The Importance of Tidal Acclimation When Assessing the Physiological Responses of *Carcinus maenas* to Environmental Stressors

by

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Abstract

Animals inhabiting the intertidal zone are exposed to abrupt changes in environmental conditions associated with the rise and fall of the tide. For convenience, the majority of laboratory studies on intertidal organisms have held these organisms in constant submerged conditions. However, this is not representative of the daily cycle of emersion and immersion that intertidal organisms experience in their natural habitat. In this thesis, I explored whether acclimation of the intertidal green crab *Carcinus maenas* to a continuous simulated tidal cycle (from herein referred to as 'tidal' crabs) resulted in different physiological responses to environmental stressors compared to crabs acclimated to permanently submerged ('non-tidal') conditions. Chapter 1 reviews the previous literature on the physiological tolerance of *C. maenas* to emersion, hypoxia and thermal stress and opens the discussion of the importance of ecological realism in experimental design for accurate physiological responses. Chapter 2 and 3 are experimental research manuscripts that investigates how tidal acclimation influences the physiology behind oxygen delivery and acid base balance during submersion and emersion (Chapter 2), and hypoxia and thermal stress (Chapter 3). Chapter 4 serves as an overall general discussion of Chapters 2 and 3 with suggestions for future research directions. Overall, the findings of my research show that acclimation to a tidal regime produces physiological adjustments such as elevated haemocyanin levels and lower oxygen consumption during submersion that can influence how crabs respond to environmental stressors. Notably, acclimation to a tidal regime allows *C. maenas* to avoid the metabolic acidosis that occurs in non-tidal crabs during emersion, likely through the buffering effects of elevated hemocyanin. The results of this study suggest that how *C. maenas* responds to common stressors in the intertidal is

complex, and that adaptation to air exposure during low tides may alter their physiological responses to other environmental stressors. In order to gain a better understanding of how intertidal organisms will respond to predicted environmental changes, laboratory experiments should incorporated a tidal regime into their design.

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Co-Authorship Statement

The work described in this thesis was conducted by Sarah Nancollas with guidance from Dr. Iain McGaw. Sarah Nancollas was responsible for all laboratory and field data collection and analysis. All chapters were written by Sarah Nancollas with intellectual and editorial input by Drs. Iain McGaw, Kurt Gamperl and Bill Driedzic. Any publication in

the primary literature resulting from this thesis, and from complementary work not presented, will be co-authored by Sarah Nancollas and Dr. Iain McGaw.

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Chapter 1. General Introduction

1.1 The tide

The rocky intertidal zone hosts a rich diversity of marine fauna and flora, and is characterised by the predictable ebb and flow of the tide. The movement of the tide has been shown to be an important component for shaping behavioural and physiological responses of intertidal organisms (De la Iglesia & Hsu, 2010 and Palmer, 2000), whereby intertidal organisms develop a circatidal rhythm in tune with the ~12.4 hour ebb and flow of the tides (Wilcockson & Zhang) to control activities such as feeding, locomotion and mating (De la Iglesia & Hsu, 2010 and Palmer, 2000). Crustaceans in particular have been a central focus for tidal research, and the ubiquitous green shore crab *Carcinus maenas* has played a pivotal role for the existence of circatidal clocks (Naylor, 1995).

1.2 Physiological responses and adaptations to environmental stressors

The ebb and flow of the tide is also responsible for the dynamic fluctuation in environmental conditions, such as respiratory medium (aquatic/aerial), oxygen content and temperature, which often presents stressful conditions for intertidal organisms during low tide. Consequently, the intertidal has become a model ecosystem for investigating the effects of climate change, with many studies using intertidal organisms to understand the physiological tolerances to numerous environmental stressors. As common inhabitants of the intertidal, crustaceans have frequently been used in environmental stress studies, and

due to its ubiquitous nature, *C. maenas* has become a particularly popular model organism for intertidal studies exploring physiological responses to environmental stress.

For many intertidal species, air exposure, hypoxia and temperature are key environmental drivers that determine the latitudinal distribution and fitness of this species within the intertidal. There are excellent reviews which detail the effects of air exposure (Truchot, 1990), hypoxia (Grieshaber et al., 1993; McMahon, 2001) and temperature (Denny et al., 2011; Hazel & Prosser, 1974) on the general physiology of intertidal animals. Below, I briefly describe the responses of crustaceans to these three stressors, but primarily focus on adjustments in oxygen transport and acid-base balance in *C. maenas.*

Responses to emersion

Exposure to air is a routine occurrence for many intertidal organisms, and is one of the main drivers that determines their spatial distribution in the intertidal zone (Truchot, 1990). Although oxygen levels are substantially higher in air than in seawater, desiccation stress that is associated with air exposure can decrease the efficiency of gas exchange and lead to the eventual collapse of gas exchange and aerobic metabolism (Burnett & McMahon, 1987; DeFur, 1988; McMahon, 1988; Truchot, 1990). The limited carbon dioxide (CO_2) solubility in air (as compared to seawater) also makes it difficult for aquatic organisms to excrete CO_2, and therefore, emersion produces a concurrent rise in internal PCO_2 which results in a respiratory acidosis (Truchot, 1975).

The tolerance to emersion exhibited by an intertidal animal is primarily determined by two factors: (1) position of the organism on the shore; and (2) the activity / mobility of the organism during air exposure. Animals that inhibit the upper intertidal zone can often

spend longer periods emersed than immersed, and have adaptations that can facilitate the maintenance of aerobic respiration in air. These include specialised structures for air breathing (Henry, 1994; Stillman & Somero, 1996) and physiological adjustments that enhance oxygen delivery to the tissues (Morris et al., 1996; Stenseng et al., 2005; Stillman, 2002; Stillman, 2003; Stillman & Somero, 1996; Stillman & Somero, 2000; Tomanek & Somero, 1999; Truchot, 1990). *Carcinus maenas* is considered an effective air breather (DeFur, 1988), and utilises a bimodal breathing strategy to facilitate gas exchange in both water and air. Similar to terrestrial crabs, the gill structures of *C. maenas* are strengthened by chitin, which provides support, and therefore, prevents these gills from collapsing during emersion as seen in other intertidal decapods (DeFur, 1988; DeFur & McMahon, 1984; Rastrick et al., 2014; Taylor & Butler, 1978). This adaptation allows *C. maenas* to exchange gases when emersed, however, there is substantial variation reported in the degree to which *C. maenas* can maintain aerobic respiration when emersed. For example, oxygen consumption values in air vs. in water vary from 75% (Newell et al., 1972) to 120% (Taylor & Butler, 1978) in the laboratory, and have been reported to be as low as 50% under simulated field conditions (Simonek & Henry, 2014).

Although oxygen consumption is sustained to some degree during emersion, the arterial partial pressure of oxygen (PaO_2) often decreases (by 50 - 75%) in comparison to that measured in immersed crabs (Depledge, 1984; Taylor & Butler, 1978). These low levels of PaO_2 during emersion suggest that O_2 diffusion across the gills is limited, and that compensatory mechanisms must be initiated to sustain O_2 delivery and aerobic metabolism. For example, in the intertidal crab *Hemigrapsus nudus,* the proportion of oxygen delivered

to the tissues by haemocyanin increases from 50% in submerged conditions to 85% when emersed (Morris et al., 1996).

When aerobic metabolism can no longer exclusively satisfy energy demands, *C. maenas* utilises anaerobic metabolism to provide additional energy. Lactate is commonly produced during emersion in decapod crustaceans (DeFur & McMahon, 1984; McGaw et al., 2009; Rastrick et al., 2014; Stillman & Somero, 1996; Taylor & Whiteley, 1989), and while *C. maenas* primarily uses aerobic respiration during short-term air exposure (1-3 hours), it also relies on anaerobic metabolism during prolonged air exposure (4+ hours) (Johnson & Uglow, 1985; Santos & Keller, 1993; Simonik & Henry, 2014; Taylor & Butler, 1978). The production of lactate has benefits in *C. maenas* in addition to ATP production. Lactate increases the oxygen affinity of haemocyanin (Truchot, 1980), which enhances oxygen uptake at the gills, and subsequently, oxygen delivery to the tissues.

In addition to pH changes associated with the production of anabolic end products, *C. maenas* experiences a rapid increase in PCO_2 levels during emersion and this causes a further decrease in haemolymph pH (Burnett, 1988; Taylor & Butler, 1978; Truchot, 1975). This decrease in haemolymph pH can be buffered by haemocyanin (Whiteley, 2011) and / or mobilized carbonate from its calcified exoskeleton to increase (Cameron, 1986).

Whether caused by an accumulation of CO_2, lactate, or both, the decrease in pH that occurs during emersion results in a Bohr shift which decreases the affinity of haemocyanin for oxygen (Taylor & Butler, 1978). Even with a decreased haemocyanin affinity for oxygen, oxygen is still able to be delivered to the tissues relatively efficiently. This is primarily due to venous pH being lower than arterial pH, and a further left shift of

the haemocyanin O_2-binding curve which enhances oxygen delivery to the tissues (DeFur, 1988; Taylor & Butler, 1978).

Responses to hypoxia

Hypoxia occurs frequently in the intertidal zone, especially in isolated tide pools where oxygen levels can vary between 2 and 435 mm Hg in a single day (Richards, 2011; Truchot & Jouve-Duhamel, 1980). Tide pools become hypoxic during the night due to high levels of bacterial and algal respiration. There are two main ways in which animals respond to hypoxia. In oxyconformers, oxygen consumption decreases in proportion to environmental oxygen levels, whereas oxyregulators can maintain oxygen consumption independent of environmental oxygen levels until they reach their critical oxygen tension (P_{crit}) (McMahon, 1988). At this PO_2, increases in ventilation and / or the extraction of oxygen from the haemolymph can no longer meet metabolic demands, and oxygen consumption falls in direct proportion with environmental oxygen levels. *Carcinus maenas* is an oxyregulator, and is able to maintain its oxygen consumption until PO_2 falls to 30-60 mm Hg (McGaw & Nancollas, 2018; Taylor, 1976).

Hypoxia represents a similar challenge as emersion, in that animals have difficulty in providing enough oxygen to their tissues to meet metabolic demands. Consequently, several physiological responses to hypoxia can also be observed during emersion in decapods. *Carcinus maenas* partially emerge to breathe air when aquatic oxygen tensions fall below P_{crit}, which is partially dependent on water temperature (Taylor et al., 1973; Taylor & Wheatly, 1979). By reversing the direction of scaphognathite beating, air can be drawn into the branchial chambers where it bubbles through the remaining water (Taylor

et al., 1973). This mechanism allows *C. maenas* to exploit the elevated oxygen levels available in air, without the risk of desiccation. Moreover, crabs are able to restore their haemolymph PaO_2 and O_2 content to levels found in normoxic water (Taylor et al., 1973).

When hypoxia cannot be avoided, *C. maenas* displays a number of other compensatory physiological mechanisms. Initially, ventilatory rates increase (Burnett & Johansen, 1981; Taylor, 1976; Taylor et al., 1977) to maintain oxygen supply to the gills (McMahon, 1988), which is a typical response to hypoxia in crustaceans (McMahon, 2001). In addition, aerobic metabolism is initially maintained in the face of decreasing PaO_2 via adjustments in haemocyanin oxygen affinity (Taylor, 1976). In normoxic conditions, as much as 50% of oxygen delivered to the tissues is dissolved in the haemolymph. However, as the environmental oxygen levels decreases further, the proportion of oxygen bound to haemocyanin increases, with 90% of oxygen delivered by haemocyanin at oxygen levels of 30 mm Hg. The increased binding of oxygen to haemocyanin allows oxygen delivery per unit of blood flow to be maintained in *C. maenas* during hypoxia (McMahon, 1988).

Once the P_{crit} is reached, a shift to anaerobic metabolism usually occurs, and this results in an increase in circulating lactate and urate concentrations in various crustaceans (Burke, 1979; Bridges & Brand, 1980; Gäde, 1983: Maciel et al., 2008; McMahon, 2001). Large amounts of lactate only accumulate during prolonged exposure (10 hours) to severe hypoxia (i.e. 16.8 mm Hg) in *C. maenas* (Lallier et al., 1987), and therefore, lactate levels only have a modest effect on the oxygen affinity of haemocyanin unless hypoxia is severe (< 16.8 mm Hg) (Lallier et al., 1987; Lallier & Truchot, 1989). However, the concentration

of urate gradually increases when *C. maenas* is exposed to hypoxic conditions, reaching 0.185 mmol at 20 mm Hg (Lallier et al., 1987), and thus it modulates haemocyanin oxygen binding over a wider range of oxygen levels than lactate (Lallier & Truchot, 1989).

During emersion, crustaceans have difficulty excreting CO_2, and this results in hypercapnic acidosis (see above). In contrast, exposure to progressive hypoxia results in an increase in ventilation and a decrease in haemolymph PCO_2 (Burnett & Johansen, 1981). This alkalosis results in a reverse Bohr effect (i.e., a left shift of the haemolymph O_2 binding curve), and consequently an increase in the affinity of haemocyanin for oxygen. (Burnett & Johansen, 1981). This allows *C. maenas* to take up more oxygen at the gills in hypoxic water (McMahon, 1988).

Responses to temperature

Temperature is a key abiotic factor determining population ranges and species' distributions (Firth & Williams, 2009; Gilman et al., 2006; Helmuth et al., 2002; Helmuth et al., 2006a; Somero, 2002; Somero, 2005). Within the intertidal zone, organisms can experience large scale and abrupt fluctuations in temperature due to the ebb and flow of the tide. In aquatic ectotherms, increases in temperature result in an increase in metabolic demand and a concomitant decrease in environmental oxygen levels (Mark et al., 2002; Melzner et al., 2006; Peck et al., 2004; Pörtner et al., 1999; Sartoris et al., 2003; Sommer et al., 1997; Schröer et al., 2009; Van Dijk et al., 1999). Oxygen consumption tends to increase with temperature, eventually reaching a plateau at a critical temperature where an organism's maximum metabolic rate is reached (i.e., where there is no metabolic scope),

and thus, cannot increase any further. At this critical temperature, the organism must meet additional temperature-related metabolic demands through anaerobic metabolism, but this can quickly lead to irreversible damage and eventually death (Pörtner, 2010; Verbeck et al., 2016). In the last two decades, many studies have suggested that restrictions in aerobic metabolism is the limiting factor behind thermal tolerance, and these have given rise to the oxygen capacity limited thermal tolerance (OCLTT) concept (Frederich & Pörtner, 2000; Pörtner, 2001; 2010; Pörtner & Knust, 2007; Clark et al., 2013; Ern et al., 2014; Bjelde et al., 2015). However, growing evidence has shown that the OCLTT concept is unable to explain cold thermal limits, and many arthropod studies have not shown an increase in thermal tolerance in hyperoxia situations (Verbeck et al., 2016). As such, this concept is by no means universally accepted (e.g. see Clark et al., 2013; Jutfeldt et al., 2018; Verbeck et al; 2016).

Carcinus maenas has a broad thermal tolerance. It can survive at temperatures between 0°C and 35°C+ (Klassen & Locke, 2007; Madeira et al., 2012), and is capable of reproducing between 9°C and 26°C (Cohen & Carlton, 1995; Roff et al., 1984). Similar to what occurs in hypoxia, *C. maenas* will leave the water at temperatures > 28°C even if the air is of a relatively similar temperature (Taylor & Wheatly, 1979). This transition into air allows for evaporative heat loss over the gas exchange areas, which contributes to a decrease in body temperature, and subsequently, metabolic rate and oxygen demand (Fusi et al., 2016; Taylor & Wheatly, 1979). This 'emersion response' (Taylor & Whiteley, 1979) has since been shown in other crustaceans, and is thought to be a mechanism to reduce the effects of acute temperature (McGaw, 2003).

Ventilation and oxygen consumption rates for *C. maenas* increase when exposed to acute warming (i.e., at 1ºC hr^{-1}) (Giomi & Pörtner, 2013; Truchot, 1973), and *C. maenas* generally exhibits a linear relationship between oxygen consumption and temperature, rather than the exponential one which is observed in some marine ectotherms (Giomi & Pörtner, 2013; Taylor & Butler, 1978; Taylor & Wheatly, 1979). Subsequently, *C. maenas* has a relatively low Q_{10} of approximately 1.3 between 15°C and 25 ºC (Giomi & Pörtner; 2013; Taylor & Wheatly, 1979; Wallace, 1972).

When exposed to an acute increase in temperature from 10ºC to 25ºC at 1ºC hr^{-1}, the PaO_2 of *C. maenas* declines proportionately due to an increase in oxygen demand (Giomi & Pörtner, 2013). Thus, similar to emersion and hypoxia, *C. maenas* depends more on haemocyanin, rather than dissolved O_2, to deliver oxygen as temperature increases. This is a result of the decreased partial pressure of venous blood (PvO_2), which facilitates increased unloading of oxygen from haemocyanin at the tissues (Giomi & Pörtner, 2013; Weber et al., 2008).

Lactate production has been used as an indicator of the critical temperature of marine species (Frederich & Pörtner, 2000). However, there are only a few studies that have assessed this concept in decapods (Frederich et al., 2009; Frederich & Pörtner, 2000; Jost et al., 2012; Lallier et al., 1987). With respect to *C. maenas*, Jost et al. (2012) reported that only extreme heat stress (34ºC+) resulted in an increase lactate production in *C. maenas*. Similarly, Lallier et al. (1987) failed to see an increase in lactate production when *C. maenas* was exposed to temperatures between 15ºC and 32ºC.

1.3 Comparison of experimental procedures and the role of acclimation in the physiological responses of intertidal organisms

The physiological responses of intertidal organisms to environmental stressors have been evaluated using a number of different approaches. These can broadly be grouped into laboratory, field and natural experiments (Spicer, 2014). Each approach plays an essential role in developing our understanding of physiological processes in intertidal organisms, and there are advantages and limitations with each approach. These are effectively summarised by Spicer (2014) (Fig. 1.1). The control and manipulation possible in a laboratory setting allow for careful examination of the organism's physiological responses to a certain set of conditions, but can often lack ecological context. Field studies often incorporate laboratory-type manipulations (e.g., caging to maintain position / depth), and therefore, provide a certain degree of ecological realism. However, the interaction of multiple environmental and biotic stressors makes it difficult / impossible to draw tangible conclusions. Natural experiments take advantage of the fact that the environmental variable(s) of interest vary naturally (e.g., El Niño events, hypoxic fjords), and that their effect(s) on a physiological mechanism or response can be recorded (Spicer, 2014). These types of experiments are perhaps the most valuable as they are based on *in situ* conditions and without manipulation, but they are often opportunistic and impossible to standardize. Further, in the absence of prior knowledge of the organism / mechanism in question, it can be difficult to interpret the data.

A combination of these experimental approaches is often necessary to develop a true understanding of the physiology of intertidal animals. However, most researchers have

chosen to control and manipulate the organism's surroundings to answer these questions, and this has resulted in the majority of experiments being conducted in the laboratory. For convenience, almost all of these studies acclimate intertidal organisms to constant ambient conditions, including keeping them permanently submerged rather than exposing them to a naturally occurring cycle of immersion and emersion (e.g., Burnett, 1988; Burnett et al., 2002; DeFur & McMahon, 1984; Lagos et al., 2014; McGaw et al., 2009; Rastrick et al., 2014). Despite being sound scientific practice, that has provided a wealth of valuable information about the physiology of intertidal organisms (e.g., Bertness et al., 2001; Denny et al., 2011; Helmuth et al., 2006a,b; Pörtner 2001; 2002; 2010; Tomanek & Helmuth, 2002), it is not representative of the conditions these organisms experience in their native habitats.

In the last decade, it has been increasingly recognized that assessing the responses of intertidal organisms exposed to conditions more representative of their natural habitat is imperative before we can fully understand their physiology (e.g., Drake et al., 2017; Paganini et al., 2014; Yin et al., 2017). For example, some articles have highlighted the importance that regular periods of emersion have on the physiological capacity of molluscs to respond to temperature or hypoxic stress (Altieri, 2006; Drake et al., 2017). Further, research on heat shock proteins (HSP's) in *Mytilus californianus* determined that air exposure (desiccation, anaerobiosis) was the predominant driver of the heat shock (stress) response, rather than temperature stress (Roberts et al., 1997). This was identified by acclimating *M. californianus* to permanently submerged conditions in the laboratory and noting that the stress response was abolished (Roberts et al., 1997).

More importantly, this plasticity of physiological mechanisms and tolerance limits can occur on an intraspecific level. This was demonstrated in a study using *Mytilus edulis,* where individuals were reciprocally transferred between the intertidal and subtidal, and maintained at these respective shore heights for seven weeks (Altieri, 2006). After this period, emersion and hypoxia tolerance were assessed in the laboratory. Intertidal acclimated individuals had higher tolerance to air exposure and hypoxia than subtidal individuals. Furthermore, tolerance to emersion and hypoxia was gained by subtidal individuals acclimated to intertidal conditions and lost in intertidal individuals acclimated to subtidal conditions. This highlights that tolerance to aerial exposure is a plastic (inducible) response that can be lost or gained depending on the acclimation regime used (Altieri, 2006).

This research suggests that taking individuals out of the intertidal and acclimating them to permanently submerged conditions, as is often done in the lab, could result in a loss / reduction in physiological capacity and ability to tolerate environmental stressors. In an attempt to understand this association, and to incorporate some ecological realism into laboratory experiments, some studies have acclimated intertidal organisms to simulated tidal cycles in the lab (Altieri, 2006; Dong & Williams, 2011; Drake et al., 2017; Han et al., 2013; Jimenez et al., 2016; Marshall & McQuaid, 1992; McMahon et al., 1991; Paganini et al., 2014; Widdows & Shick, 1985; Yin et al., 2017). The objective of the majority of these studies was to determine how repeated exposure to air affects the physiological responses to thermal or hypoxic stress, and the mechanisms controlling them. Routine exposure to air was more important than acclimation temperature in determining

upper temperature tolerance (Drake et al., 2017), resulted in the synthesis of more stress resistant proteins (Han et al., 2013; Yin et al., 2017), and allowed individuals to perform better under aerial exposure (McMahon et al., 1991; Widdows & Shick, 1985) than those acclimated to non-tidal conditions.

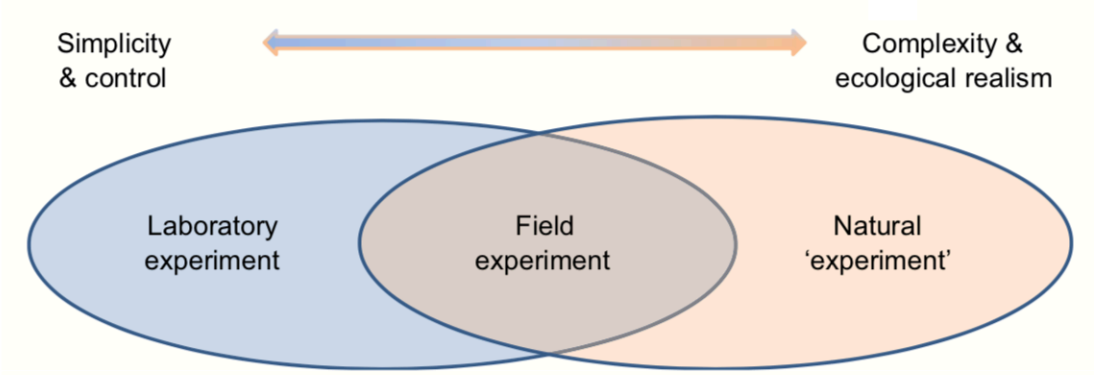
The above studies suggest that physiological responses and tolerances to environmental stressors may be plastic (inducible) traits, and that intertidal species may have the capacity to maintain or recruit physiological mechanisms when aerially emersed that better allow them to tolerate environmental stress. However, as this association between emersion and stress tolerance is still in its early stages of evaluation, additional knowledge of how routine exposure to air affects physiological responses is needed. To date, studies that have simulated tidal cycles in the lab have mainly used sessile molluscs as their model organisms, and there is limited information on how repeated exposure to air affects the physiological responses of other invertebrate taxonomic groups; particularly mobile organisms such as decapod crustaceans.

1.4 Thesis aims

The aim of these studies was to understand if *C. maenas* acclimated to continuous cycles of immersion and emersion (i.e., simulating normal tidal cycles) exhibit different physiological responses as compared to those acclimated to constant submerged conditions. This research tested two hypotheses, which are presented as separate chapters.

Chapter 2: Hypothesis - Individuals acclimated to a tidal cycle will have increased physiological capacity to tolerate emersion than individuals that had been acclimated to non-tidal conditions.

Chapter 3: Hypothesis - Based on previous work on sessile molluscs (e.g.: Drake et al., 2017; Roberts, 1997; Stenseng et al., 2005), I predicted that individuals acclimated to a tidal cycle will have different metabolic and physiological responses to acute temperature and hypoxic stressors.



Strengths		
Can draw causal conclusions	Can make some causal conclusions	Ecologically realistic
Extraneous variables minimised	More ecological realism	
Easily replicated	Little experiment effect	
Weaknesses		
Not realistic – contrived	Less control than laboratory	Cannot draw causal conclusions
Experiment effects	More ecological realism	Many extraneous factors
	Little experiment effect	'Flawed' data

Figure 1.1. Diagram illustrating the relationship between the three different, but related, experimental approaches used by ecophysiologists, and the strengths and weaknesses of each. Adapted from "What can an ecophysiological approach tell us about the physiological responses of marine invertebrates to hypoxia?" by Spicer (2014).

Chapter 2. *Carcinus maenas* acclimated to tidal conditions respond differently to emersion compared with those acclimated to non-tidal (submerged) conditions

2.1 Abstract

Animals inhabiting the intertidal zone are exposed to abrupt changes in environmental conditions associated with the rise and fall of the tide. For convenience, the majority of laboratory studies on intertidal organisms have acclimated individuals to permanently submerged conditions in seawater tanks, and not to the tidal cycle that would occur in their natural environment. In this study, the intertidal green crab *Carcinus maenas* was acclimated to either a simulated tidal regime of continuous emersion-immersion (referred to as 'tidal') or to permanently submerged conditions (referred to as 'non-tidal') to assess their physiological responses to subsequent submersion and emersion. Tidal crabs exhibited an endogenous rhythm of oxygen consumption during continuous submersion and lower oxygen consumption during periods of anticipated emersion, which was not detected in non-tidal crabs. During emersion, tidal crabs were able to buffer apparent changes in acid-base balance and exhibited no change in venous pH whereas non-tidal crabs developed an acidosis associated with a rise in lactate levels. These results indicate that tidal crabs are better able to sustain aerobic metabolism and have lower metabolic costs during emersion than non-tidal crabs. In addition, I suggest that the elevated levels of haemocyanin exhibited by tidal crabs, combined with potential buffering by the carapace, allowed them to maintain oxygen transport and buffer pH changes during emersion. This suggests that acclimation of *C. maenas* to submerged conditions, results in a loss of important physiological mechanisms that enable it to tolerate emersion. The results of this

study show that caution must be taken when acclimating intertidal organisms to submerged conditions in the laboratory, as it may abolish important physiological responses and adaptations that are critical to their performance or survival when exposed to air.

2.1 Introduction

The intertidal zone is defined as the area between the high and low water marks during the spring tide (Levington, 2009), and its defining feature is the continuous transition between aerial and aquatic environments. Organisms that inhabit the intertidal zone almost exclusively originate from the marine environment, and subsequently, one of the major challenges that they face is the shift from an aquatic to aerial respiratory medium. The dehydrating properties of air promote desiccation, which can have adverse effects on the metabolic performance of many intertidal organisms. Body water loss can lead to difficulties in ion regulation and nitrogen excretion (Truchot, 1990), and the desiccation of gas exchange surfaces can reduce or inhibit aerobic respiration (Burnett & McMahon, 1987; DeFur, 1988; McMahon, 1988; Truchot, 1990). Furthermore, desiccation is often coupled with an inability to effectively excrete carbon dioxide, which can result in hypercapnia and respiratory acidosis (Truchot, 1990). In addition to periods of emersion, the transition between aquatic and aerial environments often results in rapid and large-scale changes in temperature, salinity, oxygen availability and exposure to solar radiation (Gunderson et al., 2016; Helmuth et al., 2010).

On a temporal scale, fluctuations in environmental conditions in the intertidal are regular and predictable. Therefore, intertidal organisms have developed a variety of mechanisms to ensure survival during emersion. One common response to emersion is

avoidance, either temporally or spatially. Sessile animals use behaviour to reduce desiccation during low tide (i.e., temporal avoidance). Mussels and other bivalves exhibit shell closure to conserve water and avoid exposure to air (Byrne & McMahon, 1994; Nicastro et al., 2010). Limpets grind down a 'home scar' on the rock with their radula, returning to this depression during low tide to isolate themselves from air (Lowell, 1984), while anemones reduce evaporative water loss by withdrawing their tentacles into their body (Ottaway, 1973). Mobile species tend to exhibit spatial avoidance and seek microhabitats which have a higher humidity and smaller variations in temperature. Crabs and gastropods seek shelter in crevices, under damp rocks or seaweed, or in tidepools at low tide (Grant & McDonald, 1979; Kensler, 1967), whereas amphipods and some shrimps can construct burrows in the sediment to reduce desiccation (Williams, 1995). Intertidal fish remain in tidepools to avoid air exposure all together, and can stay within the pools even when they become hypoxic. During these periods they often rely on aquatic surface respiration or air breathing (Bridges, 1988). Although behavioural responses may be sufficient in certain species during low tide, ultimately many of them need to employ physiological mechanisms in order to maintain homeostasis (Helmuth et al., 2006a; Helmuth et al., 2006b; Hofmann & Todgham, 2010; Somero, 2002; Tomanek & Helmuth, 2002).

Despite the fact that intertidal zones are highly dynamic environments, they support a rich flora and fauna. The exposure of these organisms to large scale and rapid environmental changes, coupled with their ease of access, has resulted in intertidal organisms being an important group for studying physiological responses to environmental

change (Somero, 2002). The intricate nature and complexity of the physiological monitoring equipment required to perform these studies makes it difficult to conduct field studies, and thus, the majority of studies that have investigated the effects of air exposure on the physiology of intertidal organisms have been carried out in a laboratory setting (e.g., Burnett, 1988; Burnett et al., 2002; DeFur & McMahon, 1984; Lagos et al., 2014; McGaw et al., 2009; Rastrick et al., 2014; Widdows & Shick, 1985; Yin et al., 2017). For convenience, almost all of these studies acclimate intertidal organisms in aquaria in permanently submerged conditions rather than a routine cycle of immersion and emersion as they would typically experience in nature (e.g., Burnett, 1988; Burnett et al., 2002; DeFur & McMahon, 1984; Lagos et al., 2014; McGaw et al., 2009; Rastrick et al., 2014). Although a period of acclimation to constant conditions in the laboratory is sound scientific practice, evidence suggests that intertidal animals acclimated to a regular cycle of immersion-emersion exhibit different physiological responses when exposed to emersion than those maintained in permanently submerged conditions (Altieri, 2006; Bjelde & Todgham, 2013; Drake et al., 2017; Widdows & Shick, 1985; Yin et al., 2017). This difference in physiological responses was initially inferred by comparing intertidal (regular air exposure) and subtidal (limited / no air exposure) congener species. For example, when subjected to 12 hours of air exposure, the intertidal mussel *Geukensia demissa granosissima* is able to maintain aerobic respiration, whereas the subtidal mussel *Modiolus squamosus* displays a much greater reliance on anaerobic pathways (Nicchitta & Ellington, 1983). Likewise, large specimens of the intertidal porcelain crab *Petrolisthes cinctipes* were able to maintain a higher respiration rate than its subtidal counterpart *Petrolisthes*

eriomerus when exposed to air at 25ºC. This was facilitated by a specialized leg membrane structure, not found in *P. eriomerus* (Stillman & Somero, 1996). More importantly, this plasticity in physiological mechanisms and tolerance limits occurs on an intraspecific level. In an *in-situ* translocation study by Altieri (2006), blue mussels, *Mytilus edulis* were reciprocally transplanted between the intertidal and subtidal zones over a seven-week period, following which tolerance (survival) to emersion was determined in a laboratory experiment. Intertidal individuals were found to have higher survival rates during air exposure than mussels from the subtidal zone (Altieri, 2006). Moreover, tolerance to emersion was lost in *M. edulis* from the intertidal zone when acclimated to subtidal conditions, and vice versa. This suggests that the responses of intertidal animals acclimated to permanently submerged conditions in the laboratory may not reflect the physiological responses that occur *in situ*. Recognition of this phenomenon has attracted some interest, particularly recently, and subsequently there are some studies that have acclimated individuals to simulated tidal conditions in the laboratory (e.g., Altieri, 2006; Dong & Williams, 2011; Drake et al., 2017; Han et al., 2013; Jimenez et al., 2016; Marshall & McQuaid, 1992; McMahon et al., 1991; Paganini et al., 2014; Widdows & Shick, 1985; Yin et al., 2017). Most of these studies investigated how tolerance to regular air exposure affects the ability of molluscs to cope with either hypoxia or elevated temperature stress (e.g., Altieri, 2006; Dong & Williams, 2011; Drake et al., 2017; Han et al., 2013; Jimenez et al., 2016; Marshall & McQuaid, 1992; McMahon et al., 1991). However, as far as I am aware, there are few studies that have investigated how acclimation to a simulated tidal cycle affects an intertidal animal's response to subsequent air exposure (Drake et al., 2018;

Widdows & Shick; 1985; Yin et al., 2017). Further, this data suggests that the response to emersion is plastic. For example, these studies showed that routinely exposing bivalves to air resulted in an increased scope for growth during air exposure (Widdows & Shick, 1985) and an upregulation of antioxidant enzymes (Yin et al., 2017). Such studies are enhancing our understanding of the influence of acclimation history on the physiological capacity of intertidal organisms, as they combine the benefits of controlled laboratory experiments with ecologically realistic field observations. However, our knowledge of the effect of routine air exposure on the physiology of mobile intertidal invertebrates, such as sea stars and crabs, is still lacking.

Small decapod crustaceans are common inhabitants of the intertidal zone and the green shore crab (*Carcinus maenas*) is a typical example. *Carcinus maenas* is native to Europe, where it is found in sheltered bays and estuaries. It primarily occurs from the mid intertidal zone down to shallow subtidal habitats (5-6 m) (Klassen & Locke, 2007), but can be found at depths up to 60 m (Crothers, 1968). Because of its tolerance of a wide variety of environmental conditions, *C. maenas* has become a successful marine invader with a global distribution (Klassen & Locke, 2007). This species utilises bimodal breathing and is considered an effective air breather (DeFur, 1988), and is therefore, an ideal candidate to be studied in the context of emersion. *Carcinus maenas* is able to utilize aerobic metabolism during air exposure (Dejours & Truchot, 1988; Depledge, 1984; Newell et al., 1972; Taylor et al., 1973; Taylor & Butler, 1973, 1978; Wallace, 1972). However, the extent to which it can be maintained as compared to values when this species is submerged varies significantly. For example, aerobic respiration has been reported to vary from

between 50% (Simonik & Henry, 2014) to 120% (Taylor & Butler, 1978) of values when submerged. At the onset of emersion, *C. maenas* typically experiences a 50-75% decline in the partial pressure of arterial oxygen (PaO_2) (Dejours & Truchot, 1988; Depledge, 1984; Taylor & Butler, 1978), despite an increased availability of oxygen in air. In addition, due to the difficulty in excreting CO_2 in air, it experiences an increase in PCO_2 levels which results in a decrease in haemolymph pH (Truchot, 1975; Taylor & Butler, 1978), which is sustained throughout the duration of emersion (Burnett, 1988). However, there are discrepancies among studies as to whether emersion in crustaceans results in a shift to anaerobic metabolism, with some studies reporting a significant increase in lactate production during short-term air exposure (Santos & Keller, 1993; Simonik & Henry, 2014), while others report that no lactate build-up occurs during emersion (Johnson & Uglow, 1985; Taylor & Butler, 1978).

