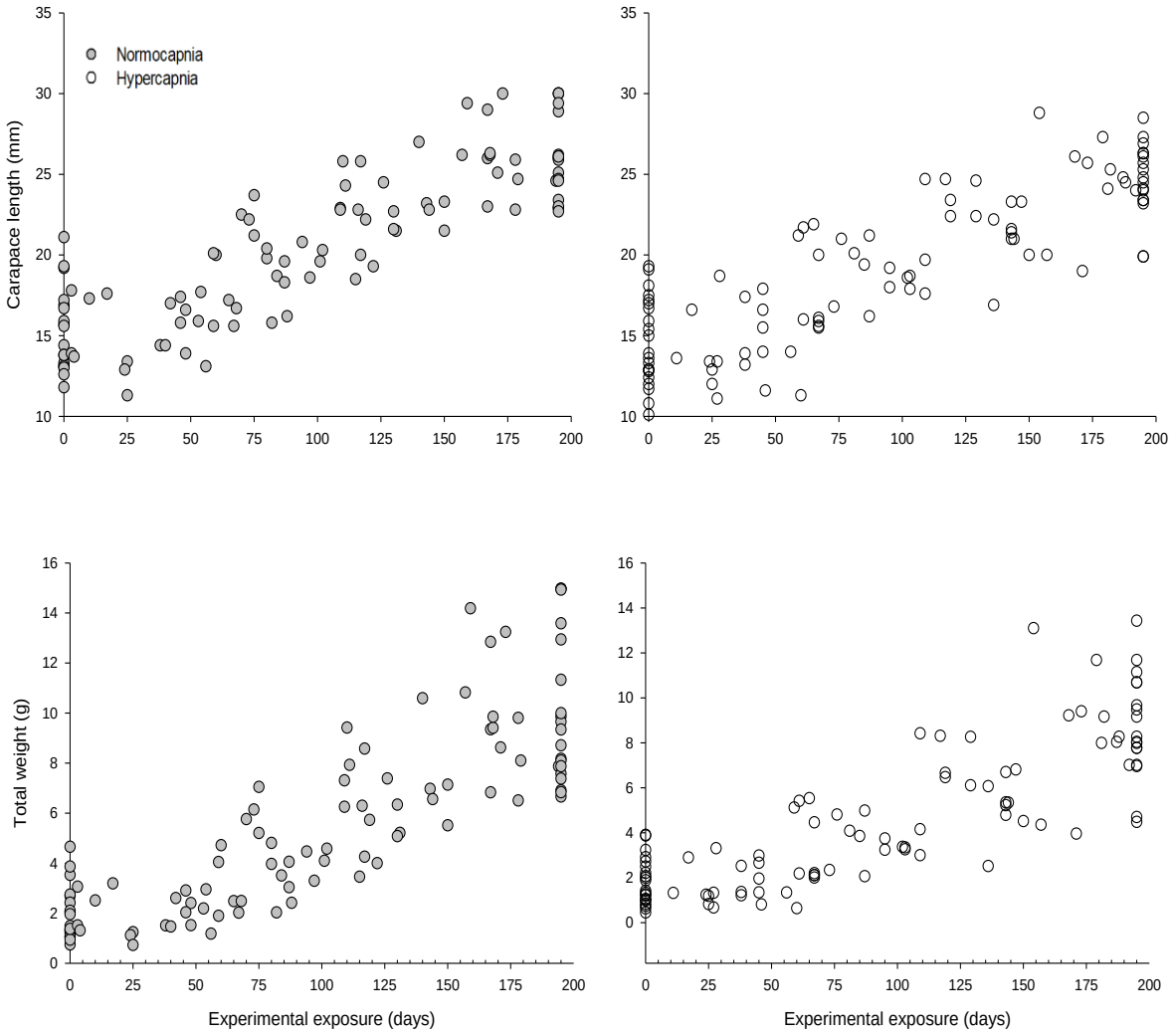

Figure 3. Raw individual growth data of carapace length (*CL*) and total weight (*w*) accumulated from juvenile lobsters before exposure, at respective moults and after termination of normo- and hypercapnia at the same temperature (18.8 °C) for a period of 28 weeks (pH 7.3).

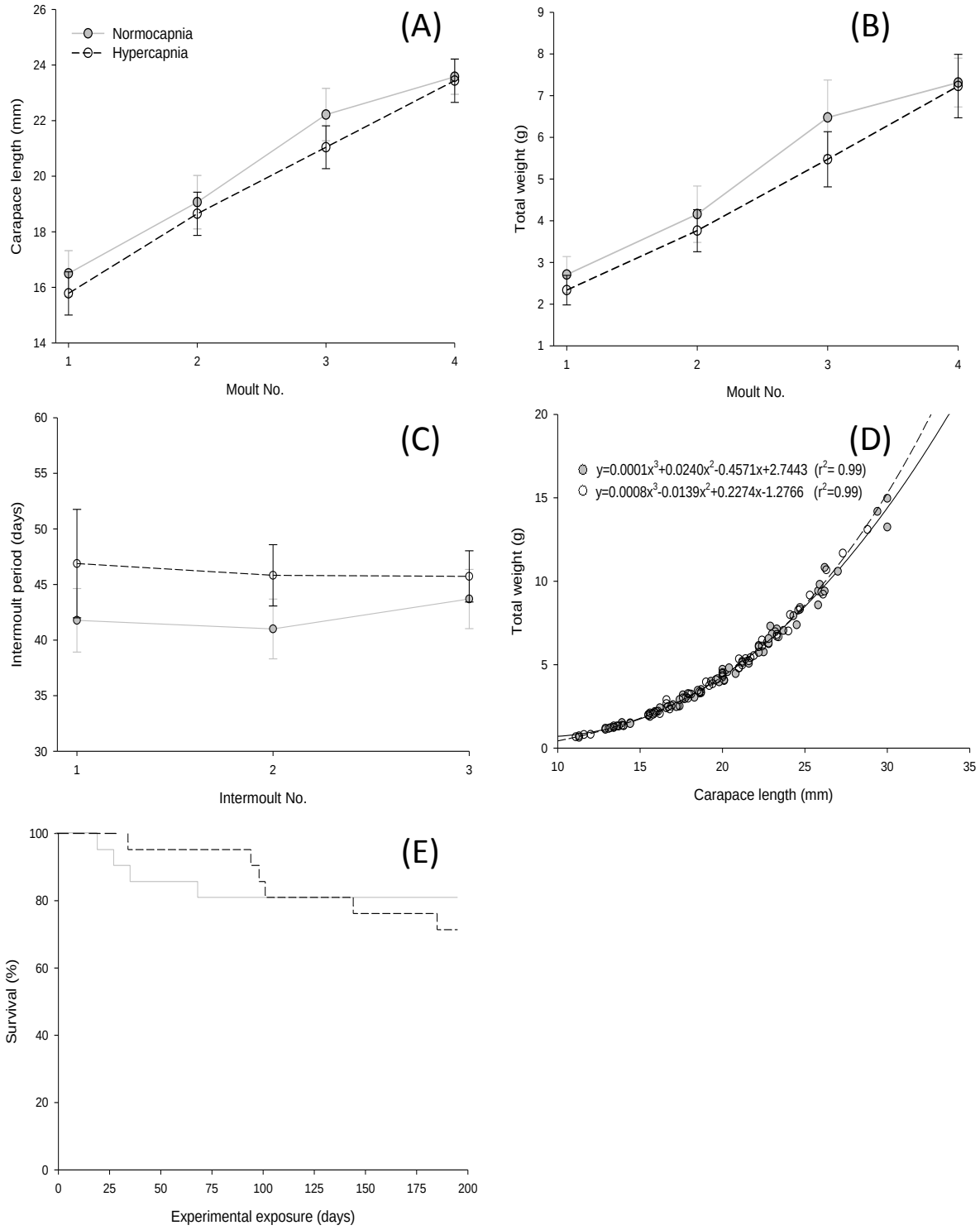

Figure 4. Mean growth and survival data accumulated (moults 1 - 4) from juvenile lobsters exposed to normo- (pH 8) and hypercapnia (pH 7.3) at the same temperature (18.8 °C) for a period of 28 weeks: A) Carapace length (*CL*), B) total weight (*w*), C) intermoult period (IMP), D) total weight (*w*) *vs.* carapace length (*CL*) (Insert: equations for regression lines of respective treatments) and E) survival (for experimental period). Values are given as mean ± S.E. No significant differences ($p > 0.05$) occurred.

Table 3. Measured parameters of juvenile lobsters after termination of chronic exposure to either a change in seawater pCO_2 (trial 1) and a combination of low/high pCO_2 with either low/high temperature (trial 2).

	Trial 1 (28 weeks)		Trial 2 (48 weeks)			
Measured parameter	Normocapnia (18.8 °C) (n = 18)	Hypercapnia (18.8 °C) (n = 15)	Hypercapnia/ Low temp. (n = 27)	Normocapnia/ High temp. (n = 28)	Normocapnia/ Low temp. (n = 20)	Hypercapnia/ High temp. (n = 25)
Final *CL* length (mm)	$25.8^{a} \pm 0.6$	$24.7^{a} \pm 0.6$	$32.3^{a} \pm 0.6$	$35.3^{b} \pm 0.5$	$30.7^{a} \pm 0.8$	$36.5^{b} \pm 0.7$
Final *w* (g)	$9.71^{a} \pm 0.65$	$8.70^{a} \pm 0.64$	$18.84^{a} \pm 1.14$	$24.31^{b} \pm 0.97$	$16.71^{a} \pm 1.27$	$26.92^{b} \pm 1.47$
Tail weight (g)	$2.61^{a} \pm 0.19$	$2.33^{a} \pm 0.19$	$5.54^{a} \pm 0.36$	$6.92^{b} \pm 0.28$	$4.98^{a} \pm 0.39$	$7.60^{b} \pm 0.44$
Hepatopancreas weight	$0.45^{a} \pm 0.033$	$0.38^{a} \pm 0.027$	$0.76^{a} \pm 0.06$	$0.84^{a} \pm 0.06$	$0.68^{a} \pm 0.06$	$0.93^{a} \pm 0.06$
Moisture content[#] (%)	$75.56^{a} \pm 0.58$	$78.68^{b} \pm 0.45$	$78.22^{a} \pm 0.47$	$77.66^{a,b} \pm 0.36$	$76.76^{b} \pm 0.39$	$77.03^{a,b} \pm 0.28$
W gain (%)	$475^{a} \pm 61$	$399^{a} \pm 64$	$1498^{a} \pm 109$	$2124^{b} \pm 194$	$1297^{a} \pm 140$	$2208^{b} \pm 172$
CL gain (%)	$71^{a} \pm 5.7$	$63^{a} \pm 6.7$	$142^{a} \pm 6$	$171^{b} \pm 8$	$130^{a} \pm 8$	$176^{b} \pm 6$
Survival (%)	82	71	90	90	67	83

Seawater parameters: Normocapnia – pH 8; hypercapnia – pH 7.3; high temperature – 19 °C; low temperature – 15.6 °C.
Abbreviations: *CL* - carapace length (mm); *w* - total weight (g).
[a,b]Values within rows per trial sharing the same superscript do not differ significantly ($p < 0.05$).
[#]Eight lobsters from each treatment were not tested for moisture content in trial 2, due to use for bacterial challenge (see Chapter 6).
Values are means ± S.E.

Table 4. Calculated average daily gain (ADG) and average moult gain (AMG) in carapace length (mm) and total weight (g) for juvenile lobsters exposed to either a change in seawater pCO_2 (trial 1) or a combination of low/high pCO_2 with either low/high temperature (trial 2).

Trial No.	Effect(s)	Treatment	ADG *CL* (*CL* gain day^{-1})	AMG *CL* (*CL* gain moult^{-1})	ADG *w* (*w* gain day^{-1})	AMG *w* (*w* gain moult^{-1})
1	pH	Normocapnia (18.8 °C)	$0.073^{a} \pm 0.005$	$2.994^{a} \pm 0.115$	$0.051^{a} \pm 0.004$	$2.218^{a} \pm 0.216$
		Hypercapnia (18.8 °C)	$0.059^{b} \pm 0.004$	$2.678^{a} \pm 0.121$	$0.038^{b} \pm 0.003$	$1.769^{a} \pm 0.170$
2	Main	pH 8	$0.068^{a} \pm 0.002$	$3.280^{a} \pm 0.063$	$0.066^{a} \pm 0.003$	$3.232^{a} \pm 0.167$
		pH 7.3	$0.073^{b} \pm 0.002$	$3.472^{a} \pm 0.070$	$0.078^{b} \pm 0.004$	$3.712^{a} \pm 0.183$
		Temperature (19.0 °C)	$0.080^{a} \pm 0.002$	$3.420^{a} \pm 0.068$	$0.087^{a} \pm 0.003$	$3.785^{a} \pm 0.181$
		Temperature (15.6 °C)	$0.061^{b} \pm 0.002$	$3.328^{a} \pm 0.067$	$0.058^{b} \pm 0.003$	$3.158^{a} \pm 0.165$
2	Combined	Hypercapnia/Low temp.	$0.063^{a} \pm 0.002$	$3.376^{a} \pm 0.089$	$0.063^{a} \pm 0.042$	$3.382^{a} \pm 0.223$
		Normocapnia/High temp.	$0.076^{b} \pm 0.003$	$3.281^{a} \pm 0.073$	$0.080^{b} \pm 0.004$	$3.529^{a} \pm 0.223$
		Normocapnia/Low temp.	$0.060^{a} \pm 0.003$	$3.280^{a} \pm 0.105$	$0.053^{a} \pm 0.034$	$2.934^{a} \pm 0.241$
		Hypercapnia/high temp.	$0.083^{b} \pm 0.003$	$3.565^{a} \pm 0.105$	$0.092^{b} \pm 0.005$	$4.032^{a} \pm 0.281$

Seawater parameters: Normocapnia – pH 8; hypercapnia – pH 7.3; high temperature – 19 °C; low temperature – 15.6 °C.
^{a} ,^{b}Values within columns per effect sharing the same superscripts do not differ significantly ($p < 0.05$).
Values are means ± S.E.

Table 5. Haemolymph parameters of juvenile lobster after chronic exposure to either a change in seawater pCO_2 (trial 1) or a combination of low/high pCO_2 with either low/high temperature (trial 2).

Trial	Haemolymph parameter	*n*	pH	cCO_2 (mmol l^{-1})	pCO_2 (Torr)	HCO_3^- (mmol l^{-1})	Ca^{2+} (mmol l^{-1})	Mg^{2+} (mmol l^{-1})	Haemocyanin (mg ml^{-1})
1	Normocapnia	12	$7.76^{a} \pm 0.03$	$4.2^{a} \pm 0.4$	$1.7^{a} \pm 0.2$	$4.1^{a} \pm 0.4$	$12.8^{a} \pm 0.8$	$17.5^{a} \pm 0.6$	$22.3^{a} \pm 2.0$
	Hypercapnia	12	$7.66^{a} \pm 0.06$	$8.5^{b} \pm 1.2$	$3.9^{b} \pm 0.2$	$8.4^{b} \pm 1.2$	$9.2^{b} \pm 0.6$	$19.4^{a} \pm 1.5$	$13.9^{b} \pm 2.1$
2	Hypercapnia/Low temp.	27	$7.70^{a} \pm 0.02$	$15.5^{a} \pm 0.9$	$6.4^{a} \pm 0.3$	$15.2^{a} \pm 0.8$	$12.3^{a} \pm 0.5$	$6.8^{a} \pm 0.9$	$8.7^{a} \pm 0.7$
	Normocapnia/High temp.	28	$7.65^{a} \pm 0.02$	$3.9^{b} \pm 0.2$	$1.9^{b} \pm 0.1$	$3.9^{b} \pm 0.2$	$11.9^{a} \pm 0.5$	$7.3^{a} \pm 0.2$	$7.9^{a} \pm 0.5$
	Normocapnia/Low temp.	20	$7.63^{a} \pm 0.02$	$3.7^{b} \pm 0.3$	$1.8^{b} \pm 0.2$	$3.6^{b} \pm 0.3$	$10.8^{a} \pm 0.6$	$7.1^{a} \pm 0.3$	$10.0^{a} \pm 0.8$
	Hypercapnia/High temp.	25	$7.68^{a} \pm 0.02$	$11.5^{c} \pm 0.6$	$5.3^{c} \pm 0.2$	$11.2^{c} \pm 0.6$	$12.0^{a} \pm 0.5$	$7.3^{a} \pm 0.4$	$8.4^{a} \pm 0.7$

Seawater parameters: Normocapnia – pH 8; hypercapnia – pH 7.3; high temperature – 19 °C; low temperature – 15.6 °C.
^{a,b,c}Values with similar superscripts per trial within columns do not differ significantly ($p < 0.05$).
Values are means ± S.E.

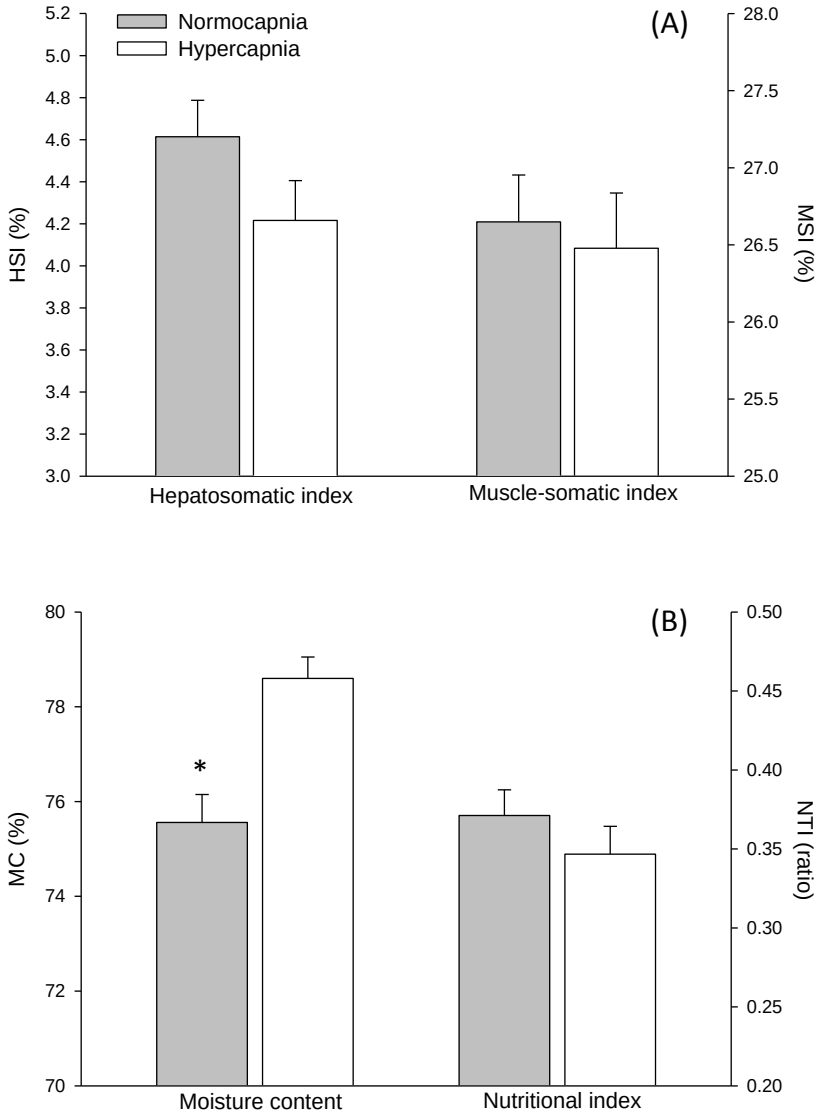

Figure 5. Body indices of juvenile lobsters calculated from endpoint data after exposure to normo- (pH 8) and hypercapnia (pH 7.3) at the same temperature (18.8 °C) after a period of 28 weeks: A) Hepatosomatic index (HSI) and muscle-somatic index (MSI), B) moisture content (MC) and nutritional index (NTI). Values are given as ± S.E. *Significant difference between treatments ($p < 0.05$).

