EFFECTS OF FOOD DEPRIVATION STATES ON BEHAVIORAL AND PHYSIOLOGICAL RESPONSES TO HYPOXIA IN ROCK CRABS (*Cancer irroratus*)

By

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A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Ocean Sciences

Memorial University of Newfoundland

April 2022

St. John's, Newfoundland and Labrador, Canada

Abstract

Hypoxia has become increasingly prevalent in benthic marine environments. Despite this fact most experiments have investigated the effects of hypoxia alone, without taking into account the routine activities of animals. Crustaceans are among the most sensitive taxa to changes in environmental oxygen. Here, I used the Atlantic rock crab (*Cancer irroratus*) to determine the interactive effects of hypoxia and feeding/food deprivation state on aerobic metabolism, hemolymph biochemistry, feeding and gastric processing as well as the behavioral responses of this shallow water decapod. The critical oxygen partial pressure (P_{crit}) was related to the food deprivation state of the crabs. Starved crabs exhibited the lowest (P_{crit}), while fed crabs had the highest P_{crit}. Interestingly, below the P_{crit} level (fasted crabs), an elevated oxygen consumption (MO_2) after feeding and increased locomotor activity indicated that the P_{crit} calculated by piecewise linear regression might represent a hypometabolic shift, which allowed some aerobic scope reserved for critical activities. Hypoxia retarded the specific dynamic action (SDA) and gastric processing of both fasted and starved crabs, resulting in lower peak MO_2 and more prolonged duration that MO_2 remained elevated. The starved crabs exhibited a lower peak MO_2, prolonged duration and higher energy expenditure of SDA, and slightly longer transit times for digesta compared with fasted crabs. It was postulated that starvation may have triggered a cross-tolerance to hypoxia, since both long-term starvation and severe hypoxia elicited similar hypometabolic responses. Changes in oxygen consumption were paralleled by changes in hemolymph biochemistry. Following feeding, only fasted

crabs exhibited a significant increase in hemolymph L-lactate concentration. This hypoxia-induced alkalosis and elevated L-lactate may have improved the hemocyanin-oxygen affinity and thus oxygen transport, below the P_{crit} level. During exposure to severe hypoxia (20% oxygen), rock crabs reduced food intake, and some crabs refused to eat. Although food deprivation state did not significantly affect food intake, more starved crabs fed in 20% oxygen than their fasted conspecifics. Although the rock crabs did not appear to exhibit a preference when offered a choice of oxygen levels, the level of hypoxia played an important role in regulating their activity levels, which was also influenced to a lesser degree by the food deprivation state. Overall, the fed crabs were less active than their fasted conspecifics in 20% oxygen (below P_{crit} of both fed and fasted crabs), likely because they could not balance the simultaneous demands of digestion and increased activity. The results of this thesis suggest that although responses to hypoxia are modulated by food deprivation states, they are unlikely to severe enough to affect the distribution or survival of rock crabs.

Acknowledgements

First and foremost, I would like to give my sincerest gratitude to my supervisor Dr. Iain McGaw for his exceptional mentorship and great tolerance during my doctoral research. Without his patient guidance and enthusiasm for research, I would not be where I am today. I also appreciated the insightful comments of my committee member Dr. Mark Abraham. I would also like to thank Dr. Kurt Gamperl and Dr. Bill Driedzic for sharing their laboratory equipment and for their expertise and feedback on the biochemistry analysis. Special thanks go to Dr. Chris Wood for his informative discussion of P_{crit}, Dr. Dirk Weihrauch for his valuable comments on Chapter Three, and Dr. Amanda Bates for her professional guidance on statistical analysis.

I would also want to thank the workshop technicians and the custodians of the ocean sciences center. Without their excellent support, this project would not have succeeded. Warm thanks go to all the graduate students at the OSC and especially to my lab members, Travis, Sarah, Gemma, Evgeni and Logan for all their encouragement and help.

Finally, I will never forget my parents and all my friends, near and far, for their mental support, encouragement and understanding all the time.

This research was funded by an NSERC grant to Dr. Iain McGaw and by fellowships of the School of Graduate Studies, Memorial University of Newfoundland, the China Scholarship Council and the Mitacs Accelerate to Qiwu Jiang.

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Co-authorship Statement

The field and laboratory work described in the present thesis was carried out by Qiwu Jiang with Dr. Iain McGaw's supervision and guidance. All chapters of this thesis were written by Qiwu Jiang with the intellectual and editorial suggestions of Dr. Iain McGaw and Dr. Mark Abraham. Any papers arising from the thesis will be co-authored as Jiang and McGaw.