Despite being a prominent model species in understanding the effects of emersion on crustacean physiology, previous researchers have maintained *C. maenas* in conditions more representative of subtidal than intertidal conditions (e.g., Dejours & Truchot, 1988; Depledge, 1984; Newell et al., 1972; Taylor et al., 1973; Taylor & Butler, 1973, 1978; Wallace, 1972). The influence of acclimation to tidal conditions on the physiological responses reported in other intertidal species (Widdows & Shick, 1985; Altieri, 2006; Jimenez et al., 2016; Drake et al., 2017, Yin et al., 2017), coupled with the disparity between physiological responses reported for this species, suggests that our understanding of *C. maenas*'s physiological response to emersion may not be accurate. Therefore, the focus of this study was to determine how acclimation to constant submergence (referred to

as ‘non-tidal’) vs. a simulated semi-diurnal tidal cycle (referred to as ‘tidal’) influenced the physiological responses of *C. maenas* to emersion. I hypothesise that *C. maenas* acclimated to a tidal regime will display physiological adjustments in oxygen delivery and acid base balance to tolerate periodic air exposure that will be absent in *C. maenas* acclimated to submerged conditions.

2.2 Materials and methods

2.2.1 Sampling site and collection

Large adult male *C. maenas* (carapace width > 50 mm) were collected at Fox Harbour (47.3209° N, 53.9082° W), Long Harbour (47.4324° N, 53.8162° W) and Fairhaven (47.5343° N, 53.8998° W) in Newfoundland (NL), Canada using dome crab traps between September and November 2015, and June and November, 2016. Only individuals with no carapace damage or missing chelae were used in experiments. The crabs were transported to the Department of Ocean Sciences, Memorial University, NL, Canada, where they were placed in a flow-through system provided with unfiltered seawater at ambient temperatures (approximately 3°C - 12°C). This seawater was ~ 100% saturated with air and had a salinity of 32‰, and the crabs were fed mackerel once a week (uneaten food removed after two days). Perforated plastic pipes (diameter: 10 cm, length: 20 cm) were placed in the holding tanks to discourage aggressive behaviour amongst conspecifics.

2.2.2 Experimental holding conditions

Prior to experiments, the crabs were transferred to two separate flow through seawater tanks (155 cm x 95 cm x 50 cm deep, 1000L) where they were held in perforated containers (37 cm x 40 cm x 17.5 cm deep, 30 L). An air stone was placed in each container to ensure that the oxygen content of the water was close to air saturation.

The crabs were maintained in either non-tidal (control) or tidal (experimental), conditions for at least 4 weeks prior to experimentation. The non-tidal tank received unfiltered seawater at 2.2 L min^{-1}, and the crabs in this tank were kept permanently submerged (Fig. 2.1.). The tidal tank had a similar seawater supply, but the water level in this tank was controlled so that it simulated a semi-diurnal six-hour tidal regime (i.e., alternating periods of six hours of immersion and then six hours of emersion), to replicate the tidal cycle at the collection sites. This was achieved by manipulating the water level via a timer-controlled solenoid valve connected to the outflow. When off, the solenoid valve closed off the outflow, allowing the tank to slowly fill up with seawater and submerge the crabs. When turned on, the valve opened the outflow, allowing the water to gradually drain, and exposing the crabs to air (Fig. 2.1). The air temperature was controlled in both tanks (using an air conditioning unit) so that it was similar to that in the water in the tanks (15°C ± 0.5°C). Humidity during emersion was monitored using a hygrometer (11-661-16, Fisher Scientific, USA), and varied between 70-80%.

Water temperature of both tanks was maintained at 15°C (± 0.5°C) via an in-tank heater and salinity remained constant at 31-32‰. A temperature of 15°C was used as it is the optimum temperature for growth and physiological processes in *C. maenas* (Robertson

et al., 2002). Water oxygen content was maintained at 100% saturation. Animals were held in constant dim red light to reduce any endogenous cycles associated with light change. Prior to experimentation, the crabs were fasted for 3-4 days to ensure that digestive processes did not affect any of the measured physiological parameters (McGaw, 2006; Robertson et al., 2002). Individual crabs from the non-tidal and tidal group were matched as closely as possible with respect to size, mass and colour morph (Styrishave et al., 2004).

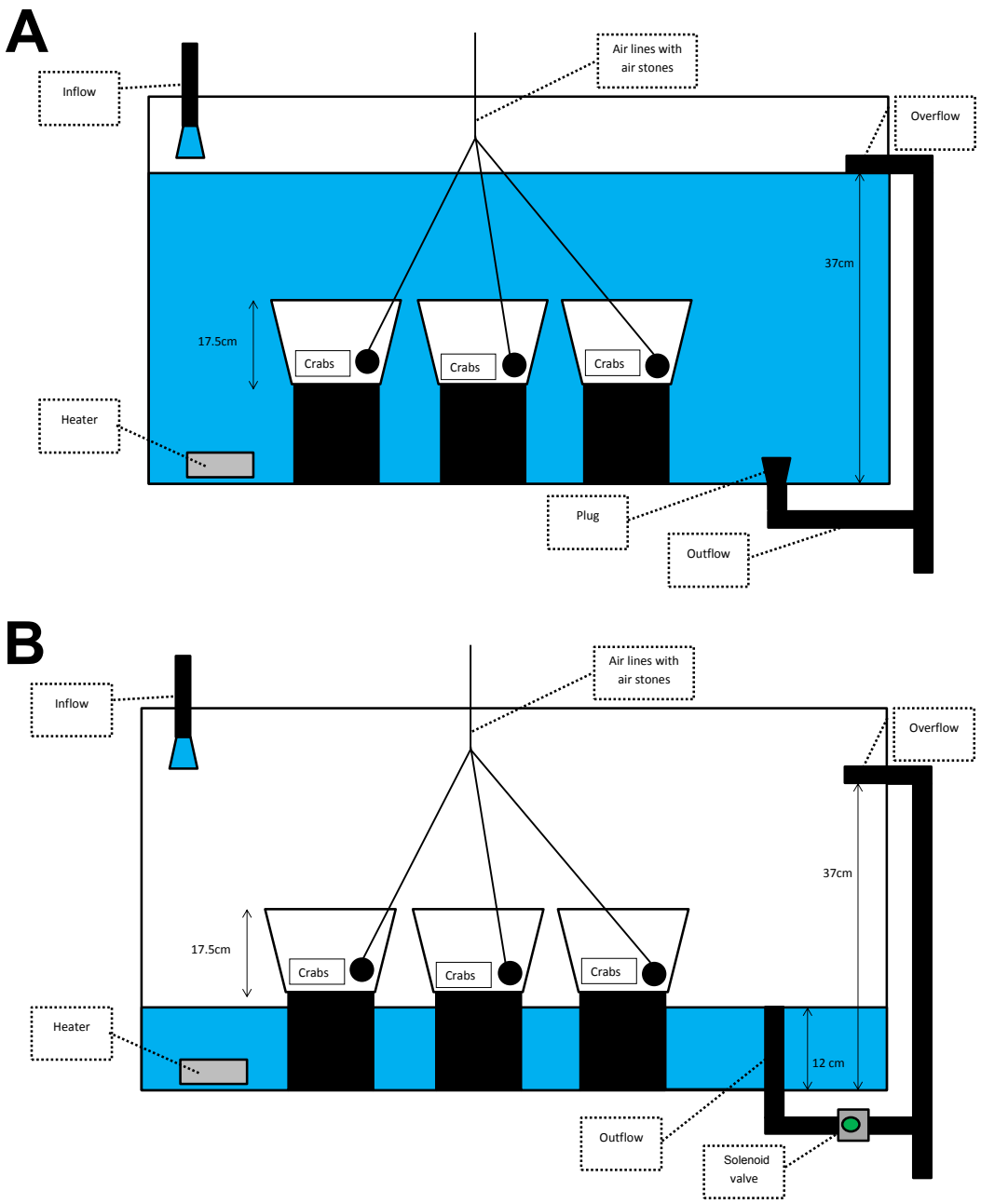


Figure 2.1. (A) Control (non-tidal) holding conditions, in which the crabs were continuously submerged. (B) Experimental (tidal) holding conditions. The height of the water in this tank was controlled by a solenoid valve in the outflow pipe which opened / closed every 6 hours, either exposing the crabs to air or immersing them. Both tanks were supplied with aerated seawater through the inflow line, and a heater maintained the seawater temperature at 15°C (± 0.5°C) Air temperature was maintained at 15°C (± 0.5°C) using an air conditioning unit.

2.2.3 Experiments

Oxygen consumption during 72 hours of submersion

To establish if acclimation to a cycle of emersion entrains any endogenous rhythms (see De la Iglesia & Hsu, 2010 and Palmer, 2000 for reviews), oxygen consumption was measured for both the non-tidal and tidal crabs (n = 12 per group) during 72 hours of immersion (100% air saturation) at a salinity of 32‰ and a temperature of 15°C. Individuals were transferred from their holding tanks to plexiglass respirometry chambers submerged in a seawater table. Although every precaution was taken to carefully transfer the animals, handling of crustaceans can result in a short-term increase in oxygen consumption (Jouve-Duhamel & Truchot, 1985). Thus, I assessed the potential impact that this handling may have had on oxygen consumption, and determined the least stressful method for transferring individual crabs from their holding tank to the respirometry chambers (see Appendix A.1 for details of how this transfer was performed).

Oxygen consumption was measured using an L-DAQ intermittent flow respirometry system (Loligo Systems, Copenhagen, Denmark), with the oxygen probes calibrated to 0 and 100% saturation using a sodium sulphite (0.01 g mL^{-1}) and air-bubbled seawater, respectively. The system consisted of 4 identical cylindrical chambers (20 cm in diameter x 12 cm deep), which were submerged in a tank (155 cm x 95 cm x 50 cm deep, 1000L) containing normoxic seawater (32‰) at 15°C. Each chamber was equipped with two pumps. The first pump continually flushed seawater through the chamber between oxygen consumption measurements. This pump was turned off when the chamber was closed (sealed) for oxygen measurements, and a second pump recirculated water through

the chamber at a rate of 5 L min^{-1} to ensure that there were no oxygen gradients in the chamber. Two crabs from each treatment, with one crab in each chamber, were used during each measurement period. Experiments were carried out in constant dim red light, and black plastic sheeting surrounded the experimental tank to avoid visual disturbance to the animal. Oxygen consumption was measured every hour; with the chamber sealed for 30 minutes (so that oxygen consumption could be measured), and then flushed with fresh seawater for the remaining 30 minutes. Oxygen levels in the chamber were continuously recorded using the AutoResp 4 (v 1.7) data acquisition system (Loligo Systems, Copenhagen, Denmark), which calculated oxygen consumption as mg O_2 kg^{-1} hr^{-1} using the following equation:

$$\dot{M}O_2 = V\left(\frac{\Delta PO_2}{t}\right) \cdot \alpha \cdot MB$$

Where V is the volume of the respirometry chamber minus the volume of the crab (where 1 kg is assumed to equal 1 L), $\Delta PO_2/t$ is the change in oxygen partial pressure (kPa) per unit time, α is the solubility coefficient for oxygen in seawater (at salinity of 32‰ and 15.0 °C) in mg O_2 kPa^{-1}, and MB is the body mass of the crab (kg). As I was interested to see if tidal acclimation lead to an endogenous rhythm, it was not possible to acclimate the crabs to the chambers before the respirometry experiments. To account for the effect of handling stress on the oxygen consumption rates, each oxygen consumption value was divided by a stress index as calculated in Appendix A.1, through preliminary tests. The experiment was also run with empty chambers and background microbial respiration was found to be negligible.

Simulated tidal cycle

In a separate series of experiments, the crabs were subjected to a simulated tidal cycle: 6 hours immersed, 6 hours emersed, followed by re-immersion for 6 hours. This was carried out in synchronisation with times of anticipated immersion and emersion in the tidal holding tank. Oxygen consumption was measured at hourly intervals during the first 6-hour immersion period using the aquatic respirometry system previously described. After 6 hours, individual crabs were carefully transferred from the aquatic respirometry system to separate blacked out airtight respiration chambers (20 cm x 27cm x 12 cm deep, 3.3 L) (a detailed account of transfer methods from aquatic to aerial chambers is provided in Appendix A.1) and housed in an incubator (MIR-254-PE, Panasonic Biomedical, Europe) at 15°C and a relative humidity of 70%. These chambers were sealed for 30 minutes, which allowed a measurable drop in chamber oxygen level, without exposing the crabs to hypoxia. Following these measurements, the chambers were opened for 30 minutes before being sealed for the next reading. Samples of air in the chamber were taken every hour during the six-hour emersion period using a 60 ml syringe by inserting the syringe's 18-gauge needle through a small hole in the lid that was sealed with dental wax. The syringe was pumped in and out three times to circulate the air in the chamber before withdrawing an air sample. The sample was injected through a Drierite® column (to remove any moisture) and into a Q-S102 O_2 analyser (Qubit Systems, Ontario, Canada). The analyser was pre-calibrated to 100% air saturation (20.95% oxygen saturation) with air from the incubator, while nitrogen gas was used to achieve 0% oxygen saturation. Following this

period of emersion, the crabs were carefully transferred back into their aquatic respiration chamber, and oxygen consumption was monitored at hourly intervals for a further 6 hours.

Aerial oxygen consumption was calculated using the following formulas: which take into consideration incubator temperature, the volume of air displaced by the crab in the chamber, and the length of time the chamber remained closed:

$$u\dot{M}O_2 = V\left(\frac{\Delta PO_2}{t}\right) \cdot MB$$

$$Corrected\ \dot{M}O_2 = \frac{(uMO_2 \cdot BP \cdot 273.15)}{(101.325 \cdot T)}$$

Where $u\dot{M}O_2$ is uncorrected oxygen consumption (mL O_2 kg^{-1} hr^{-1}) V is the volume of the respirometry chamber minus the volume of the crab (where 1 kg is assumed to equal 1 L), $\Delta PO_2/t$ is the change in oxygen partial pressure (kPa) per unit time, MB is the body mass of the crab (kg). BP is barometric pressure in kilopascals, and T is the temperature of the specimen (= incubator) in Kelvin. After STP correction, oxygen consumption was converted from millilitres per hour to milligrams per hour by multiplying by 1.43 (32 g mol^{-1} divided by 22.4 L mol^{-1} at STP). Preliminary tests were completed to assess the effects of handling stress on oxygen consumption of *C. maenas* in air (see Appendix, A.1). The results of these tests showed an effect of handling on oxygen consumption during the first hour of emersion. Therefore, to account for this effect, the oxygen consumption values from hour one of emersion for non-tidal and tidal crabs were divided by a 'stress index' as calculated in Appendix A.1.

In a third series of experiments, separate animals were subjected to the same 18 hour simulated tidal cycle (used above) and haemolymph samples were collected and analysed for partial pressure of arterial oxygen (PaO_2), venous pH (pH_v), and haemocyanin and lactate concentrations. To avoid adversely affecting animals by the repeated collection of large samples from the same crab, separate animals (n = 7 per group) were used at each time point. Samples were collected at the start, middle and end of each stage in the simulated tidal cycle, which corresponded to hours 1, 3, 6, 7, 9, 12, 13, 15, and 18 hours after the experiment began. Lactate was determined at 1, 6, 7, 9, 12, 13, 15 and 18 hours.

For PaO_2 measurements, at least three days before sampling, a small hole was drilled directly over the heart which pierced through the carapace but left the pericardial membrane intact. A section of dental dam was placed over the hole and secured with cyanoacrylate glue. During the experiment, an arterial blood sample (400 µL) was taken by inserting a 21-gauge needle attached to a 1 mL airtight Hamilton syringe through the dental dam and into the pericardial cavity. Samples were taken within 30 seconds of removing the animal from their chamber, and approximately 200 µL of arterial haemolymph was injected below a layer of mineral oil in an Eppendorf® tube and immediately transferred to a water bath at 15°C. PaO_2 was measured using a Fibox-3 O_2 analyser (PreSens, Regensburg, Germany). This meter was calibrated using fully aerated seawater as 100 % air saturation and seawater with sodium sulphite (0.06 g mL^{-1}) as 0% air saturation. The dipping probe was inserted into the sample, and readings were taken once PaO_2 had stabilised (after 3 minutes) using OxyView software (PreSens, Regensburg, Germany) running on a laptop computer (Dell Inspiron).

Once the arterial haemolymph sample was collected (for PaO_2 analysis), approximately 400 µL of venous haemolymph was withdrawn from the same individual from the arthrodial membrane at the base of a walking leg. A 200 µL sample was injected below a layer of mineral oil in an Eppendorf® tube and transferred immediately to the water bath at 15°C. pH_v was measured using a pH mini-V2 analyser (PreSens, Regensburg, Germany). This was calibrated using colourless pH reference buffers (Ricca Chemical Company, Arlington, Texas, USA). The dipping probe was inserted into the sample and readings were taken once levels had stabilised (3 minutes) using pH 1-view software (PreSens, Regensburg, Germany) running on a laptop computer (Dell Inspiron) The remaining 200 µL aliquots of arterial and venous haemolymph were immediately transferred to Eppendorf® tubes, and placed on ice before being transferred to a -80°C freezer for later haemocyanin and lactate analyses, respectively.

Haemocyanin concentration was determined spectrophotometrically using a Spectramax M5 multimode microplate reader (Molecular Devices, California, USA) and an assay adapted from Pascke et al. (2010). Arterial haemolymph was thawed at room temperature, then vortexed for 5 seconds to evenly distribute the protein. A 1:20 dilution was made with deionized water and vortexed for a further 5 seconds (n = 7 per time point). Haemocyanin concentrations were estimated using the Beer – Lambert law from the peak absorbance measured at 335 nm, and using an extinction coefficient of 17.5 mmol L^{-1} cm^{-1} based on the specific absorbance ($A_{1\%,1cm}$) value of 2.33 reported for *C. maenas* (Nickerson & Van Holde, 1971), and a molecular mass of 75·kDa.

Lactate concentration was determined using an assay that was adapted from Clow et al. (2016) using thawed venous haemolymph samples. Samples were deproteinized using 6% perchloric acid with a dilution ratio of 1:10 (n = 7 per time period). The samples were vortexed and then centrifuged at 10,000 x g for 10 minutes. The subsequent supernatant was then extracted, and 25 µL of this extract was added to 200 µL of assay medium containing glycine buffer (Sigma, G5418) and 2.5 mmol L^{-1} NAD^{+}, pH 9.0. Absorbance was determined at 340 nm using a DTX 880 microplate reader (Beckman Coulter, Ontario, Canada) before the addition of 10 IU mL^{-1} of lactate dehydrogenase (Sigma, L2500). Absorbance was read after 30 min or until stable. Lactate concentrations (mmol L^{-1}) were then calculated from a standard curve.

2.2.4 Data and statistical analyses

All data passed the assumptions of normality, independence and homogeneity, except for the lactate data which was square root transformed before statistical analysis. Periodicity in oxygen consumption in the submersion experiment was determined by a Lomb-Scargle periodogram using the software PAST (Hammer et al., 2001). Periods of periodicity were determined using the equation (1 / x) where 1 is total frequency, and x is the frequency of peak power. Differences in oxygen consumption between groups were identified using a two-way repeated measures ANOVA, while PaO_2, pH_v and haemolymph lactate and haemocyanin concentrations were analysed using two-way ANOVAs. One-way ANOVAs, or Tukey's HSD post-hoc tests were used when applicable to detect significant differences between levels of individual factors. One-way ANOVAs were performed to

compare immersion with re-immersion oxygen consumption values. A two-sample t-test was used to compare the drop in oxygen consumption from immersion to emersion in both acclimation groups. The statistical analyses were carried out using GraphPad Prism (version 5.3 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). In all cases, a p value of < 0.05 was utilised as the criteria for statistical significance.

2.3 Results

2.3.1 Oxygen consumption during seventy-two hours of submersion

There was considerable variation in oxygen consumption rates during the 72-hour period of submersion, both within individual animals and between the non-tidal and tidal crabs. However, this variation was more pronounced in non-tidal crabs. The mean oxygen consumption of non-tidal crabs was 53.4 ± 7.8 mg O_2 kg^{-1} hr^{-1} during the 72-hour experiment, whereas that of individuals from the tidal treatment was 45.8 ± 4.2 mg O_2 kg^{-1} hr^{-1}. There was a significant interaction between acclimation and time (two-way RM ANOVA, df = 71, F = 1.433, p = 0.0118, Fig. 2.2). When tested separately for main effects, this was due to significant variation in the oxygen consumption of tidal crabs (one-way RM, ANOVA, df = 71, F = 3.129, p = 0.0108), but not in non-tidal crabs (one-way RM ANOVA, df = 71, F = 0.9391, p = 0.4762) over time. Tidal crabs appeared to have an endogenous rhythm, and exhibited a small but significant, decrease in oxygen consumption during times of anticipated air exposure in comparison to anticipated immersion (two-way RM ANOVA, df = 1, F = 6.329, p = 0.0306). This periodicity in tidal crabs was confirmed

using a Lomb-Scargle periodogram (Fig. 2.3), which revealed a significant peak ($p < 0.01$) in the spectrum at 0.0809 cycles per hour (i.e., 12.36 hours per cycle).

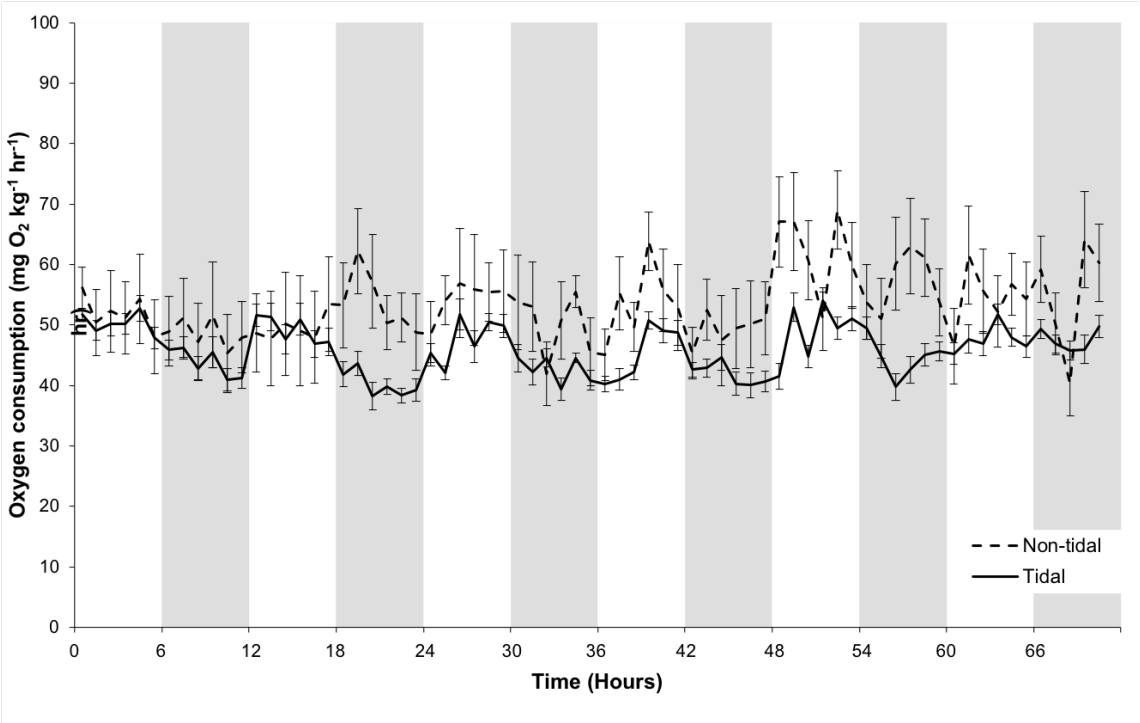


Figure 2.2. Oxygen consumption of green crab (*Carcinus maenas*) that had been previously acclimated to non-tidal (dashed) or tidal (solid) cycles during a 72-hour period in seawater at 15ºC. The values represent the mean ± SEM of 12 individuals for each treatment. White bars indicate times when 'tidal' crabs would be anticipating immersion in their acclimation regime, and grey bars indicate periods of anticipated emersion.

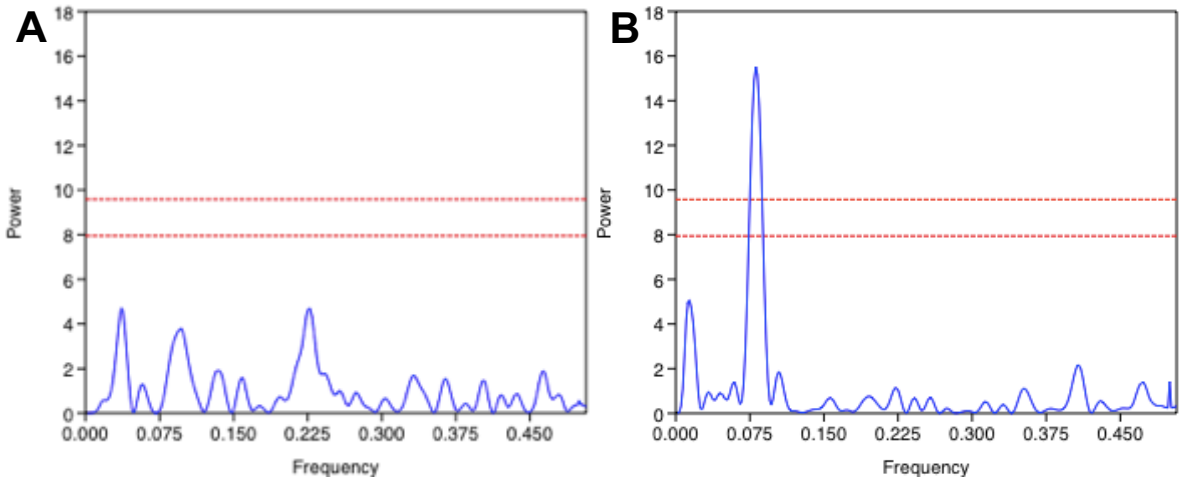


Figure 2.3. Lomb-Scargle periodogram used to test for the presence of periodicity in oxygen consumption of *Carcinus maenas* during a 72-hour period of immersion in seawater at 15ºC. These crabs were previously acclimated to non-tidal (A) and tidal (B) cycles for 4 weeks. Power represents statistical power, where the lower red dotted line indicates statistical significance at $p < 0.05$, while the upper line indicates significance at $p < 0.01$. Frequency is scaled from of 0 – 1 cycles per hour, where 1 represent the duration of the submersion period (72 hours), but the upper limit is plotted in terms of the Nyquist frequency, and thus, is half of the sampling frequency (0.5).

2.3.2. Physiological responses of tidal and non-tidal crabs subjected to a simulated tidal cycle

Oxygen consumption

During the initial immersion phase, the oxygen consumption of non-tidal and tidal crabs was similar, varying between 40.02 ± 4.62 and 51.41 ± 5.79 mg O_2 kg^{-1} hr^{-1}. The oxygen consumption of both non-tidal and tidal crabs decreased considerably when exposed to emersion (Fig. 2.4), with non-tidal crabs experiencing a larger drop (54%) than tidal crabs (45%) (two-sample t-test, df = 22, T = 2.171, p = 0.0410) and both groups maintained comparable oxygen consumption throughout the duration of emersion (two-way RM ANOVA, df = 1, F = 0.2655, p = 0.6115). When the crabs were re-immersed, there was a significant increase in oxygen consumption in both the non-tidal and tidal crabs (two-way ANOVA, df = 1, F = 86.9, p = < 0.0001), oxygen consumption returning to initial immersion values in both groups (one-way ANOVA, non-tidal: df = 11, F = 0.3393, p = 0.9753; tidal: df = 11, F = 0.805, p = 0.6325). However, the oxygen consumption of non-tidal individuals was significantly elevated over that in the tidal group for the majority of the re-immersion period (two-way RM ANOVA, df = 1, F = 7.808, p = 0.0106).

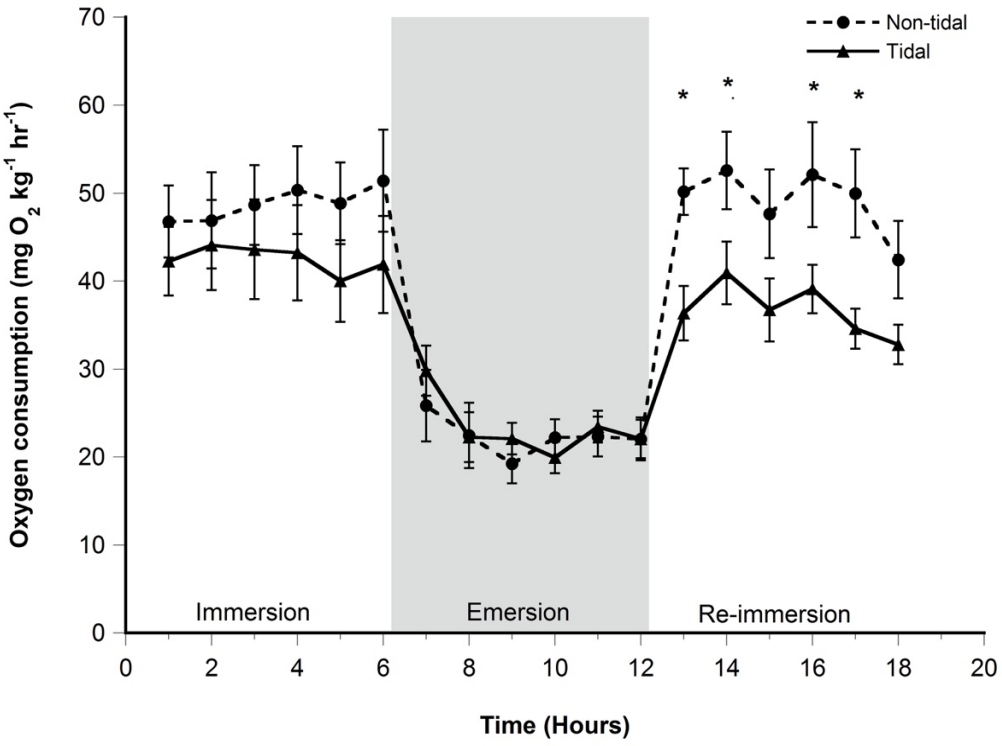


Figure 2.4. Oxygen consumption of *Carcinus maenas* during a simulated tidal cycle; immersion in seawater at 15ºC for 6 hours, followed by a 6-hour period of emersion in air at 15ºC, and a subsequent 6-hour recovery period in seawater at 15ºC. The data represent the mean $\pm$ SEM of 12 crabs that were previously acclimated to a non-tidal (dashed line) or a tidal (solid line) regimen for > 4 weeks before measurements began. Asterisks indicate a significant difference ($p < 0.05$) between acclimation groups at a given time point.

PaO_2

The PaO_2 of the crab's haemolymph did not change significantly during the simulated tidal cycle in either group (two-way ANOVA, df = 8, F = 1.868, p=0.0725), and values were not significantly different between the two groups (two-way ANOVA, df = 1, F = 1.294, p= 0.2578); with values averaging between 93.60 ± 4.46 mm Hg and 75.57 ± 5.96 mm Hg for non-tidal crabs and 97.75 ± 5.73 and 76.66 ± 11.1 mm Hg for tidal crabs during immersion, emersion and re-immersion (Fig. 2.5).

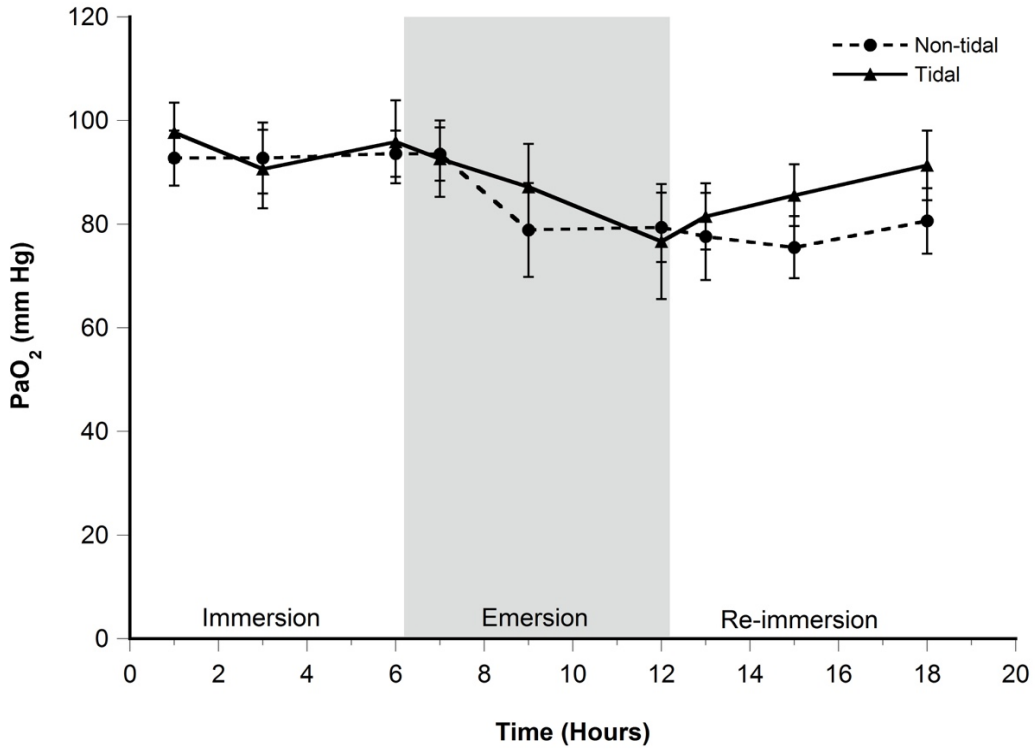


Figure 2.5. Arterial oxygen partial pressure (PaO_2) of *Carcinus maenas* haemolymph during a simulated tidal cycle of immersion in seawater at 15ºC for 6 hours, followed by a 6-hour period of emersion in air at 15ºC, and finally a 6-hour period of re-immersion in seawater at 15ºC. The data represent the mean $\pm$ SEM of 7 crabs previously acclimated to a non-tidal (dashed line) or tidal (solid line) regimen for > 4 weeks.