3.2. Trial 2

Measured body parameters collected of juvenile lobster exposed to a combination of factors (seawater pH × T_A) for the duration of this trial are depicted in Figures 6 and 7, whilst the growth data collected on the final day is summarised in Table 3. During the trial, when accumulated per respective moult number, some differences became visible regarding growth in terms of *CL* and *w*, as the experiment progressed (Figures 8A, B). At moult 5, *CL* was different ($p < 0.05$) between seawater pH treatments, irrespective of temperature, with the hypercapnic treatment having a greater *CL*. At moult 6, *CL* was different ($p < 0.05$) between seawater pH treatments, as well as different temperature treatments. Of these, the low pH and high temperature had the largest *CL* values. The same was true for *w* at this moult stage. Moreover, Inter moult periods (IMP) of the high temperature treatments, irrespective of pH, are clearly shorter ($p < 0.05$) throughout all moults (approximately 12 days), whereas different pH levels had little effect ($p > 0.05$, Figure 8C). Once again, as in trial 1, when *CL* and *w* data, collected throughout the experiment, were plotted, the exponential growth of *w* in relation to *CL* in all treatments was observed (Figure 8D). Survival was lowest in the treatment at normocapnia and low temperature (60%) and highest in both, combinations of normocapnia and high temperature (90 %) as well as hypercapnia and low temperature (90%), Figure 8E). Mortalities started to occur between 105 and 211 days of exposure in all treatments. Calculation of AMG (moult 1 - 6), which does not take IMP into account, reflects only small differences regarding growth, apparent in Figures 8A and 8B. ADG (moults 1 - 6), however, ADG which incorporates length of IMP, revealed a different result per main effect: While there were no differences ($p > 0.05$) in the AMG for *CL* and *w*, there were clear effects on ADG in the following order: high temperature (31% *CL*, 49% *w* higher than low temperature) > hypercapnia (20% *CL*, 34% *w*) > normocapnia (11% *CL*, 14% *w*) > low temperature (Table 4). The contribution of these effects are reflected in the calculation of AMG and ADG per treatment: in terms of *CL* and *w* gain, treatments with higher temperature, irrespective of pH, had the highest daily gains (27 – 32% *CL*, 46 – 51% *w* higher than respective low temperature treatment), whereas exposure to hypercapnia caused a higher growth (5 – 9% *CL*, 15 – 19% *w*) compared with the respective normocapnic treatment (Table 4).

At termination of the experiment, clear differences are apparent and reflect growth rates described above: For example, the highest weight gain occurred under high temperature and hypercapnia (2208%), whereas the lowest was in the low temperature and normocapnia treatment (1279%). Indices calculated on the final day of trial 2 are depicted in Figure 9. There were differences ($p < 0.05$) in calculated Hepatosomatic- (HSI) and Muscle-somatic (MSI) indices, both were higher in the lower temperature treatments, irrespective of pH (Figures 9A, B). Moisture content (MC) showed an interaction ($p < 0.05$) between seawater pH and temperature, resulting in a 1.5% higher MC in the normocapnic/low temperature treatment compared with the low temperature/hypercapnic treatment (Figure 9C). The nutritional index (NTI) was lower ($p < 0.05$) in both groups that were exposed to low

temperatures compared with those from high temperature (Figure 9D), i.e. the low temperature lobsters were heavier per length.

Each experimental group consisted of a cohort of lobsters that were in a similar range of moult stages. These stages varied as follows: In the hypercapnic/low temperature treatment eight were at stage C, twelve at D_0, six at D_1, and one at D_2. In the normocapnic/high temperature two were at stage C, nineteen at D_0, and seven at D_1. In the normocapnic/low temperature seven were at stage C, nine at D_0, three at D_1, and one at D_2. In the hypercapnic/high temperature four were at stage C, eleven at D_0, nine at D_1, and one at D_{3-4} (depicted in Chapter 6).

Acid-base balance parameters measured at the end of both trial 1 and 2 are summarized in Table 5, only results for trial 2 are described here because those of trial one are the topic of Chapter 5. In trial 2, haemolymph pH was similar in all treatments, whereas total CO_2 (cCO_2), partial CO_2 pressure (pCO_2) and bicarbonate concentration (HCO_3^-) showed an interaction ($p < 0.05$) between seawater pH and temperature, so that no main effects could be determined. In spite of the similar pH, cCO_2 showed differences ($p < 0.05$) between treatments where pH differed, as well as between hypercapnic treatments (4 mM), with a difference of 7.6 mM between high temperature treatments and an 11.8 mM difference between cold treatments. Accordingly, calculated pCO_2 and HCO_3^- showed a corresponding trend. This is also apparent when a Henderson-Hasselbalch diagram was plotted (Figure 10). Calcium (Ca^{2+}), magnesium (Mg^{2+}) and haemocyanin concentration did not differ ($p > 0.05$, Table 5). Ca^{2+} levels ranged from 10.8 to 12.3 mM, whereas Mg^{2+} was very low at approximately 7 mM. Small differences are observed in haemocyanin concentration at a generally very low level of about 8 – 10 mg/ml. Deformities, initially noted were still present at termination of the trial, even after consecutive moults. Some additional deformities were noted however, no trend between treatments became apparent.

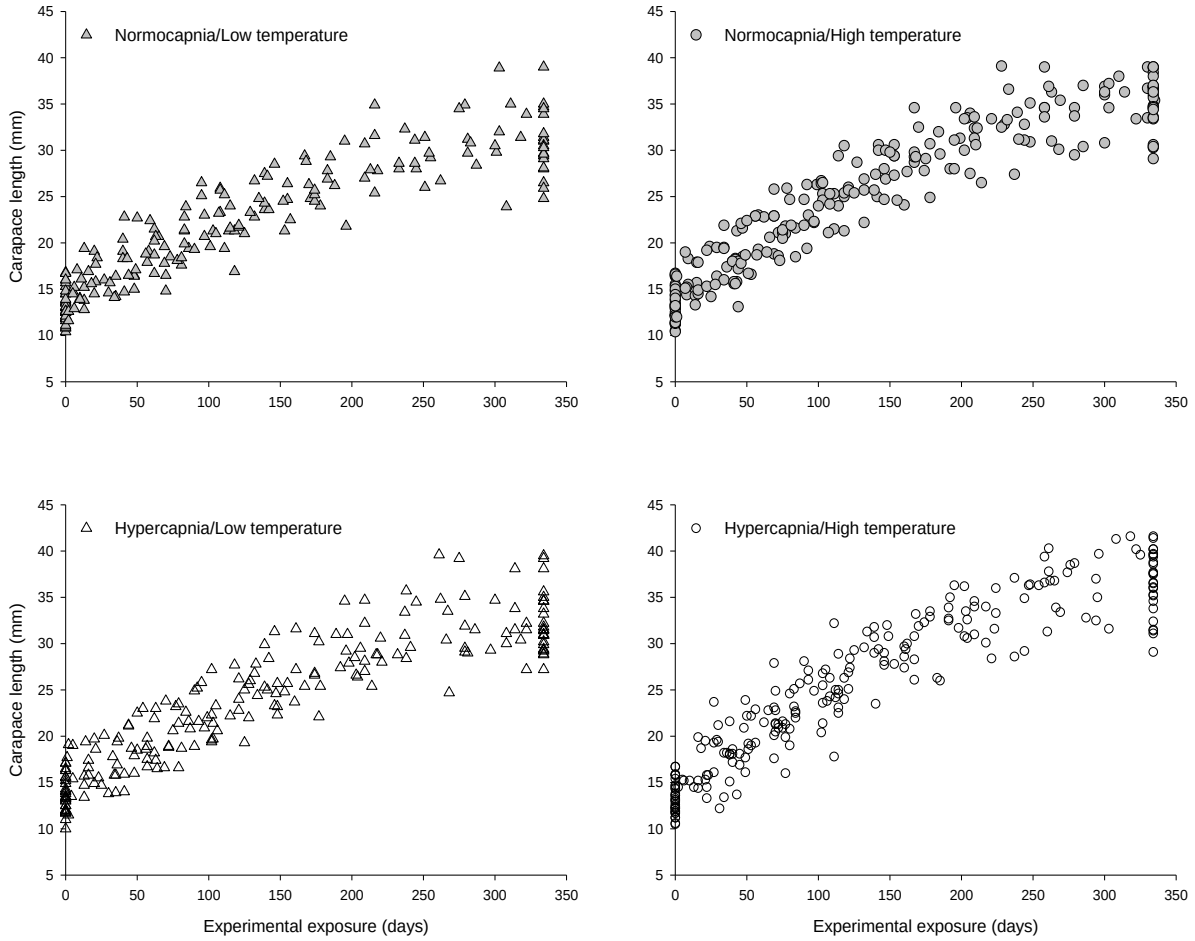

Figure 6. Raw individual growth data for carapace length (*CL*) accumulated (as described in Figure 3) from juvenile lobsters exposed to four combinations of two stressors (seawater pH and temperature: pH 7.3, 8 and 15.6 and 19 °C, respectively) for a period of 48 weeks.

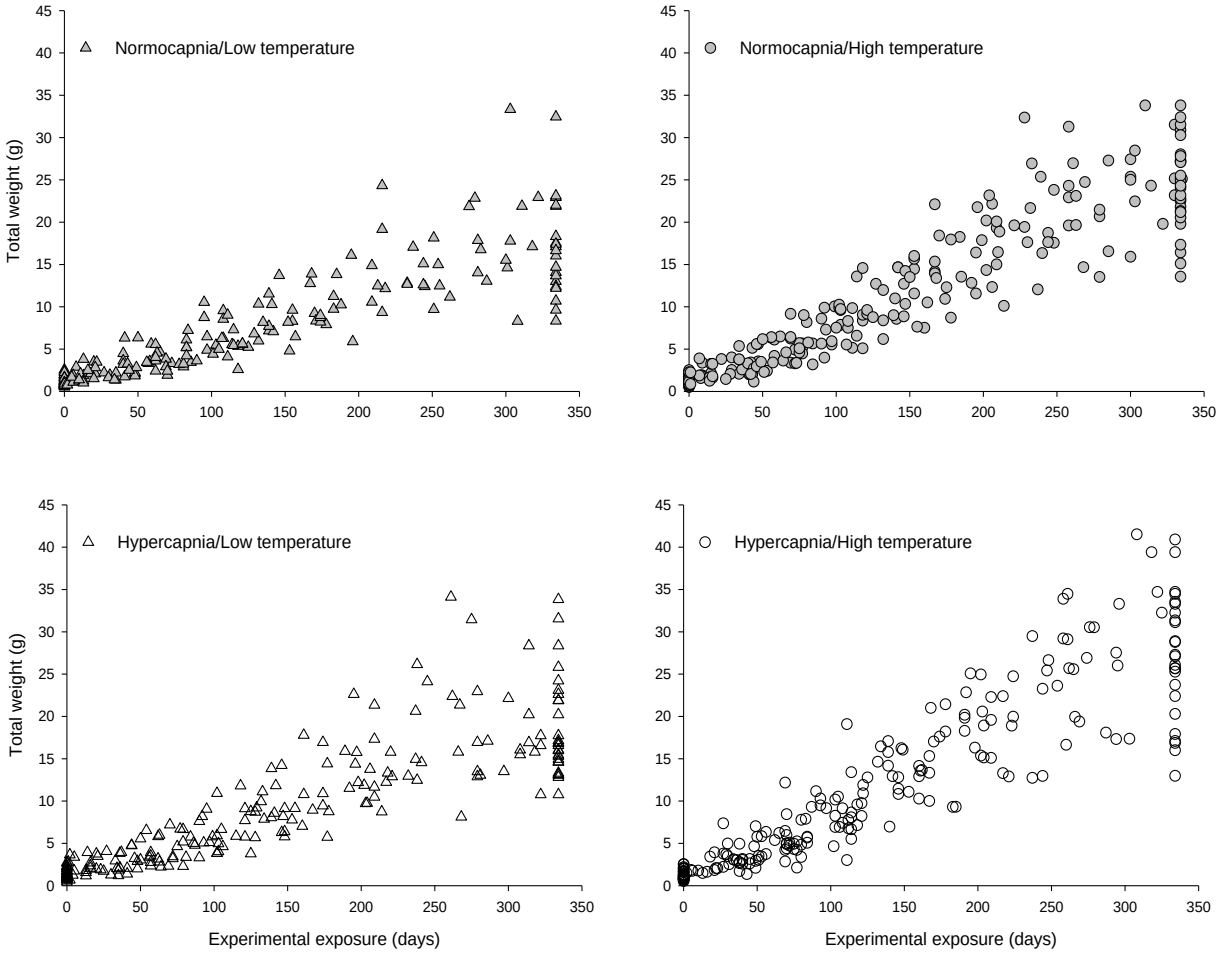

Figure 7. Raw individual growth data for total weight (*w*) accumulated (as described in Figure 3) from juvenile lobsters exposed to four combinations of two stressors (seawater pH and temperature: pH 7.3, 8 and 15.6 and 19 °C, respectively) for a period of 48 weeks.

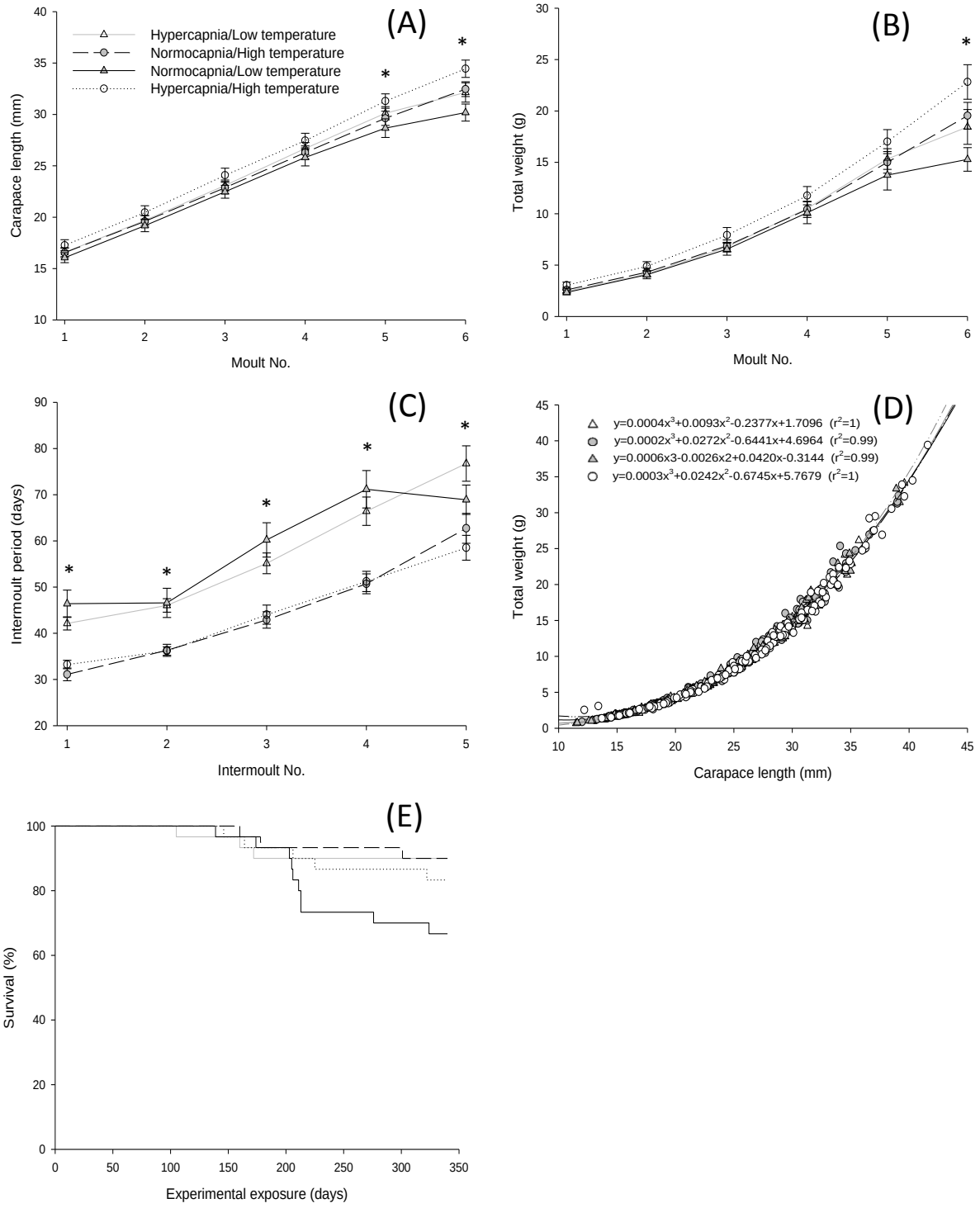

Figure 8. Mean growth and survival data accumulated (moults 1 - 6) from juvenile lobsters exposed to four combinations of two stressors (seawater pH and temperature: pH 7.3, 8 and 15.6 and 19 °C) for a period of 48 weeks: A) Carapace length (*CL*), B) total weight (*w*), C) intermoult period (IMP), D) total weight (*w*) *vs.* carapace length (*CL*) (moult 1 – 6, Insert: equations for regression lines of respective treatments) and E) survival (for experimental exposure). Values are given as mean ± S.E. *Significant difference between treatments ($p < 0.05$).