Chapter 1. General Introduction

1.1 Oceanic hypoxia and general effects

Under the background of global climate change, ocean deoxygenation has long intrigued scientists (Breitburg et al., 2018; Diaz and Rosenberg, 1995; Vaquer-Sunyer and Duarte, 2008). The global oceanic oxygen content of $227.4 \pm 1.1 \times 10^{15}$ mol has decreased by more than 2% since 1960 as a consequence of global warming and anthropogenic activity (Schmidtko et al., 2017). Furthermore, ocean models under different climate change scenarios all predict a continuous decline in the dissolved oxygen inventory (Bopp et al., 2013; Cocco et al., 2013), resulting in widespread oxygen deficiency of the global ocean in the next thousand years (Watson, 2016). This oxygen loss is associated with significant variations at different depths and between open oceans and coastal waters (Figure 1.1). The general dissolved oxygen concentration in normoxic seawater (at 33ppt, 15°C and 1 atm) is 8.30 mg/L (equal to 5.81 ml/L or 259.48 µmol/L) (Green and Carritt, 1967), while a typical threshold for hypoxia is approximately 63 µmol/L for many macro-organisms in the ocean (Gray et al., 2002; Vaquer-Sunyer and Duarte, 2008).

1.1.1 Open oceans

The oxygen concentration of open oceans has decreased by a mean rate of 1–3 µmol/L per decade in the past 50 years, and it is predicted that the global ocean oxygen inventory will decline by 1–7% over the next century (Keeling et al., 2010). Two main causes for the deoxygenation of open oceans are the reduced oxygen solubility in warmer seawater and the reduced penetration of oxygen into deeper oceans due to enhanced stratification in the upper oceans (Breitburg et al., 2018). The reduced solubility of oxygen

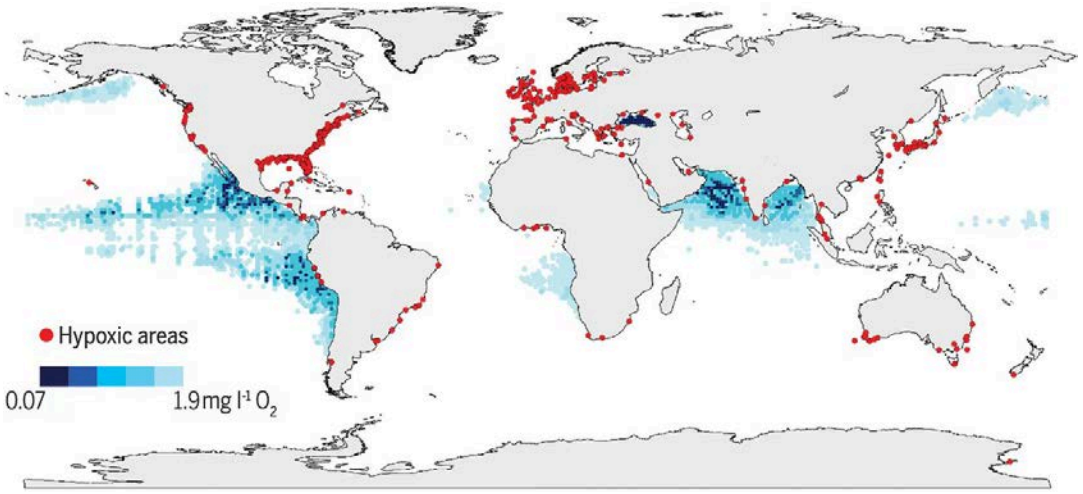

Figure 1.1 Low and declining oxygen levels in the open ocean and coastal waters affect processes ranging from biogeochemistry to food security. The global map indicates coastal sites where anthropogenic nutrients have exacerbated or caused O_2 declines to < 2 mg/L (< 63 µmol/L) (red dots), as well as ocean oxygen-minimum zones at 300 m of depth (blue shaded regions). The figure was adapted from Breitburg et al. (2018).

explains about 15% of current overall oxygen loss and over 50% of the oxygen loss in the upper 1000 m of open oceans (Helm et al., 2011). Meanwhile, intensified stratification may account for the remaining 85% of global ocean oxygen loss (Breitburg et al., 2018). Global warming directly influences thermal stratification (thermoclines) and indirectly enhances salinity-driven stratification (haloclines) through ice melt and precipitation, which further alters ocean currents and the oxygen transport into the ocean interior (Schmidtko et al., 2017).