Venous pH (pH_v)

The pH_v of tidal and non-tidal crabs was stable during the initial period of immersion, with mean values of 7.78 ± 0.04 and 7.80 ± 0.04 in the two groups, respectively (Fig. 2.6). The pH_v of tidal crabs did not change during the experiment. However, the pH_v of non-tidal crabs decreased significantly (by 0.17 pH units) during emersion, and then returned to values similar to those recorded during the initial immersion period. This difference in the pattern of changes in pH_v resulted in the pH_v of non-tidal crabs being significantly lower than measured in tidal crabs (two-way ANOVA, df = 1, F = 4.666, p = 0.0330). This was due to significant decline in pH during the 3rd (Tukey HSD, p = 0.0151) and 6th (Tukey HSD, p = 0.0111) hour of emersion.

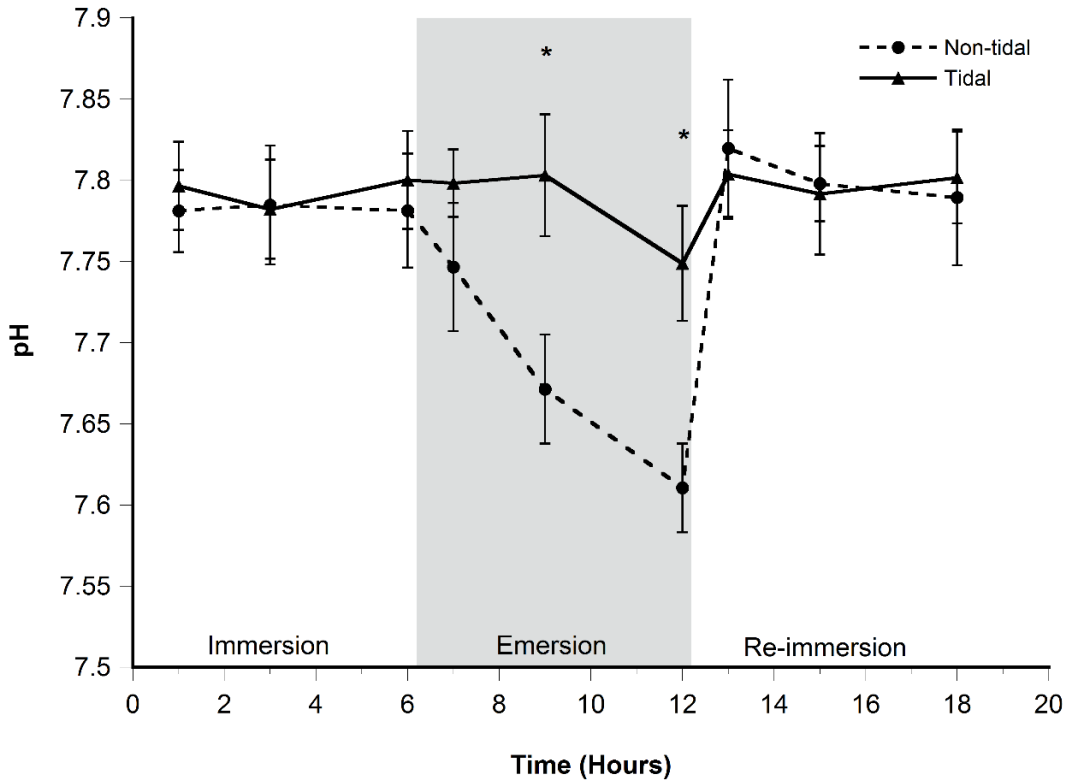


Figure 2.6. Venous pH of *Carcinus maenas* haemolymph during a simulated tidal cycle of immersion in seawater at 15ºC for 6 hours, followed by a 6-hour period of emersion in air at 15ºC, and a subsequent 6-hour period of re-immersion in seawater at 15ºC. The data represent the mean $\pm$ SEM of 7 crabs previously acclimated to a non-tidal (dashed line) or to a tidal (solid line) regimen for > 4 weeks. Asterisks indicate a significant difference ($p < 0.05$) between acclimation treatments at a given time point.

Haemocyanin concentration

The haemocyanin concentration of both groups remained relatively stable over the course of the simulated tidal cycle (two-way ANOVA, df = 8, F = 0.7726, p = 0.6277; Fig, 2.7), with the haemocyanin concentration of non-tidal crabs varying from 0.54 mmol L^{-1} to 0.75 mmol L^{-1}, and that of tidal crabs varying from 0.7 mmol L^{-1} to 0.85 mmol L^{-1}. However, the haemocyanin concentration of tidal acclimated individuals was significantly higher overall as compared to non-tidal individuals (two-way ANOVA, df = 1, F = 19.32, p = < 0.0001), and this was largely driven by significant differences between non-tidal and tidal crabs at hours 1, 7, 9 and 15 (Tukey's HSD test, p = 0.0466; 0.0105; 0.0106; 0.0442, respectively).

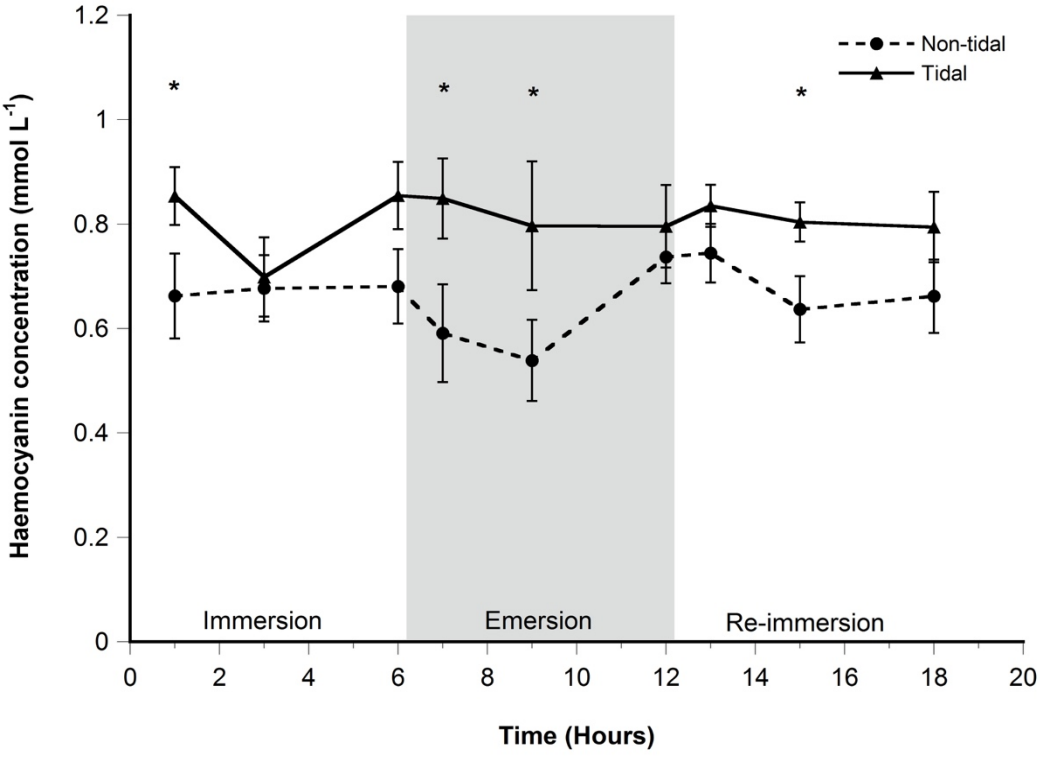


Figure 2.7. Haemolymph haemocyanin concentration in *Carcinus maenas* during a cycle of immersion in seawater at 15ºC for 6 hours, followed by a 6-hour period of emersion in air at 15ºC, and a subsequent 6-hour period of immersion in seawater at 15ºC. The data represent the mean $\pm$ SEM of 7 crabs previously acclimated to a non-tidal (dashed line) or to a tidal (solid line) regimen for > 4 weeks. Asterisks indicate a significant difference ($p < 0.05$) between acclimation treatments at a given time point.

Lactate concentration

There was considerable inter-individual variation in haemolymph lactate concentration in both non-tidal and tidal crabs (Fig. 2.8), and a significant interaction effect between acclimation group and time (two-way ANOVA, df = 7, F = 2.619, p = 0.0164). When the main effect of time (sampling point) was analysed separately, it was revealed that the haemolymph lactate concentration of non-tidal crabs changed significantly over the course of the experiment (one-way ANOVA, df = 7, F = 2.226, p = 0.0495), whereas it was not significantly different in tidal crabs (one-way ANOVA, df = 7, F = 1.274, p = 0.2835). Haemolymph lactate concentration increased from 1.34 mmol L^{-1} at the beginning of emersion (hour 7) to 2.22 mmol L^{-1} at the end of this period (hour 12) in non-tidal crabs (Tukey HSD, p = 0.0461); which was also significantly higher than that measured in tidal individuals at this time point (Tukey, HSD, p = 0.0018). During re-immersion the haemolymph lactate concentration of non-tidal crabs returned to pre-treatment levels (Tukey HSD, p = 0.9993), and was significantly lower than measured in tidal crabs after 3 hours of re-immersion (i.e., at hour 15) (two-way ANOVA, df = 1, F = 5.255, p = 0.0282).

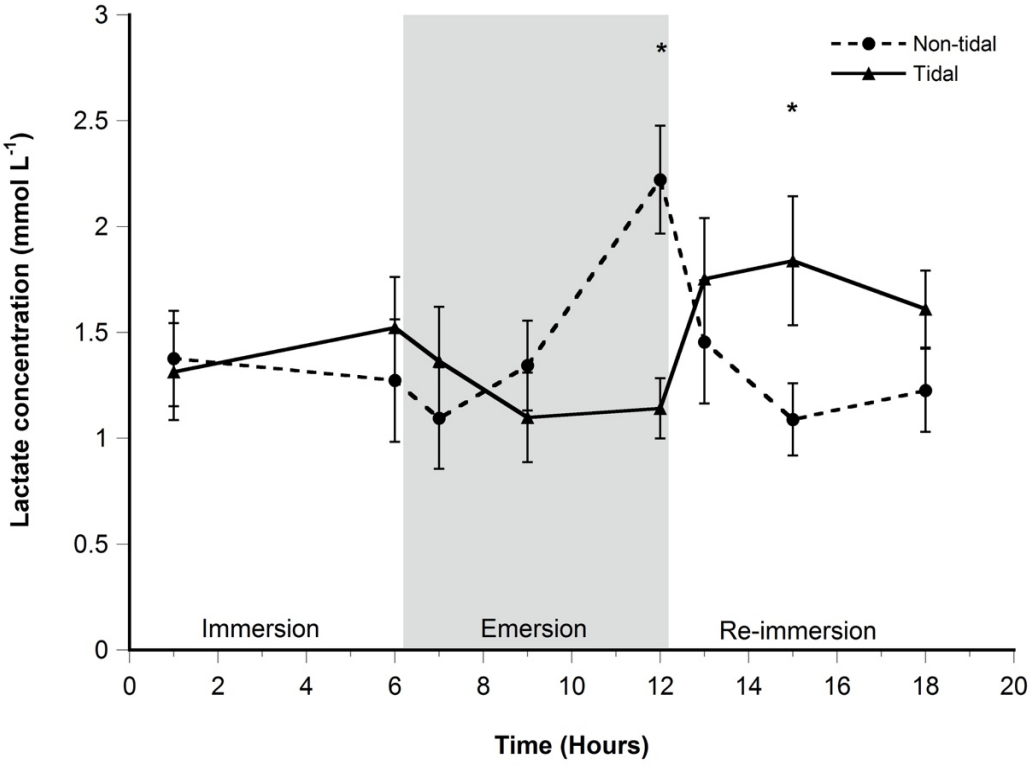


Figure 2.8. Lactate concentration of *Carcinus maenas* haemolymph during a cycle of immersion in seawater at 15ºC for 6 hours, followed by a 6-hour period of emersion in air at 15ºC, and a subsequent 6-hour re-immersion period in seawater at 15ºC. The data represent the mean $\pm$ SEM of 7 crabs acclimated to a non-tidal regime (dashed line) and 7 crabs acclimated to a tidal regime (solid line). Asterisks indicate significant ($p < 0.05$) differences between acclimation treatments at a given time point.

2.4 Discussion

Endogenous rhythm

In the present study, oxygen consumption had a periodicity of 12.35 hours in tidal crabs (Fig. 2.3), very close to that of a circatidal rhythm (~12.4 hour), with lower oxygen consumption rates during times of expected emersion (Fig. 2.3). Conversely, non-tidal crabs showed no evidence of periodicity in oxygen consumption, suggesting that acclimation to permanent submersion eliminates important stimuli that initiate circatidal rhythms. Circatidal rhythms allow intertidal organisms to anticipate and respond to predictable daily changes in tidal height (Tessmar-Raible et al., 2011; Wilcockson & Zhang, 2008), and play a key role in preparing physiological mechanisms for anticipated periods of stress (Schnytzer et al., 2018). Circatidal rhythms in various physiological parameters have been shown in a number of intertidal groups. For example, the Californian mussel *Mytilus californianus* exhibits notable metabolic rhythms during simulated tidal cycles (Connor & Gracey, 2012), and several studies report biological rhythms in locomotor activity and oxygen consumption in a variety of decapod species (e.g., Leiva et al., 2016; Naylor, 2010; Palmer, 1995). Circatidal rhythms can be entrained by a number of environmental variables such periodic inundation, changes in salinity, hydrostatic pressure, water turbulence, temperature and food availability (De la Iglesia & Hsu, 2010; Palmer, 1973; Reid & Naylor, 1990). As many of these entraining variables (e.g., salinity, food availability, and air-water temperatures) were maintained at constant levels in both acclimation treatments, periodic emersion and subsequent pressure increases associated with re-immersion were the most likely zeitgebers entraining this rhythm (Chabot et al.,

2013; Harris & Morgan, 1984; Williams & Naylor, 1969). The cyclic fluctuations in oxygen consumption of tidal crabs were most likely due to changes in locomotor activity. Tidal and non-tidal crabs showed similar levels of activity during times when tidal crabs were expected to be immersed, but tidal crabs became noticeably quiescent during periods of expected emersion, even though they were continuously immersed. Locomotor activity is considered the primary mechanism underlying changes in cardiorespiratory responses (McGaw & McMahon, 1998), and this circatidal pattern of locomotor activity, and concomitant changes in oxygen consumption, are well established in *C. maenas* (Arudpragasam & Naylor, 1964; Naylor, 1958, 1996; Warman et al., 1993). This suggests that a reduction of locomotor activity is a key behavioural response to air exposure in this species.

Responses to a simulated tidal cycle

Overall, non-tidal crabs had elevated levels of oxygen consumption (aerobic metabolism) during immersion but showed similar levels as tidal crabs during emersion (Fig. 2.4). However, non-tidal crabs experienced a decline in pHv (Fig 2.6) and an increase in lactate production (Figure 2.8), whereas tidal crabs did not utilize anaerobic metabolism and were able to maintain acid-base homeostasis during emersion. These results suggest that acclimation to permanently submerged conditions may result in the loss of important compensatory mechanisms that allow *C. maenas* to tolerate air exposure.

Routine metabolic rate and activity

Both non-tidal and tidal crabs were able to maintain oxygen consumption at approximately 50% of the rate measured during the initial period of immersion (Fig. 2.4). This is comparable to previous work on *C. maenas* under simulated field conditions (50%; Simonik and Henry, 2014), but lower than other studies with *C. maenas* (75%-120% of immersion values; Newel, 1972; Taylor & Butler, 1978). The maintenance of oxygen consumption during emersion in both non-tidal and tidal acclimated crabs was likely facilitated by the strengthened gill lamellae possessed by this species, which unlike most aquatic decapod crustaceans such as *Callinectes sapidus* (DeFur, 1988), *Cancer productus* (DeFur & McMahon, 1984) and *Homarus gammarus* (Taylor & Whiteley, 1989) do not collapse when in air, thus allowing some gas exchange to continue when emersed (Truchot, 1975). This hypothesis is supported by the values for PaO_2, which were similar to those measured during the initial period of immersion, and thus, it is likely that the decline in oxygen consumption partially due to a decline in locomotor activity, which is a typical response of aquatic decapods to emersion (see DeFur, 1988 and references therein). This behaviour has also been reported in *C. maenas* in response to emersion (Depledge, 1984; Simonik & Henry, 2014;) and was anecdotally observed with both non-tidal and tidal crabs during this study. Additionally, as previously discussed, periods of quiescence during emersion are often the predominant driver behind circatidal rhythms in oxygen consumption in this species (Arudpragasam & Naylor, 1964; Naylor, 1958, 1996; Warman et al., 1993).

Although oxygen consumption during emersion was similar between tidal and non-tidal crabs, the production of lactate only in non-tidal crabs suggests that their metabolic

demands could not be met aerobically during constant emergence, and that they had to supplement oxygen consumption during emersion with anaerobic metabolism. For example, non-tidal crabs had a higher RMR than tidal crabs (which was apparent both before and after emersion, and statistically significant for the latter, Fig. 2.4), and the capacity to transport and consume oxygen during emersion may not have been sufficient alone to support this elevated rate of metabolism. This hypothesis would be consistent with the larger drop in oxygen consumption for the non-tidal crabs (Fig. 2.4), and the reported lower metabolic demands in intertidal organisms than their subtidal (permanently submerged) conspecifics. There is evidence that acclimation to cyclic conditions can result in lower values for RMR. For example, diel fluctuating temperature regimens depress the oxygen consumption of mud crab *Panopeus herbstii* and fiddler crab *Uca pugilator* (Dame & Vernberg, 1978). In the rainbow trout (*Oncorhynchus mykiss*), exposure to diel cycling hypoxia results in lower oxygen consumption (Williams et al., 2019). Finally, in juvenile sea cucumber *Apostichopus japonicus,* a lower RMR occurred when acclimated to fluctuating temperatures, and this was proposed as a mechanism to make more energy available for growth (Dong et al., 2006). However, it is also possible that the elevated energy demand (and thus requirement for anaerobic metabolism) of non-tidal crabs was due to increased costs specifically related to emersion. In the anemone *Anthopleura elegantissima*, intertidal individuals were shown to have lower metabolic costs during air exposure in comparison to subtidal individuals (Shick, 1981; Shick & Dykens, 1984). When acclimated to intertidal conditions (continuous cycle of 7 hours immersed, 5 hours emersed) for greater than two weeks, intertidal *Mytilus edulis* reduced their daily rate of

heat dissipation (a proxy for metabolism) by 39% in comparison to only 17% in subtidal individuals during five hours of emersion (Shick et al., 1985), and this was attributed to a reduction in metabolic costs related to nitrogenous excretion (Shick et al., 1985; Shick et al., 1988). Further, while crabs from both the non-tidal and tidal groups appeared quiescent throughout the emersion period, this was not directly measured / analysed in this study and therefore possible than non-tidal crabs were more active (or agitated / stressed), which could have contributed towards the elevated metabolic costs observed in comparison to tidal individuals.

The inability of non-tidal crabs to support their metabolic costs using aerobic metabolism may also have been partially related to an issue with oxygen delivery to the tissues. For example, haemocyanin concentration was higher in the tidal crabs (Fig. 2.7), and changes in haemocyanin concentration are often observed in response to cyclic or stochastic environmental variations in oxygen concentration (Giomi & Beltramini, 2007). *C. maenas* sampled *in situ* have a higher haemocyanin concentration than those acclimated to permanently submerged conditions in the laboratory (Massabuau & Forgue, 1996), which may reflect the need for increased oxygen delivery during environmental fluctuations. Increases in the contribution of O_2 bound to haemocyanin to oxygen delivery also occur in response to both acute and prolonged exposure to increases in temperature, hypoxia and low salinity (Baden et al., 1990; Boone & Schoffeniels, 1979; DeFur et al., 1990; Gilles, 1977; Giomi & Pörtner, 2013; Hagerman et al., 1990; McMahon,1988). Further, exposure to air for three or more hours has been shown to result in a higher percentage of oxygen delivered bound to haemocyanin as compared to that dissolved in the

haemolymph (Hsia et al., 2013; Lorenzon et al., 2007; 2008; Mangum et al., 1975; Morris et al., 1996; Taylor & Whiteley, 1989). For example, oxygen delivered by haemocyanin in the intertidal crab *Hemigrapsus nudus* increases from < 50% during submersion to over 85% when exposed to air (Mangum et al., 1975; Morris et al., 1996). Even in subtidal crustaceans, such as *Homarus gammarus*, haemocyanin's participation in oxygen delivery increases when exposed to air, with 94% of oxygen delivered under this condition (Taylor & Whiteley, 1989). The dependence on haemocyanin for oxygen transport during emersion could stem from a reduced PaO_2-PvO_2 difference that can occur when gill integrity is compromised, or bradycardia, which reduces cardiac output, and thus, the amount of haemolymph that is delivered to the tissues (DeFur, 1988). Here, as PaO_2 in the tidal and non-tidal crabs was maintained at relatively high levels during emersion, and the oxygen consumption of the two groups of crabs was similar under this condition, it is unlikely that an arterial – venous PaO_2 difference led to a discrepancy in the importance of haemocyanin for oxygen transport between the two groups. However, haemocyanin levels were lower in the non-tidal crabs, and this would have resulted in a lower level of O_2 delivery overall to the tissues to meet metabolic demands. Bradycardia commonly occurs during emersion, and has been reported in several subtidal (DeFur, 1988; Truchot, 1990) and intertidal (Airriess & McMahon, 1996; Depledge, 1984; Greenaway et al. 1995) decapod species, and has been reported in *C. maenas* with comparable emersion durations (Styrishave et al., 2003; Wallace, 1972; Newell et al., 1972). Therefore, while not investigated in this study, it is possible that a more severe bradycardia in non-tidal crabs (i.e., a greater reduction in

circulatory capacity) in conjunction with lower haemocyanin levels, could have imposed a limitation on aerobic metabolism during emersion in non-tidal crabs.

pH regulation

During emersion tidal crabs were able to maintain pH_v at pre-emersion levels, whereas non-tidal crabs exhibited a progressive decline, suggesting that either tidal crabs do not experience acidosis during emersion, or they have developed adaptations to efficiently buffer pH changes. The decline in the pH_v displayed by non-tidal crabs was likely to be largely caused by the progressive accumulation of lactate in the haemolymph, particularly towards the end of emersion. As such, the absence of lactate accumulation in the haemolymph of tidal crabs aided in maintaining a constant pH during emersion. As significant levels of lactate did not accumulate in non-tidal crabs until hour 6 of emersion (Fig. 2.8), the prior decline in pH exhibited by non-tidal crabs, could be due to an increase in PCO_2. In addition to bradycardia, a respiratory acidosis would place limitations on haemocyanin function in non-tidal crabs via the Bohr effect, and therefore, could be a precursor for the initiation of anaerobic metabolism and the production of lactate to supplement aerobic respiration. Although not directly measured in this study, it is important to consider the influence of carbon dioxide (CO_2) on acid-base balance within the context of emersion. As oxygen is much more available in air than in water, but CO_2 is much harder to excrete, it has been well established that decapod crustaceans experience an increase in PCO_2 during emersion (see DeFur, 1988; Truchot, 1990 for reviews). This is due to the difficulty of excreting CO_2 across the gills in air (Truchot, 1990) and reduced

ventilation (Morris, 1991; Truchot, 1983), even when oxygen consumption and PaO_2 are maintained (Luquet & Ansaldo, 1998; McMahon et al., 1991). The increase in PCO_2 results in an increase in H^+, and thus a decrease in pH, unless buffered by HCO_3^- derived from the dissolution of $CaCO_3$ in the exoskeleton or by haemolymph proteins (Whiteley, 2011). Evidence indicates that low intertidal / subtidal species are more limited in their ability to compensate for increased PCO_2 during emersion than species that experience regular exposure to air. For example, the velvet crab (*Necora puber*; a low intertidal / subtidal species) has a limited capacity to mobilise HCO_3^-, (Rastrick et al., 2014), and *C. maenas* acclimated to submerged conditions take 100 hours to compensate for PCO_2 increases during emersion (Truchot, 1975). Conversely, intertidal species such as the purple rock crab (*Leptograpsus variegatus*) and *Neohelice granulata* (previously *Chasmagnathus granulata*) can better regulate PCO_2 levels and restore pH within 1-2 hours of air exposure (Luquet et al., 1998; Butler & Morris, 1990). This is likely facilitated by elevated carbonic anhydrase (CA) activity in the gills, which has been shown to be 10-fold higher in intertidal crustacean species in comparison to subtidal species (Henry et al., 1984), and shows circatidal rhythmic activity, with higher activity during low tide (Connor & Gracey, 2012). Additionally, as haemocyanin makes up the majority of the protein component of crustacean plasma (Pascual et al., 2003) it is an important buffer of pH changes (Whiteley, 2011; Rastrick et al., 2014). Therefore, it is possible that while both tidal and non-tidal crabs experienced a rise in PCO_2 at the onset of emersion, tidal crabs can limit haemolymph pH changes due to elevated levels of haemocyanin and CA activity, and thus, its effects on haemocyanin affinity during emersion.

Haemocyanin mechanism

The underlying mechanism promoting the elevated haemocyanin concentration in tidal crabs during acclimation to cyclic emersion could be hypoxia inducible factor (HIF-1), which has shown to be upregulated during emersion in the swimming crab *Portunus trituberculatus* (Lu et al., 2016). HIF-1 is an important transcription factor that acts as a global regulator of oxygen homeostasis (Semenza, 1999). HIF-1 has also been identified as the primary regulator behind the increased synthesis of haemoglobin in *Daphnia* (Gorr et al., 2004; Hoogewijs et al., 2007) and haemocyanin in *Cancer magister* (Head et al., 2010) during hypoxia, as well as selectively upregulating subunits that have a high affinity for oxygen (Head et al., 2010). Moreover, HIF-1 is one of the main factors coordinating anaerobic glycolysis (Discher et al., 1998; Hochachka & Lutz, 2001; Murphy et al., 1999). Based on the above, it appears that HIF-1 could be a key coordinator of physiological responses to acute and chronic emersion responses in *C. maenas*, and assessing its role during emersion could provide valuable information towards understanding the physiological responses of intertidal organisms to emersion.

Lactate release dynamics

Although oxygen consumption and lactate levels returned to pre-treatment levels on re-immersion, haemolymph lactate levels were slightly (but not significantly) higher in tidal crabs and this could suggest that non-tidal and tidal crabs use different mechanisms, or have different capacities, to regulate oxygen consumption and lactate production during emersion. The crayfish *Austropotamobius pallipes* exhibits elevated lactate levels during

re-immersion after a 24-hour emersion period (Taylor & Wheatly, 1981; Jackson et al., 2001). This is due to the crayfish sequestering lactate and protons in the calcified skeleton (Jackson et al., 2001), which results in a reduction of lactate in the haemolymph during emersion. Upon re-immersion, there is a lactate wash-out into the haemolymph, that results in elevated haemolymph lactate levels. A similar phenomenon may have occurred in the present study, with lactate produced by tidal crabs being sequestered during emersion but released into the haemolymph during re-immersion. Sequestration by the exoskeleton could also aide in acid-base buffering capacity by the formation of calcium and bicarbonate from calcium carbonate and hydrogen (Jackson et al., 2001).

Conclusions

This study showed that *C. maenas* acclimated to simulated tidal conditions respond differently to emersion than crabs acclimated to permanently submerged conditions. For example, my data shows that while oxygen consumption is similar in tidal and non-tidal crabs during emersion, the non-tidal crabs have higher haemolymph lactate levels and a lower pH_V during emersion and an elevated oxygen consumption when re-immersed. Although this study cannot conclusively identify the contributions of various factors / mechanisms to the above differences, it is probable that both behavioural (lower activity, and thus RMR) and physiological (better regulation of PCO_2, elevated haemocyanin levels, differences in HIF- 1 expression, buffering of plasma lactate and haemolymph pH levels) factors contributed to the differences between groups.

Importantly, the results of this study on *Carcinus maenas* supports previous studies showing that caution must be taken when acclimating intertidal organisms to laboratory conditions. Although acclimation to constant conditions allows us to associate physiological responses with a particular stressor, it also risks abolishing important physiological responses and adaptations that may play a critical role in physiological performance of organisms *in situ*. With respect to intertidal studies, the cyclic exposure to air is a predictable (daily) occurrence, and it is clearly an important modulator of the physiology of intertidal animals. Moving forward, it is paramount that organisms are exposed to realistic ecological scenarios that incorporate a multifaceted design within the laboratory environment if we are to gain an accurate understanding of how these species respond *in situ.*

With regards to the mechanisms that may enable tidal crabs to better balance aerobic metabolism vs. energy demands during emersion, the potential role played by HIF-1 is particularly interesting, and may provide insights into the roles played by this important molecule in regulating the energy available for long-term performance in terms of growth, reproduction and survival. Such information will be essential to understanding how additional environmental stressors impact the physiological limits and population shifts of intertidal organisms.

Chapter 3. The role of tidal acclimation in the physiological responses of *Carcinus maenas* to hypoxia and thermal stress

3.1 Abstract

Animals inhabiting the intertidal zone are exposed to abrupt changes in environmental conditions associated with the rise and fall of the tide. For convenience, the majority of laboratory studies on intertidal organisms have acclimated individuals to permanently submerged conditions, and thus we currently know little about how acclimation to cyclic emersion-immersion that occurs in the intertidal influences the ability of intertidal organisms to cope with fluctuations in environmental conditions. In the present study, the intertidal green crab *Carcinus maenas* was acclimated to either a simulated tidal regime of continuous emersion-immersion (tidal) or to permanently submerged conditions (non-tidal). Oxygen consumption was assessed when crabs were submersed and exposed to hypoxia (100% - 20% of normoxia), cooling (15°C - 5°C) or warming (15°C - 25°C) over 5 hours and during 12 hours of recovery. Tidal crabs generally had a lower oxygen consumption at their acclimation temperature (15°C) and during recovery from hypoxia. However, whether a crab was exposed to immersion or cyclical immersion-emersion did not have an effect on its response to acute hypoxia. During acute thermal stress, the Q_{10} values for oxygen consumption of non-tidal crabs increased as temperature declined to 5°C, whereas tidal crabs maintained relatively consistent Q_{10} values across the temperature range (5°C-25°C). These results show that non-tidal and tidal crabs had different physiological responses to acute changes in water oxygen level and temperature. Further,

they suggest that while acclimation to a simulated tidal cycle may result in different physiological responses to environmental stressors, anticipation of emersion has few effects on these responses during submersion.

3.2 Introduction

The intertidal zone is one of the most intensively studied ecosystems (Paine, 1974; Helmuth et al., 2006a). Large fluctuations in abiotic parameters that occur during the ebb and flood of the tide, such as temperature and oxygen availability, provide a unique opportunity to study the physiological and ecological responses of organisms to environmental stressors (Denny et al., 2011). Many of these organisms function at the limits of their physiological tolerance and regular exposure to fluctuating conditions means that these organisms can be useful indicators of the impact of climate change (Barry et al., 1995; Harley et al., 2006; Helmuth et al., 2006b; Mieszkowska et al., 2006; Sagarin et al., 1999; Thompson et al., 2002, 2004).

Organisms that inhabit the intertidal zone are almost exclusively marine in origin, subsequently, the major stressor that they have to adapt to is the shift from an aquatic to an aerial respiratory medium. The dehydrating properties of air promote desiccation which can have adverse effects on metabolic performance. For example, body water loss can lead to difficulties in ion regulation and nitrogen excretion (Truchot, 1990), while desiccation of gas exchange surfaces can reduce or inhibit aerobic respiration (Burnett & McMahon, 1987; DeFur, 1988; McMahon, 1988; Truchot, 1990). Furthermore, emersion is often coupled with an inability to effectively excrete carbon dioxide which can result in hypercapnia and respiratory acidosis (Truchot, 1990). On a cellular level, the internal

hypoxia that often occurs during emersion results in the induction of hypoxia-inducible factors (HIFs) to help regulate metabolism, as well as heat-shock proteins (HSPs), inhibitors of apoptosis proteins and endoplasmic reticulum chaperones to ensure that cell homeostasis is maintained (Kawabe & Yokoyama, 2009; Lu et al., 2016; Zhang et al., 2012; Zhang et al., 2016). Moreover, emersion can induce an increase in the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Yin et al., 2017) to protect against free radical damage during re-immersion.

In addition to the risk of desiccation, the transition between aquatic and aerial environments often results in rapid and large-scale fluctuation in temperature, salinity and oxygen availability (Gunderson et al., 2016; Helmuth et al., 2010). In temperate environments, daily temperature fluctuations typically exceed 20ºC as the tide ebbs and floods (Fangue et al., 2006). Crevices and tidepools can often provide refuge for intertidal animals seeking to avoid desiccation (Fangue et al., 2006). However, these tidepools can also experience large daily variations in oxygen availability (2 - 435 mm Hg) due to imbalances in the rate of photosynthesis vs. respiration (Truchot & Jouve-Duhamel, 1980; Richards, 2011), and thus, animals residing here can experience alternating periods of hyperoxia, hypoxia and related pH changes.

Due to the complexity of this environment, and the difficulty in investigating the integrative effects of these stressors *in situ,* much of our understanding of the physiology of intertidal organisms to environmental stressors has come from laboratory experiments where individuals are submerged and acclimated to constant conditions. Despite being sound scientific practice that has provided a wealth of valuable information with regards

to our understanding of animal physiology (e.g., Bertness et al., 2001; Denny et al., 2011; Helmuth et al., 2006a; Pörtner 2001; 2002; 2010; Tomanek & Helmuth, 2002), such conditions are not representative of the cyclical conditions that intertidal organisms experience in nature, and acclimation to fluctuating or cyclic conditions often results in different physiological phenotypes (Todgham et al., 2006). For example, acclimation to a normal tidal cycle results in the depression of oxygen consumption in the blue mussel *Mytilus edulis* and the crab *Panopeus herbstii* when they are subjected to a subsequent increase in temperature as compared to individuals that were constantly submerged. This reduction in oxygen consumption may allow for more scope for growth in comparison to individuals acclimated to constant conditions (Dame & Vernberg, 1978; Widdows, 1976). Likewise, acclimation to a cyclical thermal regime results in an increase in thermal tolerance in the coral *Acropora hyacinthus* (Oliver & Palumbi 2011). This phenomenon, however, is not unique to the effects of temperature. Juvenile qingbo (*Spinibarbus sinensis*) acclimated to diel-cycling hypoxia (12 hours at 130 mm Hg, 12 hours at 55 mm Hg cycle) have a lower P_{crit} (critical oxygen tension), and subsequently increased tolerance to hypoxia, than fish acclimated to constant moderate hypoxia (93 mm Hg) (Dan et al., 2014). Additionally, spot (*Leiostomus xanthurus*) acclimated to diel-cycles of hypoxia show a higher expression of hypoxia inducible factor-1 (HIF-1) than individuals acclimated to constant hypoxia (Smith et al., 2012). This data suggests that acclimation to permanently submerged conditions (as usually occurs in controlled lab conditions) instead of a cyclic regime of immersion and emersion could impair the physiological performance and

environmental tolerance of intertidal organism, and thus, may lead to misleading or inaccurate conclusions with regards to how these organisms respond *in situ*.