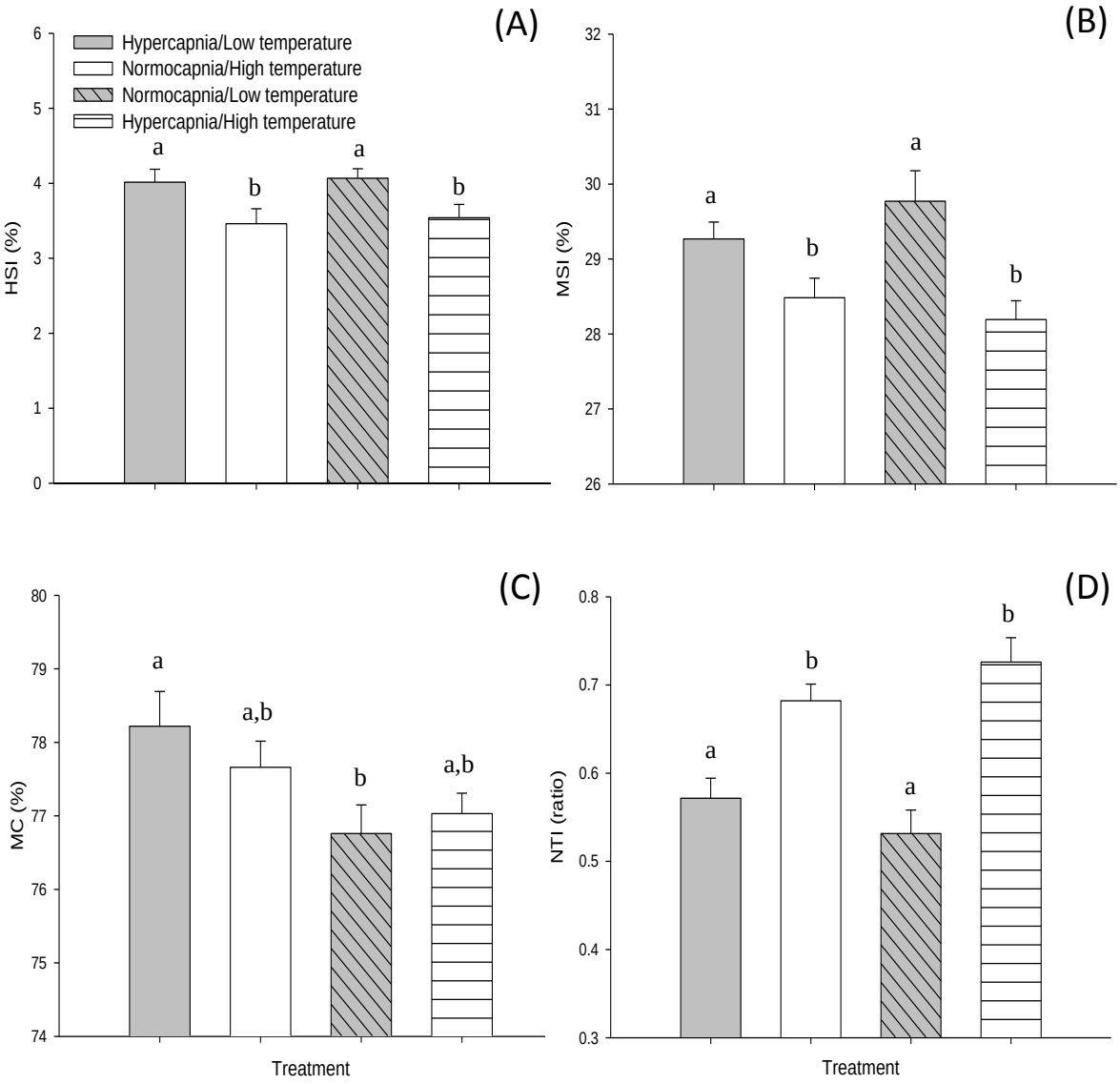

Figure 9. Body indices of juvenile lobsters calculated from endpoint data after exposure to a combination of two stressors, namely seawater pH (7.3, 8) and temperature (15.6 and 19 °C) after a period of 48 weeks: A) Hepatosomatic index (HSI), B) muscle-somatic index (MSI), C) moisture content (MC) and D) nutritional index (NTI). Values are given as mean ± S.E. Same lower case letters are not significantly different ($p > 0.05$).

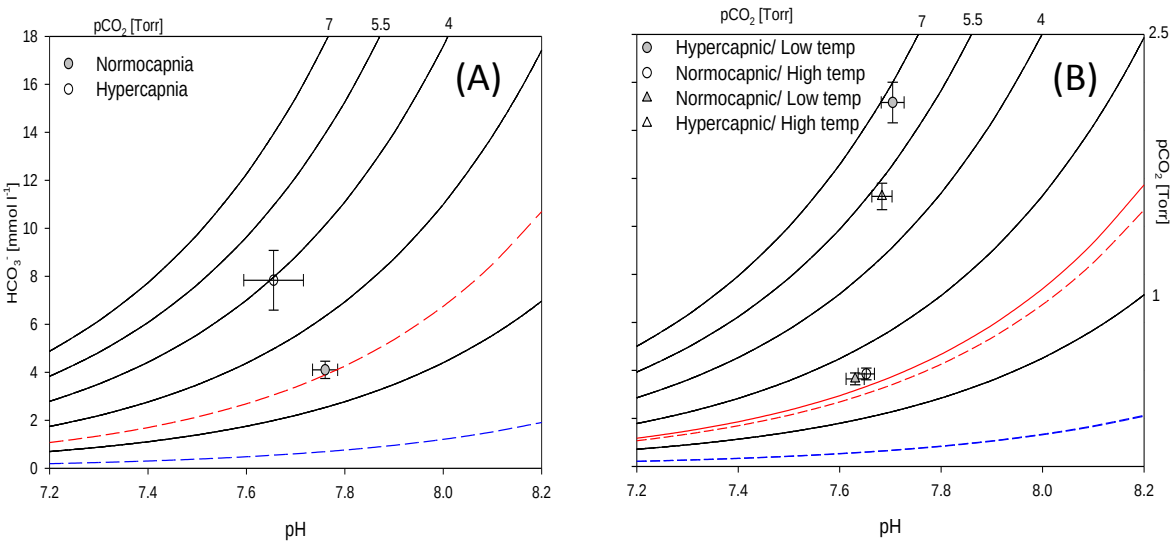

Figure 10. Henderson-Hasselbalch (pH-bicarbonate) diagrams for haemolymph of juvenile lobsters incubated in trial 1 (A, Chapter 5) and 2 (B). pCO_2 isopleths were derived from the Henderson-Hasselbalch equation. Values for the first dissociation constant (pK'_1) and solubility coefficient (αCO_2) were derived from Truchot, (1976) using appropriate inputs. For isopleths an average pK'_1 and αCO_2 was used as temperature differed between treatments (provides an indication of approximate pCO_2) Blue dashed line = normocapnic (19 & 15.6 °C) seawater isopleth, red dashed line = hypercapnic seawater (19 °C) isopleth, red solid line= Hypercapnic seawater (15.6 °C).

4. Discussion

Globally, as a result of anthropogenic CO_2 increase in the atmosphere, the oceans are warming and "acidifying". It is not clear, however, into what direction the Benguela Current Large Marine Ecosystem (BCLME) is headed, at least with regards to surface temperature (see Chapter 2). The present chapter investigates, therefore, the impact of possible physicochemical properties of seawater, namely a high and a "low" temperature and a "high" and a low pH, each in combination with each other, on growth and physiology of juvenile West Coast rock lobster (WCRL). The "low" temperature level used is more or less in the middle of the natural temperature range of the WCRL, whereas the high one is close to the upper end (Dubber et al., 2004). The high pH level is the current "normal" level (normocapnia) experienced outside upwelling episodes and the low level was chosen to emulate global scenarios that could arrive earlier in the BCLME due to its already low pH levels. In this regard, a single factor (pH) trial was carried out first, followed by a two-factor (pH and temperature) trial. The main objective was to determine the sensitivity of the WCRL to these environmental perturbations and allow for a proxy from which future management directions pertaining to the resource could be determined.

4.1. Growth and survival

Studying growth in crustaceans is complex, as growth and feeding are interrupted by moult (Lockwood, 1968). During intermoult, lobsters do not increase in *CL* (Oliver and MacDiarmid, 2001). Generally growth data is recorded, at set time intervals, after the event of moulting or at the beginning and end of a trial (Bordner and Conklin, 1981; Crear et al., 2000; Kemp and Britz, 2008). In juvenile *J. lalandii*, however, timing of moult is not synchronized, as that of adults, where it is needed for tight coupling of moult with the reproductive cycle (Cockcroft and Goosen, 1995). Timing of moult therefore varies, and so, in general when used for experimental purposes, the period before first moulting in an experiment needs to be excluded (Dubber et al., 2004; Hazell et al., 2001), as it will potentially lead to an inaccuracy in determining growth parameters. This is due to the influence of differential reserve built-up pre-experiment (i.e. in the field or acclimation tanks) under different environmental parameters to those tested in the trials. In addition, each individual is, in fact, exposed for a different duration at respective moult stages. In the present study, individuals were tracked over the duration of the experimental period (Figures 3, 6 and 7) which allowed for calculation of average daily gain (ADG, Table 4). This is considered a better approach to distinguish between treatments as it is not only a representation of two measured points (initial and final), but takes every data point accumulated over the experimental period of the trial into consideration.

In trial 1, ADG was higher in the normocapnic- than in the hypercapnic treatment (Table 4). Currently, to the best of my knowledge, there is no literature describing the effect of hypercapnia on somatic growth in *J. lalandii* or other spiny lobsters. There are, however, two separate studies on clawed lobsters in which the effect of seawater pH on larval development was assessed. Their results were contrasting: In the European lobster (*Homarus gammarus*), growth was not affected by a pH of 8.1, in comparison to 8.4 which was considered the "control" (Arnold et al., 2009), whereas in the American lobster (*Homarus americanus*) IMP increased and growth increment decreased contributing to an overall reduction in growth rate (pH 7.7 *vs*. 8.1, Keppel et al., 2012). In the former however, a reduction in carapace weight was observed and the authors speculated that this was due to the environmental pH interfering with internal homeostasis, rather than a direct impact of the alteration in seawater carbonate chemistry, as treatments seawater was not undersaturated with calcium carbonate species (due to high experimental pH). In the latter, reduced growth rate was attributed to a diversion of energy away from growth, to ensure calcification of the exoskeleton or acid-base regulation. Wood et al. (2008) found that maintenance of calcification under several reduced seawater pH exposures (pH 7.7 - 6.8) came at a substantial cost (muscle-wastage) in the brittle star (*Amphiura filiformis*). This may have been the case in *J. lalandii* too, as on further analysis of the Nutritional index (NTI), lobsters from the hypercapnic treatment were on average 7% higher (i.e. > ratio of *CL* to *w*; Table 3). To strengthen this hypothesis, although both in a similar range to that observed in adult WCRL (Cockcroft, 1997), moisture content (MC) was significantly higher in the hypercapnic treatment (Figure 5B), even after moult stage was

accounted for. The combined results of these two parameters, therefore, point to a potential muscle break-down, or the diversion of energy to other mechanisms, such as acid-base regulation. Trial 1 also showed that chronic hypercapnic exposure at a much reduced pH level does not cause elevated mortality in juvenile *J. lalandii* (Figure 4E).

In the second trial, the combination of hypercapnia and high temperature resulted in the greatest growth rate in terms of *CL*-, *w* gain and IMP (shorter), while exposure to normocapnia and low temperature caused the slowest growth rate (Figure 8). Temperature was by far the largest factor positively influencing ADG in *J. lalandii*, followed by hypercapnia, superseding both normocapnia and cold temperature (Table 4). Exposure to the high temperature in the present experiment resulted in the greatest growth rates in *J. lalandii*, although the temperature of 19 ºC is close to the upper end of their natural temperature range (Dubber et al., 2004). As in other organisms, metabolism in palinurids increases with temperature up till their upper temperature limit is reached (Chittleborough, 1975; Thomas et al., 2000). An elevated metabolism could therefore explain the much stronger behavioural feed response (personal visual observation) in lobsters exposed to high-temperature treatments, irrespective of pH.

Up to now, I am only aware of two studies published in which the impact of a combination of hypercapnia and temperature were tested on lobsters, the Norway lobster (*Nephrops norvegicus*), and the European lobster (*H. gammarus*). The former assessed the effects of these parameters on embryonic development (Styf et al., 2013), whereas the latter investigated stage-specific tolerance to the induced stressors (Small et al., 2015). In the first, embryos grew faster at a higher temperature, irrespective of environmental pH. In the second, temperature change lead to a stage-specific sensitivity of metabolic rate, while, an elevation in pCO_2 increased C: N ratios and, in combination with elevated temperature, shell mineralization was decreased. In *H. gammarus*, these physiological changes were associated with slower growth rates and higher mortality. Response patterns of juvenile WCRL, although at a later ontogenetic stage and exposed to a far greater increase in pCO_2 and exposure period, were similar to *N. norvegicus*. The above authors attribute the positive response to the natural variability of the habitat of *N. norvegicus*, and the same could be true for *J. lalandii*. It lives in the BCLME, a system which shows high variability in terms of several physicochemical parameters, such as drastic short-term decreases in seawater pH (to pH 6.6), large temperature fluctuations (8.4 – 19 °C) and extreme reductions in oxygen concentration ($< 0.1 ml^{-1}$) to name just a few (Blamey and Branch, 2012; Blamey et al., 2015; Hutchings et al., 2009; Pitcher and Probyn, 2010; Pitcher et al., 2014; Summerhayes et al., 1995).

In juvenile American lobster (*H. americanus*), an increase in seawater pCO_2 was associated with an increased calcification rate (Ries et al., 2009). The author postulated that this was due to a number of physiological attributes, namely: 1) The greater concentration of dissolved inorganic carbonate (DIC), primarily in the form of HCO_3^- in seawater, 2) the ability of crustaceans to increase the pH at the calcification site above that of the external seawater, translating to an increased availability of HCO_3^{-1}

and so a greater pool of CO_3^{2-} available for calcification and 3) protection of the high magnesium-calcite exoskeleton by a protective membrane, the epicuticle. Although calcification rate was not quantified in *J. lalandii*, it is possible that it was increased here, too, on the base of the high NTI (length- to weight ratio) and relative *CL* gain observed in hypercapnic/high- and low temperature exposed lobsters in comparison with the normocapnic treatments.

On the day of termination of the experiment, it was clear that survival was not negatively affected by either high temperature or hypercapnia. In fact, highest mortality occurred in the treatment that was most "normal", normocapnia and low temperature. The rapid loss of lobsters from this treatment between moults 5 and 6 (leading to a smaller IMP, Figures 8C, E) occurred within several days after moult. The reason for this is unclear, as water parameters were not abnormal and on analysis of immune parameters in eight lobsters from this treatment, immune response was not negatively affected (Chapter 6). Although differences were observed in HSI, MSI and NTI, MC was the only parameter that showed a statistical interaction between pH and temperature with two of the treatments being significantly different. The difference was too small (1.5%), however, to draw any meaningful biological conclusion. Simon et al. (2015) found that in the juvenile spiny lobster (*Sagmariasus verreauxi*), the hepatopanceas and abdominal muscle are the two primary energy reserves used during starvation. The significantly reduced HSI and MSI observed between treatments, where temperature differed, may therefore be linked to increased metabolic activity and, in turn, diminished energy stores.

The calculated AMGs (*CL*) throughout the two growth trials (Table 4), are in a similar range as values obtained from other growth experiments with juvenile WCRL: In Hazell et al. (2001) average moult increment (AMI) for juveniles (*CL* 20 - 25 mm) at similar temperatures to those tested here (15 and 19 °C), were on average ~3.8 and ~2.5 mm, indicating an increased AMI in the lower temperature (Note: only over two IMPs). The results from the present trials, however, exceeded growth rates reported in Dubber et al. (2004) for a period of 77 days (~ 2 moults) at two temperatures (16 to 18 °C), where IMP and AMI were on average ~40 days and 2.4 mm, respectively.

Results regarding the effect of hypercapnia on the juvenile WCRL were contrasting between trials 1 and 2. This is thought to be due to the slight variation in methodology used, namely differing diets and additional stress in trial 1, due to seawater exchange twice a week. Diet-related growth trials in lobsters show a strong species-specific dietary requirement (Johnston et al., 2007). In some, where growth rate and diet have been assessed, formulated feeds in combination with mussel have shown lower growth rates in comparison with mussel-only diets (Dubber et al., 2004; Johnston et al., 2007). These differences were attributed to the pellets dissolving in the water column and their lower palatability compared with mussels, leading to a decreased energy accumulation and, therefore, reduced growth. If so, in the field, combined with the higher energy cost associated with foraging (Pörtner, 2010) a further reduction in growth rate could occur. Stress associated with tank water replenishment most likely led to an increased locomotory activity and a higher energy demand. Recently, a study on the European

spiny lobster (*Palinurus elephas*) showed boat noise to significantly affect locomotor behaviour, along with several physiological parameters (Filiciotto et al., 2014), and so displaying the sensitivity of lobsters to small agitations. In addition, fluctuating water temperatures in trial 1 during water exchange could have led to additional stress for the hypercapnic treatment, since acid-base regulation is temperature-dependent (Truchot, 1978). These factors combined may have contributed to the slower growth rates observed in trial 1.

Although hypercapnia showed small but significant effects between growth parameters, high energy expenditure is expected in order to fuel the large energy requirement of maintaining acid-base balance via pumping HCO_3^- from the seawater for such an extended period (Pörtner et al., 2004).

4.2. Acid-base balance

The haemolymph pH measured (Table 5) in lobsters from the hypercapnic treatments were well within the resting pH range observed in *J. lalandii* (Haupt et al., 2006, Chapter 3). In contrast to the first trial, haemolymph pH from hypercapnic treatments in the second trial were in fact higher than that of the normocapnic treatments in spite of the increased environmental pCO_2. This shows that juvenile *J. lalandii* were able to actively maintain acid-base regulation in an environment which had a hydrogen ion (H^+) concentration that was ~401% greater (see Chapter 2) than that in the normocapnic treatments. The highest bicarbonate concentration observed during sampling was 28 mmol l^{-1}. This lobster was in the hypercapnia/low temperature treatment and had moulted 10 days prior to sampling. This may indicate that it was still actively taking up bicarbonate for possible shell calcification (Neufeld and Cameron, 1993), contributing to a haemolymph pH of 7.95.

The slight difference in seawater pCO_2 between the two normocapnic treatments and two hypercapnic can be explained by the increased solubility of CO_2 in cooler water (Rahn, 1966). Extracellular pCO_2 is maintained above that of seawater pCO_2 in order to maintain an outward gradient of pCO_2, and so this would account for the difference observed between hypercapnic and normocapnic treatments (256% on average, Figure 10). The greater pCO_2 gradient between the seawater and haemolymph observed however in lobsters from the hypercapnic treatments may be indicative of an increased metabolism in comparison with the normocapnic treatments.

The haemocyanin (hc) concentration was low in all trial 2 lobsters when compared with results from trial 1 (Table 5, Chapter 5), and those reported for other crustaceans (Bridges et al., 1984; Chen and Cheng, 1993). The reason may have been a combination of 1) a decrease in stress/agitation, compared to trial 1 (associated with seawater replenishment), which may have decreased locomotory and respiratory activity of the lobsters, and 2) the fact that the duration of the trial was far greater. These two factors may have led to a down-regulation in the concentration of circulating haemocyanin, as observed under hyperoxica (Terwilliger, 1998). The low magnesium levels in the second trial may also

have been a result of lower locomotory and respiratory activity compared with trial 1 (Morritt and Spicer, 1993).