The reduced oxygen solubility and intensified stratification changes result in an expansion of the oxygen minimum zones (OMZs, where most large mobile organisms are unable to inhabit) vertically and horizontally (Stramma et al., 2008). In many tropical regions, oxygen concentrations have decreased at an average rate of 2 to 3 μmol/L per decade over the last 50 years, and the OMZs (O_2 < 72 μmol/L at a depth of 200 m or 18% oxygen saturation at 21°C) have expanded by 4.5 million km^2 (Stramma et al., 2010).

1.1.2 Coastal waters

The oxygen decline rates are more severe in coastal regions than those in the open oceans. The oxygen concentration declined four times faster in the coastal waters (0.35 ± 0.12 μmol/L per year) than the speed in the open ocean (0.09 ± 0.06 μmol/L per year) in the upper 300 m layer between 1976 and 2000 (Gilbert et al., 2010). Additionally, since 1950, oxygen dead zones (≤ 63 μmol/L) in the global coastal oceans have increased by 90% (Diaz and Rosenberg, 2008).

Similar to open oceans, the adverse effects of global warming (reduced oxygen solubility and enhanced stratification) contribute to deoxygenation in coastal waters (Watson, 2016). Additionally, there are natural and anthropogenic causes (Diaz and Rosenberg, 2008). In continental margins of the eastern Pacific, the southeastern Atlantic, the Arabian Sea and the Bay of Bengal, natural upwellings bring nutrients, as well as hypoxia from bottom waters (Helly and Levin, 2004). Furthermore, a semi-enclosed hydrogeomorphology, such as the Baltic, Kattegat, Black Sea, Gulf of Mexico, and the East China Sea, would minimize water exchange, and this combined with water-column stratification makes hypoxic events more severe (Diaz and Rosenberg, 2008). Human activities have also aggravated the situation. Anthropogenic eutrophication (excess inputs of nitrogen and phosphate and organic matter from continental watersheds) affects ocean nutrient cycles (Watson, 2016). The enriched nutrients encourage the growth of photosynthetic plankton and enhance primary and secondary production in surface waters. The death of these organisms eventually accumulates degradable organic matter in the bottom waters, where microbial decomposition increases the consumption of dissolved oxygen by aerobic respiration (Breitburg et al., 2018).

1.1.3 Ecological consequences

Oxygen is fundamental for all aerobic organisms. It acts as an electron acceptor in order to drive the production of ATP, which provides energy for organisms (Richards, 2009). All aerobic organisms have limits to the severity or duration of hypoxia that they can tolerate. Generally, the empirical sublethal and lethal oxygen thresholds for many

marine species are reached when oxygen concentrations fall below 24% (5.1 kPa at 33 ppt, 15°C) (Vaquer-Sunyer and Duarte, 2008). Oxygen levels below these thresholds may lead to the shrinkage of suitable habitats for pelagic species (Eby and Crowder, 2002; Gibson and Atkinson, 2003). Although some hypoxia-tolerant species might thrive because they can avoid their predators and competitors in OMZs (Seibel, 2011), severe hypoxia can adversely affect organisms: increasing mortality (Eggleston et al., 2005; Haselmair et al., 2010), reducing growth rates (Dan et al., 2014; Stierhoff et al., 2009) and altering their behaviors (Broughton et al., 2017; Riedel et al., 2014). Thus, in order to understand the impacts of hypoxia on the marine ecosystem, it is crucial to study the responses and adaptations of different marine species (Breitburg et al., 2018). Crustaceans are among the most sensitive marine taxa to hypoxia, with high median lethal oxygen concentrations (LC_{50}: 30.8 ± 0.02%) and the short median lethal times (LT_{50}: 55.5 ± 12.4 h); therefore, they are good indicator species for ecological criteria with respect to hypoxia stress (Vaquer-Sunyer and Duarte, 2008).

1.2 Effects of hypoxia on crustaceans

1.2.1 Physiology

1.2.1.1 Respiration and circulation

As ambient oxygen levels decline, crustaceans will either reduce oxygen consumption (oxygen conformer) in line with that of the external medium or maintain their oxygen uptake (oxygen regulator) at stable levels (Pörtner and Grieshaber, 1993).