Experimental evidence also suggests that exposure to an initial stressor can confer increased tolerance to a different, subsequent stressor; an effect known as cross tolerance (Todgham & Stillman, 2013). This association is based on the initiation of similar mechanisms of cellular protection to a number of stressors (Kültz, 2005). For instance, exposure to a 12ºC heat shock conferred increased tolerance to severe hypoxia and osmotic shock in tidepool sculpin (*Oligocottus maculosus*), and it was suggested that this could be due to the priming of heat-shock proteins (HSPs) (Todgham et al., 2005). Chronic acclimation to particular stressors can produce similar physiological adjustments, and these can increase aerobic scope (Giomi & Pörtner, 2013). For example, acclimating *Callinectes sapidus* to either low salinity (Mason et al., 1983) or hypoxia (DeFur et al., 1990) results in an increase in haemocyanin concentration, and this suggests that exposure to low salinity could confer tolerance to hypoxia and vice-versa. As environmental stressors tend to be more pronounced during periods of low tide (Bjelde & Todgham, 2013), tolerance to emersion has been shown to influence physiological responses to both hypoxia and heat stress (e.g., Altieri 2006, Drake et al., 2017; Roberts et al., 1997). When subtidal *Mytilus edulis* are transplanted into the intertidal zone they gain increased tolerance to hypoxia in comparison to individuals that remain constantly submerged (Altieri, 2006). This increased tolerance is thought to be due to physiological adjustments induced by long-term (weeks of) cyclic exposure to air, including switching to alternative anaerobic pathways, changes in the regulation of key metabolic enzymes (e.g., pyruvate kinase), a lowered basal

metabolic rate, and possibly higher mitochondrial content (Altieri, 2006; Demers & Guderley, 1994; De Zwaan & Wijsman 1976; Shick et al., 1988; Sukhotin & Pörtner, 1999; Widdows & Shick 1985). Consequently, intertidal congeners often have increased thermal and hypoxia tolerance compared with their subtidal counterparts (Alteri, 2006; Stillman & Somero, 2000).

To date, the majority of studies assessing the importance of tidal acclimation on tolerating other stressors has primarily focused on sessile species, and less is known about this concept in mobile intertidal organisms. Small decapod crustaceans are common inhabitants of the intertidal zone and play crucial roles in determining community structure. The green shore crab *Carcinus maenas* is native to Europe, but has become a successful marine invader with a global distribution because of its tolerance of environmental perturbations. Accordingly, it has become a model organism to investigate the physiological responses of marine organisms to environmental stressors (Hyde et al., 2012; Klassen & Locke, 2007; Leignel et al., 2014). Given the importance of the tidal cycle in influencing responses to other environmental stressors as highlighted in previous literature, coupled with the evidence of physiological adjustments in tidal acclimated green shore crabs (*Carcinus maenas*) shown in Chapter 2, I was interested to see if *C. maenas* acclimated to a tidal regime would exhibit different physiological responses with regards to hypoxia and thermal stress, typical stressors for this species in the intertidal. Furthermore, as acclimation to a tidal regime develops a metabolic endogenous rhythm (see Chapter 2), I was interested to see if timing of hypoxic or thermal stress in relation to anticipated medium conditions (air or water) influenced the metabolic response to such

stressors. I hypothesised that *C. maenas* acclimated to a tidal regime would be more tolerant to hypoxic and thermal stress due to a more efficient oxygen delivery system and better buffering of the acid base balance, and this would be most pronounced when subjected to stress in synchronisation with their acclimation regime. If differences are found between crabs exposed to immersion vs. cycles of submersion-emersion, this questions whether physiological responses derived from previous laboratory experiments are accurate representations of the responses that intertidal organisms exhibit *in situ*. Such information will be critically important for predicting shifts in the environmental tolerances, and the population distributions of species, in the era of global climate change.

3.3 Materials and methods

3.3.1 Sampling site and collection

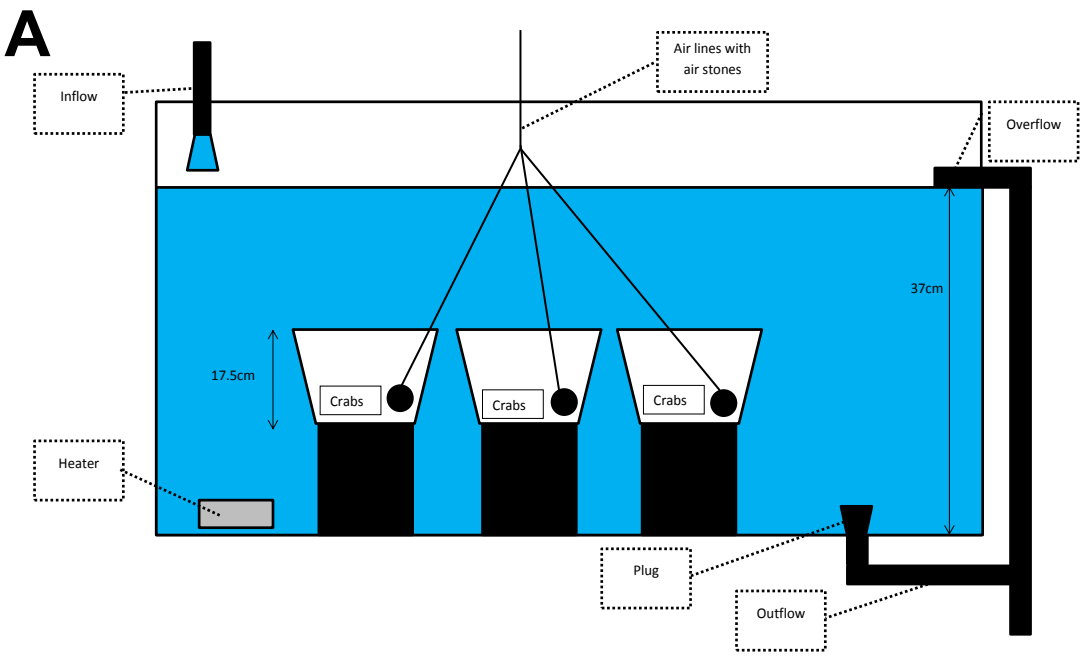
Large adult male *C. maenas* (carapace width > 50 mm) were collected at Fox Harbour, Long Harbour and Fairhaven (Newfoundland (NL), Canada) using dome crab traps between September and November 2015, and June and November 2016. Only crabs without carapace damage or missing chelae were used in experiments. Females were not taken due to permitting regulations in Newfoundland. The crabs were transported to the Department of Ocean Sciences, Memorial University, NL, Canada, where they were placed into holding tanks receiving unfiltered, aerated, seawater at ambient temperature (approximately 3°C – 12°C) and with a salinity of 32‰, and fed mackerel once a week (any uneaten food removed after two days). Perforated plastic pipes (diameter, 10 cm; length, 20 cm) were placed in the holding tanks to discourage aggressive behaviour amongst conspecifics.

3.3.2 Experimental holding conditions

The crabs were transferred to two separate flow through seawater tanks (155 cm x 95 cm x 50 cm deep, 1000 L) where they were held in perforated containers (37 cm x 40 cm x 17.5 cm deep, 30 L). An air stone was placed in each container to ensure that the water was fully saturated with oxygen, the temperature of both tanks was maintained at 15°C (± 0.5°C) via an in-tank heater, and salinity was constant at 31-32‰. A temperature of 15°C was used as it is the optimum temperature for growth and normal physiological function in *C. maenas* (Robertson et al., 2002). The crabs were acclimated to either non-tidal (control) or tidal (experimental) conditions for at least 4 weeks prior to experimentation. The non-tidal tank was constantly provided with unfiltered seawater at a rate of 2.2 L/min. Crabs were kept submerged for 24 hours a day (Fig. 3.1), which represented the conditions that intertidal organisms are typically maintained for laboratory experiments.

The tidal tank had a similar flow rate, but the water level of the tank was controlled to replicate a semi-diurnal, six-hour, tidal regime (alternating periods of six hours immersed and six hours emersed). A semi-diurnal tide was deemed the most appropriate as it is the most common tidal pattern in their natural environment (Little & Kitching, 1996). This was achieved by manipulating the water level via a timer-controlled solenoid valve connected to the tank outflow. When off, the solenoid valve closed the outflow, allowing the inflow to slowly fill up the tank, submerging the crabs. When on, the valve opened, allowing the water to gradually drain and exposing the crabs to air (Fig. 3.1). The air temperature was controlled at a similar temperature to the water (15°C ± 0.5°C) via an

air conditioning unit. Humidity during emersion was monitored using a hygrometer (11-661-16, Fisher Scientific, USA) and varied between 70-80%. Animals were held in constant dim red light to reduce any endogenous cycles associated with photoperiod. Prior to experimentation, the crabs were fasted for 3-4 days to ensure digestive processes did not affect physiological readings (McGaw, 2006; Robertson et al., 2002). Individual crabs from the non-tidal and tidal group were matched as closely as possible with respect to size, mass and colour morph (Styrishave et al., 2004).



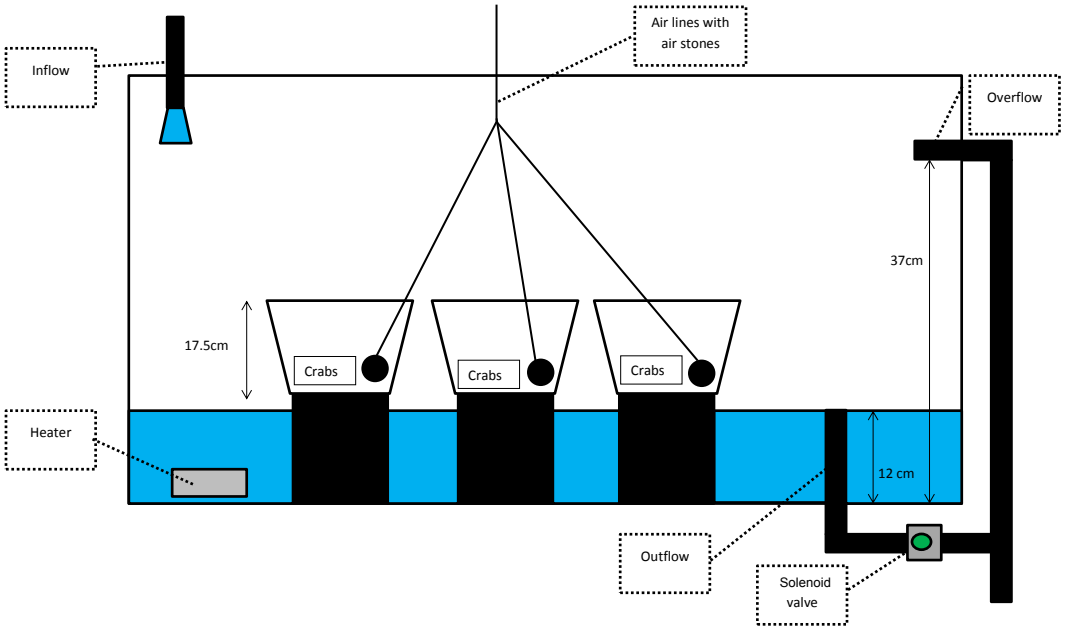


Figure 3.1. (A) Control (non-tidal) holding conditions. Crabs were permanently submerged. (B) Experimental (tidal) holding conditions. An inflow provides the tank with fully oxygenated flow through seawater. The height of the water is controlled by a timer-controlled solenoid valve which opens / closes every 6 hours, either exposing the crabs to air or immersing them.

3.3.3 Experiments

Hypoxia

To establish if acclimation to periodic emersion affected the ability of *C. maenas* to cope with hypoxic conditions, oxygen consumption was measured for both the non-tidal and tidal crabs (n = 10 per group) subjected to acute hypoxia when submerged. Crabs were transferred directly from their holding tanks to plexiglass respirometry chambers maintained at 15ºC and 32‰, and the recording of oxygen consumption began immediately. This was because the specific timing of the air / water exposure in the tidal acclimation tank prevented acclimation to the respirometry chambers prior to recording

oxygen consumption. An effort was made to assess whether this handling response may have had any effect on oxygen consumption, and a detailed method for how this transfer was carried out can be found in A.1 of the appendix. Similarly to Chapter 2, the raw oxygen consumption values were adjusted to remove the effects of handling stress on oxygen consumption, which was calculated through preliminary tests outlined in Appendix A.1.

Crabs from both acclimation groups were then exposed to progressive hypoxia. The oxygen concentration of the water was controlled using an oxygen analyser and control system (OX10000, Loligo Systems, Tjele, Denmark). The system consists of a control unit connected to a galvanic oxygen probe which monitors the oxygen partial pressure of the seawater. The control unit monitors the oxygen level in the water and opens / closes solenoid valves connected to nitrogen or air cylinders if the dissolved oxygen level deviates from the desired set point. The oxygen level of the water was decreased in increments of 40% air saturation (i.e., from 100% to 60% and then 20% air saturation) over a period of an hour. It was then maintained at each concentration for an hour during which time the oxygen consumption rate of the crabs was measured. Following this, oxygen levels were returned to 100% saturation (over an hour) and oxygen consumption was measured during a 12-hour recovery period in normoxia (15ºC, 32 ‰). This meant that the overall time the crabs were exposed to the hypoxia treatment was 5 hours with an additional hour to return to normoxic conditions.

To understand whether the endogenous rhythm developed by tidal crabs (Fig 2.2; 2.3) influenced the response to hypoxia, non-tidal and tidal crabs were subjected to progressive hypoxia at the time of anticipated immersion in the tidal acclimation regime

(referred to as HI), and then repeated with other individuals at the time of anticipated emersion (HE).

Oxygen consumption (MO_2) was measured using an L-DAQ intermittent flow respirometry system (Loligo Systems, Tjele, Denmark). The system consisted of 4 identical cylindrical chambers (20 cm diameter x 12 cm depth), which were submerged in a shallow tank (155 cm x 95 cm x 50 cm deep, 1000 L). Each chamber was equipped with two pumps. The first pump continually flushed seawater through the chamber while it was open. The chamber was automatically sealed by the computer for MO_2 measurements, and a second pump recirculated the water through the chamber at a rate of 5 L min^{-1}. A total of four replicates were used at any one time (two from each treatment), with one crab in each chamber. Experiments were carried out in constant dim red light and black plastic sheeting surrounded the apparatus to avoid visual disturbance to the animal. Oxygen consumption was calculated at 1-hour intervals; a reading was taken whilst the chamber was sealed for 30 minutes, then the chamber was continuously flushed with fresh hypoxic seawater. Data was recorded with the data acquisition system AutoResp 4 (v 1.7) (Loligo Systems, Tjele, Denmark) which calculated oxygen consumption as mg O_2 kg^{-1} hr^{-1} using the following equation:

$$\dot{M}O_2 = V\left(\frac{\Delta PO_2}{t}\right) \cdot \alpha \cdot MB$$

Where V is the volume of the respirometry chamber minus the volume of the crab (L), $\Delta PO_2/t$ is the change in oxygen partial pressure (kPa) per unit time, α is the solubility

coefficient of oxygen in water (salinity of 32‰, 15.0°C) in mg O_2 kPa^{-1}, and *MB* is the body mass of the crab (kg).

Thermal stress

In another series of experiments, oxygen consumption was measured for both non-tidal and tidal crabs (n = 10 per group) subjected to either an acute increase or decrease in water temperature. The crabs were transferred from their holding tank to the plexiglass respirometry chambers mentioned above using the method detailed in A.1 of the appendix and recording started immediately. The temperature of the tank was manipulated via in-tank heaters, and increased or decreased by 5ºC (over a period of an hour) and held at each temperature for an additional hour while the MO_2 of the crabs was recorded. In the first experiment, the crabs were subjected to an acute decrease in temperature, and oxygen consumption was recorded at 15ºC, 10ºC and 5ºC. In the second experiment, a separate set of animals were subjected to an acute increase in temperature and MO_2 was recorded at 15ºC, 20ºC and 25ºC. Following both experiments, the temperature was restored to the initial temperature of 15ºC (over an hour) and oxygen consumption was measured during a 12-hour recovery period (at normoxia, 32‰ salinity). As with the hypoxia experiment, the temperature increase or decrease occurred over a period 5 hours. This allowed the experiments to be performed at times when tidal crabs expected to be immersed in the tidal acclimation regime and when tidal crabs expected to be emersed (the latter using different individuals). This created four separate experimental scenarios: acute cooling during anticipated immersion (CI), acute cooling during anticipated emersion (CE), acute

warming during anticipated immersion (WI), acute warming during anticipated emersion (WE). Oxygen consumption was measured using the experimental apparatus described in the hypoxia experiment, and was calculated as described in Chapter 2. However, the oxygen solubility coefficient was adjusted to match each experimental temperature. Similarly to Chapter 2 and the hypoxia experiment, the raw oxygen consumption values were adjusted to remove the effects of handling stress on oxygen consumption, which was determined in the preliminary tests outlined in Appendix A.1.

The temperature coefficient (Q_{10}) for oxygen consumption was determined for all individuals at every 5°C interval (5°C – 10°C, 10°C - 15°C, 15°C - 20°C, 20°C - 25°C) for both acclimation groups during each of the four scenarios. Q_{10} was determined using the equation:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10°C}{(T_2 - T_1)}}$$

Where R_1 and R_2 are the oxygen consumption rate at the first (T_1) and second (T_2) temperatures, and Q_{10} was reported as a mean value of all individual determinations.

The thermal stress experiments revealed interesting differences between the tidal and non-tidal crabs with regards to MO_2, particularly during expected emersion. Therefore, measurements of parameters important for haemolymph oxygen delivery [arterial partial pressure of oxygen (PaO_2), haemocyanin concentration] and those indicative of anaerobic metabolism [venous pH (pHv), lactate concentration] were measured to try and understand what physiological mechanisms might be mediating these differences. In this final series of experiments, separate crabs (from the non-tidal and tidal groups) were subjected to a

temperature increase as outlined above and haemolymph samples were collected at 15°C, 20°C, 25°C and hour 1, 2, 6 and 12 of recovery. To avoid adversely affecting animals by the repeated collection of large samples, separate animals (n = 7 per acclimation group) were used at each time point.

For PaO_2 measurement, at least three days before sampling, a small hole was drilled directly over the heart which pierced through the carapace but left the pericardial membrane intact. A section of dental dam was placed over the hole and secured with cyanoacrylate glue. During sampling, an arterial blood sample (400 μL) was taken by piercing the dental dam and the pericardial membrane with a 1 mL airtight Hamilton syringe and a 21-gauge needle. Samples were taken within 30 seconds of removing the animal from the apparatus. Approximately 200 μL of arterial haemolymph was then injected below a layer of mineral oil in an Eppendorf® tube and immediately transferred to a water bath. As *C. maenas* is an ectotherm, the temperature of the water bath was adjusted throughout the experiment to parallel the changes in temperature the crabs were exposed to in the experimental system. PaO_2 was measured using a pre-calibrated Fibox-3 O_2 analyser (PreSens, Regensburg, Germany). The dipping probe was inserted into the sample and readings were taken once PaO_2 had stabilised (after 3 minutes) using OxyView software (PreSens, Regensburg, Germany) running on a laptop (Dell Inspiron). Immediately after arterial haemolymph was collected (for PaO_2 analysis), approximately 400 μL of venous haemolymph was withdrawn from the same crab from between the arthrodial membrane at the base of a walking leg. A 200 μL sample was injected below a layer of mineral oil in an Eppendorf® tube and transferred immediately to a water bath set

to the temperature of the experimental system. The venous pH (pH_v) was measured using a pH mini-V2 analyser (PreSens, Regensburg, Germany) and a probe calibrated using colourless pH reference buffers (Ricca Chemical Company, Arlington, Texas, USA). To measure pH, the dipping probe was inserted into the sample and readings began once levels had stabilised (3 minutes) using pH 1-view software (PreSens, Regensburg, Germany). The remaining 200 µL aliquots of arterial and venous haemolymph were transferred to Eppendorf® tubes and placed on ice before being transferred to a -80°C freezer for later measurement of haemolymph haemocyanin and lactate levels.

Haemocyanin concentration was determined by spectrophotometry using a Spectramax M5 multimode microplate reader (Molecular Devices, California, USA). Arterial haemolymph was thawed at room temperature, then vortexed for 5 seconds to evenly distribute the protein. A 1:20 dilution was then made with distilled water and vortexed for a further 5 seconds (n = 7 per time period). Haemocyanin concentrations were estimated using the Beer – Lambert law from peak absorbance at 335 nm, using an extinction coefficient of 17.5 mmol L^{-1} cm^{-1}, based on the specific absorbance value ($A_{1\%,\ 1\ cm}$) of 2.33 reported for *C. maenas* (Nickerson & Van Holde, 1971) and a molecular mass of 75·kDa.

Lactate concentration was determined from an assay adapted from Clow et al. (2016) using thawed venous samples. Samples were deproteinized using 6% perchloric acid at a dilution of 1:10 (n = 7 per time period). The samples were then mixed and centrifuged at 10,000x g for 10 minutes. The subsequent supernatant was then extracted, and 25 µL of this extract was added to 200 µL of assay medium containing glycine buffer

(Sigma, G5418) and 2.5 mmol L^{-1} NAD^+, pH 9.0. Absorbance was measured at 340 nm using a DTX 880 microplate reader (Beckman Coulter, Ontario, Canada) before the addition of 10 IU mL^{-1} of L-lactic dehydrogenase (Sigma, L2500). Absorbance was read after 30 min or after it stabilised, and compared with a standard curve to determine the sample's lactate concentration.

3.3.4 Statistical analyses

All data passed tests of normality, independence and homogeneity, apart from the lactate data which was square root transformed. Differences in MO_2 during hypoxia, acute warming and acute cooling were identified using separate three-way repeated measures (RM) ANOVAs, with temperature, acclimation group and timing of exposure (in relation to tidal acclimation regime) as factors. PaO_2, pHv, lactate and haemocyanin concentration were analysed using separate two-way ANOVAs, with temperature and acclimation group as factors. Differences in MO_2 during recovery from hypoxia and thermal stress were measured using separate three-way RM ANOVAs with time (hour), acclimation group and timing of exposure as factors whereas PaO_2, pHv, lactate and haemocyanin concentration were analysed using separate two-way ANOVAs. Differences in Q_{10} values between acclimation groups under the four thermal scenarios were examined using a three-way ANOVA with temperature, acclimation group, and timing of exposure as factors. To understand if hypoxia or thermal stress resulted in a prolonged change in physiological parameters, initial 15°C values were compared to recovery values for all parameters measured. This was done using a one-way RM ANOVA for MO_2 values, and a one-way

ANOVA for PaO_2, pHv, lactate and haemocyanin values. One-way ANOVAs, or Tukey HSD post-hoc tests were used when applicable to identify significant differences between factors. The statistical analyses were carried out using GraphPad Prism software (version 8.03 for Windows, GraphPad, La Jolla California USA, www.graphpad.com). In all cases, a p value of < 0.05 was utilised as the criteria for statistical significance.

3.4 Results

3.4.1 Oxygen consumption in response to acute hypoxia

Initial oxygen consumption at normoxia (100% air saturation) during the HI treatment was 49.06 ± 2.40 mg O_2 kg^{-1} hr^{-1} for non-tidal and 42.09 ± 2.06 mg O_2 kg^{-1} hr^{-1} for tidal crabs, whereas during the HE treatment MO_2 was 49.45 ± 4.61 mg O_2 kg^{-1} hr^{-1} for non-tidal crabs and 35.31 ± 2.58 mg O_2 kg^{-1} hr^{-1} for tidal crabs. Exposure to decreasing water oxygen levels resulted in a stepwise decline in oxygen consumption in both non-tidal and tidal crabs (Fig. 3.2) (three-way RM ANOVA, df = 2, F = 54.7, p = < 0.0001) with the MO_2 of non-tidal crabs dropping to 29.87 ± 2.52 mg O_2 kg^{-1} hr^{-1} (HI) and 27.60 ± 1.62 mg O_2 kg^{-1} hr^{-1} (HE), and tidal crabs dropping to 25.77 ± 1.80 mg O_2 kg^{-1} hr^{-1} (HI) and 21.86 ± 1.72 mg O_2 kg^{-1} hr^{-1} (HE), at 20% air saturation. During this decline, tidal crabs displayed a consistently lower MO_2 (three-way RM ANOVA, df = 1, F = 13.6, p = 0.0008). There was no difference in MO_2 values, or their response to hypoxia, between crabs tested during anticipated periods of immersion and emersion (three-way RM ANOVA, df = 1, F = 1.796, p = 0.1886).

Although non-tidal crabs appeared to have an elevated MO_2 during the first hour of recovery compared to initial (100% air saturation) values, this was not statistically

significant (HI: one-way RM ANOVA, df = 12, F = 1.524, p = 0.234; HE: one-way RM ANOVA, df = 12, F = 0.5972, p = 0.6659). The oxygen consumption of tidal crabs subjected to the HI treatment also returned to initial values by hour one of recovery (One-way RM ANOVA, df = 12, F = 1.184, p = 0.3344). However, tidal crabs from the HE treatment had elevated oxygen consumption (from initial 15°C and 100% air saturation) for the first two hours of recovery (One-way RM ANOVA, df = 12, F = 1.881, p = 0.0496; Tukey HSD, hour 1: p = 0.0245; hour 2: p = 0.0391). During recovery, tidal crabs had significantly lower oxygen consumption rates compared with non-tidal crabs (three-way RM ANOVA, df = 1, F = 13.63, p = 0.0007). There was also a significant difference over time (three-way RM ANOVA, df = 11, F = 2.606 p = 0.0297), with oxygen consumption at hour one elevated above hour two to five of recovery in tidal crabs (Tukey HSD, $p < 0.05$ for all). There was no difference in oxygen consumption during recovery from hypoxia between crabs tested during periods of anticipated immersion vs. emersion (three-way RM ANOVA, df = 1, F = 0.1096, p = 0.7426).

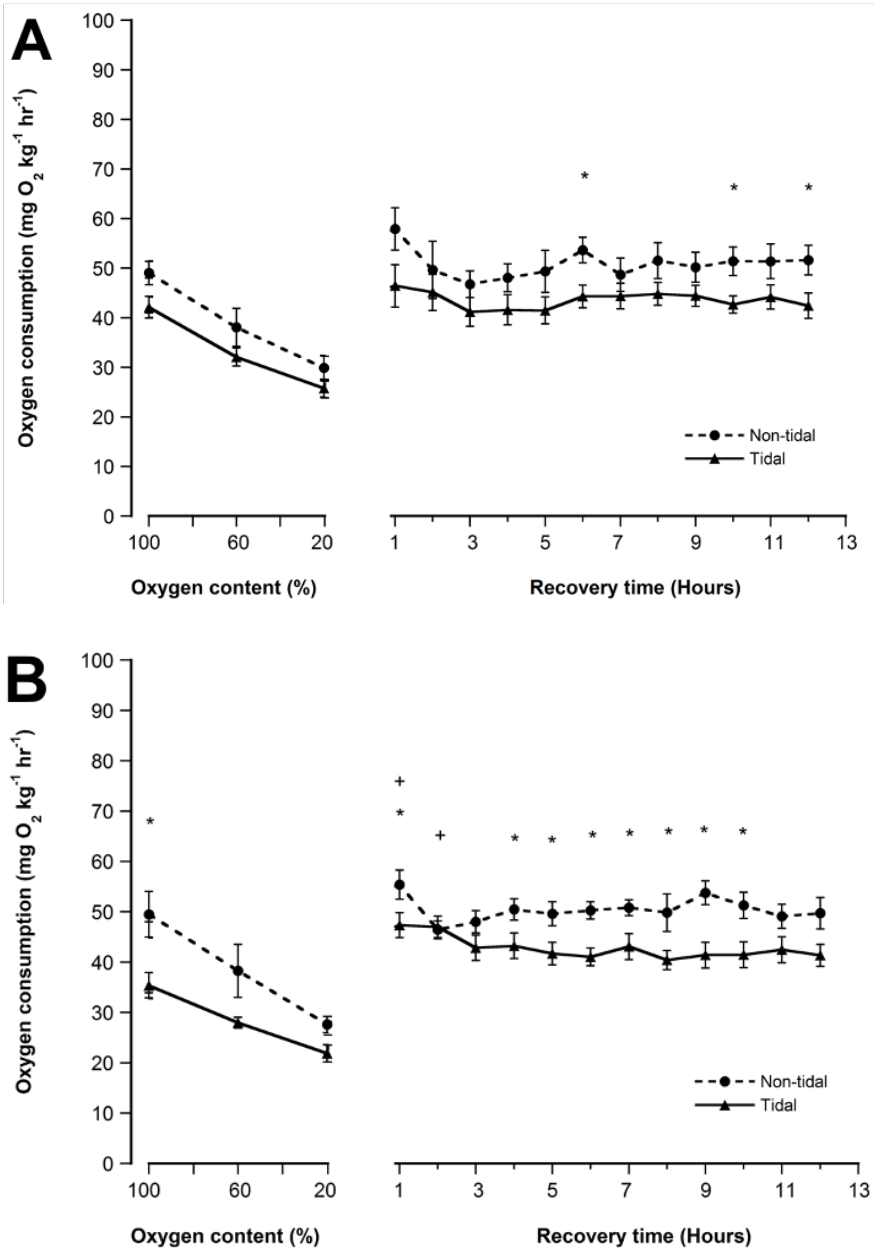


Figure 3.2. Oxygen consumption of submerged *Carcinus maenas* at 15ºC that were subjected to a decrease in seawater oxygen content (100 - 20% of air saturation) over 5 hours at times corresponding to immersion (A) and emersion (B) in the tidal crabs, followed by a 12-hour recovery period in normoxic seawater. The data are means ± SEM values for 10 crabs acclimated to non-tidal and tidal regimes. Asterisks (*) indicate significant differences between acclimation treatments ($p < 0.05$) at a particular oxygen level or time point during recovery. Plus sign (+) indicates significant difference between MO_2 at initial 15°C and MO_2 at a particular hour of recovery in tidal crabs.

3.4.2 Physiological response to acute temperature change

Oxygen consumption during cooling

The initial MO_2 (at 15°C) of non-tidal crabs was similar between the CI and CE treatments; 52.82 ± 1.53 mg O_2 kg^{-1} hr^{-1} and 51.55 ± 3.15 mg O_2 kg^{-1} hr^{-1}, respectively. In contrast, tidal crabs from the CI treatment had a higher MO_2 at 15°C (39.58 ± 3.28 mg O_2 kg^{-1} hr^{-1}) than the CE treatment (32.92 ± 1.6 mg O_2 kg^{-1} hr^{-1}). Further, the oxygen consumption of tidal crabs and non-tidal crabs responded differently to acute cooling (g 3.4). When cooled to 5°C, non-tidal and tidal crabs from both the CI and CE treatments had similar values of MO_2 at 5°C [non-tidal: 14.24 ± 0.52 mg O_2 kg^{-1} hr^{-1} (CI), 14.04 ± 1.25 mg O_2 kg^{-1} hr^{-1}(CE); tidal: 16.13 ± 2.13 mg O_2 kg^{-1} hr^{-1} (CI), 16.62 ± 1.38 mg O_2 kg^{-1} hr^{-1} (CE)], and this resulted in significant main effects for temperature (three-way RM ANOVA, df = 2, F = 162.8, p = < 0.0001) and acclimation group (three-way RM ANOVA, df = 1, F = 9.378, p = 0.004), and a significant interaction between temperature and acclimation group (three-way RM ANOVA, df = 2, F = 14.98, p = < 0.0001). Post-hoc tests revealed that this interaction was due to non-tidal crabs experiencing a stepwise decline in oxygen consumption in response to cooling, where the oxygen consumption at each temperature was significantly different (Tukey HSD, Table 3.1). However, in tidal crabs there was no difference between 15°C and 10°C in the CI or CE group (Tukey HSD, CI: p = 0.0708; CE: p = 0.4699), and no difference between 10°C and 5°C in the CE group (Tukey HSD, p = 0.2361). Anticipated immersion or emersion had no effects on oxygen consumption during the acute decrease in temperature (three-way RM ANOVA, df = 2, F = 0.478, p = 0.4970).

When the temperature was returned to 15ºC, the oxygen consumption of non-tidal crabs in both the CI and CE treatments returned to initial levels during the first hour (CI: one-way RM ANOVA, df = 12, F = 1.030, p = 0.4040; CE: one-way RM ANOVA, df = 12, F = 0.3442 p = 0.7933). This trend was similar for tidal crabs in the CI treatment (one-way RM ANOVA, df = 12, F = 0.5322, p = 0.6641), but tidal crabs from the CE treatment had elevated values for oxygen consumption as compared to initial values at 15°C for the first three hours of the recovery period (one-way RM ANOVA, df = 12, F = 3.538 p = 0.0177; Tukey HSD, hour 1: p = 0.0216; hour 2: p = 0.0389; hour 3: 0.0450). During recovery, tidal crabs had significantly lower oxygen consumption rates than non-tidal crabs (three-way RM ANOVA, df = 1, F = 7.636 p = 0.0090). There was no difference in oxygen consumption during the recovery period with regards to whether cooling was experienced during expected immersion or emersion (three-way RM ANOVA, df = 1, F = 0.6409 p = 0.4286).

Oxygen consumption during warming

As seen with the acute cooling experiment, the initial MO_2 at 15°C for non-tidal crabs was similar between the WI and WE treatments (50.15 ± 4.84 mg O_2 kg^{-1} hr^{-1} and 53.02 ± 2.61 mg O_2 kg^{-1} hr^{-1} respectively) whereas tidal crabs from the WI treatment had a higher MO_2 at 15°C (39.66 ± 1.15 mg O_2 kg^{-1} hr^{-1}) than tidal crabs from the WE treatment (31.91 ± 3.28 mg O_2 kg^{-1} hr^{-1} 1.61). During acute warming, there was a significant interaction between temperature and acclimation (three-way RM ANOVA, df = 2, F = 9.687, p = 0.0002; Fig. 3.3. C, D). This interaction was due to tidal crabs experiencing a

stepwise increase in oxygen consumption in response to warming (regardless of whether warming occurred during anticipated emersion or immersion), where the oxygen consumption at each temperature was significantly different (Tukey HSD, Table 3.1). However, in non-tidal crabs there was no difference in oxygen consumption between 15°C and 20°C (regardless of whether the crabs were anticipating immersion vs. emersion) (Tukey HSD, Table 3.1), but there was a difference between 15°C and 25°C and between 20°C and 25°C (Tukey HSD, Table 3.1). This resulted in non-tidal and tidal crabs from both the WI and WE treatment having similar MO_2 values at 25°C [non-tidal: 87.71 ± 5.26 mg O_2 kg^{-1} hr^{-1} (WI), 87.40 ± 3.59 mg O_2 kg^{-1} hr^{-1}(WE); tidal: 87.66 ± 6.13 mg O_2 kg^{-1} hr^{-1} (WI), 87.37 ± 5.74 mg O_2 kg^{-1} hr^{-1} (WE)]. Similar to acute cooling, there was also a significant difference between the two acclimation groups during the temperature change (three-way RM ANOVA, df = 1, F = 6.797, p = 0.0178). Tidal crabs in the WE group had a significantly lower oxygen consumption at 15ºC and 20ºC as compared to non-tidal crabs (Tukey HSD, 15°C: p = 0.0008; 20°C: p = 0.0431); whereas this difference was not evident at 25ºC (Tukey HSD, p = > 0.9999). Whether warming was experienced during anticipated emersion or immersion had no effect of the oxygen consumption of non-tidal or tidal crabs (three-way RM ANOVA, df = 1, F = 0.3701 p = 0.546).