Although differences in growth rates were observed in the juvenile lobsters from both trials, they were able to maintain acid-base balance within a narrow range (pH 7.63 - 7.76) and showed positive growth over the experimental period. The outcomes, however, may very well be different when other factors co-occurring with changes in pCO_2 and temperature are taken into consideration. Upwelled water in the BCLME is low in oxygen (Pitcher et al., 2014), and this level is further reduced upon collapse of large algal blooms occurring in the nearshore environment (Pitcher and Probyn, 2010). The lobsters would have to therefore deal with the ensuing hypercanic-hypoxia, as well as changes in temperature (Pitcher and Probyn, 2010). Aerobic scope is limited by thermal extremes, on either side of an organism's thermal range (Pörtner, 2010). Although the field temperatures recorded thus far are not considered to be on the thermal extremes for this species, the reduced thermal limit in aerobic scope as observed under hypercapnia in the edible crab (*Cancer pagurus*, Metzger et al., 2007) may too, play a role here. This would translate into the lobsters experiencing hypoxemia at lower temperatures than would normally be observed. Beyers et al. (1994) found that juvenile WCRL, when exposed to O_2 saturations of less than 35% at 13 °C (well within natural range), showed decreased growth rate and increased mortality. Pörtner et al. (2008) found that in the Atlantic cod (*Gadus morhua*), oxygen limitation developed earlier (i.e. at a lower temperature) as size increased, leading to a progressively decreased temperature at which growth performance was highest. Should the WCRL have a similar physiological response as the above mentioned species, the larger lobsters would therefore be negatively affected first by an increase in temperature, more specifically the spawning females, due to the additional energy cost of gonadal tissue, which would narrow their thermal window further (Pörtner and Farrell, 2008). Smaller individuals and juveniles would be favoured (Pörtner, 2008) due to their larger thermal window, as observed here, with juvenile WCRLs growth improved, at a temperature considered to be on the upper end of what they would experience naturally (Dubber et al., 2004). The WCRL resource as a whole may have already been affected, which would explain the overall decrease in body size of lobsters in the field (Cockcroft et al., 2008).

5. Conclusion

This study provided information, for the first time, on the influence of chronic hypercapnia alone and hypercapnia combined with different temperatures, on growth and physiology of a palinurid crustacean. The results show, that not only will juvenile WCRL be able to efficiently deal with the predicted environmental scenarios in terms of acid-base balance, but growth rate will in fact be higher under increased temperature and/or pCO_2. This is however, under conditions where other environmental parameters are kept constant and a continuous supply of food is provided. The lower growth rates

observed in hypercapnia-exposed individuals in trial 1 may give an indication of the effect of sub-optimal food availability and indicate limitations of adaptation due to lack of energy stores. Naturally, the lobsters will not only deal with a single or combination of stressors, but rather a multitude and therefore further research should include seawater oxygen concentration as an additional factor as this is realistically experienced along with hypercapnia and temperature change on the West Coast of South Africa.

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Chapter 5

Acid–base balance and changes in haemolymph properties of the South African rock lobsters, *Jasus lalandii*, a palinurid decapod, during chronic hypercapnia*

Abstract

Few studies exist reporting on long-term exposure of crustaceans to hypercapnia. We exposed juvenile South African rock lobsters, *Jasus lalandii*, to hypercapnic conditions of pH 7.3 for 28 weeks and subsequently analysed changes in the extracellular fluid (haemolymph). Results revealed, for the first time, adjustments in the haemolymph of a palinurid crustacean during chronic hypercapnic exposure: 1) acid-base balance was adjusted and sustained by increased bicarbonate and 2) quantity and oxygen binding properties of haemocyanin changed. Compared with lobsters kept under normocapnic conditions (pH 8), during prolonged hypercapnia, juvenile lobsters increased bicarbonate buffering of haemolymph. This is necessary to provide optimum pH conditions for oxygen binding of haemocyanin and functioning of respiration in the presence of a strong Bohr effect. Furthermore, modification of the intrinsic structure of the haemocyanin molecule, and not the presence of molecular modulators, seems to improve oxygen affinity under conditions of elevated pCO_2.

Keywords: Chronic hypercapnia, Molecular structure change, Haemoprotein haemocyanin, Acid-base balance, Ocean acidification

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1. Introduction

Exposure to acute and chronic hypercapnia leads to various responses in the few different crustacean species investigated so far (Whiteley, 2011), making generalisation difficult and more research necessary. In marine crustaceans, some responses can be observed in the extracellular fluid. Haemolymph separates the animals' environment from the intracellular space and mediates external impacts before they affect cellular metabolism and oxygen supply of the cells. For example, tight control of the extracellular pH via regulation of acid-base balance is essential for oxygen supply and gas exchange (Bridges, 2001). Marine invertebrates are exposed to natural hypercapnia during events such as upwelling episodes, low oxygen events and – in the medium to long-term – Ocean Acidification (OA).

The West Coast rock lobster, *Jasus lalandii*, is a cold-water palinurid decapod supporting a valuable commercial fishery in South Africa and Namibia (Melville-Smith and van Sittert, 2005). The species lives mainly along the West Coast of Southern Africa in the Benguela Current System in shallow coastal waters (Holthuis, 1991). This environment is characterised by 1) frequent upwelling events, 2) periods of low-oxygen in some areas due to algal decay and bacterial respiration and is potentially threatened by 3) declining pH due to OA. During 1) and 2), the pH can drop to levels as low as 6.6 for several days (Pitcher and Probyn, 2010). These events have been forecast to become more frequent and severe due to OA (Bakun, 1990; Rhein et al., 2013). Currently, global pH levels are decreasing at rates between 0.0014 and 0.0024 units yr^{-1} (Rhein et al., 2013) and by the year 2300, a global pH of about 7.3 is expected (Caldeira and Wickett, 2003). Due to the already low pH levels in the Benguela Current system, extended periods of high acidity could occur earlier.

Chronic responses to hypercapnia are currently unknown for this species and, in fact, other palinurid decapods. In general, there is a lack of chronic studies on crustacean hypercapnia that exceed a few weeks (Whiteley, 2011). The aim of the present study was therefore to elucidate the responses at the extracellular level in *J. lalandii* during true long-term exposure (28 weeks) to hypercapnia.

2. Materials and methods

2.1. Lobsters

Jasus lalandii juveniles were collected from oyster settlement plates of an offshore oyster farm in the Langebaan lagoon, Western Cape, South Africa. They were transported within ±2 h to holding tanks at the research aquarium in Cape Town in plastic bags filled to 50% with 4.5 l seawater, bubbled with oxygen before sealing. Bags were placed into a polystyrene container with ice bricks to ensure T_A below 20 °C. In Cape Town, they were maintained in flow through tanks for four months prior to experimentation (pH ranged from 7.9 to 8.1; T_A from 8.4 to 16.8 °C) and fed a mixed diet of mussel

(*Choromytilus meridionalis; Mytilus galloprovincialis*), sardines (*Sardinops sagax*) and Maasbunker (*Trachurus trachurus)* twice a week *ad libitum*. Feeding was discontinued three days before handling.

2.2. Hypercapnic exposure

Lobsters were weighed (*w*), carapace length (*CL*) measured and placed into individual perforated 750 ml plastic containers. Twelve individuals (mean ± S.E.; *w*: 2.3 ± 0.4 g; *CL*: 16.0 ± 0.8 mm) were placed into a 1000 l tank with normocapnic seawater and 12 individuals (*w*: 2.1 ± 0.3 g; *CL*: 15.5 ± 0.7 mm) into a 1000 l tank with hypercapnic seawater. Student's t-test revealed no difference between *w* and *CL* between the two groups. Tanks were well mixed and aerated. Lobsters were acclimatized for a week to around 18 °C after which the pH in one tank was lowered in two steps in five days from approximately 8.1 to 7.3 using a pH controller (7074/2, TUNZE, Germany) containing a solenoid valve (7074.111) and a pH electrode (7070.110) attached to a 9 kg CO_2 bottle (technical). Lobsters remained under these experimental conditions for 195 days (~28 weeks). Seawater (~80%) was exchanged with pre-conditioned seawater twice a week. Seawater parameters pH and T_A were measured five times a week, A_T and salinity twice weekly. These conditions are summarised in Table 1. Seawater pCO_2, HCO_3^- and CO_3^{2-} were calculated using measured pH, T_A, salinity and A_T (Sarazin et al., 1999) as constants in CO2SYS software (Pierrot et al., 2006), using dissociation constants refitted by Dickson and Millero (1987) from Mehrbach et al. (1973) and *KSO₄* from Dickson (1990). Lobsters were fed *ad libitum* five days a week, 3 days with pelleted feed (Nutrafin max, A6792U), the other two, fresh mussel (*Choromytilus meridionalis* and *Mytilus galloprovincialis*). Food and moults were removed and water quality monitored by measuring NH_4^+ concentration (Ammonia test kit, Sera, Germany). The latter never exceeded 0.4 mg l^{-1}.

2.3. Haemolymph sampling

At the time of haemolymph sampling, lobsters had the following size: Normocapnia: *w* 10.7 ± 0.8 g, *CL* 26.6 ± 0.7 mm; hypercapnia: *w* 9.6 ± 0.5 g, *CL* 25.6 ± 0.4 mm. Student's t-test revealed no differences in *w* and *CL* between the groups. Pre-branchial haemolymph (max. 0.3 ml) was extracted from the arthrodial membrane at the base of the fifth pair of pereiopods by syringe with hypodermic needle (Neomedic 1 ml, 29 G); avoiding tail flips by securing the abdomen with a firm grip. The sample was placed in a 0.5 ml microcentrifuge (Eppendorf) tube for acid-base analysis or frozen at -80 °C for haemocyanin studies. Subsequently, the moult stage of each lobster was determined microscopically from setagenic stages of pleopods, based on a moulting stage range of AB, C, through to D_4 (Marco, 2012).

Table 2. Seawater conditions recorded during exposure to normocapnic and hypercapnic conditions during a period of 28 weeks.

	Salinity (‰)	T (°C)	pH	A_T (µmol kg^{-1})	pCO_2 (Torr)	HCO_3^- (mmol l^{-1})	CO_3^{2-} (mmol l^{-1})
Normocapnia	34.4 ± 0.1	18.8 ± 0.1	8.02 ± 0.02	1903 ± 8	0.27 ± 0.01	1.58 ± 0.02	0.14 ± 0.01
Hypercapnia	34.4 ± 0.0	18.8 ± 0.1	7.32 ± 0.01	1925 ± 7	1.57 ± 0.03	1.88 ± 0.01	0.04 ± 0.00

Values are given as means ± S.E.

2.4. Haemolymph acid-base balance

pH was measured within 20 s after sampling using a Orion 3 star pH meter equipped with an Orion 8220 BNWP micro pH electrode (Thermo Scientific, USA). Calibration was performed with NBS precision buffers (Applichem, Germany) at the same temperature as that of ambient seawater of the lobster tanks. A haemolymph subsample (50 µl) was immediately injected into a de-gassing (magnetic stirrer) chamber containing 200 µl of 100 mM H_2SO_4 and liberated total CO_2 (cCO_2) determined by CO_2 analyser (SBA4, PP Systems, USA) using CO_2-free N_2 (technical) as carrier gas (50 ml min^{-1}) calibrated against freshly made Na_2HCO_3 standards (1 – 10 mM). From measured pH and cCO_2 values, pCO_2, and [HCO_3^-] were calculated using derivatives of the Henderson Hasselbalch equation (I and II). The required solubility coefficient αCO_2 and dissociation constant pK'_1 of carbonic acid were adopted as described previously for *Carcinus maenas* (Truchot, 1976a).

$$\text{I.} \quad pCO_2 = \frac{cCO_2}{10^{pH-pK'_1} \times \alpha CO_2 + \alpha CO_2}$$

$$\text{II.} \quad HCO_3^- = cCO_2 - \alpha CO_2 \times pCO_2$$

Ca^{2+}, Mg^{2+} (Diaglobal, Germany) and L-lactate concentrations (Roche, Germany) were determined by commercial kits on small subsamples from each individual. Haemocyanin concentration was determined spectrophotometrically (335 nm) in 1:50 haemolymph *vs. Jasus lalandii* Ringer solution (0.52 M NaCl, 0.015 M $MgSO_4$, 0.013 M $CaSO_4$, 0.005 M KCl, 0.005 M $NaHCO_3$, pH 7.8), calculated using an extinction coefficient ε_{335} = 0.233 ΔE units mg^{-1} ml^{-1} (Nickerson and Van Holde, 1971).

2.5. Oxygen affinity of haemocyanin

After transporting frozen samples to Düsseldorf, aliquots from each treatment were pooled due to their small individual volumes; subsequently the clotted haemolymph was re-suspended by means of a pestle, and centrifuged for 15 min at 17 100 g and 4 °C (Eppendorf, Germany). Aliquots (1.5 ml) from each pool were dialysed (1:1000 ratio haemolymph *vs.* Ringer) at 4 °C for 48 h against two changes of standard dialysis Ringer (0.017 M NaCl, 0.006 M $CaSO_4$, 0.003 M $MgSO_4$, 0.013 M KCl, and 0.012 M $NaHCO_3$, pH 7.8). Aliquots of full and dialysed haemolymph were transferred into 2 ml reaction tubes and centrifuged for 15 min at 13 000 rpm (4 °C). Subsequently, 200 µl supernatant was centrifuged (Airfuge ultracentrifuge, Beckman, USA) for 30 min at 160 000 g. The top 100 µl were removed and the remaining 100 µl re-suspended with the haemocyanin pellet, doubling the concentration of haemocyanin. From these preparations, oxygen affinity curves were established and analysed for whole and dialysed haemolymph using a spectrophotometric method on 5 µl haemocyanin samples in a diffusion chamber (Sick and Gersonde, 1969) modified as described previously (Bridge et al., 1979, 1984). The non-bicarbonate buffer capacity was calculated from carbon dioxide titration of 50 µl aliquots in a Radiometer BMS II system (Radiometer, Denmark), used to measure haemolymph pH at carbon dioxide tensions provided by gas mixing pumps (Wöstoff, Germany).

3. Results

3.1. Haemolymph acid-base balance

Measured pH in the haemolymph from hypercapnic incubated lobsters was significantly lower ($p<0.05$) than in the normocapnic group, whereas cCO_2 had doubled to 8.5 mM under hypercapnia compared with normocapnia (Table 2). Accordingly, calculated pCO_2 and $[HCO_3^-]$ were 3.9 Torr compared to 1.7 Torr and bicarbonate 8.4 mM compared to 4.1 mM under normocapnia, respectively. $[Ca^{2+}]$ was significantly lower (28%) under hypercapnic conditions, whereas $[Mg^{2+}]$ changes were slight and not significant (Table 2). Lactate concentrations were relatively low at 0.4 to 0.3 mM in both groups. pH and $[HCO_3^-]$ values were similar within the normocapnic group and most values below the 2 Torr isopleth (Figure 1A). They varied considerably, however, in both pH and $[HCO_3^-]$, in the hypercapnic group and are assembled along the 4 Torr pCO_2 isopleth with no value below the 2 Torr isopleth (Figure 1B). The non-bicarbonate buffer line of haemolymph from the hypercapnic group is elevated and shifted parallel compared with that of the normocapnic lobsters (Figure 1C). At the same time, haemocyanin concentration in the haemolymph decreased by 38% under hypercapnic exposure indicating a decrease in oxygen carrying capacity.

3.2. Oxygen affinity of haemocyanin

In figure 2 oxygen affinity is depicted in whole blood and dialysed (Figure 2A) and dialysed blood (Figure 2B) together with cooperativity measured as n_{50}. The slope of the regression lines indicate the Bohr coefficient, -0.42 in normocapnic whole haemolymph and higher at -0.66 in hypercapnic whole haemolymph. Dialysis had no effect on the Bohr coefficient of hypercapnic or normocapnic haemolymph (Figure 2A).

Oxygen binds cooperatively to *J. lalandii* haemocyanin. Maximal cooperativity (Hill coefficient n_{50} = 4.15) was found in dialysed haemolymph from normocapnic lobsters but was marginally smaller (n_{50} = 3.92) in dialysed haemolymph from hypercapnic lobsters. Cooperativity was lower in both whole haemolymph samples, again with that of hypercapnic haemolymph slightly lower (n_{50} = 3.22) than in normocapnic haemolymph.

A full oxygen saturation curve was constructed using the haemocyanin from the juvenile lobsters that were exposed to normocapnia, using a 0.1% CO_2 to set pH. The curve is described by the equation: $y = -2E\text{-}08x^5 + 7E\text{-}06x^4 - 0.0006x^3 + 0.0141x^2 - 0.0846x + 0.152$ ($r^2 = 0.9957$, pH = 7.216, 15 °C) (not shown here).

Table 3. Haemolymph parameters of juvenile lobsters after exposure to normocpanic and hypercapnic conditions for 28 weeks.

Haemolymph parameter	pH	cCO_2 (mmol l^{-1})	pCO_2 (Torr)	HCO_3^- (mmol l^{-1})	Ca^{2+} (mmol l^{-1})	Mg^{2+} (mmol l^{-1})	L-lactate (mmol l^{-1})	Haemocyanin (mg ml^{-1})
Normocapnia	7.760 ± 0.025	4.2 ± 0.4	1.7 ± 0.2	4.1 ± 0.4	12.8 ± 0.8	17.5 ± 0.6	0.4 ± 0.1	22.3 ± 2.0
Hypercapnia	7.655 ± 0.061	8.5 ± 1.2*	3.9 ± 0.2*	8.4 ± 1.2*	9.2 ± 0.6*	19.4 ± 1.5	0.3 ± 0.1	13.9 ± 2.1*

Values are mean ± S.E. (n = 12). *denotes significant difference to normocapnic value ($p < 0.05$, Student's t-test).

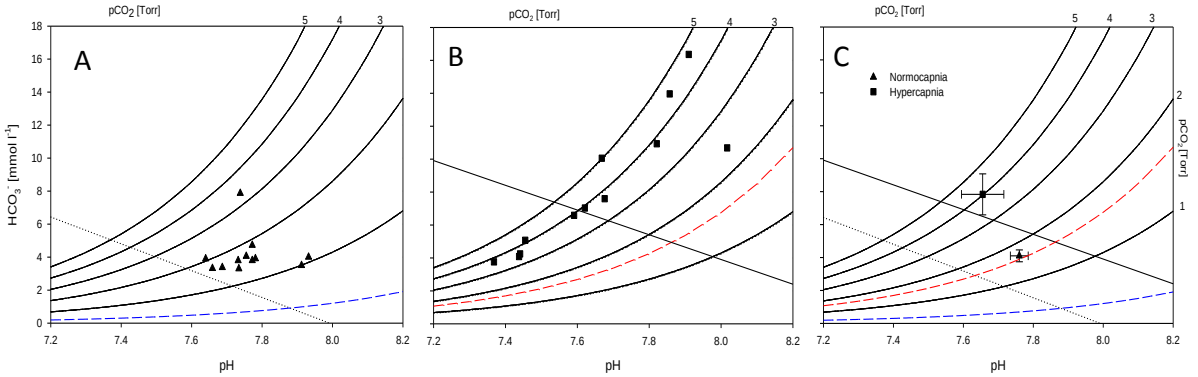

Figure 1. Henderson-Hasselbalch (pH-bicarbonate) diagrams for haemolymph of juvenile lobsters incubated for 28 weeks. (A) Individual values from 12 lobsters exposed to normocapnic conditions, (B) individual values from 12 lobsters exposed to hypercapnic conditions and (C) mean ± S.E. from values depicted in (A) and (B). pCO_2 isopleths were derived from the Henderson-Hasselbalch equation. Values for the first dissociation constant (pK'_1) and Solubility coefficient (αCO_2) were derived from (Truchot, 1976b) and were: $pK'_1 = 6.01$, $\alpha CO_2 = 0.044$ (18 °C). Blue dashed line = normocapnic seawater isopleth, red dashed line = hypercapnic seawater isopleth. Dotted black line = normocapnic non-bicarbonate buffer line, solid black line = hypercapnic non-bicarbonate buffer line (see also materials and methods).