Most crustaceans are oxygen regulators, and true oxygen conformers are rare within the taxa (Brill et al., 2015; McMahon, 2001b). Crustaceans, like most other oxygen regulators, cannot maintain a steady rate of oxygen consumption when the amount of environmental oxygen declines below a certain point. At the certain point – the critical oxygen tension (P_{crit}) – physiological mechanisms can no longer supply the aerobic demand, and oxygen consumption falls in line with ambient oxygen tension (Brill et al., 2015; McMahon, 2001b; Pörtner and Grieshaber, 1993; Taylor, 1976). The P_{crit} is not a set value and varies depending on the environmental conditions (e.g., temperature, salinity) or the state of the animal (e.g., food deprivation state, ontogeny, reproductive state). This has led to some confusion about the oxyregulatory ability of species. For example, the blue crab (*Callinectes sapidus*) was reported to be a partial oxygen conformer (Batterton and Cameron, 1978); however, recent work shows it to be a typical oxygen regulator with a median critical oxygen level of 20% oxygen at 23°C (Brill et al., 2015).

Immediate compensatory responses to declining ambient oxygen occur in the respiratory and cardiovascular systems of crustaceans, while slower changes in hemolymph parameters can also assist in maintaining oxygen delivery during chronic hypoxic exposure (McGaw, 2005; McMahon, 2001b; Spicer, 2014; Whiteley and Taylor, 2015). The frequency and depth of scaphognathite (gill bailer) beating increases immediately in response to acute hypoxia, which can increase ventilation volume by 5 to 10-fold and facilitate oxygen supply (Airriess and McMahon, 1994; Taylor, 1976; Wilkes and McMahon, 1982). There is also a concomitant increase in frequency and duration of ventilatory reversals to irrigate the dorsal areas of the posterior gills (Arudpragasam and

Naylor, 1964; Massabuau and Burtin, 1984; Uglow, 1973). However, the elevated ventilatory performance may be depressed during prolonged hypoxia (> 24 h) as it is energetically expensive (McMahon et al., 1974). For instance, the crayfish (*Orconectes virilis*) increases the frequency of scaphognathite beat by 254% during acute hypoxia (0–24 h), but this declines to 149% during chronic hypoxic exposure (4–8 days). This reduces the metabolic cost of the scaphognathite ventilation from approximately 12% down to 4% of total oxygen consumption (Burggren and McMahon, 1983). Similar results are found in the crayfish (*Orconectes rusticus*), where scaphognathite pumping frequency increases up to 4-fold over control levels after 24 h hypoxic exposure but decreases by 50% after 72 h (Wilkes and McMahon, 1982).

Oxygen uptake can also be augmented by increasing branchial hemolymph flow (McMahon, 2001a). During hypoxia, hemolymph is diverted from the viscera to the sternal circuit supplying the limbs and gills. This further increases the branchial hemolymph flow because hemolymph traveling to the sternal circuit returns to the pericardial cavity via the gills (Airriess and McMahon, 1994). Branchial hemolymph flow can also be enhanced by increasing cardiac output, but mechanisms of this compensation differ between crustaceans. For large crustaceans, hypoxia may induce a progressive bradycardia (decline in heart rate), which can be explained by an inherent response of the cardiac ganglion to intraventricular hypoxia (Taylor et al., 1973; Wilkens, 1993). However, despite the decline in heart rates, the cardiac output of the crayfish (*Procambarus clarkii*) and the American lobster (*Homarus americanus*) is compensated by a concomitant increase in cardiac stroke volume, which results from increasing time for filling of the

heart (Reiber and McMahon, 1998). In contrast, the cardiac output of the small grass shrimp (*Palaemonetes pugio*) is maintained by down to 13.3 kPa via an increased heart rate, despite a decline in cardiac stroke volume (Harper and Reiber, 1999).

During long-term hypoxia, the decrease in ventilation rate and heart rate may be compensated by improved oxygen transport efficiency by augmenting respiratory protein concentration or increasing its oxygen affinity (Giomi and Beltramini, 2007; Hagerman, 1986). For example, the prawn (*Macrobrachium rosenbergii*) can increase hemocyanin concentration by 7.4% within 24 h and 22.5% after 8 days of exposure to oxygen tensions of 5.4 kPa (Chen and Kou, 1998; Cheng et al., 2003). In the Norway lobster (*Nephrops norvegicus*), the amount by which the Hemocyanin (Hc) increases during hypoxia is negatively related to the initial individual Hc concentration (Spicer and Baden, 2001): this result suggests an "optimum" Hc concentration. The rationale is that higher Hc concentrations may raise the colloidal osmotic pressure and viscosity of hemolymph, which increases the energetic cost of circulation (Mangum and Johansen, 1975). There are also reports of chronic hypoxia causing a decline in Hc. For example, low Hc concentrations in blue crabs (*Callinectes sapidus*) in the wild are correlated with chronic hypoxia (Engel et al., 1993). This may be caused by a reduction in protein synthesis, including Hc, which is produced in the hepatopancreas (Gellissen et al., 1991; Mente et al., 2003; Senkbeil and Wriston, 1981). This reduction may be induced by the limited oxygen supply (Carlos et al., 1998; McGaw, 2005), as well as a possible poor nutritional condition associated with diminished food intake during chronic hypoxia (McGaw, 2008; Paschke et al., 2009).