When the temperature was decreased back to 15ºC, oxygen consumption of non-tidal acclimated crabs in both the WI and WE treatments returned to pre-treatment levels after one hour (WI: one-way RM ANOVA, df = 12, F = 1.311, p = 0.297; WE: one-way RM ANOVA, df = 12, F = 0.7663, p = 0.512). This trend was similar for tidal crabs in the WI treatment (one-way RM ANOVA, df = 12, F = 2.632, p = 0.0674), but tidal crabs from

the WE treatment exhibited an elevated oxygen consumption (above initial 15°C) for the first three hours of the recovery period (one-way RM ANOVA, df = 12, F = 3.542, p = 0.0116; Tukey HSD, hour 1: p = 0.0007; hour 2: p = 0.0029; hour 3: p = 0.0081). During recovery, although tidal crabs also had lower oxygen consumption than non-tidal crabs, this difference was not statistically significant (three-way RM ANOVA, df = 1, F = 2.846 p = 0.1002).

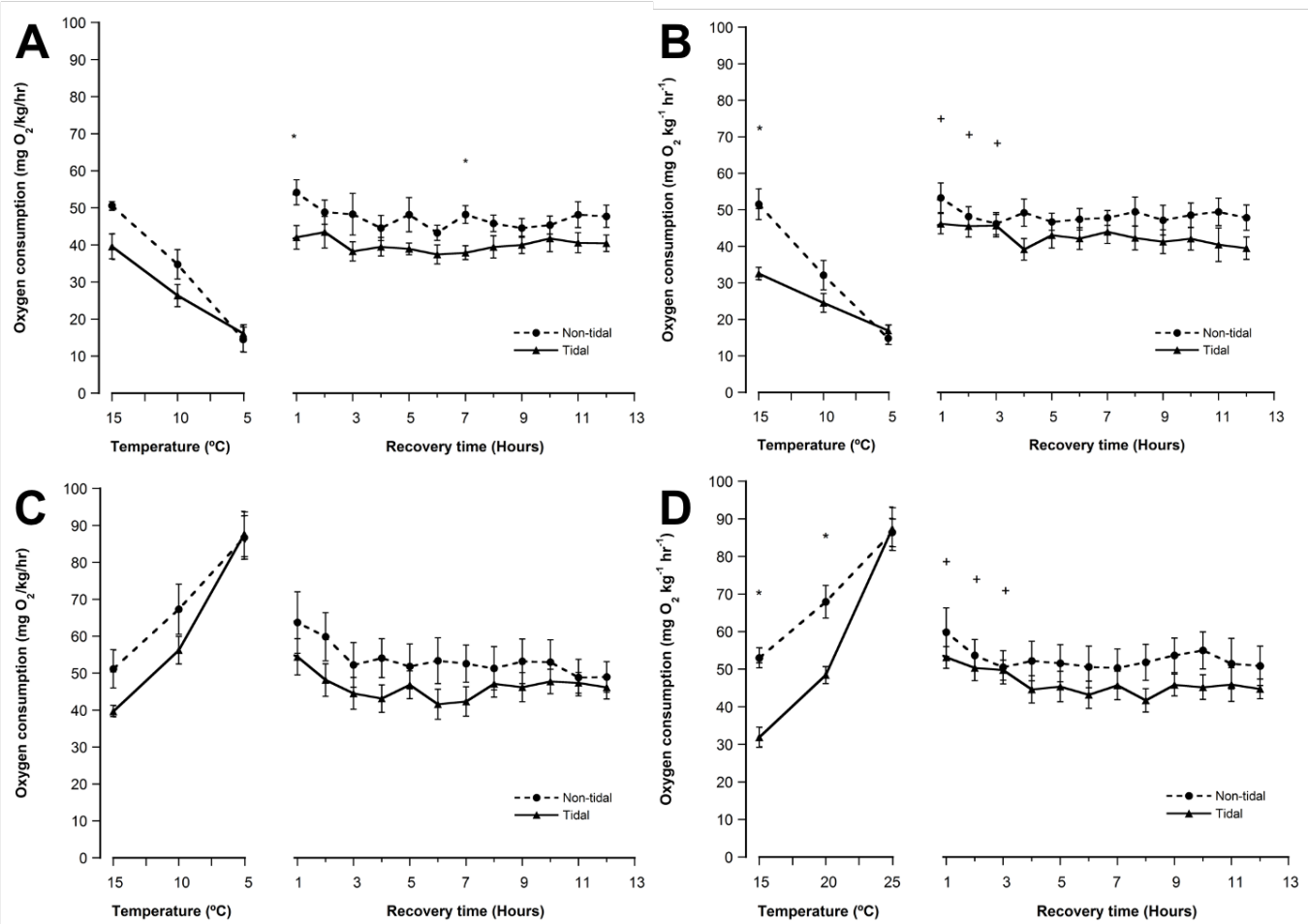


Figure 3.3. Oxygen consumption of submerged *Carcinus maenas* subjected to an acute temperature decrease from 15ºC – 5ºC during anticipated immersion (CI, A and emersion (CE, B, and an acute temperature increase from 15ºC – 25ºC during anticipated immersion (WI, C) and emersion (WE, D) over 5 hours followed by a 12 hour recovery at 15ºC. The data are means $\pm$ SEM values for 10 crabs acclimated to non-tidal and tidal regimes. Asterisks (*) indicate significant ($p < 0.05$) differences between acclimation treatments at a particular temperature or time point during recovery. Plus sign (+) indicates significant difference between MO_2 at initial 15°C and MO_2 at a particular hour of recovery in tidal crabs.

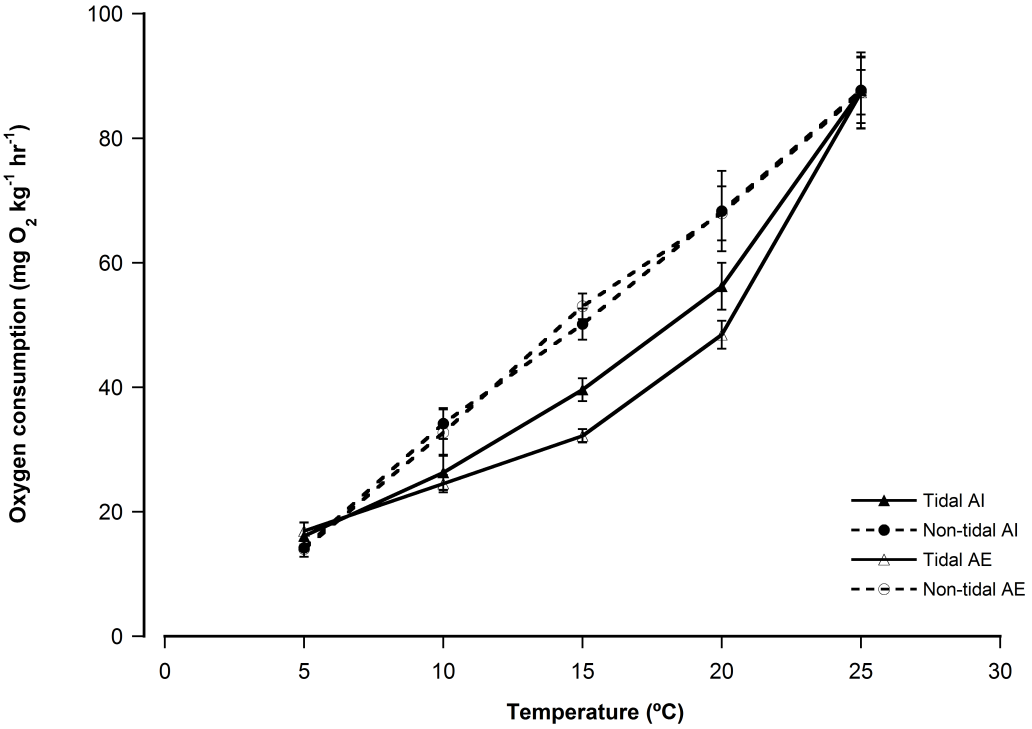


Figure 3.4. Thermal performance curves for non-tidal and tidal *Carcinus maenas* subjected to temperatures of 5°C-25°C during anticipated immersion (AI) and emersion (AE). As oxygen consumption at 15°C was recorded during both warming and cooling, these values were averaged to produce one oxygen consumption value at 15°C for each acclimation group – timing combination.

Table 3.1. Summary of Tukey's multiple comparison tests comparing oxygen consumption for *Carcinus maenas* acclimated to non-tidal and tidal conditions (at 15ºC) and subjected to decreases (C) or increases in temperature (W) when crabs were anticipating immersion (I) or emersion (E). Significantly different ($p < 0.05$) oxygen consumption values between the two temperatures are indicated by an asterisk. Ns = Non-significant.

Treatment	Acclimation	Adjusted P value	Summary
CI			
15°C - 10°C	Non-tidal	0.0152	*
	Tidal	0.0708	Ns
15°C - 5°C	Non-tidal	<0.0001	*
	Tidal	0.0003	*
10°C - 5°C	Non-tidal	0.0184	*
	Tidal	0.0031	*
CE			
15°C - 10°C	Non-tidal	0.0337	*
	Tidal	0.4699	Ns
15°C - 5°C	Non-tidal	<0.0001	*
	Tidal	0.0005	*
10°C - 5°C	Non-tidal	0.0176	*
	Tidal	0.2361	Ns
WI			
15°C - 20°C	Non-tidal	0.3420	Ns
	Tidal	0.0229	*
15°C - 25°C	Non-tidal	0.0092	*
	Tidal	0.0008	*
20°C - 25°C	Non-tidal	0.0041	*
	Tidal	0.0003	*
WE			
15°C - 20°C	Non-tidal	0.0572	Ns
	Tidal	0.0009	*
15°C - 25°C	Non-tidal	0.0005	*
	Tidal	<0.0001	*
20°C - 25°C	Non-tidal	0.0106	*
	Tidal	0.0002	*

Q_{10}

Assessment of Q_{10} values revealed a significant two-way interaction (three-way ANOVA, df = 1, F = 9.58, p = < 0.0001; Fig. 3.5) suggesting that Q_{10} was influenced by acclimation group and temperature. Regardless of the timing (E vs. I) of acute thermal stress, non-tidal crabs had the highest Q_{10} between 5°C-10°C (CI: 5.80 ± 0.60, CE: 5.81 ± 0.79; Fig. 3.5), and this was significantly higher than the Q_{10} at all other temperature ranges (Tukey HSD, p = < 0.0001 for all comparisons), where Q_{10} was between 3.17 ± 0.60 and 1.67 ± 0.13. The Q_{10} of non-tidal crabs between 5°C-10°C was also significantly higher than tidal crabs (CI:3.22 ± 0.57, CE: 2.41 ± 0.50; Fig. 3.5; Tukey HSD, p = 0.0001), but similar across all other temperatures (Tukey HSD, 10°C-15°C: p = 0.9617; 15°C - 20°C: p = 0.9617; 20°C - 25°C: p = 0.3257). Tidal crabs exhibited a more consistent Q_{10} across all temperatures, with Q_{10} varying between 2.08 ± 0.18 and 3.23 ± 0.60, with no significant difference between temperatures (Tukey HSD, p > 0.05 for all interactions). The Q_{10}'s of both tidal and non-tidal crabs were not significantly affected by whether they experienced a temperature change during expected immersion or emersion (three-way ANOVA, df = 1, F = 0.049, p = 0.824).

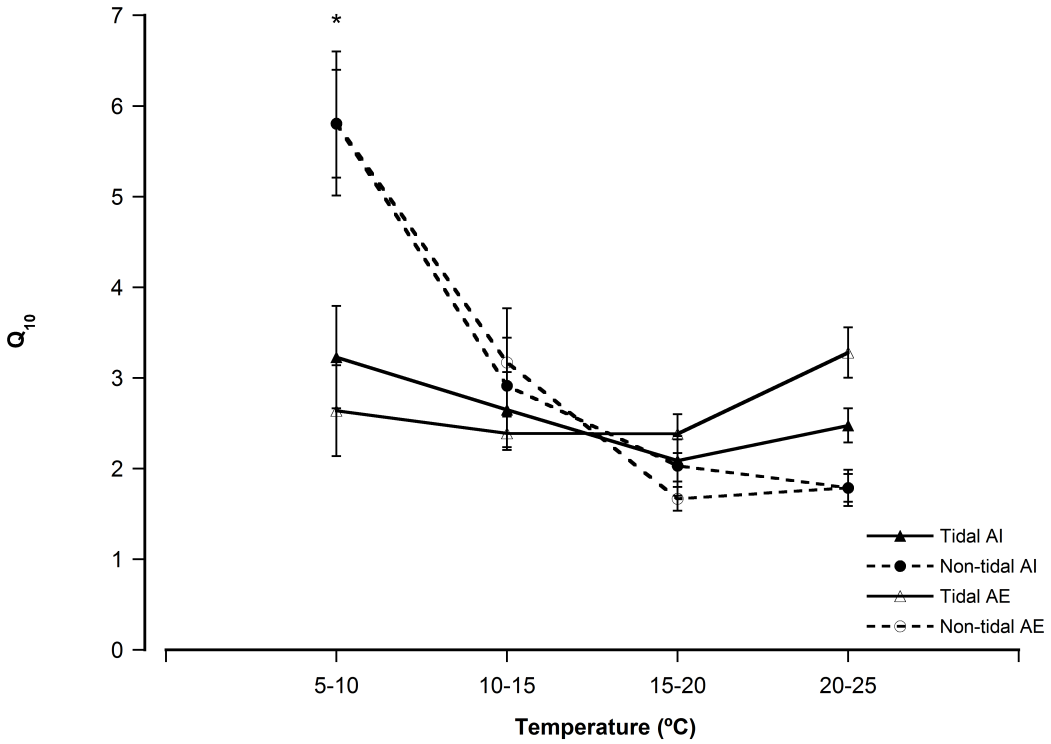


Figure 3.5. Mean Q_{10} values (± SEM) calculated for every 5°C period from 5°C to 25°C for non-tidal and tidal crabs subjected to warming or cooling over 5 hours during anticipated immersion (AI) and emersion (AE). Asterisks (*) indicate significant ($p < 0.05$) difference in Q_{10} between acclimation treatments at the given temperature range.

PaO_2 during WE

There was a significant decline in PaO_2 in response to the temperature increase (two-way ANOVA, df = 2, F = 12.22, p = < 0.0001; Fig. 3.6). PaO_2 in non-tidal crabs declined from 126.85 ± 8.56 mm Hg at 15°C to 87.38 ± 8.13 mm Hg at 25°C, and that of tidal crabs declined from 114.05 ± 8.75 mm Hg at 15°C to 71.33 ± 6.86 mm Hg at 25°C. However, there were no statistically significant differences between the PaO_2 of non-tidal and tidal acclimated crabs (two-way ANOVA, df = 1, F = 3.195, p = 0.0823) either before or during warming. When the temperature was returned to 15ºC, the PaO_2 of both groups increased back to pre-exposure levels (one-way ANOVA, non-tidal: df = 4, F = 0.724, p = 0.401; tidal: df = 4, F =0.067, p = 0.797). Although there was a trend for PaO_2 to decline somewhat in the tidal crabs after 3 hours, this was not statistically significant (two-way ANOVA, df = 3, F = 0.586, p = 0.6263). However, this apparent decline resulted in the PaO_2 of tidal crabs resulted in these values being significantly lower than those measured in the tidal group at hour 6 and 12 of recovery (two-way ANOVA, df = 1, F = 6.91, p = 0.0117).

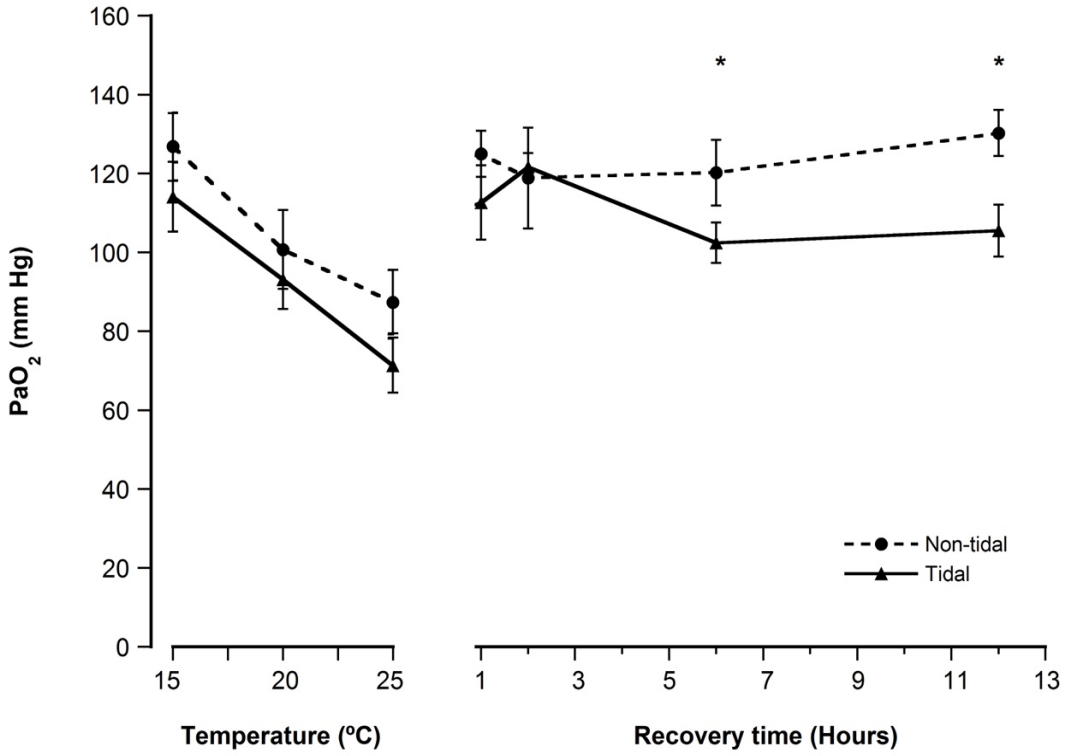


Figure 3.6. Arterial oxygen partial pressure (PaO_2) in *Carcinus maenas* in normoxic seawater during exposure to an acute temperature increase from 15ºC to over 5 hours followed by a 12 hour recovery at 15ºC. The crabs were exposed to the temperature increase when emersion was anticipated. The data represent the mean $\pm$ SEM of 7 crabs acclimated to non-tidal and tidal regimes. Asterisks indicate significant differences between acclimation groups ($p < 0.05$).

Venous pH (pH_v) during WE

Both non-tidal and tidal crabs experienced a similar stepwise decrease in venous pH (pH_v) when subjected to the acute temperature increase (two-way ANOVA, df = 2, F = 26.81, p = < 0.0001; Fig. 3.7). The pH_v of non-tidal crabs declined from 7.76 ± 0.02 at 15°C to 7.52 ± 0.05 at 25°C, and the pH_v of tidal crabs declined from 7.76 ± 0.03 at 15°C to 7.49 ± 0.03 at 25°C. The initial (15°C) pH_v of both non-tidal (one-way ANOVA, df = 4, F = 1.550, p = 0.2141) and tidal acclimated crabs (one-way ANOVA, df = 4, F = 0.2658, p = 0.8976) was re-restored within 1 hour of the temperature being lowered back to 15ºC. These values remained unchanged during the 12-hour recovery period (two-way ANOVA, df = 3, F = 1.265, p = 0.2971). There was no significant difference in pH_v between non-tidal and tidal crabs either before or after the temperature challenge (two-way ANOVA, df = 1, F = 0.1977, p = 0.6592).

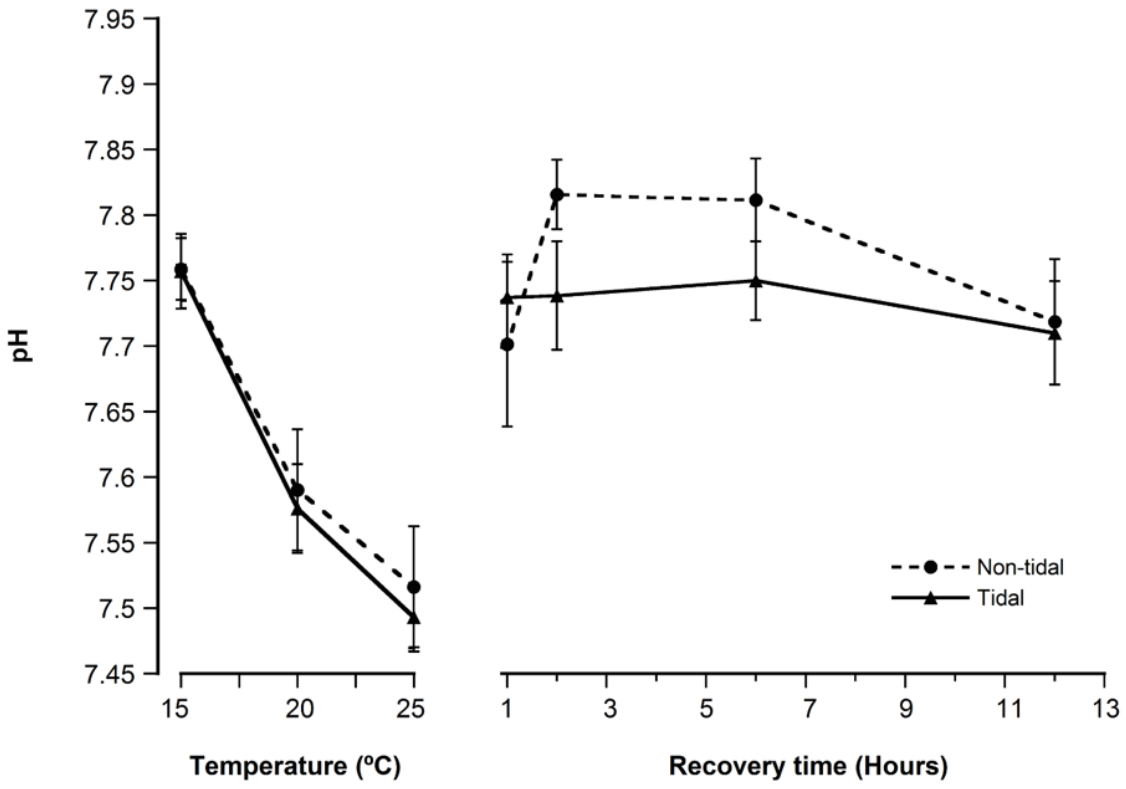


Figure 3.7. Venous pH in *Carcinus maenas* during exposure to an acute temperature increase from 15°C to 25ºC over 5 hours followed by a 12 hour recovery period at 15ºC. The crabs were exposed to the temperature increase when emersion was anticipated. The data are means $\pm$ SEM of 7 crabs for each group.

Haemocyanin concentration during WE

Tidal crabs had a significantly higher haemocyanin concentration at 15ºC and this difference was largely maintained as temperature increased; levels ranging in tidal crabs between 0.75 ± 0.03 mmol L^{-1} and 0.80 ± 0.06 mmol L^{-1} in comparison to 0.47 ± 0.07 mmol L^{-1} and 0.63 ± 0.09 mmol L^{-1} in non-tidal crabs (two-way ANOVA, df = 1, F = 0.8036, p = < 0.0001; Fig. 3.8). This difference between the two groups was significantly different at15°C (Tukey HSD, p = 0.0155) and at 25°C (Tukey HSD, p = 0.001). There was no significant change in haemocyanin concentration during the temperature increase in non-tidal or tidal crabs (two-way ANOVA, df = 2, F = 0.8036, p = 0.4556).

When the temperature was returned to 15°C for the recovery period, there was no significant change in haemocyanin concentration from initial 15°C values in either the tidal or non-tidal crabs (non-tidal: one-way ANOVA, df = 4, F = 1.512 p = 0.2239; tidal: one-way ANOVA, df = 4, F = 1.535 p = 0.2174). Tidal crabs continued to maintain a higher haemocyanin concentration than non-tidal crabs (two-way ANOVA, df = 1, F = 11.04, p = 0.0017), and this was most apparent between the two acclimation groups after 6 (Tukey HSD, p = 0.0139) and 12 hours of recovery (Tukey HSD, p = 0.0434).

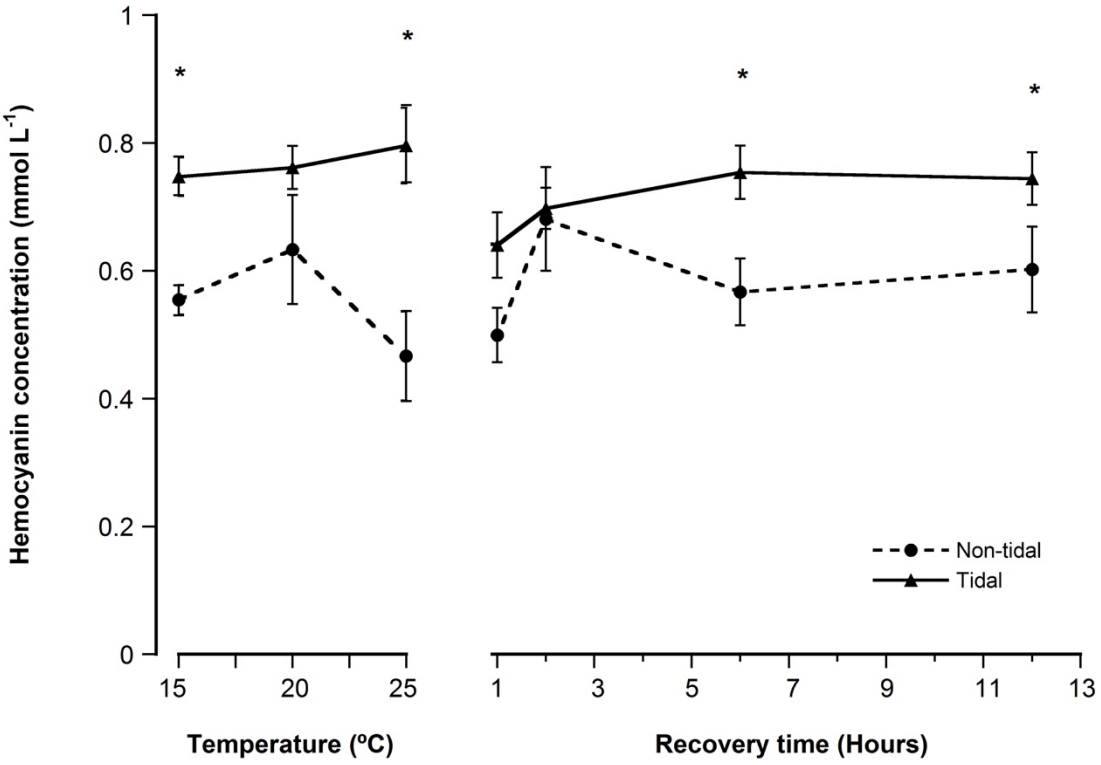


Figure 3.8. Haemolymph haemocyanin concentration in *Carcinus maenas* during exposure to an acute temperature increase from 15°C to 25ºC over 5 hours followed by a 12 hour recovery period at 15ºC. The crabs were exposed to the temperature increase when emersion was anticipated. The data are means ± SEM for 7 crabs for each group. Asterisks indicate significant differences between acclimation groups ($p < 0.05$).

Lactate concentration during WE

Initially (i.e., at 15°C) non-tidal and tidal crabs had similar haemolymph lactate concentrations [1.30 ± 0.13 mmol L^{-1} (non-tidal) and 1.36 ± 0.14 mmol L^{-1} (tidal)]. No statistically significant difference in lactate concentration due to temperature (two-way ANOVA, df = 2, F = 0.3859, p = 0.6826) or between the two acclimation groups (two-way ANOVA, df = 1, F = 1.082, p = 0.3052) during the temperature increase was found.

There was a trend towards lower lactate levels after 1 hour of recovery in both tidal and non-tidal crabs, however, this proved to be statistically insignificant (non-tidal crabs, one-way ANOVA, df = 4, F = 1.377, p = 0.263; tidal crabs, one-way ANOVA, df = 4, F = 1.102, p = 0.373). There was also no change in lactate concentration over the course of the recovery period (two-way ANOVA, df = 3, F = 2.559, p = 0.0660). Tidal crabs consistently had higher lactate levels than non-tidal crabs (two-way ANOVA, df = 1, F = 5.135, p = 0.0280), but this difference only reached statistical significance between the two acclimation groups 12 hours after the crabs had been returned to 12°C (Tukey HSD, p = 0.0242).

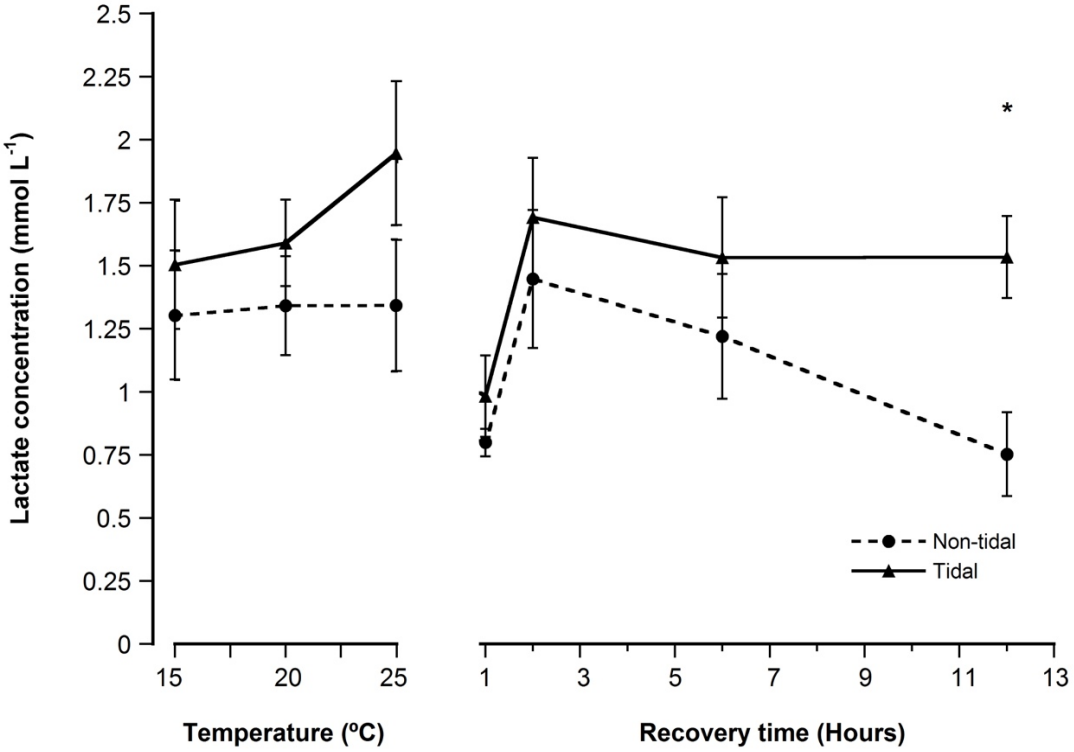


Figure 3.9. Haemolymph lactate concentrations in *Carcinus maenas* during exposure to an acute temperature increase from 15°C to 25ºC over 5 hours followed by a 12 hour recovery period at 15ºC. The crabs were exposed to the temperature increase when emersion was anticipated. The data are means $\pm$ SEM of 7 crabs. The asterisk indicates a significant difference between acclimation treatments ($p < 0.05$).

3.5 Discussion

Overall, acclimation to cyclical emersion-immersion had limited effects of the green crab's physiological responses to either hypoxia or changes in temperature when submerged. However, there were some interesting, and potentially very important, differences. These include: 1) tidal crabs had lower MO_2 values and higher haemocyanin concentrations at 15ºC; 2) while their temperature sensitivity (Q_{10} values) was / were relatively similar over the range tested, the non-tidal crabs were more sensitive to low temperatures and less sensitive to high temperatures; and 3) these changes in thermal sensitivity resulted in the oxygen consumption of crabs exposed to immersion vs. cyclical; immersion-emersion being the same at 5 and 25ºC. These results suggest that crabs exposed to cyclical increases and decreases in tidal height are less sensitive to changes in temperature, and are able to deal better with temperature fluctuations. Thus, there appears to be a complex relationship environmental stressors and oxygen consumption in non-tidal vs. tidal crabs that depends on the type of stress experienced, and over what part of the crab's thermal niche parameters are measured.

Interestingly, whether the crabs were tested during periods of anticipated immersion vs. emersion had few effects on their physiology when submerged. While this data suggests whether a crab is anticipating changes in tidal height does not affect its physiology, it does not rule out the possibility that significant differences would be observed when the crabs were tested during emersion. This is the portion of the tidal cycle where the majority of physiological challenges are expected.

Metabolism under resting conditions

Tidal crabs had a lower metabolic rate over the 15°C-20°C range as compared with non-tidal crabs. These data are consistent with that reported in Chapter 2, and collectively indicate that physiological adjustments to periodic emersion may result in a lower routine metabolic rate (RMR). There are a limited number of studies that have compared the metabolic rate of animals acclimated to cyclical variables (e.g. air exposure, hypoxia, temperature etc.) in comparison to constant conditions, and these also suggest that acclimation to a cyclic regime can lower RMR. For example, diel fluctuating temperature regimes depress the oxygen consumption of mud crab *Panopeus herbstii* and fiddler crab *Uca pugilator* (Dame & Vernberg, 1978). In the rainbow trout (*Oncorhynchus mykiss*), exposure to diel cycling hypoxia results in lower oxygen consumption (Williams et al., 2019). Finally, in juvenile sea cucumber *Apostichopus japonicus,* a lower RMR occurs when acclimated to a fluctuating temperature regime, and it was proposed that the decrease in RMR was to ensure that energy was available for growth (Dong et al., 2006). In the present study, cyclical immersion-emersion resulted in increased haemocyanin levels in the tidal crabs (Fig. 3.8). It is possible that elevated haemocyanin contributed to the lower RMR observed, whereby an increased oxygen carrying capacity would provide energy savings with ventilation and circulation (Giomi & Pörtner, 2013). In support of this, elevated haemocyanin levels in the lobster, *Homarus gammarus,* cause a reduction in ventilation, scaphognathite beating and heart rate (Spoek, 1974). It is important to note that differences in activity between non-tidal and tidal crabs could perhaps have contributed towards the differences in RMR reported here. Although activity was generally observed

to be similar between non-tidal and tidal crabs during acute stress and recovery, this was not quantified, and thus cannot, be ruled out as a contributing factor.