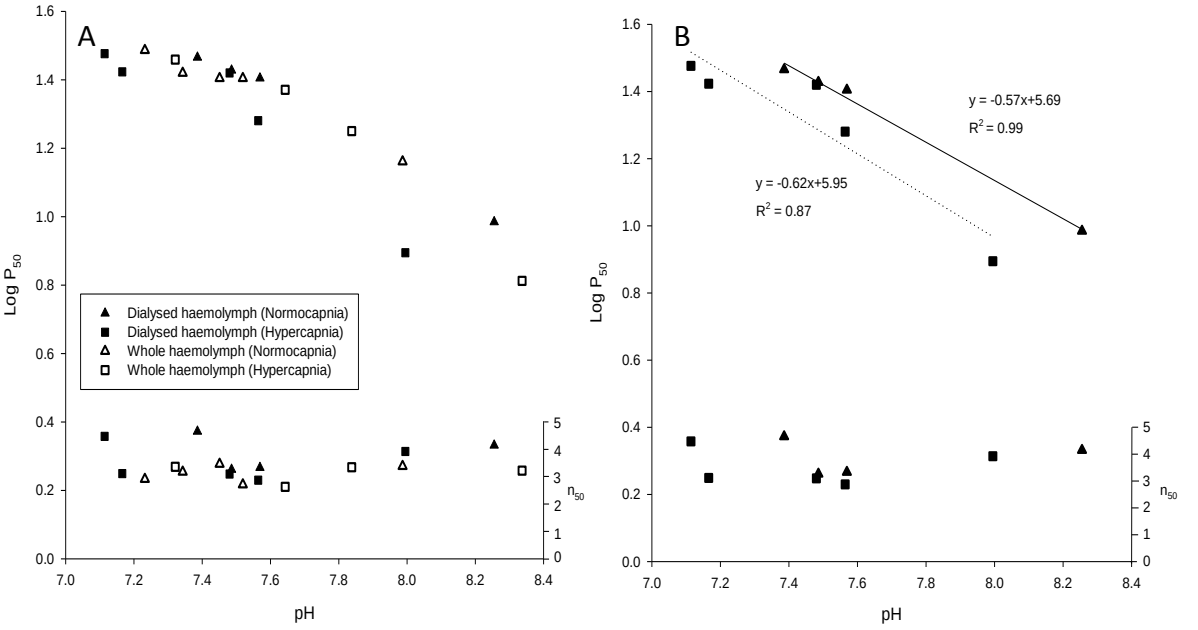

Figure 2. pH-dependence of oxygen affinity of haemocyanin from whole and dialysed haemolymph from juvenile lobsters incubated for 28 weeks in normocapnic and hypercapnic seawater, respectively. (A) Relationship of haemocyanin-oxygen affinity as log P_{50} and pH (upper part and left y-axis) and cooperativity of haemocyanin-oxygen binding and pH (lower part and right y-axis) for whole blood. (B) Bohr effect (upper part) and cooperativity (lower part) of haemocyanin from dialysed haemolymph only from juvenile lobsters after normocapnic and hypercapnic treatment, respectively.

3.3. Moult stage

Each experimental group consisted of a cohort of lobsters that were in a similar range of moult stages. In the normocapnic group, two lobsters were at stage AB, three at C, two at D_0, four at D_1 and one at D_4. In the hypercapnic group, three lobsters were at stage AB, five at C, three at D_1 and one at D_2.

4. Discussion

Our study reveals, for the first time, adjustments in the haemolymph acid-base balance of a palinurid crustacean during chronic hypercapnic exposure. During 28 weeks of exposure to a water pH level of about 7.3, the following changes in the haemolymph of juvenile *J. lalandii* occurred:

1. Adjustment of the acid-base balance by increased bicarbonate
2. A change in quantity and oxygen binding properties of haemocyanin

4.1. Regulation of acid-base balance

Extracellular pCO_2 is maintained above environmental pCO_2 to ensure an outward gradient for CO_2 removal (Cameron, 1986; Henry and Cameron, 1983) whereas oxygen affinity has to be safeguarded in the presence of a pronounced Bohr shift in *J. lalandii* haemocyanin (Figure 2B). Under hypercapnia, this requires extra buffering achieved by bicarbonate and non-bicarbonate buffers (Melzner et al., 2009). In adult *J. lalandii*, acute environmental hypercapnia causes acidosis driven by CO_2 increase but is adjusted by increased [HCO_3^-] within five hours (*unpublished data*). Here, we show that a secondary [HCO_3^-] increase (i.e. above the non-bicarbonate buffer line) is present as chronic adjustment of extracellular pH in juveniles. Compared with lobsters kept in normocapnic seawater, those under hypercapnic conditions doubled their [HCO_3^-]. Non-bicarbonate buffer capacity, however, is almost exclusively achieved by haemocyanin in crustaceans (Truchot, 1976b), whose concentration decreased under hypercapnia here. This may be compensated by an increase of other non-bicarbonate buffer substances, such as proteins or inorganic phosphate. Variation in [HCO_3^-] between individuals is large in the hypercapnic group (Figure 1B), whereas it is small in normocapnic individuals (Figure 1A). The values for hypercapnic lobsters spread along the 4 Torr pCO_2 isopleth, suggesting that individual differences in pH were primarily due to differences in [HCO_3^-]. Individual [HCO_3^-] values are not correlated to [Ca^{2+}] (not shown), indicating, as does the spread of moult stages in both groups, that this is not a moult effect. Data suggest that, after non-respiratory compensation, hypercapnic juveniles had reached a steady state at a more acidic level (i.e. only partial compensation) after exposure, similar to other crustacean species (Cameron, 1986), and maintained it thereafter. In other crustaceans, an elevated [HCO_3^-] could not be maintained over an extended period, see Whiteley (2011). The pCO_2 data for water and haemolymph from Tables 1 and 2 show that the CO_2 gradient across the gills increases from 1.4 Torr to 2.3 Torr, changing from normocapnia to hypercapnia. This could indicate that metabolism

also increased during hypercapnic exposure as would energetically be expected to fuel the ionic pumping of bicarbonate from sea water.

4.2. Properties of haemocyanin

Figure 3 summarizes the calculated changes in haemolymph oxygen affinity together with *in vivo* pH changes. In whole blood P_{50} remains relatively constant even though haemolymph pH drops by 0.1 units.

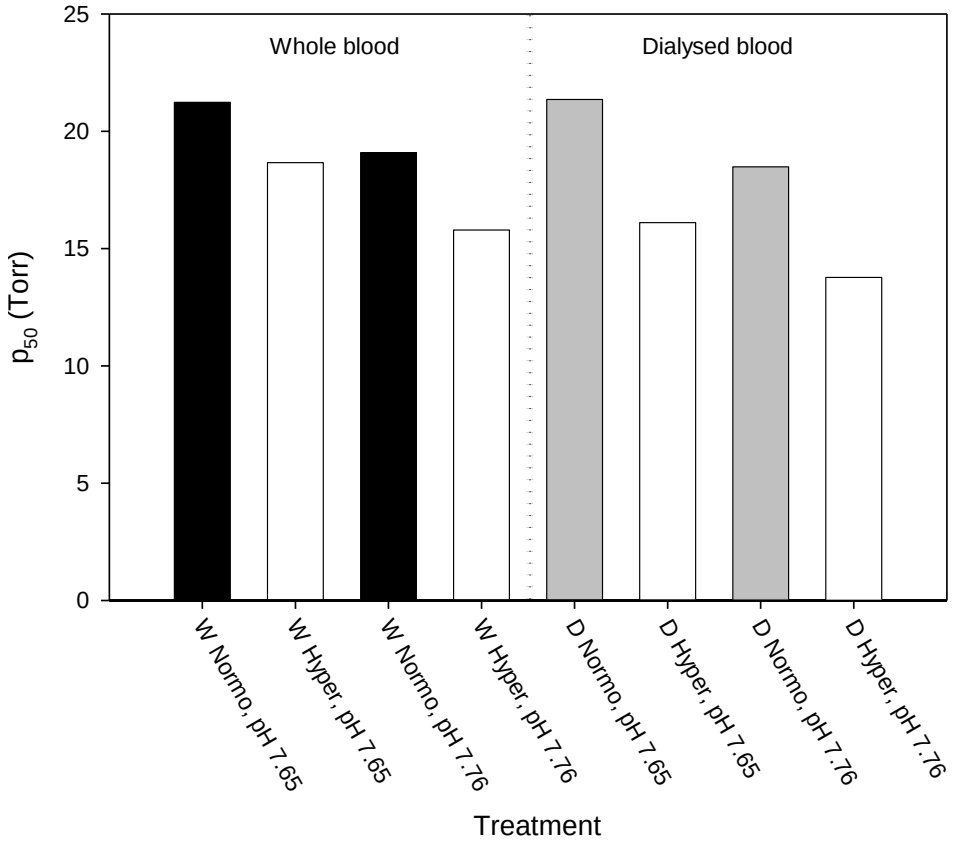

Figure 3. Calculated P_{50} values for respective treatments at *in vivo* pH conditions. Values were calculated for the different pH values using the regression equations shown in Figure 2. *In vivo* pH values (indicated in column label) were taken from Table 2. Labels: W = whole blood, D = dialysed blood, Normo = normocapnia, Hyper = hypercapnia.

Without compensation P_{50} would decrease by approximately 2.5 Torr. Since calcium levels are 28% lower in the hypercapnic group and decreases in calcium will lead to a lowering of oxygen affinity (Truchot, 1975; Wänke, 2008), one would expect an even greater change to the detriment of oxygen transport. The role of other co-factors such as lactate (Bridges, 2001) can be ruled out since these levels are similar in both normocapnia and hypercapnia. For dialysed blood at constant levels of Mg^{2+} and

Ca^{2+}, it is clear from Figure 3, that an increase in intrinsic affinity by almost 5 Torr occurred. Haemocyanin concentration was low in normocapnic juveniles compared with adult lobsters (70 mg ml^{-1}, *unpublished data*). Such differences between life- and moult stages, however, are not uncommon (Chen and Cheng, 1993; Djangmah, 1970). In terms of oxygen affinity, haemocyanin of juvenile and adult *J. lalandii* is similar (Table 3). Compared with other palinurids, affinity of *J. lalandii* haemocyanin (P_{50} ~14 Torr) is much lower than the very high affinity of the European spiny lobster, *Palinurus elephas* (P_{50} = 6.5 Torr in dialysed blood, using Tris-Buffers, Wänke, 2008) and, taking into account the difference in incubation temperature, should be in the low affinity range as its Australian relative *Jasus edwardsii* (P_{50} = 26 Torr, Morris and Oliver, 1999). Oxygen affinity is generally higher in hypoxic environments (Burggren et al., 1991) and the difference between the three palinurid species could therefore be an expression of the oxygen availability in their respective environments. Compared with crustacean species that live in shallow waters or in the intertidal, haemocyanin of juvenile *J. lalandii* has a low oxygen affinity, an indication that these juveniles are normally exposed to well-oxygenated seawater.

Haemocyanin was termed the "interface" of crustacean physiology due to its molecular and biochemical flexibility (Bridges, 2001). This flexibility is present in *J. lalandii* haemocyanin: Dialysed haemocyanin from juvenile lobsters kept in hypercapnic seawater for 28 weeks had an increased oxygen affinity compared with that from normocapnic juveniles. A possible explanation is a modification of the intrinsic structure of the haemocyanin molecule (Bridges, 2001; Truchot, 1992) and this could be a mechanism to conserve energy under environmental stress. Also, such structure change could explain the observed increase in oxygen affinity (Burnett, 1992).

In hypercapnic conditions, a decline of pH by 0.1 units (27% acidification) was observed. It is probably not energetically possible to maintain constant 'normal' pH in the long term. The lower pH could also just be a result of the increased extracellular pCO_2. The latter ensures a gradient for removal of respiratory CO_2 under hypercapnia which becomes necessary when environmental pCO_2 almost reaches normocapnic extracellular pCO_2 levels (1.6 *vs* 1.7 Torr). There is also a potential influence of molecular modulators on haemocyanin properties: The reduced [Ca^{2+}] may be due to energetic costs, too, or as a result of calcification processes in the exoskeleton. The slightly elevated [Mg^{2+}], meanwhile, may ensure an increased Bohr coefficient and oxygen affinity of haemocyanin (Truchot, 1975). In the crab *Carcinus maenas*, Ca^{2+} increases oxygen affinity whereas Mg^{2+} increases oxygen affinity AND Bohr coefficient (Truchot, 1975). Protecting the Bohr shift guarantees loading of haemocyanin with oxygen at the gills and unloading in the tissues.

Table 3. Comparison of oxygen affinity (P_{50}) of haemocyanin from different crustacean species measured at a specific pH and temperature.

Species	P_{50} (Torr)	pH	T_A (°C)	Source	whole (W) /dialysed (D)
Juvenile West Coast rock lobster (*Jasus lalandii*)	14.8	8.0	15	current study	W
Adult West Coast rock lobster (*Jasus lalandii*)	14.1	7.8	15	unpublished data	W
European spiny lobster (*Palinurus elephas)*	6.5	8.0	20	(Wänke, 2008)	D
(Australian) Spiny rock lobster (*Jasus edwardsii*)	25.8	7.8	20	(Morris and Oliver, 1999)	W
European Lobster (*Homarus vulgaris*)	9.3	7.9	15	(Bouchet and Truchot, 1985)	W
Intertidal prawn (*Palaemon elegans*)	9.0	7.8	10	(Bridges et al., 1984)	W
European shore crab (*Carcinus maenas*)	10.9	7.8	15	(Lallier and Truchot, 1989)	W

Although it was not the aim of this study to investigate the effect of the various molecular modulators of oxygen affinity in detail, the differences between full and dialysed haemolymph show that oxygen affinity is not decreased in hypercapnic juvenile lobsters when these modulators are removed. Cooperativity values were similar in all treatments.

5. Conclusion

We have shown that, to ensure functioning of respiration during prolonged hypercapnia, juvenile lobsters are capable of bicarbonate buffering of their haemolymph to provide optimum pH conditions for oxygen binding in the presence of a strong Bohr effect. In addition, modification of the intrinsic structure of the haemocyanin molecule, and possibly the presence of molecular modulators, seems to improve oxygen affinity under conditions of elevated pCO_2.

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Chapter 6

Effects of chronic hypercapnia and elevated temperature on the immune response of the spiny lobster, *Jasus lalandii*

Abstract

The West Coast rock lobster (WCRL), *Jasus lalandii*, inhabits highly variable environments. Due to upwelling events, episodes of hypercapnia and large temperature variations occur frequently. The predicted threat of ocean acidification and temperature change for the coming centuries will more than likely affect the immune response in crustaceans. We therefore tested the hypothesis that chronic exposure to hypercapnia and elevated seawater temperature alters immune function of the WCRL. The chronic effects of four combinations of two stressors: seawater pCO_2 and temperature on the total number of circulating haemocytes (THC) as well as on the lobsters' ability to clear (inactivate) a dose of *Vibrio anguillarum* injected into the haemolymph were assessed. Juvenile lobsters were held in normocapnic (pH 8.01) or hypercapnic (pH 7.34) conditions at two temperatures (15.6 and 18.9 °C) for 48 weeks (n = 30 per treatment), after which a subsample of lobsters (n = 8) at a similar moult stage were selected from each treatment for the immune challenge. Baseline levels of haemocytes (THC ml^{-1}) and bacteria (CFU ml^{-1}) in haemolymph were quantified 24 h prior to bacterial challenge. Lobsters were challenged by injecting 4×10^4 *V. anguillarum* per g body weight directly into the cardiac region of each lobster and THC ml^{-1} and CFU ml^{-1} measured again at 20 min post challenge. No differences ($p < 0.05$) were observed between any of the treatment groups for baseline THC ml^{-1}. However, after challenge, lobsters that were chronically exposed to a combination of hypercapnia and high temperature, showed a lower ($p< 0.05$) THC in comparison with the lobsters chronically exposed to hypercapnia and low temperature. Clearance of culturable *V. anguillarum* from the haemolymph was in all cases rapidly achieved. Clearance performance was in the following order: Hypercapnia/low temperature > normocapnia/high temperature > normocapnia/low temperature > hypercapnia/high temperature. The WCRL, therefore, showed a rapid immune response in spite of the chronic exposure to combinations of reduced seawater pH and high temperature.

Keywords: *Jasus lalandii*; Spiny lobster; Haemolymph; *Vibrio*; Hypercapnia; Global warming; Ocean acidification, Immune response.

1. Introduction

Crustaceans are found in a variety of habitats and are therefore naturally exposed to a wide range of environmental parameters (Henry and Wheatly, 1992; Le Moullac and Haffner, 2000), giving them, to a certain degree, some physiological plasticity. They have to respond to, for example, changes in salinity, temperature, seawater pH and oxygenation (Bridges, 2001).

The South African cold water palinurid, *Jasus lalandii*, inhabits such an environment, the Benguela Current Large Marine Ecosystem (BCLME), which displays great variability in its physicochemical makeup in the short term, primarily due to upwelling (Blamey et al., 2012; Hutchings et al., 2009; Pitcher and Probyn, 2010; Pitcher et al., 2014; Summerhayes et al., 1995). Coming centuries, however, are predicted to bring new, more sustained challenges, namely, a decrease in ocean pH, termed "ocean acidification" and seawater temperature increase (Rhein et al., 2013). It is not clear, though, into what direction the latter will develop in the BCLME (Jarre et al., 2015).

Due to the West Coast rock lobsters' (WCRL) high economic importance (Cockcroft et al., 2008), and its role as a keystone benthic predator (Barkai and Branch, 1988), various field- and aquarium studies assessing the effects of environmental change on several biological parameters have been completed (Beyers et al., 1994; Dubber et al., 2004; Hazell et al., 2001; Pollock and Beyers, 1981; Pollock et al., 1997). Studies on the lobsters' physiological-, biochemical- and molecular responses, however, are lacking, and the same is true for its immunology. Recently, *J. lalandii* was observed to be capable of efficiently maintaining its acid-base balance when exposed to acute- (Chapter 3) and chronic (Chapters 4 and 5) hypercapnia. The ability of lobsters to defend themselves against opportunistic bacteria and other pathogens, however, may be compromised under these conditions, especially when seawater chemistry changes together with ambient temperature. It has been postulated that disease prevalence in decapods will increase due to the projected climate scenarios (Stentiford et al., 2012), and several studies have observed adverse effects on the immune response of crustaceans exposed to environmental stressors, such as hypercapnia and increased temperature (Hernroth et al., 2012; Le Moullac and Haffner, 2000; Oweson and Hernroth, 2009), suggesting that similar stressors may lead to a weakened immune defence in *J. lalandii*.