Alternatively, some crustaceans are able to increase the "quality" instead of "quantity" of Hc by changing the intrinsic properties (polymorphism and subunit frequency) of Hc to increase oxygen affinity (Giomi and Beltramini, 2007). In hypoxic blue crabs (*Callinectes sapidus*), a higher ratio of the 1 × 6-meric oligomer (hexameric structure) of Hc is found, which has higher oxygen affinity than the 2 × 6-meric oligomer (dodecameric structure), which is predominant during normoxia (Defur et al., 1990; Mangum, 1994; Mangum, 1997). Moreover, changes in the frequency of the six subunits of hexamers in Hc can also alter the intrinsic oxygen affinity. Blue crabs (*Callinectes sapidus*) collected from hypoxic habitats have higher ratios of the subunit 1 and lower ratios of subunit 4, resulting in an increased hypoxia tolerance (Bell et al., 2010), and after 7-day hypoxic exposure, a decrease in the ratios of subunit 5 and 6 occurs (Defur et al., 1990).

Hemocyanin oxygen binding affinity can also be enhanced by extrinsic inorganic and organic allosteric modulators, which bind to specific sites of the hexamer (Bridges, 2001; McMahon, 2001b; Terwilliger, 2015; Truchot, 1992). For example, hypoxia-induced hyperventilation reduces the CO_2 concentration in hemolymph, resulting in respiratory alkalosis (McMahon, 2001b). The decreasing H^+ concentration enhances the oxygen affinity of Hc within a certain pH interval (Bohr effect) (Bohr et al., 1904; Truchot, 1992). If the pH is well buffered, the accumulation of L-lactate during hypoxia (independent from increased H^+) facilitates an increase in oxygen affinity of Hc without the Bohr effect (Morris, 1990; Truchot, 1980). In addition, Ca^{2+} mobilized from the carapace in response

to an internal acidosis can also act as an inorganic allosteric modulator of Hc (Taylor and Whiteley, 1989).

1.2.1.2 Anaerobic metabolism and hypometabolism

Although most crustaceans have physiological mechanisms to deal with both acute and chronic hypoxic exposure, a point will be reached where these mechanisms are no longer able to maintain the required oxygen uptake: the critical oxygen level (P_{crit}). Below the P_{crit}, anaerobic metabolism and metabolic depression are observed (Pörtner and Grieshaber, 1993; Seibel, 2011). During severe hypoxia, elevated anaerobic metabolism (e.g., fermentation) offsets the decreasing aerobic metabolism (Spicer et al., 2002; Yannicelli et al., 2013). In some burrowing species (*Corystes cassiuelaunus*), anaerobic metabolism can account for up to 50% of the overall energy budget during severe hypoxia (Bridges and Brand, 1980; Zou et al., 1996). However, anaerobic metabolism produces less ATP than oxidative metabolism and leads to the accumulation of deleterious metabolites (e.g., L-lactate), which may cause an acid-base imbalance (Hochachka and Mommsen, 1983; Hochachka and Somero, 2002). In the red swamp crayfish (*Procambarus clarkia*), the hemolymph lactate concentration reaches 28 mmol/L (220% from normoxia), causing a 0.2 decline in pH during 48 h exposure to severe hypoxia (Bonvillain et al., 2012). Euryhaline marine crustaceans maintain the extracellular acid-base balance mainly by ion regulation through the posterior gills, where contain dense patches of "chloride cells", the cellular site of ion transport (Henry and Wheatly, 1992; Wheatly and Henry, 1992; Whiteley and Taylor, 2015). The major exchange processes ($Na^+/NH_4^+/H^+$ and Cl^-/HCO_3^-) are regulated by specialized chloride cells on gills with high

activities of the Na^+/K^+ ATPase and carbonic anhydrase, while the calcified exoskeleton may be utilized as an internal source of HCO_3^- (Freire et al., 2008; Whiteley, 1999). As a result, the pH is buffered in response to the regulated HCO_3^- concentrations.