Exposure to, and recovery from, acute hypoxia

Aerial exposure and hypoxia can impose similar challenges on aquatic animals, namely difficulty in extracting and utilising environmental oxygen (Wilmer et al., 2009). Prior acclimation to hypoxia has been shown to increase the ability of crustaceans to cope with subsequent periods of low oxygen, often through the upregulation of different isoforms or increased concentrations of haemocyanin (Baden et al., 1990; DeFur et al., 1990; Hagerman et al., 1990; Senkbeil & Wriston, 1980). I, therefore, hypothesised that because tidal crabs were regularly exposed to air and had elevated levels of haemocyanin (Fig. 2.7), they would be better able to maintain oxygen consumption during hypoxia than non-tidal crabs. However, non-tidal and tidal crabs showed a similar decline in oxygen consumption when subjected to hypoxia (Fig. 3.2), suggesting that increased levels of haemocyanin did not alter how tidal crabs regulated aerobic metabolism when oxygen becomes limited. However, my results do not exclude the possibility that these elevated haemocyanin levels might improve the tolerance of green crabs to very low oxygen levels or decrease their critical oxygen tension. In support of this hypothesis, it has been shown that while tadpole shrimp *Triops longicaudatus* acclimated to normoxia, moderate hypoxia (75 – 97 mm Hg) or severe (7-22.5 mm Hg) hypoxia had a similar decrease in oxygen consumption as environmental PO_2 decreased, only the group acclimated to severe hypoxia group was able to maintain cardiac output below P_{crit} (Harper & Reiber, 2006). Similarly, while juvenile qingbo (*Spinibarbus sinensis*) acclimated to either stable or cyclical hypoxia

showed a similar decline in oxygen consumption with reductions in environmental PO_2, they had a lower P_{crit} than those acclimated to normoxia, (Dan et al., 2014). In this study, I tested hypoxia tolerance down to 20% air saturation (~30 mm Hg) to represent typical low oxygen conditions reported for tidepools (Sloman et al., 2008; Todgham et al., 2005). The reported P_{crit} of *C. maenas* is also around ~30 mm Hg (McGaw & Nancollas, 2018) and therefore while there appeared to be no difference in metabolic performance between tidal and non-tidal crabs during hypoxia, it is possible that there might have been a difference in the P_{crit} of the two acclimation groups.

Response to acute thermal stress

Despite tidal crabs having lower MO_2 values at 15- 20ºC, the oxygen consumption of non-tidal crabs was more sensitive to low temperatures and less sensitive to high temperatures as compared to tidal crabs, and this resulted in similar values for MO_2 at the measured temperature extremes (5 and 25ºC) (Figs. 3.3 and 3.4). The similarity of oxygen consumption in these two groups at these temperatures suggests that these temperatures are critical set points for *C. maenas*. This would be consistent with studies which report that their typical average thermal range is 6°C - 23°C (Kelley et al., 2011; McGaw & Whiteley, 2012; Tepolt & Somero, 2014). Low temperatures result a reduction in mitochondrial performance which causes a decrease in ATP production rates and can result in a difficulty to meet the energetic demand of ventilation and circulation (Frederich & Pörtner, 2000; Zielinski & Pörtner, 1996). Consequently, at 5°C-7°C, *C. maenas* enters a state of torpor, reduces activity (Whiteley et al., 1997) and ceases feeding (Behrens Yamada, 2001; Berrill, 1982). My results could suggest that at 5°C, non-tidal crabs are struggling to maintain

metabolic demand, and are likely going into a state of torpor. The matching of oxygen consumption at 25°C is more complex, but suggests that regardless of acclimation, *C. maenas* may be approaching the maximum capacity of its aerobic systems at 25°C, and this would correlate with the observed upper thermal limit in their native range (Tepolt & Somero, 2014).

Tidal crabs maintained a more consistent Q_{10}, than non-tidal crabs throughout the experimental temperature range, suggesting that tidal crabs may be better able to compensate for temperature changes (Magozzi & Calosi, 2015). In this study, I was interested in assessing thermal performance over the typical range of temperatures for *C. maenas* (6°C - 23°C; Kelley et al., 2011; McGaw & Whiteley, 2012; Tepolt & Somero, 2014), and the trends in Q_{10} reported here do indicate that acclimation to periodic emersion could be an important modulator of metabolic adjustments to temperature changes, and thus may play an important role this species' physiology over is typical temperature range. However, given that oxygen consumption at the two temperature extremes was the same, this does not suggest that aquatic thermal tolerance was affected by immersion-emersion. This interpretation is in contrast to the results of studies that propose that exposure to regular periods of emersion can lead to physiological adjustments that enhance the ability to extract oxygen, and support a higher thermal tolerance in aerial conditions (Bjelde & Todgham, 2013; Drake et al., 2017). Whether differences in MO_2 and Q_{10} values at high temperatures, and the thermal tolerance, of tidal and non-tidal green crabs is different awaits further studies. In this regard, based on the assumption that thermal tolerance is fundamentally linked to the ability to obtain or efficiently utilise oxygen (Frederich & Pörtner, 2000; Pörtner, 2001; Pörtner, 2010; Pörtner & Knust, 2007), one might expect that

the higher haemocyanin levels (Fig. 3.8) would enhance maximum oxygen delivery in tidal crabs, and could lead to a higher thermal tolerance.

The fact that the Q_{10} of tidal crabs was more stable could indicate that tidal crabs have a more plastic metabolism, and thus, are able to adjust their metabolic performance over a larger range of temperatures than non-tidal crabs (Magozzi & Calosi, 2015). Metabolic plasticity is an important adaptive trait for intertidal organisms as it allows for the maintenance of aerobic scope during rapid fluctuations of environmental conditions (Bozinovic et al., 2011; Via et al., 1995). Previous studies comparing the metabolic rate of intertidal and closely-related subtidal counterparts have shown similar a trend between metabolic rate and temperature as found here for *C. maenas* (Burggren & McMahon, 1981; Jost et al., 2012; Magozzi & Calosi, 2015). For example, at cooler temperatures subtidal hermit crab species *Paguristes turgidus* and *Elassochirus tenuimanus* have a significantly higher Q_{10} between 15°C and 5°C compared with similar intertidal species (*Pagurus granusirnanus, Pagurus hirsutiusculus*) (Burggren & McMahon, 1981). During warming (10°C-25 °C) a decline in Q_{10} also occurs in the subtidal prawn *Palaemon serratus*, whereas intertidal species (*Palaemon elegans*, *Palaemon varians)* either maintain or exhibit an increase in Q_{10} (Magozzi & Calosi, 2015). This association between temperature and metabolic rate has traditionally been linked to intertidal species experiencing a wider range of temperatures. However, the crabs used in the present study were acclimated to 15°C (during both immersion and emersion), suggesting that periodic emersion may also be a contributing factor that drives thermal sensitivity in intertidal crustaceans.

As expected, there was no significant effect when the non-tidal crabs experienced heating or cooling (Fig. 3.3; Fig. 3.4), because they were maintained in constantly

submerged conditions, and showed no circatidal rhythm (Fig. 2.2). Overall, the timing of the temperature change (i.e., whether it occurred during the period of anticipated emersion or immersion) also had no effect on the oxygen consumption of tidal crabs. Nevertheless, subtle decreases in oxygen consumption were noted when the tidal crabs experienced temperature changes during periods of expected emersion at 15°C and 20° (Fig. 3.3 B and D), and this led to lower MO_2 values for tidal crabs at these temperatures. These data suggest that there is an underlying endogenous rhythm that influences the metabolic responses to thermal stress in *C. maenas.* A reduction of metabolic rate during low tide is common for many intertidal animals, often due to risk of desiccation coupled with a reduction in activity (see DeFur, 1988 and Taylor, 1990 for reviews). Consequently, some intertidal organisms have been shown to have lower metabolic demands during emersion than their subtidal conspecifics (Dykens & Shick, 1981; Shick et al., 1985; Shick et al., 1988). Recent evidence also indicates that intertidal organisms may invoke other energy saving mechanisms during emersion. For example, the swimming crab *Portunus trituberculatus* increases the expression of the energy regulators AMP-activated protein kinase alpha (AMPKα) and HIF-1α during air exposure to maintain energy homeostasis (Lu et al., 2016). Similarly, the intertidal limpet *Cellana rota***,** which also exhibits an endogenous circatidal rhythm in oxygen consumption, has endogenous control over the mTOR pathway, which is a central regulator of metabolism and AMPK (Schnytzer et al., 2018). Such mechanisms are controlled by a circatidal clock, and play a key role in enabling organisms to anticipate and respond to predictable daily changes in tidal height (O'Neill et al., 2015; Schnytzer et al., 2018; Tessmar-Raible et al., 2011; Wilcockson &

Zhang, 2008; Zhang et al., 2013), and may have influenced the responses of *C. maenas* in the present study.

The circatidal production of enzymes and energy regulators may have also influenced patterns of oxygen consumption during the recovery phase, as was apparent when the crabs recovered from hypoxia and thermal stress experienced during anticipated emersion. For example, while the oxygen consumption of tidal crabs continued to be lower than measured in non-tidal crabs, it was elevated (for the first 2-3 hours of recovery) above pre-treatment levels measured at 100% oxygen saturation/15°C (Fig 3.2 B; Figs. 3.3. B and D) in tidal crabs measured when they anticipated emersion. Recovery from air exposure often results in an increase in antioxidant enzymes to reduce oxidative stress that can occur during re-oxygenation following re-immersion (Nicastro et al., 2010; Romero et al., 2007; Romero et al., 2011; Togni, 2007; Yin et al., 2017). For example, the Manilla clam *Ruditapes philippinarum*, chronically acclimated to periodic emersion had significantly higher levels of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD), and consequently a higher oxygen consumption when re-immersed in comparison to individuals that received no air exposure (Yin et al., 2017). Similarly, after 6 hours of air exposure, false king crab *Paralomis granulosa* displayed elevated levels of SOD, CAT and glutathione s-transferase (GsT) in haemolymph, muscle and gill tissues during the first 2 hours of re-immersion (Romero et al., 2011). These antioxidant enzymes act as an important defence mechanism against reactive oxygen species produced by changes in mitochondrial activity and rapid reoxygenation (Gorr et al., 2010; Pöhlmann et al., 2011).

Changes in haematological parameters

To try and elucidate the underlying mechanisms behind some of the differences in oxygen consumption between tidal and non-tidal crabs, oxygen delivery (haemolymph PaO_2, haemocyanin concentration) and acid-base (pHv, lactate concentration) parameters were assessed when warming occurred during expected periods of emersion. In each case, there were only a few time points at which statistically significant differences were observed between the two treatment groups. Nevertheless, there were consistent trends during the initial measurements, and those during the treatment and recovery periods, with tidal crabs having elevated haemocyanin and lactate levels, and a somewhat lower PaO_2 than non-tidal crabs. These data suggest that acclimation to cyclic emersion resulted in physiological adjustments, particularly in regards to oxygen delivery, and were consistent with the results found in Chapter 2.

Both tidal and non-tidal crabs exhibited a decrease in PaO_2 as the water warmed (Fig. 3.6), this was most likely related to the lower solubility of oxygen at warmer temperatures (Wilmer et al., 2009). Tidal crabs had marginally lower P_aO_2 values at the initial measurement at 15ºC, and at 20ºC and 25ºC, which became significantly different as compared to non-tidal crabs after 6 and 12 hours of recovery. Amphibious and terrestrial crabs generally have a lower PaO_2 than aquatic crabs (Adamczewska & Morris, 1994; Greenaway et al., 1988; McMahon & Burggren, 1979; Morris et al., 1996), and this has been attributed to an increased concentration of high affinity haemocyanin which facilitates substantial oxygen uptake (McMahon & Burggren, 1979; Morris, 1991), and thus reduces the need for the high ventilation rates typically associated with aquatic crustaceans (O'Mahoney & Full, 1984). A similar pattern occurs in aquatic crustaceans: lobsters *Homarus gammarus* with elevated haemocyanin reduce scaphognathite frequency beat

(ventilation) frequency, and thus oxygen consumption (Spoek, 1974). Therefore, the elevated levels of haemocyanin observed here for tidal crabs could also explain the lower PaO_2 and contributed to their lower oxygen consumption.

Venous haemolymph pH declined in both the tidal and non-tidal crabs as the water was warmed, and this is the typical pattern for pH in crustaceans due to an increase of ionization of water with increasing temperature (Wilmer et al., 2009). There were no obvious differences in pH between tidal and non-tidal crabs during warming despite the tidal crabs having somewhat higher levels of lactate. It is likely that the increased levels of haemocyanin in the tidal crabs minimised the pH changes produced by lactate, as haemolymph proteins (>90% of which are haemocyanin) are an important mechanism for buffering pH in crustaceans (Whiteley, 2011; Rastrick et al., 2014). There was more variation in pH during the recovery phase, but lack of consistency suggested that these levels were unrelated to lactate or haemocyanin levels.

Haemocyanin concentration increases in response to several environmental perturbations such as hypoxia (Baden et al., 1990; Haegerman & Oksama, 1985; Spicer & Baden, 2001) and low salinity (Boone & Schoffeniels, 1979; Gilles, 1977; Mason et al., 1983), and the adaptive modulation of haemocyanin concentration and affinity is considered to be an important adaptation for tolerating environmental change in crustaceans (Giomi & Beltramini, 2007). As discussed, this increased level of haemocyanin in tidal crabs could play several key roles. Primarily, it would carry more oxygen allowing for more enhanced delivery to the tissues (Spoek, 1974; Mangum, 1983). Furthermore, Giomi and Pörtner (2013) measured components of the oxygen transport cascade during acute warming and argued that the presence of haemocyanin extended the upper critical

temperature from 16°C to 22°C in crabs acclimated to 10°C. This implies that increases in haemocyanin concentration would increase available oxygen, and therefore, may play an important role in maintaining oxygen delivery during the temperature fluctuations that frequently occur in the intertidal.

In addition to elevated haemocyanin levels, tidal crabs had consistently higher lactate levels both prior to and during warming, and this difference became more noticeable during the recovery phase. Although greater reliance on anaerobic resources has been shown in some intertidal species (Newell et al., 1973; Shick et al., 1988), the present results (i.e., for pH_v and PaO_2) did not suggest that tidal crabs were relying on anaerobic respiration to a greater degree. Although lactate is typically regarded as an end-product of anaerobic metabolism, it may also be an important metabolic fuel (Gladden, 2004, Jayasundara & Somero, 2013). Crustaceans living in more dynamic environments display higher constitutive levels of lactate (Jost et al., 2012, Maciel et al., 2008) which may reflect the need to have fuel readily available to tolerate unpredictable conditions. Furthermore, in many crustaceans, elevated levels of lactate can increase the oxygen affinity of haemocyanin, ensuring optimal oxygen delivery during periods of stress (Truchot, 1980). Due to its role as an end product from anaerobic metabolism, it is understandable that less work has been done investigating whether lactate plays a role during normoxia (but see suggestions by Gladden, 2004; Jayasundara & Somero, 2013; Maciel et al., 2008). Studies on the fate of lactate after events such as exercise, hypoxia and temperature stress in crustaceans indicate that there is a lot of inter-specific variability (Hervant et al., 1997; Henry et al., 1994; Maciel et al., 2008; Morris and Adamczewska, 2002; Zebe, 1982; Zou et al., 1996), and thus, the potential role of lactate will largely depend on ecological and

biological factors specific to each species (Maciel et al., 2008). Nevertheless, in the crab *Neohelice granulata* [previously *Chasmagnathus granulata* (which lives in an estuarine environment)], elevated haemolymph lactate levels during normoxia are common and utilized as fuel either via oxidation through the tricarboxylic acid cycle (TCA), or conversion to glucose via gluconeogenesis (Maciel et al., 2008). Therefore, the elevated lactate observed in tidal *C. maenas* during normoxia could serve as a metabolic fuel during changes in respiratory medium, or to enhance the oxygen affinity of haemocyanin (Truchot, 1980).

Conclusion

The aim of this study was to determine if acclimation to cyclic immersion-emersion affected how the metabolic physiology of the green crab hypoxia and changes in temperature when submerged. Overall, while emersion reduced the temperature sensitivity of oxygen consumption to changes in temperature, whether the crabs were anticipating emersion or immersion when they were exposed to these stressors had only minor effects on the physiological responses of *C. maenas* to hypoxia and thermal stress (with the exception of oxygen consumption at 15 and 20^{o}C; Figs. 3.3. and 3.4). Assessment of physiological parameters associated with oxygen delivery (PaO_2) and acid base balance (pH_v, lactate) did not reveal clear explanations for the relationships observed in the oxygen consumption data, suggesting that other mechanisms must be regulating these changes. While I have speculated about potential mechanisms in the above discussion, we currently know very little about what protective mechanisms and pathways are upregulated during air exposure alone, and which of these mechanisms are endogenously controlled. I have suggested that energy mediators such as AMPK, and antioxidant enzymes such as SOD

and CAT, may play a role in the observed metabolic differences between the tidal and non-tidal crabs during recovery, but detailed experiments / measurements need to be performed to test this hypothesis. Understanding physiological responses to cyclical air exposure, and how they are regulated, would be a fruitful area for future research. Such information would be important for understanding how global environmental change may affect the growth and performance of intertidal and subtidal communities. The results of this study also indicate that more caution must be taken when inferring physiological responses of intertidal animals to environmental perturbations when species are acclimated to constant conditions. In order to gain a more accurate understanding of how intertidal species respond to environmental perturbations, a more realistic approach must be taken where animals are subjected to multiple cycling parameters, as typically occurs in nature.

Chapter 4. General Discussion

Summary of findings

The main goal of this thesis was to examine if the intertidal green crab *C. maenas* displayed different physiological responses to common intertidal environmental stressors when acclimated to continuous submersion (non-tidal) or cyclical immersion-emersion (tidal) regimes. I focused on physiological parameters associated with oxygen delivery and acid-base balance, in order to give a broad understanding of physiological adjustments associated with aerial exposure. Chapter 2 revealed that acclimation to a tidal regime of cyclic immersion-emersion entrained a circatidal rhythm of oxygen consumption in *C. maenas,* with tidal crabs displaying lower oxygen consumption during expected periods of

emersion. In contrast, non-tidal crabs displayed no evidence of a circatidal rhythm, suggesting that periodic inundation is an important zeitgeber for entraining circatidal rhythms in *C. maenas.* With regards to their physiology over a tidal cycle, tidally acclimated *C. maenas* had higher constitutive levels of haemocyanin and were able to maintain acid-base balance during periods of emersion, with no change in lactate production. In contrast, non-tidal crabs experienced an acidosis, and a partial reliance on anaerobic metabolism (as evidenced by an increased haemolymph lactate concentration).

The results of Chapter 2 led me to wonder whether the physiological differences between the two acclimation groups could affect how *C. maenas* responded to other environmental stressors that typically fluctuate over the tidal cycle, such as oxygen concentration and temperature. Initially, the oxygen consumption of both non-tidal and tidal crabs was measured when subjected to three different environmental challenges when immersed: 1) a stepwise decline in water oxygen content (100%-20% air saturation); 2) a stepwise decline in temperature (15°C – 5°C); or 3) a stepwise increase in temperature (15°C – 25°C). All these challenges occurring over a period of 5 hours with an additional hour to return to initial conditions. Due to evidence of a circatidal rhythm in tidal *C. maenas* (Fig. 2.2; 2.3), I also thought it would be interesting to see whether anticipated immersion vs. emersion would influence oxygen consumption in response to these stressors. Therefore, these three experiments were repeated, first in synchronization with the times tidal crabs would be expecting to be immersed, and secondly out of synchronization - when tidal crabs expected to be emersed. The results from these experiments indicated that the relationship between acclimation to cyclic immersion-emersion, and the response to aquatic hypoxia or temperature stress, was complex. Interestingly, while tidal crabs had a lower oxygen

consumption at 15 - both groups had sin consumption rates at the temperature extremes tested (25° C and 5° C). This was because the temperature sensitivity of tidal crabs was relatively similar (as judged by Q_{10} values) over the tested temperature range, whereas non-tidal crabs were more and less sensitive to changes in temperature from 15-5^{o}C and 20-25^{o}C, respectively (Fig. 3.5). These results suggest that, at 5 and 25^{o}C temperatures are crucial physiological set-points for *C. maenas*, and that the tidal crabs (i.e., exposed to immersion – emersion) were better able to regulate their metabolic rate of the range of temperatures they would experience in nature. However, the results also revealed that anticipation of being immersed vs. emersed has minor effects of the oxygen consumption of this species.

Measurement of parameters related to oxygen delivery and acid-balance provided few insights into the mechanisms mediating the differences in temperature sensitivity between tidal vs. non-tidal crabs; with the exception of higher haemocyanin levels in tidal crabs (Fig. 2.7; 3.8). This suggests that underlying molecular or cellular mechanisms may be responsible for the observed differences in oxygen consumption during thermal stress.

Together, the results from the experimental chapters suggest that the relationship between acclimation to cyclic emersion and physiological responses to other environmental stressors is complex, particularly when these stressors are applied in an aquatic setting.

Importance of results

In this thesis, I highlight two important points. First, *C. maenas* acclimated to cyclic immersion-emersion show different physiological responses to environmental conditions,

(particularly during emersion) than *C. maenas* acclimated to permanently submerged conditions. As the majority of previous experimental laboratory work on intertidal organisms has subjected intertidal organisms to permanently submerged conditions, this brings into question the accuracy of the physiological responses reported in these studies, and how representative they are for *in situ* responses. Clearly, future laboratory studies should try and acclimate intertidal organisms to tidal conditions within the laboratory setting in order to achieve more accurate and representative physiological responses. Furthermore, whereas many studies have looked at the combined physiological effects of emersion and thermal stress on physiology, this study has provided evidence of physiological adjustments that can be specifically attributed to air exposure. Such information is important for teasing apart how forecasted environmental change(s) (i.e. those predicted to accompany climate change) will affect the physiology of intertidal organisms.

Second, acclimation to cyclic emersion may impact how intertidal organisms respond to aquatic environmental stressors, such as hypoxia and temperature fluctuation, that can commonly occur in tidepools and coastal waters. To date, few studies have addressed whether acclimation to cyclic emersion imparts tolerance to other aquatic environmental stressors. However, this is a key area for future study as it is presumed that intertidal organisms rely on these periods of submersion to recover between low tides. Here, I just scratch the surface, but the relationships for Q_{10} suggests that acclimation to cyclic immersion-emersion may affect the performance of *C. maenas* in aquatic environments, and a more comprehensive assessment of this phenomenon will be

important to predicting the long-term effects of climate change on the fitness and survival of intertidal organisms.

Future directions

Together, the breadth of these two experimental chapters has provided a wealth of new questions and multiple avenues for further research. Below, I identify and discuss what avenues I would prioritise in building upon this research. The primary goal of this thesis was to determine whether acclimation affects the physiological responses of *C. maenas* to environmental stressors. Now that some differences have been identified, the next step should be identifying the specific mechanism(s) involved in driving these physiological differences.

One of the most important results from Chapter 2 was the higher constitutive levels of haemocyanin exhibited by tidal crabs. Due to its role as the primary regulator of increased synthesis of haemoglobin in *Daphnia* (Gorr et al., 2004; Hoogewijs et al., 2007) and haemocyanin in *Cancer magister* (Head et al., 2010) during hypoxia, assessing the role of HIF pathways could provide useful information with regards to the driver behind this change in oxygen transport capacity. Moreover, HIFs play fundamental roles in controlling energy metabolism, and may provide important information with regard to aerobic and anaerobic metabolism in non-tidal and tidal crabs. The variation in haemocyanin concentrations during temperature changes and emersion may indicate that varying haemocyanin levels could be a plastic response in crustaceans that allows them to tolerate environmental challenges as has been suggested by Spicer and Baden (2001).

The results in Chapter 3 suggested a more complex relationship between exposure to cyclical immersion-emersion and responses to aquatic environmental stressors. The mechanisms behind this were not clearly elucidated from measuring parameters involved in oxygen delivery and acid-base balance, which suggests that other mechanism/s are responsible for the observed differences in the temperature sensitivity of oxygen consumption. Assessment of other physiological mechanisms such as energy regulation, ion regulation or excretion could provide information that may shed light on the mechanism(s) responsible for these observed differences. Specifically, there could be a difference in enzyme regulation and activity between non-tidal and tidal crabs, and assessment of enzyme activity [particularly of those associated with metabolism such as AMPK, HIF-1 or ion regulation (Na^+/K^+-ATPase or carbonic anhydrase)] could provide crucial information to help elucidate the mechanisms regulating organismal performance under different temperature regimes.

In all of the experimental treatments, there was a trend for tidal crabs to have lower oxygen consumption rates in aquatic conditions than non-tidal crabs (although this was not statistically significant in all cases). From this, a question that arises is how and why acclimation to cyclical immersion-emersion produced changes in RMR. Here I have proposed that the differences in haemocyanin concentration between tidal and non-tidal crabs could have contributed to the observed differences in oxygen consumption through an enhancement of oxygen delivery, and a reduction in energetic demands with respect to ventilation and circulation. However, it is likely that there are other factors that contributed towards this trend. For example, although activity was anecdotally monitored, and appeared similar between non-tidal and tidal crabs during anticipated immersion and

exposure to air, it is possible that differences in activity could have occurred between the two acclimation groups and that this could have contributed to the differences observed in RMR. The relationship between lactate production and clearance also appeared to be different between tidal and non-tidal crabs. This finding is supported by data which suggests that tidal crabs may be able to utilize lactate as metabolic fuel during emersion, and therefore, maintain higher constitutive lactate levels than non-tidal crabs. Maciel et al. (2008) and Jost et al. (2012) have suggested that lactate is an important metabolic substrate for crustaceans, and an assessment of the production and utilization of lactate in association with metabolic rate changes could help in elucidating shifts in metabolic strategies associated with acclimation to cyclic emersion. Further, they could identify potential influences on long-term performance and fitness under a variety of environmental scenarios.

References

Adamczewska, A., & Morris, S. (1994). Exercise in the terrestrial Christmas island red crab *Gecarcoidea Natalis* – Blood gas transport. *Journal of Experimental Biology*, 188(1), 235-256.

Airriess, C., & McMahon, B. (1996). Short-term emersion affects cardiac function and regional haemolymph distribution in the crab *Cancer magister*. *Journal of Experimental Biology*, 199(3), 569-578.

Altieri, A. H. (2006). Inducible variation in hypoxia tolerance across the intertidal–subtidal distribution of the blue mussel *Mytilus edulis*. *Marine Ecology Progress Series*, 325, 295-300.

Arudpragasam, K. D., & Naylor, E. (1964). Gill ventilation volumes, oxygen consumption and respiratory rhythms in *Carcinus maenas* (L.). *Journal of Experimental Biology*, 41(2), 309-321.

Baden, S.P., Pihl, L., Rosenberg, R., 1990. Effects of oxygen depletion on the ecology, blood physiology and fishery of the Norway lobster *Nephrops norvegicus*. *Marine Ecology Progress Series*. 67, 141–155.

Barry, J. P., Baxter, C. H., Sagarin, R. D., & Gilman, S. E. (1995). Climate-related, long-term faunal changes in a California rocky intertidal community. *Science*, 267(5198), 672-675.

Behrens Yamada, S. (2001). Global invader: the European green crab. *Oregon Sea Grant, Corvallis*.

Berrill, M. (1982). The life cycle of the green crab *Carcinus maenas* at the northern end of its range. *Journal of Crustacean Biology*, 2(1), 31-39.

Bertness, M. D., Gaines, S. D., & Hay, M. E. (Eds.). (2001). *Marine community ecology* (No. QH 541.5. S3. M369 2001). Sunderland, MA: Sinauer Associates.

Bjelde, B. E., & Todgham, A. E. (2013). Thermal physiology of the fingered limpet *Lottia digitalis* under emersion and immersion. *Journal of Experimental Biology*, 216(15), 2858-2869.

Boone, W. R., & Schoffeniels, E. (1979). Haemocyanin synthesis during hypo-osmotic stress in the shore crab *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 63(2), 207-214.

Bozinovic, F., Calosi, P., & Spicer, J. I. (2011). Physiological correlates of geographic range in animals. *Annual Review of Ecology, Evolution, and Systematics*, 42, 155-179.

Bridges, C. R. (1988). Respiratory adaptations in intertidal fish. *American Zoologist*, 28(1), 79-96.

Bridges, C. R., & Brand, A. R. (1980). The effect of hypoxia on oxygen consumption and blood lactate levels of some marine Crustacea. *Comparative Biochemistry and Physiology Part A: Physiology*, 65(4), 399-409.

Burggren, W. W., & McMahon B. R. (1981). Oxygen uptake during environmental temperature change in hermit crabs: adaptation to subtidal, intertidal, and supratidal habitats. *Physiological Zoology*, 54(3), 325-333.

Burke, E. M. (1979). Aerobic and anaerobic metabolism during activity and hypoxia in two species of intertidal crabs. *The Biological Bulletin*, 156(2), 157-168.

Burnett, L. E. (1988). Physiological responses to air exposure: acid-base balance and the role of branchial water stores. *American Zoologist*, 28(1), 125-135.

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

Burnett, L. E., & McMahon, B. R. (1987). Gas exchange, haemolymph acid-base status, and the role of branchial water stores during air exposure in three littoral crab species. *Physiological Zoology*, 60(1), 27-36.

Burnett, L., Terwilliger, N., Carroll, A., Jorgensen, D., & Scholnick, D. (2002). Respiratory and acid-base physiology of the purple sea urchin, *Strongylocentrotus purpuratus*, during air exposure: presence and function of a facultative lung. *The Biological Bulletin*, 203(1), 42-50.

Butler, S. L., & Morris, S. (1996). Haemolymph respiratory gas, acid-base, and ion status of the amphibious purple shore crab *Leptograpsus variegatus* (Fabricius) in response to air-breathing. *Journal of Crustacean Biology*, 16(2), 240-252.

Byrne, R. A., & McMahon, R. F. (1994). Behavioral and physiological responses to emersion in freshwater bivalves. *American Zoologist*, 34(2), 194-204.

Cameron, J. N. (1986). Responses to reversed NH_3 and NH_4^+ gradients in a teleost (*Ictalurus punctatus*), an elasmobranch (*Raja erinacea*), and a crustacean (*Callinectes sapidus*): evidence for NH_4^+/H^+ exchange in the teleost and the elasmobranch. *Journal of Experimental Zoology*, 239(2), 183-195.

Chabot, C. C., & Watson, W. H. (2010). Circatidal rhythms of locomotion in the American horseshoe crab *Limulus polyphemus*: Underlying mechanisms and cues that influence them. *Current Zoology*, 56(5).

Clark, M. S., & Peck, L. S. (2009). Triggers of the HSP70 stress response: environmental responses and laboratory manipulation in an Antarctic marine invertebrate (*Nacella concinna*). *Cell Stress and Chaperones*, 14(6), 649-660.

Clark, T. D., Sandblom, E., & Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *Journal of Experimental Biology*, 216(15), 2771-2782.

Clow, K. A., Short, C. E., & Driedzic, W. R. (2016). Extracellular glucose supports lactate production but not aerobic metabolism in cardiomyocytes from both normoglycemic Atlantic cod and low glycemic short-horned sculpin. *Journal of Experimental Biology*, 219(9), 1384-1393.

Cohen, A. N., Carlton, J. T., & Fountain, M. C. (1995). Introduction, dispersal and potential impacts of the green crab *Carcinus maenas* in San Francisco Bay, California. *Marine Biology*, 122(2), 225-237.

Connor, K. M., & Gracey, A. Y. (2011). High-resolution analysis of metabolic cycles in the intertidal mussel *Mytilus californianus*. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 302(1), R103-R111.

Crothers, J. H. (1968). The biology of the shore crab, *Carcinus maenas* (L.). 2. The life of the adult crab. *Field Studies*, 2, 579-614.

Dame, R. F., & Vernberg, F. J. (1978). The influence of constant and cyclic acclimation temperatures on the metabolic rates of *Panopeus herbstii* and *Uca pugilator*. *The Biological Bulletin*, 154(2), 188-197.

Dan, X. M., Yan, G. J., Zhang, A. J., Cao, Z. D., & Fu, S. J. (2014). Effects of stable and diel-cycling hypoxia on hypoxia tolerance, postprandial metabolic response, and growth performance in juvenile qingbo (*Spinibarbus sinensis*). *Aquaculture*, 428, 21-28.

De la Iglesia, H.O. & Hsu, Y. W. A. (2010). Biological clocks and rhythms in intertidal crustaceans. *Frontiers in Bioscience*, 2, 1394-1404.

DeFur, P. L. (1988). Systemic respiratory adaptations to air exposure in intertidal decapod crustaceans. *American Zoologist*, 28(1), 115-124.

DeFur, P. L., & McMahon, B. R. (1984). Physiological compensation to short-term air exposure in red rock crabs, *Cancer productus* (Randall), from littoral and sublittoral habitats. I. Oxygen uptake and transport. *Physiological Zoology*, 57(1), 137-150.

DeFur, P. L., Mangum, C. P., & Reese, J. E. (1990). Respiratory responses of the blue crab *Callinectes sapidus* to long-term hypoxia. *The Biological Bulletin*, 178(1), 46-54.

Dejours, P., & Truchot, J. P. (1988). Respiration of the emersed shore crab at variable ambient oxygenation. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 158(3), 387-391.

Demers, A., & Guderley, H. (1994). Acclimatization to intertidal conditions modifies the physiological response to prolonged air exposure in *Mytilus edulis*. *Marine Biology*, 118(1), 115-122.

Denny, M. W., Dowd, W. W., Bilir, L., & Mach, K. J. (2011). Spreading the risk: small-scale body temperature variation among intertidal organisms and its implications for species persistence. *Journal of Experimental Marine Biology and Ecology*, 400(1-2), 175-190.

Depledge, M. H. (1984). Disruption of circulatory and respiratory activity in shore crabs (*Carcinus maenas* (L)) exposed to heavy metal pollution. *Comparative biochemistry and physiology. C: Comparative Pharmacology and Toxicology*, 78(2), 445-459.

De Zwaan, A., & Wijsman, T. C. M. (1976). Anaerobic metabolism in bivalvia (Mollusca) characteristics of anaerobic metabolism. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 54(3), 313-323.

Discher, D. J., Bishopric, N. H., Wu, X., Peterson, C. A., & Webster, K. A. (1998). Hypoxia regulates β-enolase and pyruvate kinase-M promoters by modulating Sp1/Sp3 binding to a conserved GC element. *Journal of Biological Chemistry*, 273(40), 26087-26093.