The innate immune response of crustaceans is composed of several effectors/mechanisms. The primary cell types responsible for defence against foreign entities or organisms are the haemocytes (Johnson, 1987; van de Braak et al., 2002), developed from haematopoietic tissue (Johansson et al., 2000), such as the hepatopancreas in lobsters (Hauton et al., 2005). Three types of haemocytes occur in crustaceans, namely hyaline-, semi-granular- and granular ones (Bauchau, 1980), each having a specific immune function (Johansson et al., 2000). The quantity of haemocytes and/or haemocyte sub-populations have been shown to vary with several physicochemical water parameters, as well as biological parameters (Cheng and Chen, 2001; Cheng et al., 2003; Hernroth et al., 2012; Lin et al., 2012; Le Moullac et al.,

1997; Ridgway et al., 2006; Verghese et al., 2007, 2008). Low circulating haemocyte numbers are strongly correlated with increased susceptibility to pathogens in crustaceans (Le Moullac et al., 1998; Persson et al., 1987).

Following challenge by bacteria, two processes occur in crustaceans: 1) Foreign particles adhere to the lining of the haemocoel (body cavity) and 2) haemocytes attach to sites where bacteria are adhered to, accordingly, a decrease in circulating haemocyte count will be observed (Martin et al., 1996). Bacteria that are not trapped will remain in circulation, and haemocytes will rapidly adhere to these, forming particles/clumps that will ultimately become trapped in small capillary networks of the gills (Ikerd et al., 2005) and/or the hepatopancreas, where they are subsequently quickly degraded (Clem et al., 1984; Martin et al., 1993, 1998; Smith and Ratcliffe, 1980). Bacterial inactivation is a mechanism which occurs far more rapidly than bacterial degradation (Burgents et al., 2005a), and in order to observe these changes in bacterial culturability (i.e. suppression) *in vitro* following injection into crustaceans, bacteria are cultured/grown on artificial media.

A strong correlation exists between environmental change and disease susceptibility, i.e. the depression of the immune system, for mussels, fish (Le Moullac and Haffner, 2000) and crustaceans (Burgents et al., 2005a; Hernroth et al., 2012; Lin et al., 2012; Macey et al., 2008). Unfortunately, the majority of these correlations have only been observed following acute exposure to environmental stressors and results cannot be used to predict chronic impacts.

In the present study, we therefore aimed at addressing this shortfall by examining the immunological response of *J. lalandii* after chronic exposure to combinations of different temperatures and seawater pHs.

2. Materials and methods

2.1. Animal collection and maintenance

Juvenile lobsters (Chapter 4) were collected in Saldanha Bay (32°59′–33°05′ S, 17°56′–18°02′ E), which is a large semi-enclosed bay on the West Coast of South Africa (Groeneveld et al., 2010). Lobsters were obtained from oyster stacks at an offshore oyster farm (Saldanha Bay Oyster Company) situated opposite the mouth of the bay. Oyster stacks were sampled during routine maintenance operations between September and December 2013. All specimens (n = 130) were placed in plastic bags filled with 4.5 l seawater, bubbled with oxygen before sealing. The bags were then placed into a polystyrene container with ice bricks to ensure T_A below 20 °C. Traction for the lobsters in the bags was provided by mesh cloth, this minimised stress during transport. These lobsters were then transported to the research aquarium of the Department of Agriculture, Forestry and Fisheries (DAFF) in Sea Point, Cape Town.

Immediately upon arrival, the juveniles were placed into glass tanks (*L × W × H: 100 × 48 × 35 cm*). Seawater flowed through at an approximate rate of ~240 l h^{-1} and seawater salinity, temperature and pH fluctuated according to incoming water (T_A ranged from 11 to 17 °C). The tanks were constantly aerated and photoperiod was maintained on a 12 hr day/night cycle.

The lobsters were pre-acclimated in these tanks for ± 4 months, prior to experimentation. During this period, lobsters were fed a mixed diet of mussel (*Choromytilus meridionalis* and *Mytilus galloprovincialis*), sardines (*Sardinops sagax*) and Horse mackerel (*Trachurus trachurus)*, once to twice a week in the afternoon *ad libitum*, excess food was removed the following day. Four days prior to commencement of the pre-conditioning and chronic exposure trial for the immune challenge, feeding was discontinued.

2.2. Pre-conditioning and chronic exposure

Pre-acclimated juvenile lobsters were removed from the holding tanks and each individual weighed to the nearest 0.001 g using an M-power electronic balance (Sartorius, Germany) and carapace length (*CL*) measured (tip of rostrum to mid caudo-dorsal margin of carapace) to the nearest 0.1 mm with a digital calliper (Mitutoyo Corp., Japan). Following measurement, lobsters were individually placed into plastic containers *(L × W × H: 10 × 10 × 7.5 cm)*, labelled with a unique number. This was done primarily for the growth trial (Chapter 4), as it allowed for precise growth monitoring of each individual, prevented cannibalism and competition for food. Each plastic container was perforated with forty-four 3 mm holes on each side to ensure sufficient water exchange with the surrounding environment. Perforation provided better traction and assisted lobsters during moulting. Once a lobster was assigned to a number and container it was placed into a tank *(L × W × H: 148× 100 × 90 cm)* with seawater parameters replicating those of the pre-acclimation tanks. After selection of similar-sized lobsters for each treatment, according to biomass (*w*), 30 individuals were placed into each of the four treatment tanks *(L × W × H: 148× 100 × 90 cm)*. Treatments consisted of a combination of: a) Hyperpercapnia/low temperature, b) normocapnia/high temperature, c) normocpania/low temperature and d) hypercapnia/high temperature. The measured and calculated (for methods, see Chapter 3) physicochemical seawater parameters are summarised in Table 1.

The pH of the hypercapnic tanks was set as described previously (Knapp et al., 2015, Chapter 3), and, to ensure sufficient mixing, a SP 103-2400 submersible pump (BOYU, China) was placed in each tank. Temperature, pH, total alkalinity and salinity were measured and pCO_2, HCO_3^- and CO_3^{2-} calculated as outlined by Knapp et al. (2015, Chapter 3) using the appropriate constants. Temperature was maintained via an universal STC-1000 Digital Temperature Controller (DTC, AGPtek, USA), connected to two 300 W aquarium heaters (Eheim Jäger, Germany) for treatments where high temperature was set as an environmental parameter. In treatments where low temperature was set, a combination of heaters (as stated above) and a HS 66-A chiller (Hailea, China) were used to ensure a stable temperature throughout

the trial. The latter variables were also controlled via a DTC. Each tank functioned as its own unit, being set up to be a partial recirculation system. Water exchange rates were set for approximately a full tank exchange every two days, oxygen concentration was determined using a Multi 350i meter set (WTW, Germany) and water quality was monitored by measuring NH_4^+ concentration (Ammonia test kit, Sera, Germany). The latter never exceeded 0.5 $mg \cdot l^{-1}$.

Table 1. Seawater conditions recorded during exposure of juvenile lobsters to respective treatments during a period of 48 weeks.

Treatment	Salinity ‰	T_A (°C)	A_T (µmol kg^{-1})	pH	pCO_2 (Torr)	HCO_3^- (mmol l^{-1})
Hypercapnic/ Low temp.	35.1 ± 0.1	15.6 ± 0.1	2063 ± 12	7.34 ±0.01	1.60 ±0.03	2.03 ±0.01
Normocapnic/ High temp.	35.0 ± 0.0	18.9 ± 0.1	2051 ± 11	8.00 ± 0.01	0.30 ± 0.01	1.71 ± 0.01
Normocapnic/ Low temp.	35.0 ± 0.1	15.6 ± 0.1	2065 ± 13	8.02 ± 0.01	0.29 ± 0.01	1.75 ± 0.02
Hypercapnic/ High temp.	35.0 ± 0.1	19.0 ± 0.1	2037 ± 23	7.35 ± 0.01	1.55 ± 0.03	1.99 ± 0.02

Values are means ± S.E.

After assignment of juvenile lobsters to respective treatments, water parameters were adjusted to the various experimental parameters (combinations) over a period of two weeks. Initially, seawater parameters remained unchanged for two days (pH ranged from 8.0 to 8.1; T_A ranged from 17 to 19 °C) in order to allow lobsters to settle. Subsequently, pH was dropped in two steps from 8.0 to 7.3 over five days in treatments where the effect of hypercapnia was one of the experimental variables. Subsequently, the pH of the seawater in the latter tanks was further reduced to 7.3. Thereafter, temperature was decreased from 18 °C, at a rate of approximately 1 °C a day, to a temperature of 15.5 °C in the treatments where low temperature was an experimental variable. Once these parameters had stabilised, the chronic trial began and continued for 334 days from this point. Juveniles were fed twice a week, with fresh or frozen mussel (*Choromytilus meridionalis* and *Mytilus galloprovincialis*), depending on availability. Lobsters' individual containers, per treatment, were all suspended in the water column by a basket. In all cases, the juveniles were fed *ad libitum*. Every evening of the day after feeding, all excess feed was removed from the containers. As the lobsters grew, feed rations were increased accordingly. When lobsters reached a size of 27 mm (*CL*), they were transferred into larger containers *(∅ × H: 11 × 16.3 cm).*

In an effort to account for potential "tank effects", approximately mid-way through the trial (day146 of exposure) treatments along with their respective water parameters were switched between tanks, i.e. lobsters environmental conditions did not change, however, their tanks did.

2.3. Preparation of bacterial solution for injecting lobsters

The bacterium used in this study was a strain of *Vibrio anguillarum*, a gram negative, rod bacterium, and the main causative agent of *vibriosis* in fish (Alsina et al., 1994). This particular strain was transfected with the *Vibrio*-derived stable plasmid pEVS146 (provided by Prof. Eric Stabb, University of Georgia), coding for chloramphenicol- (Cm) and kanamycin (Kan) antibiotic resistance. Working stocks of the bacterium and the bacterial solution for injections were prepared as described by Macey et al. (2008), with the following modifications: Tryptic soya agar (TSA) plates were supplemented with 2.0% NaCl (w/v), Cm (5 µg ml^{-1}) and Kan (100 µg ml^{-1}) and the inoculated plates were grown overnight at 30 °C. Following incubation, a small amount of bacteria was transferred from the plate into 5 ml of sterile HEPES solution (10 mM, pH 7.6) supplemented with 2.5% NaCl (w/v), hereafter referred to as HEPES-buffered saline. This solution was further diluted as required with HEPES-buffered saline to an optical density of 0.1 at 540 nm, which was determined to be equal to 4×10^7 colony-forming units (CFU) ml^{-1}. This solution was used for injecting lobsters for the bacterial clearance assays.

2.4. Pre-trial baseline circulating THCs and clearance of culturable bacteria

Due to the novelty of this immune work in *J. lalandii*, two parameters had first to be quantified before the pre-condition lobsters could be assessed for their immune response: firstly, baseline circulating total haemocyte counts (THC), and secondly, the clearance (inactivation) ability of juvenile WCRL to the introduced bacterium (*V. anguillarum*). From these results, an optimal time point for post-challenge sampling of haemolymph could be determined, where both, the change in circulating THCs and a measurable number of recoverable bacteria could be quantified.

Juveniles (n = 5, *w*: 27.3 ± 6.0 g), which had been maintained in the pre-acclimation glass tanks while the chronic pre-conditioning was underway, were carefully removed from their individual containers, and firmly placed on a towel, this prevented tail flip (Figure 1). Their eyes were subsequently covered with a wet cloth to minimize stress. For each extraction, a fresh 1 ml syringe fitted with a 26-gauge x ⅝ inch needle was used and 100 µl of haemolymph was extracted from the cardiac sinus of each individual. A 50 µl aliquot was then fixed in a microcentrifuge (Eppendorf) tube containing 450 µl of 10% Neutral formalin buffer (NBF) for counting THCs (described below). Ten minutes after the initial THC extraction, each lobster was injected with 4×10^4 *V. anguillarum* per g body weight directly into the cardiac sinus. Subsequently, haemolymph extractions (100 µl) took place at 10, 20, 40 and 120 min intervals. The 100 µl sample was divided into two aliquots (50 µl), the first for THC, the second was diluted with 300 µl HEPES-buffered saline (10 mM) and used to determine CFU ml^{-1} (described below).

2.5. Injection of bacteria into haemolymph in J. lalandii

For bacterial injections, a Hamilton syringe (50 µl) was used (above-mentioned needle size fitted, and replaced between injections), this allowed for accurate dose administration to individual lobsters. Prior

to extraction of haemolymph from the first lobster, the syringe was rinsed thoroughly with HEPES-buffed solution (10 mM, pH 7.6). In order to ensure sufficient and even distribution of *Vibrio* throughout the circulatory system of the spiny lobster, injections where made directly into the cardiac region of the lobster. *Vibrio* which was suspended in HEPES-buffered saline (10 mM, pH 7.6) was injected carefully at a slow rate to ensure sufficient dilution into the haemolymph flowing through the cardiac region. This is important to prevent clotting around the injection site and clumping of the bacteria (Macey et al., 2008).

2.6. Pre-challenge circulating THCs and CFUs

Approximately 24 h pre-challenge, lobsters were removed from their experimental containers and a haemolymph sample extracted (100 µl) from the cardiac sinus, as described above (emersion time ± 45 s). This was done to measure baseline THCs and to determine whether any culturable bacteria were present. The 100 µl sample was then allocated as follows: 1) A 50 µl aliquot was fixed in 450 µl of 10% NBF (within 20 s after haemolymph extraction) and a second aliquot (50 µl) was transferred into an microcentrifuge (Eppendorf) tube containing 300 µl of HEPES-buffer saline. Both aliquots were then vortexed and the sample for determining THC (described below) was immediately placed on ice, while two 150 µl aliquots were removed from the second aliquot (350 µl) and each spread onto TSA agar plates (2.0% NaCl) and incubated at 30 °C for 48 h to determine the number of live, culturable bacteria in the hemolymph (see below).

2.7. Experimental design and protocol

After 48 weeks of exposure to seawater conditions described above (Table 1), individuals (n = 8) from each treatment were removed (Table 2). Two criteria determined which of the individuals from the chronic study were used, namely: an approximate calculation of moult stage (C – $D_{1'''}$) and weight (mean representative weight for the specific treatment). This was done as moult stage plays a vital role in several aspects related to the physiological response to pathogens. Haemocyte numbers are known to differ between stages, specifically prior and after moulting (Le Moullac et al., 1997). Each individual was then weighed (blotted dry before weighing) to allow for the dose of *Vibrio* to be calculated, with 1 µl of saline containing *Vibrio* being injected per gram of lobster. After determination of their weight, immediately prior to haemolymph sampling, the extraction site was swabbed clean with absolute ethanol and 100 µl haemolymph was removed in order to determine THC and CFU ml^{-1} (described below). These individuals were then placed back into experimental treatments and allowed to recover for 24 h.

Table 2. Total weight (*w*) and carapace length (*CL*) of juvenile lobsters used in bacterial challenge.

	Initial		Final	
Treatment	*w* (g)	*CL* (mm)	*w* (g)	*CL* (mm)
Hypercapnia/Low temp.	1.39[a] ± 0.21	13.7[a] ± 0.6	19.10[a,b] ± 2.09	32.6[a,b] ±1.2
Normocapnia/High temp.	1.21[a] ± 0.17	13.1[a] ± 0.5	22.49[a,b] ± 2.08	34.3[a,b] ± 1.2
Normocapnia/Low temp.	1.31[a] ± 0.20	13.4[a] ± 0.7	17.43[a] ± 1.97	31.3[a] ± 1.3
Hypercapnia/High temp.	1.12[a] ± 0.16	12.9[a] ± 0.5	27.64[b] ± 2.70	36.9[b] ± 1.3

Seawater parameters: Normocapnia – pH 8; hypercapnia – pH 7.3; high temperature – 19 °C; low temperature – 15.6 °C.
[a,b]Values sharing same superscript within columns do not differ significantly different ($p < 0.05$).
Values are means ± S.E. (n = 8).

Subsequently, in preparation for the challenge, while being kept in their respective individually marked containers, each individual was transferred into a vessel *(D × H: 23.5 × 25cm)* containing seawater taken from the respective treatment (emersion time of < 5 s, Table 3). Each lobster then received a challenge dose of 4×10^4 *V. anguillarum* (i.e. 1 µl of the injection preparation) g^{-1}. This is below the lethal dose for *J. lalandii*, as no mortalities were observed in pre-trial experimentation. An optimal incubation time of 20 min post injection was identified previously (described above). At 20 min post-challenge, 100 µl haemolymph was removed from each individual and 50 µl fixed for THC in 450 µl NBF on ice and another 50 µl was then added to 300 µl HEPES-buffered saline on ice in order to determine CFU.

Table 3. Water parameters measured and calculated during the challenge trial.

Treatment	Salinity ‰	T_A (°C)	pH	A_T (µmol kg^{-1})	pCO_2 (Torr)	HCO_3^- (mmol l^{-1})
Hypercapnia/ Low temp.	34.9 ± 0.1	15.5 ± 0.1	7.32 ± 0.04	1967 ± 9	1.61 ± 0.16	1.94 ± 0.01
Normocapnia/ High temp.	35.0 ± 0.00	18.9 ± 0.03	8.04 ± 0.03	1948 ± 8	0.25 ± 0.02	1.60 ± 0.02
Normocapnia/ Low temp.	34.9 ± 0.13	15.7 ± 0.00	8.07 ± 0.03	1962 ± 15	0.24 ± 0.02	1.64 ± 0.03
Hypercapnia/ High temp.	34.9 ± 0.13	19.1 ± 0.04	7.36 ± 0.04	1956 ± 11	1.49 ± 0.15	1.91 ± 0.02

Values are means ± S.E.

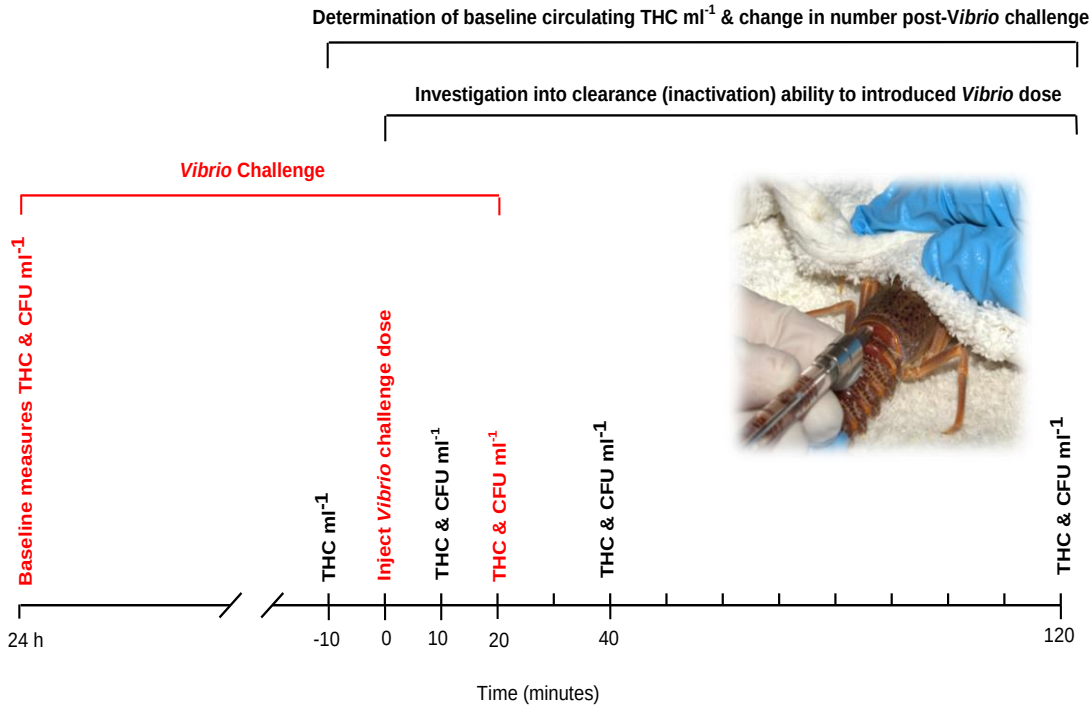

Figure 1. Schematic diagram of experimental protocol/design. Insert: Haemolymph withdrawal- and injection site of a juvenile lobster.