Metabolic depression (hypometabolism) is the last defense to survive in severe hypoxia, where anaerobic metabolism is insufficient to offset the energy deficit of basal aerobic metabolism (Seibel, 2011). Such mechanisms are often employed by animals living in extreme environments, such as the OMZs (Auel et al., 2005; Kiko et al., 2016). The pelagic red crab (*Pleuroncodes planipes*) can suppress aerobic metabolism by about 70% during daytime forays into OMZs (Seibel et al., 2018). Likewise, the hyperiid amphipod (*Phronima sedentaria*) can reduce the total metabolism by 78% when exposed to hypoxia with low temperature (Elder and Seibel, 2015). The metabolic depression is achieved primarily by downregulating expensive intercellular processes, such as Na^+/K^+-ATPase activity (Buck and Hochachka, 1993; Seibel et al., 2018). This metabolic depression is accompanied by a concomitant decrease in protein synthesis (transcription and translation) (Hand, 1998; Storey, 2015; Storey and Storey, 2004). In contrast, increasing levels of heat-shock proteins act as chaperones to stabilize the existing cellular proteome (Seibel et al., 2018; Storey and Storey, 2011).

1.2.2 Behavior

Crustaceans can also use behavior to avoid hypoxic water. Mobile crabs may increase their activity levels during declining ambient oxygen levels in order to find oxygenated water. For example, the blue crab (*Callinectes sapidus*) increases activity, orienting down-

current to avoid declining oxygen levels (Bell et al., 2009; Bell et al., 2003). In the lab, both fasted and fed Dungeness crabs (*Cancer magister*) prefer the highest oxygen regime, while in the field, fed crabs tend to avoid severe hypoxic zones, settling and remaining quiescent in areas of higher oxygen content (Bernatis et al., 2007). In the northwestern Gulf of Mexico, the brown shrimp (*Farfantepenaeus aztecus*) avoids seasonal hypoxia and aggregates in oxygenated nearshore habitats, even though this puts them at increased risk of predation (Craig, 2012).

Amphibious crustaceans may be able to obtain oxygen by breathing in air (Henry, 1994). In shallow hypoxic water, the green crab (*Carcinus maenas*) partially emerges into air (Taylor et al., 1973). In this way, it can raise the mean oxygen content of postbranchial hemolymph 2.4-fold, using ventilatory reversals, sucking in air through the mouth over the gills and expelling it through the Milne-Edwards openings on the lower edge of the branchiostegite (Taylor et al., 1973). Other species, such as the freshwater crayfish (*Paranephrops zealandicus*), fully emerge from the water and respire in air when oxygen tension in water reaches 0.6 kPa (Broughton et al., 2017). This aerial ventilation is also common in the red swamp crayfish (*Procambarus clarkii*), which spends 75% of its time in humid air when the water is hypoxic (McMahon and Stuart, 1999).

Although avoidance behaviors may be an efficient defense, hypoxia can often be too severe or widespread to enable an adequate oxygen uptake to fuel increased rates of locomotor activity (Haselmair et al., 2010). In such instances, crustaceans may halt routine activities in order to reduce oxygen consumption (Wu 2002; Eriksson and Baden, 1997). These activities may include, but are not limited to, loss of intraspecific aggression and

habitat segregation and alteration of predator-prey interactions (Haselmair et al., 2010; Riedel et al., 2014). For example, the predator and prey interactions of the isopod (*Saduria entomon*) and adult amphipod (*Monoporeia affinis*) are negatively affected by hypoxia as they both become less active (Sandberg and Bonsdorff, 1996). Meanwhile, the sediment dwelling amphipods (*Monoporeia affinis* and *Pontoporeia femorata*) exhibit a trade-off behavior: when oxygen levels drop to 5% saturation, they move up to the sediment surface where oxygen levels are higher even though this puts them at a higher risk of predation (Johansson, 1997). However, the alternative - prolonged anoxia - would cause animals to become moribund and eventually perish (Haselmair et al., 2010).

1.3 Effects of food deprivation state on crustaceans

The intrinsic physiological state of animals also significantly affects their responses to environmental stressors. For instance, feeding and subsequent digestion, or food deprivation can all affect the physiological responses and biochemical parameters of crustaceans. These may, therefore, impact how crustaceans react to subsequent environmental change.