Dong, Y. W., & Williams, G. A. (2011). Variations in cardiac performance and heat shock protein expression to thermal stress in two differently zoned limpets on a tropical rocky shore. *Marine Biology*, 158(6), 1223-1231.

Dong, Y., Dong, S., Tian, X., Wang, F., & Zhang, M. (2006). Effects of diel temperature fluctuations on growth, oxygen consumption and proximate body composition in the sea cucumber *Apostichopus japonicus* Selenka. *Aquaculture*, 255(1-4), 514-521.

Drake, M. J., Miller, N. A., & Todgham, A. E. (2017). The role of stochastic thermal environments in modulating the thermal physiology of an intertidal limpet, *Lottia digitalis*. *Journal of Experimental Biology*, 220(17), 3072-3083.

Ern, R., Phuong, N. T., Wang, T., & Bayley, M. (2014). Oxygen delivery does not limit thermal tolerance in a tropical eurythermal crustacean. *Journal of Experimental Biology*, 217(5), 809-814.

Fangue, N. A., Hofmeister, M., & Schulte, P. M. (2006). Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. *Journal of Experimental Biology*, 209(15), 2859-2872.

Firth, L. B., & Williams, G. A. (2009). The influence of multiple environmental stressors on the limpet *Cellana toreuma* during the summer monsoon season in Hong Kong. *Journal of Experimental Marine Biology and Ecology*, 375(1-2), 70-75.

Frederich, M., & Pörtner, H. O. (2000). Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 279(5), R1531-R1538.

Frederich, M., O'Rourke, M. R., Furey, N. B., & Jost, J. A. (2009). AMP-activated protein kinase (AMPK) in the rock crab, *Cancer irroratus*: an early indicator of temperature stress. *Journal of Experimental Biology*, 212(5), 722-730.

Fusi, M., Cannicci, S., Daffonchio, D., Mostert, B., Pörtner, H. O., & Giomi, F. (2016). The trade-off between heat tolerance and metabolic cost drives the bimodal life strategy at the air-water interface. *Scientific reports*, 6, 19158.

Fusi, M., Giomi, F., Babbini, S., Daffonchio, D., McQuaid, C. D., Porri, F., & Cannicci, S. (2015). Thermal specialization across large geographical scales predicts the resilience of mangrove crab populations to global warming. *Oikos*, 124(6), 784-795.

Gäde, G. (1983). Energy metabolism of arthropods and mollusks during environmental and functional anaerobiosis. *Journal of Experimental Zoology*, 228(3), 415-429.

Gilles, R. (1977). Effects of osmotic stresses on the proteins concentration and pattern of *Eriocheir sinensis* blood. *Comparative Biochemistry and Physiology Part A: Physiology*, 56(2), 109-114.

Gilman, S. E., Wethey, D. S., & Helmuth, B. (2006). Variation in the sensitivity of organismal body temperature to climate change over local and geographic scales. *Proceedings of the National Academy of Sciences*, 103(25), 9560-9565.

Giomi, F., & Beltramini, M. (2007). The molecular heterogeneity of haemocyanin: its role in the adaptive plasticity of Crustacea. *Gene*, 398(1-2), 192-201.

Giomi, F., & Poertner, H. O. (2013). A role for haemolymph oxygen capacity in heat tolerance of eurythermal crabs. *Frontiers in Physiology*, 4, 110.

Giomi, F., Fusi, M., Barausse, A., Mostert, B., Pörtner, H. O., & Cannicci, S. (2014). Improved heat tolerance in air drives the recurrent evolution of air-breathing. *Proceedings of the Royal Society B: Biological Sciences*, 281(1782), 20132927.

Gladden, L. B. (2004). Lactate metabolism: a new paradigm for the third millennium. *The Journal of Physiology*, 558(1), 5-30.

Gorr, T. A., Cahn, J. D., Yamagata, H., & Bunn, H. F. (2004). Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor-dependent. *Journal of Biological Chemistry*, 279(34), 36038-36047.

Gorr, T. A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A. F., Terwilliger, N., ... & Deten, A. (2010). Hypoxia tolerance in animals: biology and application. *Physiological and Biochemical Zoology,* 83(5), 733-752.

Grant, J., & McDonald, J. (1979). Desiccation tolerance of *Eurypanopeus depressus* (Smith)(Decapoda: Xanthidae) and the exploitation of microhabitat. *Estuaries*, 2(3), 172.

Greenaway, P., Morris, S., Sanders, N., & Adamczewska, A. (1992). Blood gas transport and oxygen consumption in a supralittoral crab, *Leptograpsus variegatus* (Crustacea: Brachyura). *Marine and Freshwater Research,* 43(6), 1573-1584.

Grieshaber, M. K., Hardewig, I., Kreutzer, U., & Pörtner, H. O. (1993). Physiological and metabolic responses to hypoxia in invertebrates. In *Reviews of Physiology, Biochemistry and Pharmacology*, Volume 125 (pp. 43-147). Springer, Berlin, Heidelberg.

Grosholz, E., & Ruiz, G. (2002). Management plan for the European green crab. *Aquatic Nuisance Species Task Force*, 55.

Gunderson, A. R., Armstrong, E. J., & Stillman, J. H. (2016). Multiple stressors in a changing world: the need for an improved perspective on physiological responses to the dynamic marine environment. *Annual Review of Marine Science*, 8, 357-378.

Hagerman, L., & Oksama, M. (1985). Haemocyanin concentration, carrying capacity and haemolymph pH under hypoxia in *Mesidothea entomon* (L.)(Isopoda, Crustacea). *Ophelia*, 24(1), 47-52.

Hagerman, L., Søndergaard, T., Weile, K., Hosie, D., & Uglow, R. F. (1990). Aspects of blood physiology and ammonia excretion in *Nephrops norvegicus* under hypoxia. *Comparative Biochemistry and Physiology. Part A. Physiology*, (1).

Hammer, Ø., Harper, D. A., & Ryan, P. D. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, 4(1), 9.

Han, G. D., Zhang, S., Marshall, D. J., Ke, C. H., & Dong, Y. W. (2013). Metabolic energy sensors (AMPK and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion. *Journal of Experimental Biology*, 216(17), 3273-3282.

Harley, C. D., Randall Hughes, A., Hultgren, K. M., Miner, B. G., Sorte, C. J., Thornber, C. S., ... & Williams, S. L. (2006). The impacts of climate change in coastal marine systems. *Ecology Letters*, 9(2), 228-241.

Harper, S. L., & Reiber, C. L. (2006). Metabolic, respiratory and cardiovascular responses to acute and chronic hypoxic exposure in tadpole shrimp *Triops longicaudatus*. *Journal of Experimental Biology*, 209(9), 1639-1650.

Harris, G. J., & Morgan, E. (1984). Entrainment of the circatidal rhythm of the estuarine amphipod *Corophium volutator* (pallas) to non-tidal cycles of inundation and exposure in the laboratory. *Journal of Experimental Marine Biology and Ecology*, 80(3), 235-245.

Hazel, J. R., & Prosser, C. L. (1974). Molecular mechanisms of temperature compensation in poikilotherms. *Physiological Reviews*, 54(3), 620-677.

Head, J. M. (2010). The effects of hypoxia on haemocyanin regulation in *Cancer magister*: Possible role of Hypoxia-Inducible Factor-1. *Journal of Experimental Marine Biology and Ecology,* 386(1-2), 77-85.

Helmuth, B., Broitman, B. R., Blanchette, C. A., Gilman, S., Halpin, P., Harley, C. D., ... & Strickland, D. (2006a). Mosaic patterns of thermal stress in the rocky intertidal zone: implications for climate change. *Ecological Monographs,* 76(4), 461-479.

Helmuth, B., Broitman, B. R., Yamane, L., Gilman, S. E., Mach, K., Mislan, K. A. S., & Denny, M. W. (2010). Organismal climatology: analyzing environmental variability at scales relevant to physiological stress. *Journal of Experimental Biology*, 213(6), 995-1003.

Helmuth, B., Harley, C. D., Halpin, P. M., O'Donnell, M., Hofmann, G. E., & Blanchette, C. A. (2002). Climate change and latitudinal patterns of intertidal thermal stress. *Science*, 298(5595), 1015-1017.

Helmuth, B., Mieszkowska, N., Moore, P., & Hawkins, S. J. (2006b). Living on the edge of two changing worlds: forecasting the responses of rocky intertidal ecosystems to climate change. *Annual Reviews Ecology Evolution and Systematics*, 37, 373-404.

Henry, R. P. (1984). The role of carbonic anhydrase in blood ion and acid-base regulation. *American Zoologist*, 24(1), 241-251.

Henry, R. P. (1994). Morphological, behavioral, and physiological characterization of bimodal breathing crustaceans. *American Zoologist,* 34(2), 205-215.

Henry, R. P., Booth, C. E., Lallier, F. H., & Walsh, P. J. (1994). Post-exercise lactate production and metabolism in three species of aquatic and terrestrial decapod crustaceans. *Journal of Experimental Biology*, 186(1), 215-234.

Henry, R. P., Kormanik, G. A., Smatresk, N. J., & Cameron, J. N. (1981). The role of $CaCO_3$ dissolution as a source of HCO_3^- for the buffering of hypercapnic acidosis in aquatic and terrestrial decapod crustaceans. *Journal of Experimental Biology*, 94(1), 269-274.

Hervant, F., Mathieu, J., & Messana, G. (1997). Locomotory, ventilatory and metabolic responses of the subterranean *Stenasellus virei* (Crustacea, Isopoda) to severe hypoxia and subsequent recovery. *Comptes Rendus de l'Académie des Sciences-Series III-Sciences de la Vie*, 320(2), 139-148.

Hochachka, P. W., & Lutz, P. L. (2001). Mechanism, origin, and evolution of anoxia tolerance in animals. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 130(4), 435-459.

Hofmann, G. E., & Todgham, A. E. (2010). Living in the now: physiological mechanisms to tolerate a rapidly changing environment. *Annual Review of Physiology*, 72, 127-145.

Hoogewijs, D., Terwilliger, N. B., Webster, K. A., Powell-Coffman, J. A., Tokishita, S., Yamagata, H., ... & Abele, D. (2007). From critters to cancers: bridging comparative and

clinical research on oxygen sensing, HIF signaling, and adaptations towards hypoxia. *Integrative and Comparative Biology*, 47(4), 552-577.

Hsia, C. C., Schmitz, A., Lambertz, M., Perry, S. F., & Maina, J. N. (2013). Evolution of air breathing: oxygen homeostasis and the transitions from water to land and sky. *Comprehensive Physiology*, 3(2), 849-915.

Hyde, D., Qari, S., Hopkin, R. S., & Bowler, K. (2012). Thermal acclimation, heat shock and photoperiod: Do these factors interplay in the adaptive responses of crab neuromuscular systems to temperature?. *Journal of Thermal Biology*, 37(3), 164-170.

Jackson, D. C., Wang, T., Koldkjaer, P., & Taylor, E. W. (2001). Lactate sequestration in the carapace of the crayfish *Austropotamobius pallipes* during exposure in air. *Journal of Experimental Biology*, 204(5), 941-946.

Jayasundara, N., & Somero, G. N. (2013). Physiological plasticity of cardiorespiratory function in a eurythermal marine teleost, the longjaw mudsucker, *Gillichthys mirabilis*. *Journal of Experimental Biology*, 216(11), 2111-2121.

Jimenez, A. G., Alves, S., Dallmer, J., Njoo, E., Roa, S., & Dowd, W. W. (2016). Acclimation to elevated emersion temperature has no effect on susceptibility to acute, heat-induced lipid peroxidation in an intertidal mussel (*Mytilus californianus*). *Marine Biology,* 163(3), 55.

Johnson, I., & Uglow, R. F. (1985). Some effects of aerial exposure on the respiratory physiology and blood chemistry of *Carcinus maenas* (L.) and *Liocarcinus puber* (L.). *Journal of Experimental Marine Biology and Ecology*, 94(1-3), 151-165.

Jost, J. A., Podolski, S. M., & Frederich, M. (2012). Enhancing thermal tolerance by eliminating the pejus range: a comparative study with three decapod crustaceans. *Marine Ecology Progress Series*, 444, 263-274.

Jouve-Duhamel, A., & Truchot, J. P. (1985). Ventilatory responses to ambient oxygen in stressed and fully settled shore crabs *Carcinus maenas* (L.). *Journal of Experimental Marine Biology and Ecology*, 90(2), 107-118.

Kankondi, S. L., McQuaid, C. D., & Tagliarolo, M. (2018). Influence of respiratory mode on the thermal tolerance of intertidal limpets. *PloS one*, 13(9), e0203555.

Kawabe, S., & Yokoyama, Y. (2009). cDNA cloning and expression of grp94 in the Pacific oyster *Crassostrea gigas*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 154(3), 290-297.

Kelley, A. L., de Rivera, C. E., & Buckley, B. A. (2011). Intraspecific variation in thermotolerance and morphology of the invasive European green crab, *Carcinus maenas*, on the west coast of North America. *Journal of Experimental Marine Biology and Ecology,* 409(1-2), 70-78.

Kensler, C. B. (1967). Desiccation resistance of intertidal crevice species as a factor in their zonation. *The Journal of Animal Ecology*, 391-406.

Klassen, G. J., & Locke, A. (2007). *A biological synopsis of the European green crab, Carcinus maenas*. Moncton, New Brunswick, Canada: Fisheries and Oceans Canada.

Kültz, D. (2005). Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology*, 67, 225-257.

Lagos, M., Cáceres, C. W., & Lardies, M. A. (2014). Geographic variation in acid–base balance of the intertidal crustacean *Cyclograpsus cinereus* (Decapoda, Grapsidae) during air exposure. *Journal of the Marine Biological Association of the United Kingdom*, 94(1), 159-165.

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

Lallier, F., Boitel, F., & Truchot, J. P. (1987). The effect of ambient oxygen and temperature on haemolymph Lactate and urate concentrations in the shore crab *Carcinus maenas*. *Comparative Biochemistry and Physiology Part A: Physiology*, 86(2), 255-260.

Leignel, V., Stillman, J. H., Baringou, S., Thabet, R., & Metais, I. (2014). Overview on the European green crab *Carcinus* spp.(Portunidae, Decapoda), one of the most famous marine invaders and ecotoxicological models. *Environmental Science and Pollution Research*, 21(15), 9129-9144.

Leiva, F. P., Niklitschek, E. J., Paschke, K., Gebauer, P., & Urbina, M. A. (2016). Tide-related biological rhythm in the oxygen consumption rate of ghost shrimp (*Neotrypaea uncinata*). *Journal of Experimental Biology*, 219(13), 1957-1960.

Levinton, J. S., & Levinton, J. S. (1995). *Marine biology: function, biodiversity, ecology* (Vol. 420). New York: Oxford University Press.

Little, C., & Kitching, J. A. (1996). *The biology of rocky shores*. Oxford University Press, USA.

Liu, Y., Hui, M., Cui, Z., Luo, D., Song, C., Li, Y., & Liu, L. (2015). Comparative transcriptome analysis reveals sex-biased gene expression in juvenile Chinese mitten crab *Eriocheir sinensis*. *PLoS One*, 10(7), e0133068.

Lorenzon, S., Giulianini, P. G., Libralato, S., Martinis, M., & Ferrero, E. A. (2008). Stress effect of two different transport systems on the physiological profiles of the crab *Cancer pagurus*. *Aquaculture*, 278(1-4), 156-163.

Lorenzon, S., Giulianini, P. G., Martinis, M., & Ferrero, E. A. (2007). Stress effect of different temperatures and air exposure during transport on physiological profiles in the American lobster *Homarus americanus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 147(1), 94-102.

Lowell, R. B. (1984). Desiccation of intertidal limpets: effects of shell size, fit to substratum, and shape. *Journal of Experimental Marine Biology and Ecology*, 77(3), 197-207.

Lu, Y., Zhang, D., Wang, F., & Dong, S. (2016). Hypothermal effects on survival, energy homeostasis and expression of energy-related genes of swimming crabs *Portunus trituberculatus* during air exposure. *Journal of Thermal Biology*, 60, 33-40.

Luquet, C. M., & Ansaldo, M. (1997). Acid–base balance and ionic regulation during emersion in the estuarine intertidal crab *Chasmagnathus granulata* Dana (Decapoda Grapsidae). *Comparative Biochemistry and Physiology Part A: Physiology*, 117(3), 407-410.

Luquet, C. M., Cervino, C. O., Ansaldo, M., Pereyra, V. C., Kocmur, S., & Dezi, R. E. (1998). Physiological response to emersion in the amphibious crab *Chasmagnathus granulata* Dana (Decapoda Grapsidae): biochemical and ventilatory adaptations. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 121(4), 385-393.

Maciel, J. E. S., Souza, F., Valle, S., Kucharski, L. C., & da Silva, R. S. M. (2008). Lactate metabolism in the muscle of the crab *Chasmagnathus granulata* during hypoxia and post-hypoxia recovery. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 151(1), 61-65.

Madeira, D., Narciso, L., Cabral, H. N., & Vinagre, C. (2012). Thermal tolerance and potential impacts of climate change on coastal and estuarine organisms. *Journal of Sea Research*, 70, 32-41.

Magozzi, S., & Calosi, P. (2015). Integrating metabolic performance, thermal tolerance, and plasticity enables for more accurate predictions on species vulnerability to acute and chronic effects of global warming. *Global Change Biology*, 21(1), 181-194.

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

Mangum, C. P., & Weiland, A. L. (1975). The function of haemocyanin in respiration of the blue crab *Callinectes sapidus*. *Journal of Experimental Zoology*, 193(3), 257-263.

Mark, F. C., Bock, C., & Pörtner, H. O. (2002). Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and 31P-MRS. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 283(5), R1254-R1262.

Marshall, D. J., & McQuaid, C. D. (1992). Comparative aerial metabolism and water relations of the intertidal limpets *Patella granularis* L.(Mollusca: *Prosobranchia*) and *Siphonaria oculus* Kr.(Mollusca: *Pulmonata*). *Physiological Zoology*, 65(5), 1040-1056.

Marshall, D. J., Dong, Y. W., McQuaid, C. D., & Williams, G. A. (2011). Thermal adaptation in the intertidal snail *Echinolittorina malaccana* contradicts current theory by

revealing the crucial roles of resting metabolism. *Journal of Experimental Biology*, 214(21), 3649-3657.

Mason, R. P., Mangum, C. P., & Godette, G. (1983). The influence of inorganic ions and acclimation salinity on haemocyanin-oxygen binding in the blue crab *Callinectes sapidus*. *The Biological Bulletin*, 164(1), 104-123.

Massabuau, J. C., & Forgue, J. (1996). A field versus laboratory study of blood oxygen status in normoxic crabs at different temperatures. *Canadian Journal of Zoology*, 74(3), 423-430.

McGaw, I. J. (2003). Behavioral thermoregulation in *Hemigrapsus nudus*, the amphibious purple shore crab. *The Biological Bulletin*, 204(1), 38-49.

McGaw, I. J. (2006). Feeding and digestion in low salinity in an osmoconforming crab, *Cancer gracilis* I. Cardiovascular and respiratory responses. *Journal of Experimental Biology*, 209(19), 3766-3776.

McGaw, I. J., & McMahon, B. R. (1998). Endogenous rhythms of haemolymph flow and cardiac performance in the crab *Cancer magister*. *Journal of Experimental Marine Biology and Ecology*, 224(1), 127-142.

McGaw, I. J., & Nancollas, S. J. (2018). Experimental setup influences the cardiovascular responses of decapod crustaceans to environmental change. *Canadian Journal of Zoology*, 96(9), 1043-1052.

McGaw, I. J., & Whiteley, N. M. (2012). Effects of acclimation and acute temperature change on specific dynamic action and gastric processing in the green shore crab, *Carcinus maenas*. *Journal of Thermal Biology*, 37(8), 570-578.

McGaw, I. J., Curtis, D. L., Ede, J. D., Ong, K. J., van Breukelen, F., & Goss, G. G. (2009). Physiological responses of postprandial red rock crabs (Cancer *productu*s) during emersion. *Canadian Journal of Zoology*, 87(12), 1158-1169.

McMahon, B. R. (2001). Respiratory and circulatory compensation to hypoxia in crustaceans. *Respiration Physiology*, 128(3), 349-364.

McMahon, B. R., & Burggren, W. W. (1979). Respiration and adaptation to the terrestrial habitat in the land hermit crab *Coenobita clypeatus*. *Journal of Experimental Biology*, 79(1), 265-281.

McMahon, B. R., Burggren, W. W., Pinder, A. W., & Wheatly, M. G. (1991). Air exposure and physiological compensation in a tropical intertidal chiton, *Chiton stokesii* (Mollusca: Polyplacophora). *Physiological Zoology*, 64(3), 728-747.

McMahon, R. F. (1988). Respiratory response to periodic emergence in intertidal molluscs. *American Zoologist*, 28(1), 97-114.

Melzner, F., Bock, C., & Pörtner, H. O. (2006). Temperature-dependent oxygen extraction from the ventilatory current and the costs of ventilation in the cephalopod *Sepia officinalis*. *Journal of Comparative Physiology B*: *Biochemical, Systemic, Environmental Physiology*, 176(7), 607.

Mieszkowska, N., Kendall, M. A., Hawkins, S. J., Leaper, R., Williamson, P., Hardman-Mountford, N. J., & Southward, A. J. (2006). Changes in the range of some common rocky shore species in Britain—a response to climate change?. In *Marine Biodiversity* (pp. 241-251). Springer, Dordrecht.

Morris, S. (1991). Respiratory gas exchange and transport in crustaceans: ecological determinants. *Memoirs of the Queensland Museum*, 31, 241-261.

Morris, S., & Adamczewska, A. M. (2002). Utilisation of glycogen, ATP and arginine phosphate in exercise and recovery in terrestrial red crabs, *Gecarcoidea natalis*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 133(3), 813-825.

Morris, S., & Butler, S. L. (1996). Haemolymph respiratory gas, acid-base, and ion status of the amphibious purple shore crab *Leptograpsus variegatus* (Fabricius) during immersion and environmental hypoxia. *Journal of Crustacean Biology*, 16(2), 253-266.

Morris, S., Greenaway, P., & McMahon, B. R. (1988). Adaptations to a Terrestrial Existence by the Robber Crab *Birgus Latro*: I. An In Vitro Investigation of Blood Gas Transport. *Journal of Experimental Biology,* 140(1), 477-491.

Morris, S., Greenaway, P., & McMahon, B. R. (1996). Air breathing by the purple shore crab, *Hemigrapsus nudus* (Dana). III. Haemocyanin function in respiratory gas transport. *Physiological Zoology*, 69(4), 839-863.

Murphy, B. J., Andrews, G. K., Bittel, D., Discher, D. J., McCue, J., Green, C. J., ... & Webster, K. A. (1999). Activation of metallothionein gene expression by hypoxia involves metal response elements and metal transcription factor-1. *Cancer Research*, 59(6), 1315-1322.

Naylor E. (1958). Tidal and diurnal rhythms of locomotory activity in *Carcinus maenas* (L.). *Journal of Experimental Biology*, 35:602–610.

Naylor, E. (1996). Crab clockwork: the case for interactive circatidal and circadian oscillators controlling rhythmic locomotor activity of *Carcinus maenas*. *Chronobiology International*, 13(3), 153-161.

Naylor, E. (2010). *Chronobiology of marine organisms*. Cambridge University Press.

Newell, R. C. (1973). Factors affecting the respiration of intertidal invertebrates. *American Zoologist*, 13(2), 513-528.

Newell, R. C., Ahsanullah, M., & Pye, V. I. (1972). Aerial and aquatic respiration in the shore crab *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology Part A: Physiology*, 43(2), 239-252.

Nicastro, K. R., Zardi, G. I., McQuaid, C. D., Stephens, L., Radloff, S., & Blatch, G. L. (2010). The role of gaping behaviour in habitat partitioning between coexisting intertidal mussels. *BMC Ecology*, 10(1), 17.

Nicchitta, C. V., & Ellington, W. R. (1983). Energy metabolism during air exposure and recovery in the high intertidal bivalve mollusc *Geukensia demissa granosissima* and the subtidal bivalve mollusc *Modiolus squamosus*. *The Biological Bulletin,* 165(3), 708-722.

Nickerson, K., & Van Holde, K. E. (1971). A comparison of molluscan and arthropod haemocyanin-I. Circular dichroism and absorption spectra. *Comparative Biochemistry and Physiology--Part B: Biochemistry & Molecular Biology*, 39(4), 855-872.

O'Mahoney, P. M., & Full, R. J. (1984). Respiration of crabs in air and water. *Comparative Biochemistry and Physiology Part A: Physiology*, 79(2), 275-282.

O'Neill, J. S., Lee, K. D., Zhang, L., Feeney, K., Webster, S. G., Blades, M. J., ... & Wilcockson, D. C. (2015). Metabolic molecular markers of the tidal clock in the marine crustacean *Eurydice pulchra*. *Current Biology*, 25(8), R326-R327.

Oliver, T. A., & Palumbi, S. R. (2011). Do fluctuating temperature environments elevate coral thermal tolerance?. *Coral Reefs*, 30(2), 429-440.

Ottaway, J. R. (1973). Some effects of temperature, desiccation, and light on the intertidal anemone, *Actinia tenebrosa* Farquhar (Cnidaria: Anthozoa). *Marine and Freshwater Research,* 24(1), 103-126.

Paganini, A. W., Miller, N. A., & Stillman, J. H. (2014). Temperature and acidification variability reduce physiological performance in the intertidal zone porcelain crab *Petrolisthes cinctipes*. *Journal of Experimental Biology*, 217(22), 3974-3980.

Paine, R. T. (1974). Intertidal community structure. *Oecologia*, 15(2), 93-120.

Palmer, J. D. (1973). Tidal rhythms: the clock control of the rhythmic physiology of marine organisms. *Biological Reviews*, 48(3), 377-418.

Palmer, J. D. (1995). Review of the dual-clock control of tidal rhythms and the hypothesis that the same clock governs both circatidal and circadian rhythms. *Chronobiology International,* 12(5), 299-310.

Palmer, J. D. (2000). The clocks controlling the tide‐associated rhythms of intertidal animals. *BioEssays*, 22(1), 32-37.

Pascual, C., Gaxiola, G., & Rosas, C. (2003). Blood metabolites and haemocyanin of the white shrimp, *Litopenaeus vannamei*: the effect of culture conditions and a comparison with other crustacean species. *Marine Biology*, 142(4), 735-745.

Peck, L. S., Webb, K. E., & Bailey, D. M. (2004). Extreme sensitivity of biological function to temperature in Antarctic marine species. *Functional Ecology*, 18(5), 625-630.

Pöhlmann, K., Koenigstein, S., Alter, K., Abele, D., & Held, C. (2011). Heat-shock response and antioxidant defense during air exposure in Patagonian shallow-water limpets from different climatic habitats. *Cell Stress and Chaperones*, 16(6), 621.

Pörtner, H. (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften*, 88(4), 137-146.

Pörtner, H. O. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 132(4), 739-761.

Pörtner, H. O. (2010). Oxygen-and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *Journal of Experimental Biology*, 213(6), 881-893.

Pörtner, H. O., & Knust, R. (2007). Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science,* 315(5808), 95-97.

Pörtner, H. O., Hardewig, I., & Peck, L. S. (1999). Mitochondrial function and critical temperature in the Antarctic bivalve, *Laternula elliptica*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 124(2), 179-189.

Rastrick, S. P., Calosi, P., Calder-Potts, R., Foggo, A., Nightingale, G., Widdicombe, S., & Spicer, J. I. (2014). Living in warmer, more acidic oceans retards physiological recovery from tidal emersion in the velvet swimming crab, *Necora puber*. *Journal of Experimental Biology*, 217(14), 2499-2508.

Ray, G. L. (2005). *Invasive marine and estuarine animals of the Pacific northwest and Alaska (*No. ERDC/TN-ANSRP-05-6). Aquatic Nuisance Species Program Vicksburg MS.

Reid, D. G., & Naylor, E. (1990). Entrainment of bimodal circatidal rhythms in the shore crab *Carcinus maenas*. *Journal of Biological Rhythms*, 5(4), 333-347.

Richards, J. G. (2011). Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. *Journal of Experimental Biology*, 214(2), 191-199.

Roberts, D. A., Hofmann, G. E., & Somero, G. N. (1997). Heat-shock protein expression in *Mytilus californianus*: acclimatization (seasonal and tidal-height comparisons) and acclimation effects. *The Biological Bulletin*, 192(2), 309-320.

Robertson, R. F., Meagor, J., & Taylor, E. W. (2002). Specific dynamic action in the shore crab, *Carcinus maenas* (L.), in relation to acclimation temperature and to the onset of the emersion response. *Physiological and Biochemical Zoology*, 75(4), 350-359.

Roff, J. C., Davidson, K. G., Pohle, G., & Dadswell, M. J. (1984). *A guide to the marine flora and fauna of the Bay of Fundy and Scotian Shelf: larval Decapoda: Brachyura*. Department of Fisheries and Oceans, Fisheries and Environmental Sciences, Biological Station.

Romero, M. C., Ansaldo, M., & Lovrich, G. A. (2007). Effect of aerial exposure on the antioxidant status in the subantarctic stone crab *Paralomis granulosa* (Decapoda: Anomura). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 146(1-2), 54-59.

Romero, M. C., Tapella, F., Sotelano, M. P., Ansaldo, M., & Lovrich, G. A. (2011). Oxidative stress in the subantarctic false king crab *Paralomis granulosa* during air exposure and subsequent re-submersion. *Aquaculture*, 319(1-2), 205-210.

Sagarin, R. D., Barry, J. P., Gilman, S. E., & Baxter, C. H. (1999). Climate‐related change in an intertidal community over short and long time scales. *Ecological Monographs*, 69(4), 465-490.

Santos, E. A., & Keller, R. (1993). Effect of exposure to atmospheric air on blood glucose and lactate concentrations in two crustacean species: a role of the crustacean hyperglycemic hormone (CHH). *Comparative Biochemistry and Physiology Part A: Physiology*, 106(2), 343-347.

Sartoris, F. J., Bock, C., Serendero, I., Lannig, G., & Pörtner, H. O. (2003). Temperature‐dependent changes in energy metabolism, intracellular pH and blood oxygen tension in the Atlantic cod. *Journal of Fish Biology*, 62(6), 1239-1253.

Schnytzer, Y., Simon-Blecher, N., Li, J., Ben-Asher, H. W., Salmon-Divon, M., Achituv, Y., Hughes, M.E., & Levy, O. (2018). Tidal and diel orchestration of behaviour and gene expression in an intertidal mollusc. *Scientific Reports*, 8(1), 4917.

Schröer, M., Wittmann, A. C., Grüner, N., Steeger, H. U., Bock, C., Paul, R., & Pörtner, H. O. (2009). Oxygen limited thermal tolerance and performance in the lugworm *Arenicola marina*: a latitudinal comparison. *Journal of Experimental Marine Biology and Ecology*, 372(1-2), 22-30.

Semenza, G. L. (1999). Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annual Review of Cell and Developmental Biology*, 15(1), 551-578.

Senkbeil, E. G., & Wriston Jr, J. C. (1981). Haemocyanin synthesis in the American lobster, *Homarus americanus*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 68(1), 163-171.

Shick, J. M. (1981). Heat-Production And Oxygen-Uptake In Inter-Tidal Sea-Anemones From Different Shore Heights During Exposure To Air. *Marine Biology Letters*, 2(4), 225-236.

Shick, J. M., & Dykens, J. A. (1984). Photobiology of the symbiotic sea anemone *Anthopleura elegantissima*: photosynthesis, respiration, and behavior under intertidal conditions. *The Biological Bulletin*, 166(3), 608-619.

Shick, J. M., Gnaiger, E., Widdows, J., Bayne, B. L., & De Zwaan, A. (1986). Activity and metabolism in the mussel *Mytilus edulis* L. during intertidal hypoxia and aerobic recovery. *Physiological Zoology*, 59(6), 627-642.

Shick, J. M., Widdows, J., & Gnaiger, E. (1988). Calorimetric studies of behavior, metabolism and energetics of sessile intertidal animals. *American Zoologist*, 28(1), 161-181.

Simonik, E., & Henry, R. P. (2014). Physiological responses to emersion in the intertidal green crab, *Carcinus maenas* (L.). *Marine and Freshwater Behaviour and Physiology*, 47(2), 101-115.

Sloman, K. A., Mandic, M., Todgham, A. E., Fangue, N. A., Subrt, P., & Richards, J. G. (2008). The response of the tidepool sculpin, Oligocottus maculosus, to hypoxia in laboratory, mesocosm and field environments. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 149(3), 284-292.

Smith, M. J., Gelsleichter, J., & Smith, K. J. (2012). Protein expression of hypoxia-inducible factor 1-alpha (HIF-1α) in spot (*Leiostomus xanthurus*) exposed to constant and diel-cycling hypoxia. *Journal of Experimental Marine Biology and Ecology*, 424, 1-4.

Somero, G. N. (2002). Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. *Integrative and Comparative Biology*, 42(4), 780-789.

Somero, G. N. (2005). Linking biogeography to physiology: evolutionary and acclimatory adjustments of thermal limits. *Frontiers in Zoology*, 2(1), 1.

Sommer, A., Klein, B., & Pörtner, H. O. (1997). Temperature induced anaerobiosis in two populations of the polychaete worm *Arenicola marina* (L.). *Journal of Comparative Physiology B: Biochemical, Systemic and Environmental Physiology*, 167(1), 25-35.

Spicer, J. I. (2014). What can an ecophysiological approach tell us about the physiological responses of marine invertebrates to hypoxia?. *Journal of Experimental Biology*, 217(1), 46-56.

Spicer, J., & Baden, S. (2001). Environmental hypoxia and haemocyanin between-individual variability in Norway lobsters *Nephrops norvegicus* (L.). *Marine Biology*, 139(4), 727-734.

Spoek, G. L. (1974). The relationship between blood haemocyanin level, oxygen uptake, and the heart-beat and scaphognathite-beat frequencies in the lobster *Homarus gammarus*. *Netherlands Journal of Sea Research,* 8(1), 1-26.

Stenseng, E., Braby, C. E., & Somero, G. N. (2005). Evolutionary and acclimation-induced variation in the thermal limits of heart function in congeneric marine snails (genus *Tegula*): implications for vertical zonation. *The Biological Bulletin,* 208(2), 138-144.

Stillman, J. H. (2002). Causes and consequences of thermal tolerance limits in rocky intertidal porcelain crabs, genus *Petrolisthes*. *Integrative and Comparative Biology,* 42(4), 790-796.

Stillman, J. H. (2003). Acclimation capacity underlies susceptibility to climate change. *Science,* 301(5629), 65-65.

Stillman, J. H., & Somero, G. N. (2000). A comparative analysis of the upper thermal tolerance limits of eastern Pacific porcelain crabs, genus *Petrolisthes*: influences of latitude, vertical zonation, acclimation, and phylogeny. *Physiological and Biochemical Zoology*, 73(2), 200-208.