2.8. Total haemocyte counts

The fixed haemocyte suspension was transferred to a haemocytometer and THC determined via a light microscope. Three separate aliquots (10 µl each) of the haemocyte suspension were counted and averaged for each lobster. In preliminary work, THC samples were assessed to be valid for approximately two weeks in storage at 5 °C.

2.9. Selective plating

In order to determine CFU in the haemolymph, selective plating was used. Briefly: Aliquots (150 µl) of the diluted haemolymph samples were pipetted onto TSA plates supplemented with 2.0% NaCl (w/v) Cm (5 µg ml) and Kan (100 µg ml). These plates were incubated for 48 h at 30 °C, after which the number of bacterial colonies were counted and recorded for each plate. Counts were averaged from duplicates.

2.10. Moult stage determination

Carried out according to a modified method from Marco (2012), described in Chapter 4.

2.11. Statistical analysis of data

Data analysis was generated using SAS software (Statistical Analysis System, Version 9.3, SAS institute Inc., Cary, NC, USA). For the clearance curve, percentage change in CFU values from the

initial concentration (ml^{-1}) were arc sin square root- transformed, after which time point 0 for the CFU data analysis was removed.

For the pre-challenge data, the assumptions of normality and equal variance were met and therefore one-way ANOVA was carried out on THC and CFU (transformed) in order to assess whether significant differences ($p < 0.05$) were present between time points 10, 20, 40 and 120.

For the challenge data, CFU was log transformed. If the assumptions of normality and equal variance were met, a two-way ANOVA was used in order to assess whether a significant ($p < 0.05$) interaction existed between the main effects (temperature and pH). If the interaction was not significant, the main effects on baseline THC, post challenge THC and CFU where assessed. If assumptions were not met, a Kruskal-Wallis test was performed. A one-way ANOVA was used in order to assess the significance of difference observed between pre- and post THC.

3. Results

3.1. Pre-trial circulating THCs and clearance of culturable V. anguillarum

Pre-trial experimentation revealed baseline circulating THC of $10 \pm 2 \times 10^6$ cells ml^{-1} for juvenile lobsters (n = 5). Following injection of *V. anguillarum*, circulating haemocyte numbers increased by 65% above pre-injection levels after 10 min and declined steadily thereafter until they returned to pre-injection levels after 120 min (Figure 2). Juvenile lobsters rapidly rendered the injected dose of *V. anguillarum* non-culturable. Within the first 10 min, more than 95% of the theoretical challenge dose of 4×10^7 *V. aguillarum* per ml of haemolymph had been inactivated. Subsequently, CFU numbers continued to decline to 2343 ± 798 CFU ml^{-1} haemolymph after 120 min.

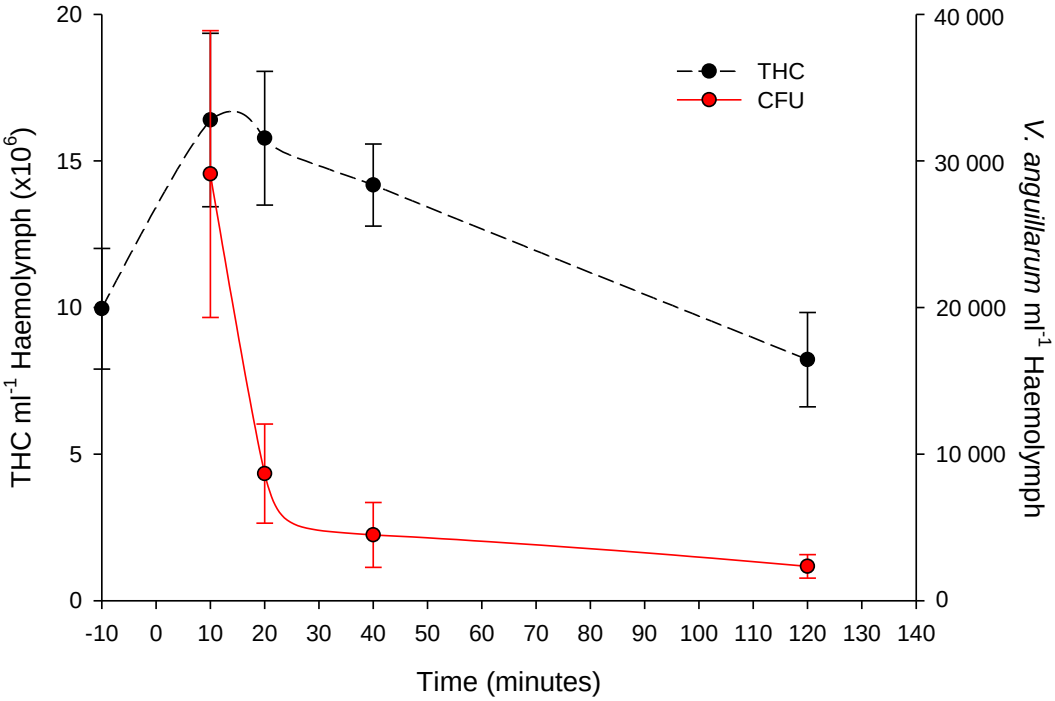

Figure 2. Time course of THC and CFU of culturable *V. anguillarum* in *J. lalandii* haemolymph during a pre-trial experiment. Baseline THC was measured at time point -10 min and then 10, 20, 40 and 120 min post challenge. Lobsters were challenged (4.6×10^4 g^{-1}) at time point 0 and sampled for CFU at 10, 20, 40 and 120 min post challenge. Values are mean ± S.E. (n = 5).

3.2. Impact of treatment on pre- and post-challenge circulating THCs

Baseline circulating THC between treatments ranged from $6 – 7 \times 10^6$ ml^{-1} (Figure 3A). No differences ($p > 0.05$) were observed between any of the treatment groups. Following bacterial challenge, circulating THC increased by between 49 – 60% relative to baseline levels in three of the treatment groups (Figure 3B), whereas lobsters chronically exposed to a combination of hypercapnia and high temperature showed a 7% reduction in circulating THC. Post-challenge circulating THC showed an interaction ($p < 0.05$) between temperature and pH so that the main effects could not be determined. Although, the interaction lead to a difference ($p < 0.05$) between the two hypercapnic treatments, indicating that temperature may have contributed more than hypercapnia here. The hypercapnic treatment with the lower temperature of the two had an 85% higher circulating THC than the latter. Increases ($p < 0.05$) from pre- to post-circulating THC within treatments of 60 and 49%, respectively, were found in both low-temperature treatments (Figure 3A).

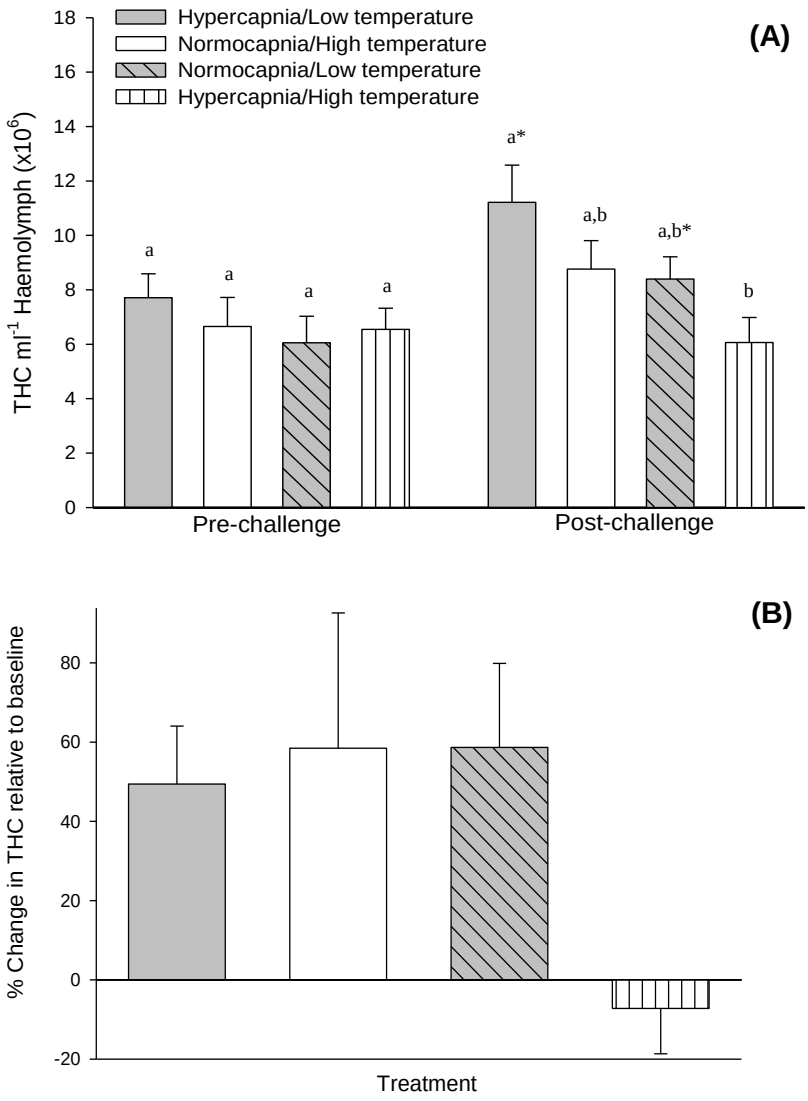

Figure 3. A) Baseline circulating THCs in juvenile WCRL compared to post-challenge levels for respective treatments. B) Relative change in circulating THC post-challenge with *V. anguillarum*. Values are mean ± S.E. (n = 8). *Denotes a significant difference relative to baseline value of THC ($p < 0.05$). Treatments sharing same lower case letters do not differ significantly ($p < 0.05$).

3.3. Impact of treatment on clearance of culturable V. anguillarum

Twenty-four hours prior to the bacterial challenge, haemolymph of the WCRL showed no or low numbers of circulating CFU. One lobster from the hypercapnic-,low temperature and one from the hypercapnic-, high temperature treatments had a CFU of 1×10^3 ml^{-1}. However, measured immune parameters (THC and clearance ability) were not different in comparison with other lobsters from their respective treatments. Twenty minutes post-injection, lobsters had reduced CFU to a range of ~7600 (hypercapnia/low temperature) to ~11 200 (hypercapnia/high temperature) ml^{-1} haemolymph (Figure

4). No differences ($p > 0.05$) were observed for the interaction between seawater pH and temperature or either of the main effects for CFU. Clearance performance of culturable *V. anguillarum* from the haemolymph after 20 min was as follows: Hypercapnia/low temperature > normocapnia/high temperature > normocapnia/low temperature > hypercapnia/high temperature (Figure 4, $p > 0.05$).

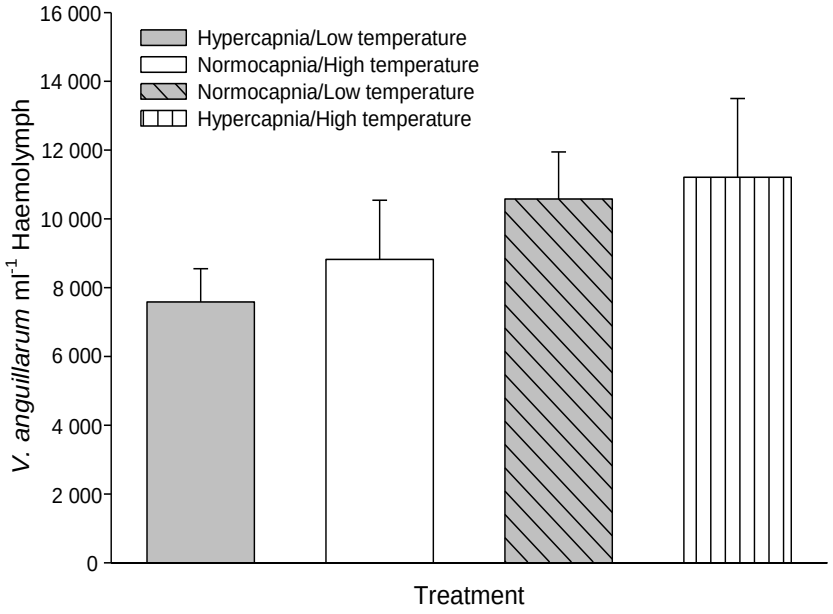

Figure 4. Post-challenge CFU ml^{-1} for respective treatments. Values are mean ± S.E. (n = 8). Values were not significantly different ($p > 0.05$)

3.4. Moult stage

Each experimental group consisted of a cohort of lobsters that were in a similar range of moult stages. These stages varied as follows: In the hypercapnic/low temperature treatment, six were at stage D_0, one at D_1, and one at $D_{1''}$. In the normocapnic/high temperature group, four were at stage D_0, and four at D_1. In the normocapnic/low temperature treatment, three were at stage C, four at D_0, one at D_1. In the hypercapnic/high temperature treatment, one was at stage C, three at D_0, three at D_1 and one at $D_{1''}$. The juvenile WCRLs used in the bacterial challenge trial provided a good representation of each treatment, as THCs were similar to that of their respective treatment cohorts (Figure 5, Addendum).

4. Discussion

The present results revealed, for the first time, that 1) juvenile *J. lalandii* are very efficient in clearing introduced bacteria from their haemolymph, independent of treatment, and 2) that chronic exposure to a combination of high temperature and hypercapnia led to a significantly lowered circulating total haemocyte count (THC) in juvenile WCRL post-bacterial challenge.

4.1. Pre-trial evaluation of immune response

The initial, pre-experiment investigation into two aspects relating to the immune response in juvenile WCRL provided some important insight into basic immune cell counts (haemocytes) as well as the lobsters ability to clear (inactivate) an introduced bacteria from its haemolymph. Total haemocyte counts vary amongst and within species due to a number of factors, such as moult stage, diet and temperature (Fotedar and Evans, 2012; Le Moullac et al., 1997). The basal circulating THCs obtained (Figure 2) in this study are in a similar range of those measured in other lobster species, such as the Western rock lobster (*Panulirus cygnus*) and the Mediterranean spiny lobster (*Palinurus elephas*), where counts were ~12 × 10^6 and 10 × 10^6 cells ml^{-1}, respectively (Filiciotto et al., 2014; Jussila et al., 1997). Shortly after administering the challenge dose of *V. anguillarum*, circulating haemocyte concentrations increased until approximately 15 min post-challenge, after which they decreased continuously for the remainder of the trial. A similar response has been recorded in the American lobster (*Homarus. americanus*) and Norway lobster (*Nephrops norvegicus*) when challenged with a bacterium (Shields et al., 2006; Stewart, 1980). The increased number of circulating THCs and subsequent decrease have been attributed to the following: 1) A migration of haemocytes to the injection site ensures rapid bacterial degradation and wound sealing (van de Braak et al., 2002). 2) The subsequent decrease is associated with haemocytes adhering to bacteria, as observed in the Ridgeback prawn (*Sicyonia ingentis*) and American lobster (*H. americanus*, Martin et al., 1998). The loose clumps of bacteria and haemocytes then get lodged into narrow spaces, especially in the fine vasculature of the gills (Martin et al., 1993, 1998) after which these clumps become melanised and are eliminated when the exoskeleton is shed (Martin et al., 2000). Another site in which bacteria are accumulated and then degraded is the hepatopancreas (Burgents et al., 2005b).

The rapid decrease in culturable bacteria from circulation, which is attributed to the break-down and/or transport to other tissue compartments (Ikerd et al., 2005), is similar to that found in the penaeid shrimp (*S. ingentis*), where the bacteria *Bacillus ceres* and *Aerococcus viridans* (challenge dose 1× 10^6 ml^{-1}) were completely removed from circulation within 10 min (Martin et al., 1993).

4.2. Effect of treatment on immune response

The effects of hypercapnia and elevated temperature on the immune response of several crustaceans have previously been studied separately from each other. Increased temperature has led to contrasting results on circulating THCs, some studies report an increase, for example in the sand fiddler crab (*Uca pugilator*), mole crab (*Enierita asiatica*), green crab (*Carcinus maenas*) and giant freshwater prawn (*Macrobrachium rosenbergii*, Cheng and Chen, 2001; Dean and Vernberg, 1966; Ravindranath, 1977; Truscott and White, 1990), whereas others have noted a decrease (Cheng and Chen, 2005; Smith and Chrisholm 1992). Difference between species, and within species, have been attributed to 1) a difference in natural temperature ranges (Cheng and Chen, 2001) and 2) acclimation conditions (Smith and

Chisholm, 1992). While there is a far greater body of literature on temperature, immune studies investigating hypercapnia in marine crustaceans have often been simulated in combination with hypoxia (Burgents et al., 2005a; Holman et al., 2004; Macey et al., 2008), making it difficult to determine the effects of hypercapnia alone on the immune system. In a few cases, where the effects of hypercapnia alone on marine organisms have been investigated, various changes in the immune system have been observed, namely: decreased haemocyte functionality (Bibby et al., 2008; Hernroth et al., 2011), reduced haemocyte number, bacteriolytic- and antibacterial activity, and increased phenoloxidase activity (Lu-Qing et al., 2005).

In contrast to the above-mentioned reports, the present study investigated the impact of chronic exposure to the two environmental factors on immune response in combination. None of the combinations of the two environmental parameters, however, influenced baseline THCs in the WCRL. The resting THCs observed in the lobsters from their respective treatment groups are lower, but not out of the range of those measured in the pre-trial lobsters. This may be caused by a combination of different moult stages and diet, as both are known to affect THCs (Fotedar and Evans, 2011; Le Moullac et al., 1997). When exposed to a decreased seawater pH, an associated decrease in haemolymph pH follows, due to the increased pCO_2 of the seawater (Chapters 3, 4, 5) and the need to maintain a gradient between the internal and external environmental pCO_2, to ensure CO_2 removal. In organisms that were not able to maintain extracellular pH during hypercapnic exposure, such as the sea star (*Asterias rubens*), green sea urchin (*Strongylocentrotus droebachiensis*), and the Norway lobster (*N. norvegicus*), immune response was depressed (Dupont and Thorndyke, 2012; Hernroth et al., 2011, 2012). Dupont and Thorndyke (2012) suggested a relationship between cellular immune response and extracellular pH. Several days prior to the immune challenge of the present study, the haemolymph acid-base parameters in these lobsters were therefore assessed (Chapter 4). The results showed that they were still actively maintaining haemolyph pH within a relatively narrow range (pH 7.72 - 7.77) across treatments after 48 weeks. It can therefore be assumed that the ability of *J. lalandii* to preserve its *in vivo* pH homeostasis ensures an efficient and rapid clearance of *V. anguillarum* in the present study. Prior to bacterial challenge, THCs did not seem affected by temperature either, as observed in the Norway lobster (*N. norvegicus*) when exposed for a four month period to a host of temperatures, ranging from 5 to 18 °C (Hernroth et al., 2012). After 20 min, the ability of the lobsters to clear culturable bacterium from their haemolymph, irrespective of treatment, was within a similar range to that observed in pre-trial investigation (Figures 2 and 4).