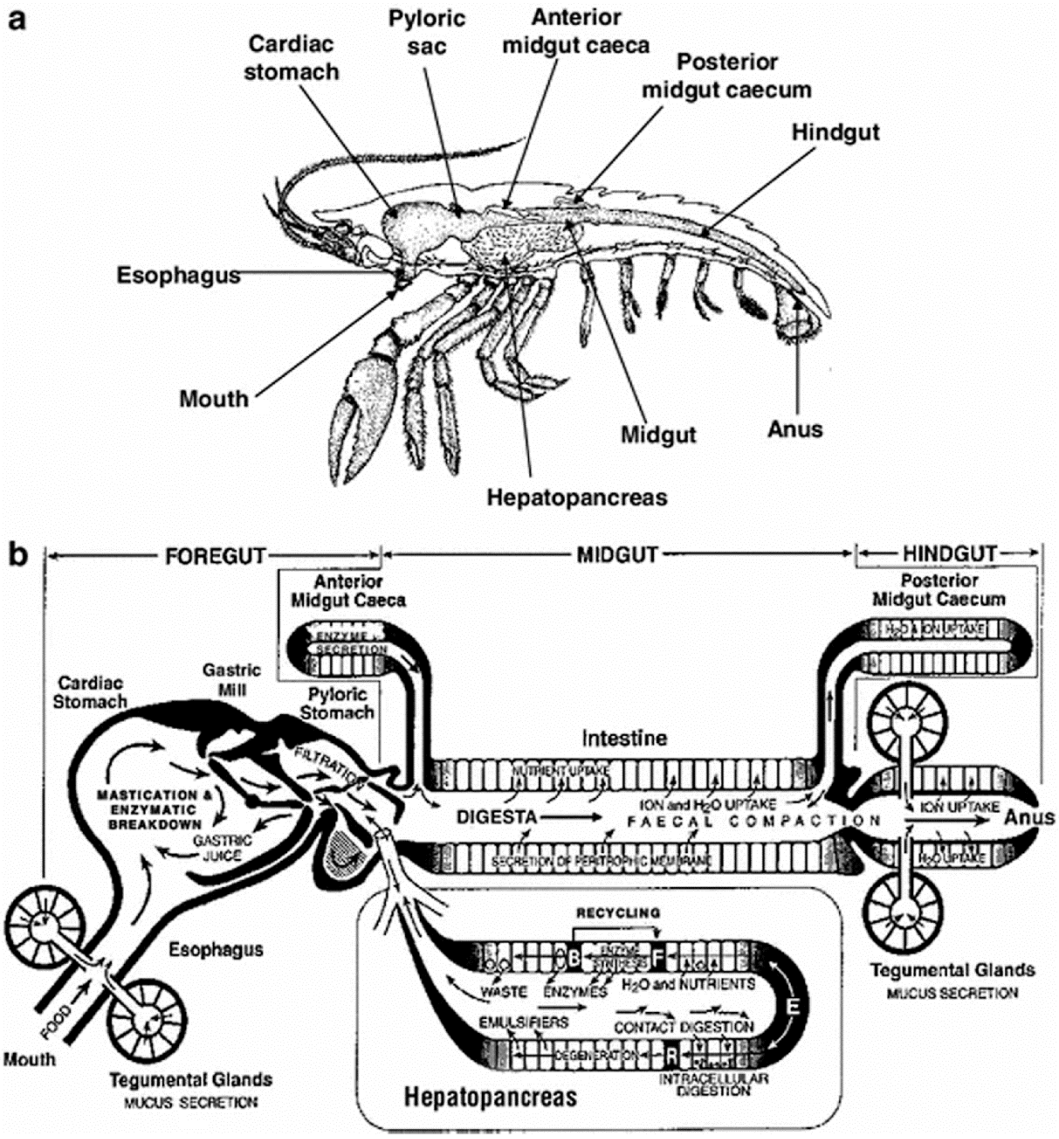

Figure 1.2 The digestive system of crustaceans (McGaw and Curtis, 2013): a. Anatomy of the digestive system of a decapod crustacean, showing the foregut, midgut, and hindgut regions. b. Schematic diagram of digestive processes occurring in the decapod crustacean gut.

1.3.1 Feeding and digestion

In general, all organisms increase oxygen consumption after feeding. This increase in the postprandial energy expenditure is associated with the ingestion, digestion, absorption, and assimilation of a meal and is termed the specific dynamic action (SDA) (McCue, 2006; Secor, 2009). In crustaceans, after food is ingested (Figure 1.2), over 40 muscles of the foregut and gastric mill are used for cutting, grinding and movement of digesta (McGaw and Curtis, 2013). The mechanical digestion of the meal may account for up to 30% of the overall SDA response (McGaw and Penney, 2014; McGaw and Van Leeuwen, 2017).