Stillman, J., & Somero, G. (1996). Adaptation to temperature stress and aerial exposure in congeneric species of intertidal porcelain crabs (genus *Petrolisthes*): correlation of physiology, biochemistry and morphology with vertical distribution. *Journal of Experimental Biology,* 199(8), 1845-1855.

Styrishave, B., Andersen, O., & Depledge, M. H. (2003). In situ monitoring of heart rates in shore crabs *Carcinus maenas* in two tidal estuaries: effects of physico-chemical parameters on tidal and diel rhythms. *Marine and Freshwater Behaviour and Physiology*, 36(3), 161-175.

Styrishave, B., Rewitz, K., & Andersen, O. (2004). Frequency of moulting by shore crabs *Carcinus maenas* (L.) changes their colour and their success in mating and physiological performance. *Journal of Experimental Marine Biology and Ecology*, 313(2), 317-336.

Sukhotin, A. A., & Pörtner, H. O. (1999). Habitat as a factor involved in the physiological response to environmental anaerobiosis of White Sea *Mytilus edulis*. *Marine Ecology Progress Series*, 184, 149-160.

Taylor, A. C. (1976). The respiratory responses of *Carcinus maenas* to declining oxygen tension. *Journal of Experimental Biology*, 65(2), 309-322.

Taylor, E. W., & Butler, P. J. (1973). The behaviour and physiological responses of the shore crab *Carcinus maenas* during changes in environmental oxygen tension. *Netherlands Journal of Sea Research*, 7, 496.

Taylor, E. W., & Butler, P. J. (1978). Aquatic and aerial respiration in the shore crab, *Carcinus maenas* (L.), acclimated to 15 C. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology,* 127(4), 315-323.

Taylor, E. W., & Wheatly, M. G. (1981). The effect of long-term aerial exposure on heart rate, ventilation, respiratory gas exchange and acid-base status in the crayfish *Austropotamobius pallipes*. *Journal of Experimental Biology*, 92(1), 109-124.

Taylor, E. W., & Whiteley, N. M. (1989). Oxygen transport and acid-base balance in the haemolymph of the lobster, *Homarus gammarus*, during aerial exposure and resubmersion. *Journal of Experimental Biology*, 144(1), 417-436.

Taylor, E. W., Butler, P. J., & Al-Wassia, A. (1977). Some responses of the shore crab, *Carcinus maenas* (L.) to progressive hypoxia at different acclimation temperatures and salinities. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 122(3), 391-402.

Taylor, E. W., Butler, P. J., & Sherlock, P. J. (1973). The respiratory and cardiovascular changes associated with the emersion response of *Carcinus maenas* (L.) during environmental hypoxia, at three different temperatures. J*ournal of Comparative Physiology*, 86(2), 95-115.

Tepolt, C. K., & Somero, G. N. (2014). Master of all trades: thermal acclimation and adaptation of cardiac function in a broadly distributed marine invasive species, the European green crab, *Carcinus maenas*. *Journal of Experimental Biology*, 217(7), 1129-1138.

Tessmar‐Raible, K., Raible, F., & Arboleda, E. (2011). Another place, another timer: marine species and the rhythms of life. *Bioessays,* 33(3), 165-172.

Thompson, R. C., Crowe, T. P., & Hawkins, S. J. (2002). Rocky intertidal communities: past environmental changes, present status and predictions for the next 25 years. *Environmental Conservation*, 29(2), 168-191.

Thompson, R. C., Norton, T. A., & Hawkins, S. J. (2004). Physical stress and biological control regulate the producer–consumer balance in intertidal biofilms. *Ecology*, 85(5), 1372-1382.

Todgham, A. E., & Stillman, J. H. (2013). Physiological responses to shifts in multiple environmental stressors: relevance in a changing world. *Integrative and Comparative Biology*, 53(4), 539-544.

Todgham, A. E., Iwama, G. K., & Schulte, P. M. (2006). Effects of the natural tidal cycle and artificial temperature cycling on Hsp levels in the tidepool sculpin *Oligocottus maculosus*. *Physiological and Biochemical Zoology*, 79(6), 1033-1045.

Todgham, A. E., Schulte, P. M., & Iwama, G. K. (2005). Cross-tolerance in the tidepool sculpin: the role of heat shock proteins. *Physiological and Biochemical Zoology*, 78(2), 133-144.

Togni, V. G., Hermes-Lima, M., & Freire, C. A. (2007). 15. P3. Different responses of the antioxidant systems of the crabs *Callinectes danae* and *C. ornatus* after exposure to air or

change in water salinity. Comparative Biochemistry and Physiology, *Part A: Molecular & Integrative Physiology,* (148), S63.

Tomanek, L., & Helmuth, B. (2002). Physiological ecology of rocky intertidal organisms: a synergy of concepts. *Integrative and Comparative Biology*, 42(4), 771-775.

Tomanek, L., & Somero, G. N. (1999). Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. *Journal of Experimental Biology,* 202(21), 2925-2936.

Truchot, J. P. (1975). Blood acid-base changes during experimental emersion and re-immersion of the intertidal crab *Carcinus maenas* (L.). *Respiration Physiology*, 23(3), 351-360.

Truchot, J. P. (1978). Mechanisms of extracellular acid-base regulation as temperature changes in decapod crustaceans. *Respiration Physiology*, 33(1), 161-176.

Truchot, J. P. (1980). Lactate increases the oxygen affinity of crab haemocyanin. *Journal of Experimental Zoology*, 214(2), 205-208.

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

Truchot, J. P. (1990). Respiratory and ionic regulation in invertebrates exposed to both water and air. *Annual Review of Physiology*, 52(1), 61-74.

Truchot, J. P., & Jouve-Duhamel, A. (1980). Oxygen and carbon dioxide in the marine intertidal environment: diurnal and tidal changes in rockpools. *Respiration Physiology*, 39(3), 241-254.

Van Dijk, P. L., Tesch, C., Hardewig, I., & Pörtner, H. O. (1999). Physiological disturbances at critically high temperatures: a comparison between stenothermal Antarctic and eurythermal temperate eelpouts (Zoarcidae). *Journal of Experimental Biology,* 202(24), 3611-3621.

Via, S., Gomulkiewicz, R., De Jong, G., Scheiner, S. M., Schlichting, C. D., & Van Tienderen, P. H. (1995). Adaptive phenotypic plasticity: consensus and controversy. *Trends in Ecology & Evolution*, 10(5), 212-217.

Wallace, J. C. (1972). Activity and metabolic rate in the shore crab, *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology Part A: Physiology*, 41(3), 523-533.

Warman, C. G., Reid, D. G., & Naylor, E. (1993). Variation in the tidal migratory behaviour and rhythmic light-responsiveness in the shore crab, *Carcinus maenas*. *Journal of the Marine Biological Association of the United Kingdom*, 73(2), 355-364.

Weber, R. E., Behrens, J. W., Malte, H., & Fago, A. (2008). Thermodynamics of oxygenation-linked proton and lactate binding govern the temperature sensitivity of O2 binding in crustacean (*Carcinus maenas*) haemocyanin. *Journal of Experimental Biology*, 211(7), 1057-1062.

Whiteley, N. M. (2011). Physiological and ecological responses of crustaceans to ocean acidification. *Marine Ecology Progress Series*, 430, 257-271.

Whiteley, N. M., Taylor, E. W., & El Haj, A. J. (1997). Seasonal and latitudinal adaptation to temperature in crustaceans. *Journal of Thermal Biology*, 22(6), 419-427.

Widdows, J. (1976). Physiological adaptation of *Mytilus edulis* to cyclic temperatures. *Journal of Comparative Physiology*, 105(2), 115-128.

Widdows, J., & Shick, J. M. (1985). Physiological responses of *Mytilus edulis* and *Cardium edule* to aerial exposure. *Marine Biology*, 85(3), 217-232.

Wilcockson, D., & Zhang, L. (2008). Circatidal clocks. *Current Biology*, 18(17), R753-R755.

Williams, B. G. (1967). Laboratory rearing of the larval stages of *Carcinus maenas* (L.)[Crustacea: Decapoda]. *Journal of Natural History*, 2(1), 121-126.

Williams, B. G., & Naylor, E. (1969). Synchronization of the locomotor tidal rhythm of *Carcinus*. *Journal of Experimental Biology,* 51(3), 715-725.

Williams, J. A. (1995). Burrow-zone Distribution of the Supralittoral Amphipod *Talitrus Saltator* on Derbyhaven Beach, Isle of Man—a Possible Mechanism for Regulating Desiccation Stress?. *Journal of Crustacean Biology*, 15(3), 466-475.

Williams, K. J., Cassidy, A. A., Verhille, C. E., Lamarre, S. G., & MacCormack, T. J. (2019). Diel cycling hypoxia enhances hypoxia tolerance in rainbow trout (*Oncorhynchus mykiss*): evidence of physiological and metabolic plasticity. *Journal of Experimental Biology*, 222(14), jeb206045.

Wilmer, P., Stone, G., & Johnston, I. (2009). Environmental physiology of animals. John Wiley & Sons. Senkbeil, E. G., & Wriston Jr, J. C. (1981). Haemocyanin synthesis in the American lobster, *Homarus americanus*. *Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular Biology,* 68(1), 163-171.

Yamada, S. B., Dumbauld, B. R., Kalin, A., Hunt, C. E., Figlar-Barnes, R., & Randall, A. (2005). Growth and persistence of a recent invader *Carcinus maenas* in estuaries of the northeastern Pacific. *Biological Invasions*, 7(2), 309-321.

Yin, X., Chen, P., Chen, H., Jin, W., & Yan, X. (2017). Physiological performance of the intertidal Manila clam (*Ruditapes philippinarum*) to long-term daily rhythms of air exposure. *Scientific Reports*, 7, 41648.

Zebe, E. (1982). Anaerobic metabolism in *Upogebia pugettensis* and *Callianassa californiensis* (Crustacea, Thalassinidea). *Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular Biology,* 72(4), 613- 617.

Zeng, C., & Naylor, E. (1996). Endogenous tidal rhythms of vertical migration in field collected zoea-1 larvae of the shore crab *Carcinus maenas*: implications for ebb tide offshore dispersal. *Marine Ecology Progress Series*, 132, 71-82.

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., ... & Xiong, Z. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature*, 490(7418), 49.

Zhang, G., Li, L., Meng, J., Qi, H., Qu, T., Xu, F., & Zhang, L. (2016). Molecular basis for adaptation of oysters to stressful marine intertidal environments. *Annual Review of Animal Biosciences*, 4, 357-381.

Zhang, L., Hastings, M. H., Green, E. W., Tauber, E., Sladek, M., Webster, S. G., ... & Wilcockson, D. C. (2013). Dissociation of circadian and circatidal timekeeping in the marine crustacean *Eurydice pulchra*. *Current Biology*, 23(19), 1863-1873.

Zielinski, S., & Pörtner, H. O. (1996). Energy metabolism and ATP free-energy change of the intertidal wormSipunculus nudus below a critical temperature. *Journal of Comparative Physiology B*, *166*(8), 492-500.

Zou, E., Du, N., & Lai, W. (1996). The effects of severe hypoxia on lactate and glucose concentrations in the blood of the Chinese freshwater crab *Eriocheir sinensis* (Crustacea: Decapoda). *Comparative Biochemistry and Physiology Part A: Physiology*, 114(2), 105-109.

Appendices

A.1 Stress Response

Oxygen consumption in water

To determine the least stressful method to transfer individuals from the holding tanks to the respirometry chambers, I examined how hand vs. cage transfer affected oxygen consumption in *C. maenas,* Twenty-four hours before the trial, four individuals were removed from the non-tidal holding tank, weighed, measured, labelled and had their colour morph noted. Two of the four individuals were then placed in a perforated plastic cage (15 cm x 9 cm x 6 cm deep) and placed back into their container in the non-tidal holding tank. The perforated nature of the cages allowed for the free flow of water. Individuals that would be transferred by hand were placed back into containers in the holding tank.

For the experiment, the two caged individuals and two free individuals were removed from the holding tank and placed in four respirometry chambers submerged in a tank supplied with normoxic seawater (at 15ºC and 32‰). For all individuals, transfer occurred in three stages. Stage 1: the individuals were transferred from the container in the holding tank to a bucket that had been submerged in the tank. Stage 2: this bucket was then removed from the holding tank and submerged in the experimental tank where the respirometers were situated. Stage 3: individuals were taken out of the bucket (while remaining submerged) and placed in the respirometry chamber. This method was used to ensure that the crabs were not exposed to air at all during the transfer process. Individuals subjected to hand transfer were transferred to the bucket and subsequently to the respirometry chamber by being picked up from behind by pinching the abdomen and the carapace. Individuals transferred by cage were moved into the bucket, the cage was then

placed into the respirometry chamber where one side was opened to allow the crabs to leave the cage and enter the chamber of their own accord. Once this occurred, the plastic cage was removed, the chamber was sealed and recordings began immediately.

This experiment was repeated many times, and thus, also allowed me how three levels of stress affected their metabolism / oxygen consumption.:

1. Minimal Stress:
    a. Stage 1: Crabs were gently and slowly moved into the bucket within five seconds.
    b. Stage 2: The bucket was very carefully submerged into the experimental tank within eight seconds.
    c. Stage 3: Individuals were carefully taken out of the bucket and placed slowly into the chamber within five seconds.
2. Moderate Stress:
    a. Stage 1: Crabs were moved into the bucket in less than three seconds.
    b. Stage 2: Bucket was submerged in five seconds.
    c. Stage 3: Individuals taken out of bucket and moved into chamber in within approximately three seconds.
3. Maximum Stress:
    a. Stage 1: Crabs were quickly moved into bucket (within a second).
    b. Stage 2: Bucket was submerged in less than three seconds.
    c. Stage 3: Crabs taken out of the bucket, shaken (under water) for five seconds before placing in chamber, and the transfer process took less than

six seconds. If caged crabs did not leave of their own accord, the cage was opened up completely to allow individuals to fall into the chamber.

This experiment was repeated twelve times so that eight individuals were tested for each of six transfer x stress level combinations (n=8 per treatment, 48 individuals in total). Oxygen consumption of individuals was then taken every hour for 24 hours at 15°C in normoxia as described in the Materials and Methods section of Chapter 2.

To identify the least stressful method of handling, a two-way repeated measures ANOVA was performed using GraphPad Prism (version 5.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). To determine the duration of stress, Tukey HSD post-hoc tests were used to compare oxygen consumption values for every hour of each combination, and to establish when values became stable. Stable values were considered to be indicative of 'resting' oxygen consumption.

Results

Overall, crabs transferred by hand with maximal stress levels had the highest initial oxygen consumption (143.8 mg O_2 kg^{-1} hr^{-1}). This was followed by the 'hand moderate' (132.8 mg O_2 kg^{-1} hr^{-1}), and then the 'cage maximal' (127.0 mg O_2 kg^{-1} hr^{-1}) groups. Crabs exposed to the 'hand minimal' protocol had the next highest initial oxygen consumption (117.6 mg O_2 kg^{-1} hr^{-1}), but appeared to stabilise quicker than the other treatments. The 'cage moderate' group had the next lowest oxygen consumption (113.8 mg O_2 kg^{-1} hr^{-1}), while crabs in the 'cage minimal' treatment had the lowest initial oxygen consumption (107.19 mg O_2 kg^{-1} hr^{-1}). Statistical analysis concluded that there was no overall difference

between the transfer method type at any stress level (two-way RM ANOVA, df = 5, F = 1.58, p = 0.2408). However, there were significant differences between the treatments of ‘cage minimal’, ‘cage moderate’ and ‘hand minimal’, and ‘hand maximum’ at hours 1-3 (Tukey HSD, $p < 0.05$).

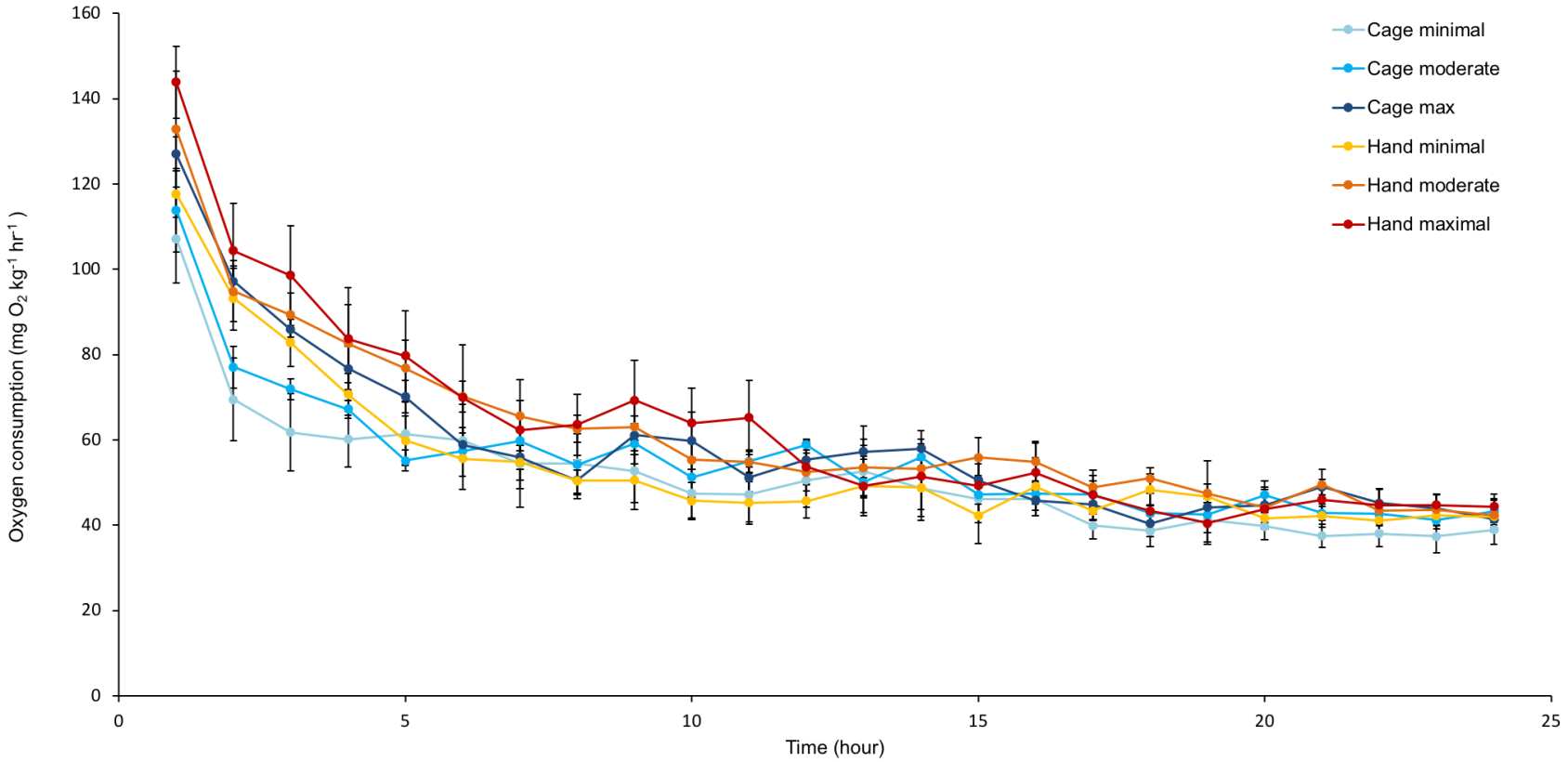


Figure A.1. Oxygen consumption of *Carcinus maenas* exposed to two different handling treatments (cage vs. hand) at three different stress levels (minimal, moderate, maximal) when submersed for 24 hours. The data are means ± SEM for 8 crabs for each handling/stress combination (n=48 in total).

From Figure A.1, it is clear that: 1) the decline between hours 1 and 6 accounted for the majority of elevated oxygen consumption values; and that 2) there was a significant difference between treatments (two-way RM ANOVA, df = 5, F = 2.926, p = 0.0235; Fig. A.2) and over time (two-way RM ANOVA, df = 5, F = 105.9; p = <0.0001). Crabs exposed to the cage-minimum stress protocol had a significantly lower oxygen consumption than those in the 'hand-moderate stress' (Tukey HSD, p=0.0170) and hand-maximum stress (Tukey HSD, p = 0.0007) groups. The oxygen consumption of the cage-moderate stress group was also significantly lower than those exposed to the hand-moderate stress (Tukey HSD, p = 0.039) and hand-maximum stress (Tukey HSD, p = 0.0074) protocols. Finally, crabs in the hand- minimum stress group had a lower value for oxygen consumption as compared to those in the hand-maximum stress group (Tukey HSD, p = 0.0329).

After 15 hours, oxygen consumption reached a baseline value (Tukey HSD, $p < 0.05$). After 15 hours the average resting oxygen consumption for *C. maenas* at 15 ºC in normoxia as 44.5 mg O_2 kg^{-1} hr^{-1}. Average oxygen consumption values for hours 1 – 14 for each treatment group were then compared to the average resting value to quantify the magnitude of stress placed on an individual at each hour after handling (Table A.1). This was done by calculating a stress index; i.e. the fold increase in oxygen consumption value at a particular hour in comparison to the resting value.

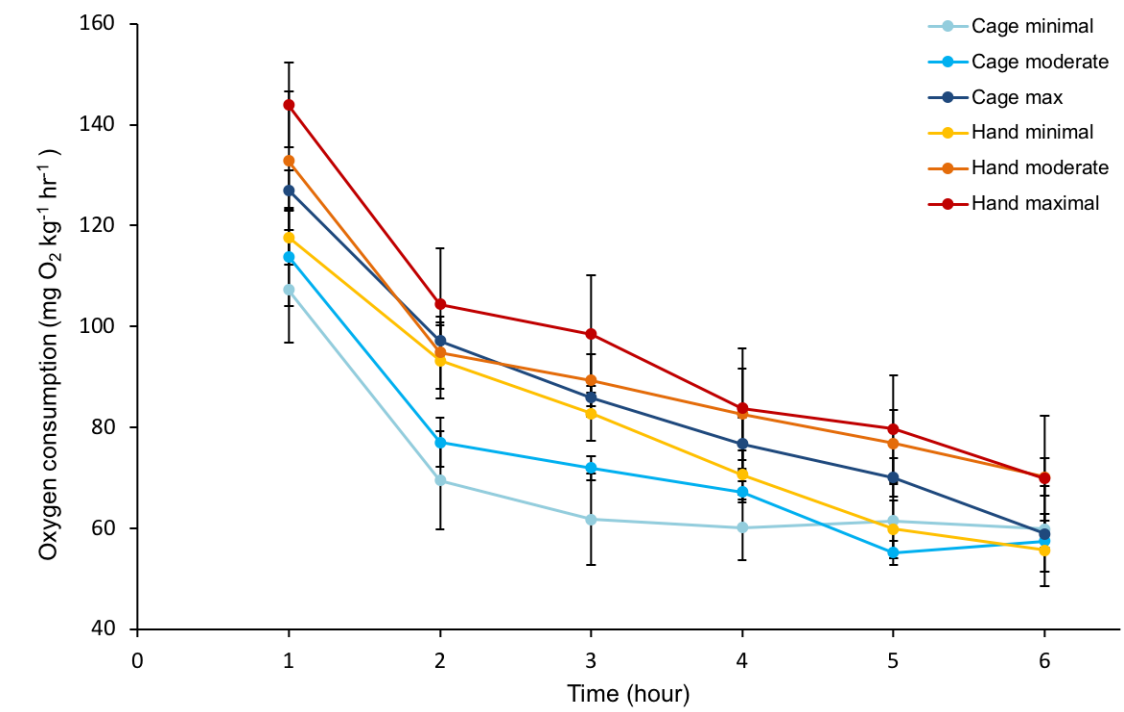


Figure A.2. A close-up examination of the first six hours of the stress experiment depicted in Figure A.1.

Table A.1. Comparison of stress indices at hours 1-14 for individuals transferred by cage and hand, and subjected to minimal, moderate and maximal stress in comparison to resting values of oxygen consumption.

	Cage			Hand		
Hour	Minimal	Moderate	Maximal	Minimal	Moderate	Maximal
1	2.406	2.555	2.851	2.641	2.982	3.230
2	1.560	1.729	2.181	2.093	2.129	2.343
3	1.387	1.615	1.928	1.858	2.005	2.211
4	1.350	1.508	1.721	1.585	1.854	1.880
5	1.378	1.238	1.573	1.343	1.724	1.789
6	1.343	1.289	1.321	1.249	1.575	1.569
7	1.221	1.342	1.255	1.230	1.471	1.398
8	1.223	1.215	1.134	1.131	1.405	1.426
9	1.183	1.326	1.371	1.133	1.414	1.554
10	1.064	1.149	1.342	1.025	1.242	1.435
11	1.059	1.234	1.149	1.015	1.229	1.464
12	1.133	1.322	1.242	1.025	1.177	1.206
13	1.180	1.124	1.284	1.103	1.201	1.104
14	1.093	1.255	1.301	1.096	1.195	1.154
15	1.036	1.059	1.134	0.948	1.254	1.106

Conclusion

From these results, it was determined that transferring the individuals by cage would result in the least amount of stress, as oxygen consumption rates and subsequent stress indices were lower than measured in those crabs transferred by hand. The transfer method described for the cage moderate-stress group was used in the experiment, thus, the stress values calculated for this method were used to correct for the effect of handling stress placed on individuals used in Chapters 2 and 3. This was done by dividing oxygen consumption by the stress value for each specific hour after stress in Table A.1. This produced oxygen consumption values that were independent of the effect of handling stress.

Aerial oxygen consumption

An effort was also made to determine the handling stress associated with removing a crab from the aquatic chamber and transferring them directly to the aerial chamber. For aerial oxygen consumption, to separate handling stress from the emersion response, two separate treatments were carried out. In the first treatment individuals (n = 6) were transferred by hand from the non-tidal holding tank into an "aerial" respirometry chamber (4.8 L) that was also submerged in the non-tidal holding tank. This ensured the animal was not exposed to air. The chamber was sealed and the crab in this chamber (that remained filled with seawater) was removed from the holding tank and carefully placed into in an incubator (MIR-254-PE, Panasonic Biomedical, Europe) set to 15°C. As the period of emersion in Experiment 2.3.2 was 6 hours in duration, aerial handling stress was only assessed for the first 6 hours. Therefore, and the crab was left submerged for six hours to settle in the chamber. Air was injected into chamber every 20 minutes using a 60 mL syringe with an 18-gauge needle via the sampling hole on the lid of the chamber. This allowed the individual to recover from handling stress, but was not long enough for the water to become hypoxic. After six hours, the chamber was slowly drained at a rate of 2 L min^{-1} via a small hole in the bottom. The chamber was then sealed, and oxygen consumption was taken every hour for six hours as described in the Methods and Materials section of Chapter 2. In the second treatment, individual crabs (n = 6) of similar size and colour morph were transferred directly by hand from the aquatic chamber to the empty aerial chamber. The chamber was then carefully placed in the incubator, sealed, and aerial oxygen consumption measurements started immediately.

Statistical analysis was performed in GraphPad Prism (version 5.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). A two-way repeated measures ANOVA was used to compare individuals acclimated to the chamber to those that were transferred immediately to determine if transfer caused an elevated level of oxygen consumption. Tukey HSD post-hoc analysis was then used to identify differences over time, and therefore, the duration of the elevation in oxygen consumption.

Results

There was a significant interaction between time and acclimation group (two-way RM ANOVA, df = 5, F = 2.442, p = 0.0468), and post-hoc tests revealed this was due to the oxygen consumption of the group directly to respirometry chambers containing air being significantly higher than that of the group initially placed into respirometers containing seawater (Tukey HSD, p = 0.039) .

Due to the significant difference in oxygen consumption between hour 1 and all other hours that occurred in the direct transfer group, but not the group of crabs acclimated to the respirometry chamber containing seawater, it was determined that the effect of handling stress needed to be accounted for at 1 hour post-transfer. Therefore, a stress index was created at hour one post-transfer by comparing the average oxygen consumption value of the directly transferred group to that of the acclimated group (Table A.2). This stress index was 1.34, and represents how many fold the oxygen consumption of the directly transferred group was above that in crabs that were acclimated to the chambers initially.

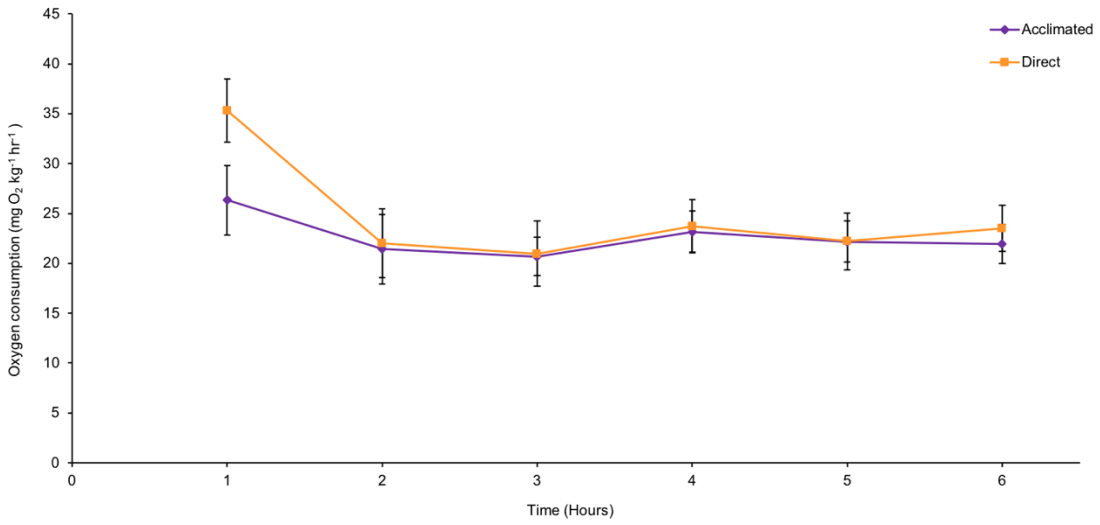


Figure A.3. Mean (± SEM, N = 6) oxygen consumption (mg O_2 kg^{-1} hr^{-1}) of individuals previously acclimated to the 'aerial' respirometry chamber (purple), and not acclimated to this chamber (orange), prior to the measurement of oxygen consumption in air over 6 hours.

Table A. 2. Mean oxygen consumption (mg O_2 kg^{-1} hr^{-1}) of individuals previously acclimated and to the aerial chamber and those directly transferred at 1 hour post-transfer. 'Direct' is divided by 'acclimated' to calculate the stress index.

Acclimated	Direct	Stress value
26.322	35.306	1.341

Conclusion

To correct for the effect of handling stress placed on crabs when directly transferring them from aquatic respirometry chamber to the aerial respirometry chamber, the raw aquatic oxygen consumption values obtained in hour one of emersion in Chapter 1 were divided by the stress index value of 1.341. This produced oxygen consumption values that were independent of the effect of handling stress, and these were subsequently used in analysis of the oxygen consumption data in this chapter.

A.2 Chapter 2 Experimental Design

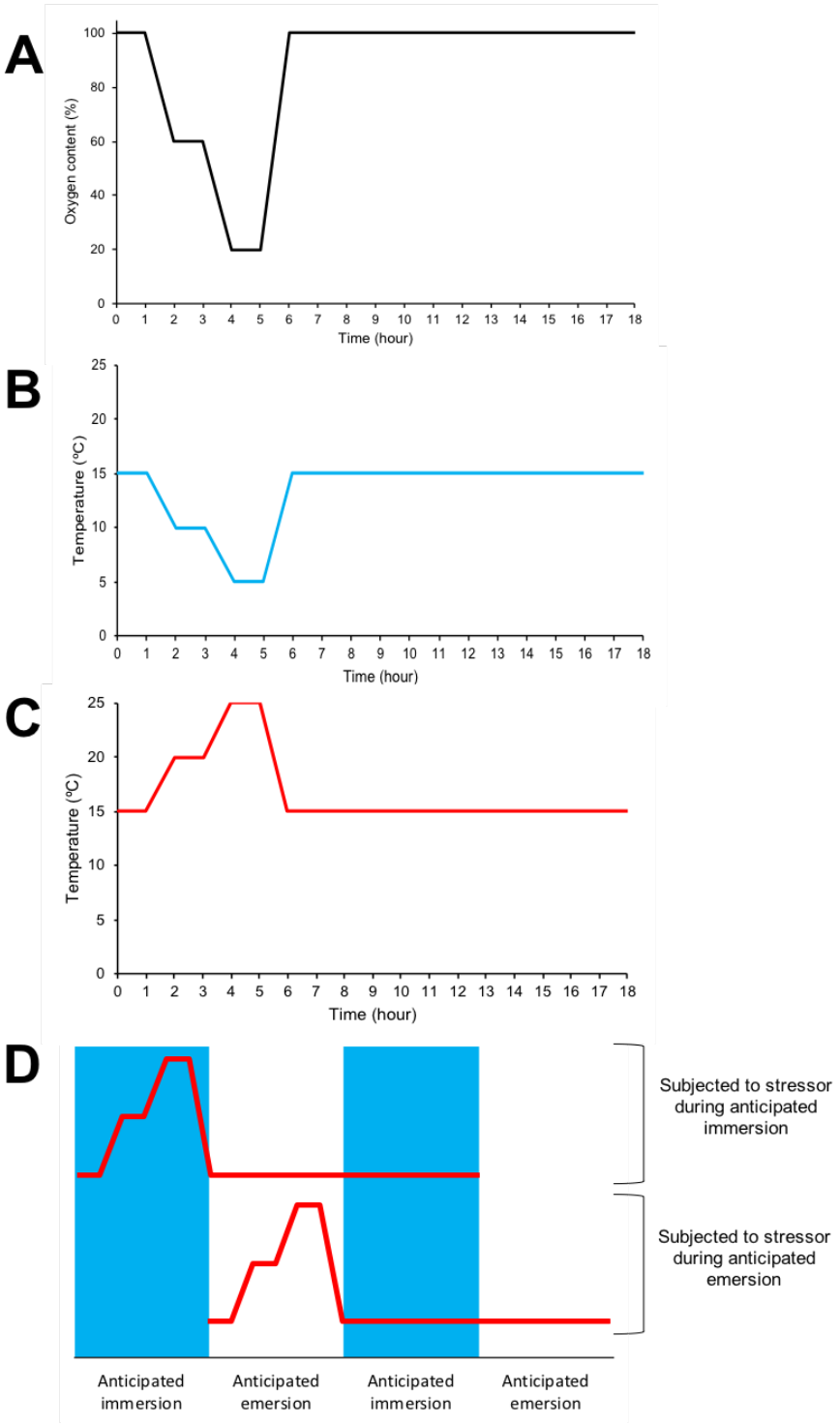


Figure A.4. Graphical representation of the experimental design for the manipulation of (A) hypoxia, (B) an acute temperature decrease, and (C) acute temperature increase to examine how tidal and non-tidal crabs respond to these stressors. (D) is a graphical representation of how the experiments were repeated at two different times (using an acute temperature increase as an example): i.e., during anticipated immersion (upper red line) and anticipated emersion (lower red line).