After lobsters were challenged with a dose of *V. anguillarum*, the number of circulating haemocytes increased above that of pre-challenge levels in three of the treatments, following a similar trend to that described for the pre-trial experiment, as well as other crustaceans. The hypercapnic-, high temperature treatment, however, revealed a decreased THC relative to pre-challenge (Figure 3). There are a number of factors which may have contributed to this result and they are summarised as follows: 1) Growth in

these lobsters was greater than in other treatments (i.e. possible increase in metabolism, Chapter 4). 2) Acid-base regulation for such an extended period (48 weeks) would be energetically expensive (Pörtner et al., 2004). 3) Aerobic scope is lost upon bacterial challenge (Burnett et al., 2006), due to rapid forming of nodules in the small vasculature of the gills (within 10 min, Martin et al., 1998), in the Atlantic blue crab, a 43% reduction in oxygen flux was observed (Burnett et al., 2006), leading to an increased energy cost associated with maintaining haemolymph pO_2. 4) The additional energy requirement needed for the immune response (Lochmiller and Deerenberg, 2000). Therefore, a high energy requirement would be needed in order to maintain all the above mentioned mechanisms. Due to the additional role of haemocytes in nutrient digestion and transport (Oubella et al., 1993), it is therefore hypothesised that the decreased post-challenge THC is due to the reversible migration of haemocytes from the haemolymph to tissues to fuel the above mentioned ongoing processes (Mounkassa and Jourdane, 1990; Oubella et al., 1993), and so reducing the number of haemocytes available from reserves (e.g. specialized haematopoietic tissue).

5. Conclusion

This was the first study whereby the immune competency of *J. lalandii* was tested and revealed that the species is able to efficiently deal with an acute immunological challenge after chronic exposure to environmental conditions that are on the limits of the range experienced in nature. However, a combination of hypercapnia and high temperature has a markedly negative effect on total haemocyte numbers after being challenged with a dose of *Vibrio anguillarum*.

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Addendum

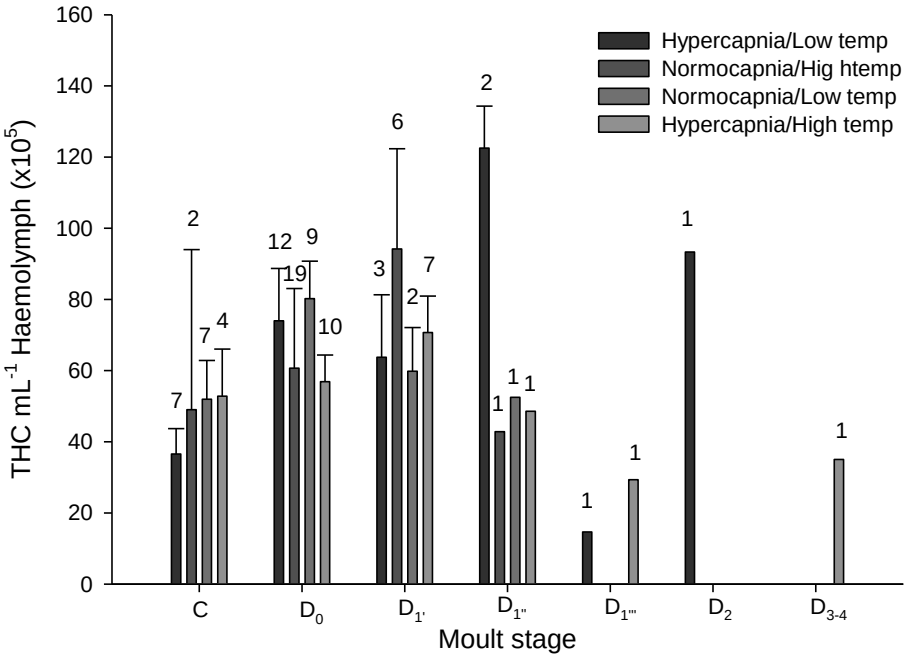

Figure 5. Mean THC for respective moult stages within treatments (entire chronic exposure cohort, n = 97). Number of observations for each value is indicated above bars. Values are mean ± SE.

Chapter 7

General discussion, future directions and conclusion

The West Coast rock lobster (WCRL) is not only a keystone benthic predator (Barkai and Branch, 1988) but also supports one of South Africa's oldest and largest fisheries. In order to ensure sustainable utilization of the resource, various management strategies have been implemented over the years (Chapter 2). In recent years, there have been two major events that impacted the WCRL resource, namely a decrease in somatic growth rates and a southward "shift" of the resource (Cockcroft, 1997; Cockcroft et al., 2008). These phenomena have attracted interest, as the reasons are not entirely understood (Blamey et al., 2015; Pollock and Shannon, 1987; Pollock et al., 1997; Shannon et al., 1992). The resource is currently at its lowest level recorded, 2.6% of pristine (DAFF, 2014). Should biological or physiological aspects of the WCRL be negatively affected by environmental changes, it will be at the detriment of both the industry and various fishing communities in South Africa.

The WCRL is a delicate resource because it is slow growing, has a complex life cycle involving an extended larval phase, is not suited for aquaculture, and moulting- and reproductive cycles are tightly coupled (Cockcroft and Goosen, 1995; Cruywagen, 1999; Kittaka, 1988; Silberbauer, 1971). The looming threat of ocean acidification and temperature change in our oceans, combined with the relatively unexplored physiology and other biology aspects of the WCRL with regards to these stressors, warranted further research. It is of critical importance to know how these environmental stressors may influence the resource in order to allow the WCRL Scientific Working Group to make informed decisions and develop future scenarios for the sustainable management of this already over-exploited resource. There will not only be direct effects that will need to be considered but also indirect effects that these environmental anomalies may have on the resource during local phenomena.

Research was initiated with the study of short-term responses of the WCRL to reduced seawater pH on the physiological and molecular level, i.e. on acid-base balance and haemocyanin oxygen affinity of adult WCRL. This provided valuable insight into the biochemical response mechanisms utilized under acute hypercapnia. Results showed that an initial, uncompensated acidosis occurred, whereby haemolymph pH decreased to a similar range to that of the seawater pH. This decrease however, was rapidly compensated for by an increase in bicarbonate concentration, returning haemolymph pH values to above resting levels. This protects functioning of respiration, which is sensitive to low pH due to the large Bohr effect that the present study revealed, of the respiratory protein (haemocyanin). The acid-base regulation observed in this study was far more rapid than in other marine crustaceans, such as the Dungeness crab (*Cancer magister*, Pane and Barry, 2007). This response was expected however, due to the seasonal occurrence of upwelling events in the Benguela Current Large Marine Ecosystem (BCLME, Pitcher et al., 2010), the WCRL's habitat, where corrosive (low pH) water moves into the nearshore environment in 3-10 day cycles (Hill et al., 1998). The present study provided new insight

into the efficiency of WCRL to deal with such short-term changes in seawater pH. Over the long term, however, the energy requirement needed to fuel the energy-costly process of actively pumping HCO_3^- ions from seawater to maintain internal equilibria, may lead to a diversion of energy from other process, such as growth.

This question warranted investigation. To study the impact of hypercapnia alone, juvenile WCRL were chosen, because, in contrast to the annual moult of adults, they moult (and grow) several times a year. Subsequently, in order to replicate field conditions more closely, a combination of effects, namely hypercapnia and different temperatures on the growth of juvenile WCRL were assessed. The two growth trials extended over 195 and 334 days, respectively. This allowed for the incorporation of several moult cycles. The initial trial revealed, for the first time, long-term biological and physiological response mechanisms to hypercapnia in a palinurid crustacean. It also showed that growth, but not survival, of juvenile WCRL was negatively affected by exposure to a reduced seawater pH. In contrast, the second trial showed that lobsters exposed to a reduced seawater pH, had a significantly higher growth than those at the same temperature exposed to a "natural" (normocapnic) seawater pH. Both seawater pH and temperature had significant effects in terms of growth in this trial, while temperature contributed to almost all differences in parameters measured at the end of the trial, irrespective of pH. Again, this trial revealed no impact on survival by any of the two tested factors. The contrasting results in terms of growth between the trials are thought to be due to a combination of two factors, namely diet and secondly experimental design. During the first trial, lobsters received a mixed, potentially sub-optimal diet, while in the second, lobsters were fed a single diet of mussels (proven to be the better food source, Dubber et al., 2004). The lobsters in the second trial also had longer access to their food rations than in the first trial, which is important since lobsters are known to be periodic feeders (Bordner and Conklin, 1981). However, this aspect of diet warrants further research in an experiment designed specifically to test dietary effects on growth of WCRL. Secondly, the agitation and slight temperature change associated with tank replenishment may have also contributed to the decreased growth rates in the hypercapnia-exposed lobster of the first trial. These growth differences under hypercapnia from both studies indicate that the physiological response mechanisms may be compromised if food quality is "sub-optimal" and/or lacking in quantity, both leading to insufficient energy supply to fuel response mechanisms associated with environmental stressors and growth. This could in the field, lead to a shift in the energy budget and so a narrowing of the thermal window in the species, which would be especially critical during low oxygen events (Pörtner, 2010). Any decrease in growth rate will influence the size of lobsters when reaching sexual maturity due to the age-specific-, rather than size-specific relationship that exists with regard to the onset of sexual maturity in the female lobsters (Beyers and Goosen, 1987). This would firstly lead a decreased growth rate of female lobsters from a smaller size than previously, due to the energy requirement of gonad tissue (Pörtner and Farrell, 2008), and secondly, as in *J. edwardsii*, to a decrease in clutch size of females (MacDiarmid, 1989), subsequently leading to

a decreased recruitment of juvenile lobsters. The slower growth would also mean that harvestable male lobsters would enter the fishing resource later. The fishery would therefore be immensely affected by these changes as they would, according to the Total Allowable Catch (TAC) calculations, lead to a reduced TAC.

The acid-base response to chronic hypercapnia of juvenile lobsters in trial 1 was similar to the acute response of adults (Chapter 3): A reduced environmental pH was compensated for by increased HCO_3^-, several mmol l^{-1} higher compared with the respective normocapnic treatment. This ensured that haemolymph pH remained relatively close to that of the normocapnic individuals, and an outward pCO_2 gradient was maintained. The latter is essential for removal of metabolic CO_2. The second trial, however, confirmed that the juvenile lobsters are able to maintain haemolymph pH over extended periods, far longer than that of the initial trial. The three experiments showed, therefore, that WCRL can 1) react swiftly to declining pH, important in dealing with upwelling events, and 2) can sustain this physiological adjustment for sustained periods as is expected in future scenarios of ocean acidification. The last experiment also showed that 3) this long-term acid regulation can be maintained at different temperature levels, even close to the upper temperature limit.

In addition to HCO_3^-, there is a second buffer system in the form of the respiratory protein in crustaceans, haemocyanin (Henry and Wheatly, 1992). Chapter 3 provided some information on the O_2 binding affinity of adult lobsters haemocyanin (Hc) under various pCO_2 concentrations, whereby the strong Bohr effect was observed. Due to a number of interlinking questions that warranted further research, the haemocyanin of juveniles from trial 1 were further analysed. These questions were: 1) how similar is the Bohr effect in juveniles compared to adults, and 2) has chronic exposure negatively affected the haemocyanins' affinity for oxygen, as concentration had been significantly reduced in the hypercapnia-exposed lobsters. Juvenile WCRL too, showed a relatively strong Bohr effect. Interestingly, haemocyanin showed an intrinsic change of its molecular structure, which translated into an increased affinity for oxygen under long-term exposure to hypercapnia, this was attributed to a possible energy conservation mechanism. The reduced Hc concentration in the hypercapnic relative to the normocapnic treatment, although not known at the time, was most likely due to the effect of an sub-optimal diet and subsequent break down of haemocyanin for energy (Djangmah, 1970). Therefore, the suggestion that the structural change may have been to conserve energy is not entirely incorrect, however, it may have in actual fact been attributed to compensate for the reduced concentration of haemocyanin in the haemolymph. Nonetheless, it still revealed the physiological plasticity of this species.

The above chapters revealed already that the WCRL has a great physiological plasticity. In Chapter 6, another, yet un-researched, physiological aspect was investigated: immune competency. This was important since a compromised immune response would directly translate into increased prevalence of diseases, slower growth and higher mortality, i.e. all leading eventually to a compromised fishing

resource (see above). A correlation of environmental change and immune suppression in marine animals, such as crustaceans, is reported in the literature (Le Moullac and Haffner, 2000). It was therefore important to investigate the effect of prolonged exposure to hypercapnia at low- and high temperatures on the immune mechanisms of the WCRL and its and ability to clear/inactivate an introduced bacterium (*Vibrio anguillarum*). Initially, non-experimental lobsters showed similar total haemocyte counts (THC) to that of other lobster species, and were equipped with a rapid immune response whereby an introduced bacteria was efficient degraded/cleared from the haemolymph. Moreover, this capability was sustained after prolonged exposure to the various treatment conditions. Post-bacterial challenge, however, the hypercapnic/high temperature treatment showed a reduced THC. The reason is unknown, but it is speculated that it may have been linked to an increased metabolic demand in these lobsters.

Overall, my results demonstrate that the WCRL is very well equipped to not only deal with the present, changeable conditions, but also with those predicted in the near and distant future. My results also show that potential impacts, such as change in somatic growth, mortality etc., are variables for which the current OMP provides already sufficient entry points to be accounted for. It therefore ensures in the present form that the resource can be sustainably managed for the future. To make predictions, however, as to how the resource will be affected in the field, would be unrealistic as the lobsters will be exposed to not only one or two factors, but rather a multitude of factors at any one time (i.e. pH, temperature and low oxygen). Also, although the effect of feed quality and quantity on the lobsters' ability to physiologically respond to changes in its environment were not specifically tested, the study provides some evidence that feed may very well play an important role in the WCRLs ability to deal with the looming environmental changes (as mentioned above, this needs further investigation).

Due to the obvious constrains of trying to monitor growth in adults, juveniles were used in both growth trials. Thus the effect of the two factors tested (pH and temperature) on the growth rate of larger (adult) lobsters cannot be predicted. It is, however, alluring to propose that the reduced growth rates observed in adult male- and female lobsters in the field (Cockcroft et al., 2008) may very well indicate that the population has already begun to physiologically adjust, to better deal with changes in their environment. Pörtner (2008) suggested that smaller individuals of a species may be favoured in thermally variable environments due to their larger thermal tolerance window, and so allowing for greater physiological plasticity, specifically if hypercapnia negatively influences this window. This could lead to (or may have happen already – see above) a reduction in the average size of adult lobsters over time. If this is the case, a decreased recruitment could be expected due to smaller females at sexual maturity and therefore smaller clutches, also smaller males would produce less sperm and so a decrease in fecundity could be expected. Unfortunately the only measure currently of recruitment is that based on lobsters attaining a carapace length of 75 mm. This does not, however, provide much of an early warning system,

and I would propose that recruitment of juveniles be monitored on an annual basis at sites where high numbers of individuals are known to accumulate and this be factored into the OMP.

Although the study provides insight into various biological, physiological and biochemical aspects with regards to the response of WCRL to reduced seawater pH and increased temperature, there are still several facets which warrant further research, namely: 1) impact on larval stages, 2) settlement success of post-larvae (pueruli), 3) impact of low oxygen episodes and emersion, 4) immune mechanisms.

Larval phases: The WCRL has a complex life cycle, and the early ontogeny is comprised of several larval stages that take a minimum of 10 months to develop into a puerulus (post-egg to settlement; Kittaka, 1988). Future investigation is needed, as it its normally this phase in the life cycle of crustaceans that is relatively exposed, and at the mercy of the environment, for instance, a delay in puerulus development will not only slow the recruitment process but will also increase the time when its most vulnerability to predation (Lawton and Lavilli, 1995). The development of a method is warranted whereby a continuous supply of larvae can be produced, this will allow for a better understanding of the fragility of these larvae to a host of environmental challenges through lab-based trials.

Settlement of juvenile WCRL: The reliance of settlement on a host of environmental factors, such as wind direction, water temperature and upwelling strength (Groeneveld et al., 2010), all of which are likely to change under predicted scenarios, are somewhat worrisome. Very limited work has been carried out on larvae of the WCRL. As far as could be ascertained, there are six studies which focused specifically on pueruli and early development of the WCRL. These consisted of embryonic, larval and settlement studies (Grobler and Ndjaula, 2001; Groeneveld et al., 2010; Keulder, 2005; Kittaka, 1988; Pollock, 1973; Silberbauer, 1971). Further studies focussing on the effect of specific environmental stressors on pueruli settlement are needed.

Low oxygen water and emersion: Local phenomena such as low oxygen events are predicted to become more pronounced on the West Coast. Studies that have assessed the effects of emersion, as well as oxygen minima of the species, show that the conditions experienced during these events are detrimental to the lobster (Beyers et al., 1994; Haupt et al., 2006). More in-depth physiological studies are needed, building on the work by Haupt et al. (2006), combined with information of the present dissertation. Additionally, studies assessing the thermal tolerance limits of the species will be valuable in assessing the points at which aerobic scope may be negatively influenced (Pörtner, 2010). For low oxygen events, a contingency plan is prepared but the outcome of some measures is unknown due to lack of research. For example, live lobsters are collected and transferred to areas with sufficient oxygen levels, but recovery of those lobsters is uncertain. Answering of these questions would assist in setting up a management strategy that would ensure that during these events the survivability of those returned lobsters would be improved.

Immune mechanisms: Although aspects with regards to immune mechanisms and clearance/inactivation efficiency were addressed in the present study, a more in-depth investigation is needed, in which, for example, THCs during specific moult stages and basal counts for each of the respective haemocyte sub-populations. It is also important to know, how efficiently WCRL can clear/inactivate bacteria after short-term exposure to naturally occurring events such as emersion or low oxygen conditions (as experienced during "walkouts"). The latter is often associated with extremely low seawater pH and high temperatures. In general, there is more immunological work required on animals from the field. This could help to monitor the condition of the WCRL resource and indicate problems early.

The study provides essential data in terms of biological, physiological and biochemical response mechanisms of the WCRL to current and potential future environmental stressors, namely changing seawater chemistry (declining pH, under saturation with carbonate ions) and temperature. In summary, the research revealed:

1) The WCRL can respond very quickly, and reversibly, to acute hypercapnia.
2) The WCRL can maintain this response for sustained periods and has the ability to adjust respiratory capacity at the molecular level.
3) These chronic adjustments do not compromise growth, survival and immune response under optimal food availability.
4) Food availability and -quality will potentially influence this ability of the WCRL to deal with environmental stressors.

These results demonstrate the great plasticity of the WCRL at the molecular, biochemical and physiological level. This is important initial information for government fisheries scientists to aid in predicting future development of and potential threats to the WCRL resource.

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