During biochemical assimilation, substrates that have been absorbed in the hepatopancreas are transported to the cells and stimulate an increase in protein synthesis (McGaw and Curtis, 2013). Protein synthesis is a major component of biochemical assimilation (Carter and Mente, 2014), accounting for 20–50% of the postprandial increase in oxygen consumption in crustaceans (Mente et al., 2003). For example, in the green crab (*Carcinus maenas*), protein synthesis increases up to 2-fold and remains elevated for 16 h, accounting for 20–37% of the total postprandial oxygen consumption (Houlihan et al., 1990). A recent study shows the oxygen consumption rates of fed spiny lobster (*Sagmariasus verreauxi*) are reduced by 96% after injection of a protein synthesis inhibitor (cycloheximide), confirming earlier reports that the postprandial protein synthesis is the major contributor to the rise in oxygen consumption following feeding (Wang et al., 2019).

During hypoxic exposure, a postprandial crab would exhibit an increase in oxygen consumption at the very time oxygen was limiting. Some species may show compensatory responses: the Dungeness crab (*Cancer magister*) reduces both the amount of food eaten and the time spent feeding in oxygen tensions below 5.3 kPa and ceases feeding below 3.2 kPa (Bernatis et al., 2007). A similar reduction in prey-handling and feeding behavior occurs in the green crab (*Carcinus maenas*) (Brante and Hughes, 2001). When hypoxic exposure is experienced after feeding, *C. magister* exhibits a 2-fold increase in gastric transit time, which is associated with a decrease in digestive efficiency (McGaw, 2005; McGaw, 2008). These changes in gastric processing may be paralleled by changes in the SDA (postprandial oxygen consumption). For example, the peak oxygen consumption of postprandial blue crab (*Callinectes sapidus*) is reduced by half, while the duration of SDA response is significantly prolonged when this species is subjected to hypoxia (3–4 kPa) (Brill et al., 2015). Similarly, in the green crab (*Carcinus maenas*), severe hypoxia (3 kPa) reduces the postprandial peak oxygen consumption from 2.4 (in normoxia) to 1.3-fold of prefeeding rates (Mente et al., 2003). This decline is associated with a downregulation of protein synthesis in the hepatopancreas (Mente et al., 2003).

1.3.2 Starvation

While feeding and digestion are associated with specific physiological changes, food deprivation can also impart a suite of different effects, and an entire science has grown up around starvation physiology (McCue, 2010; Mente et al., 2003; Wang et al., 2006). All animals continuously expend energy to survive, and survival during starvation inevitably

requires mobilization of body energy reserves in the form of carbohydrates, lipids or proteins (Kooijman, 2010). To adapt to this energetic limitation, animals can reduce their metabolic rate by behavioral or physiological mechanisms (Brown et al., 2019; Staples and Buck, 2009).

Crustaceans experience periods of starvation during ecdysis or seasonal food shortage (Moreira, 2015; Simon et al., 2015). During food deprivation, crustaceans downregulate metabolism and activity, but the degree to which this occurs is dependent on the species, life stage and duration of food deprivation (Sacristán et al., 2017; Sánchez-Paz et al., 2006). For example, 10-day food deprivation in *Cancer pagurus* induces a progressive decrease in activity with a concomitant decrease in the maximum and resting metabolic rates and heart rates (Ansell, 1973). The brown shrimp (*Crangon crangon*) exhibits a gradual reduction in oxygen consumption during 14 days of food deprivation, which remains depressed (60% below initial levels) during an additional 16 days of food deprivation (Regnault, 1981). A similar pattern is found in the green crab (*Carcinus maenas*), which also exhibits a 60% decline in metabolism after 21 days of food deprivation (Wallace, 1973). This suppressed metabolism is associated with reduced activity rates and down-regulation of protein synthesis rates (Wang et al., 2019).

During food deprivation, crustaceans utilize glycogen, lipid and protein reserves (Sánchez-Paz et al., 2006), but their order of utilization differs depending on the species and the length of food deprivation (Sacristán et al., 2017; Vinagre et al., 2007). During short-term starvation (5 days), the Pacific white shrimp (*Litopenaeus vannamei*) uses hepatopancreatic glycogen as the primary energy resource (Sánchez-Paz et al., 2007). The

brown shrimp (*Crangon crangon*) also uses carbohydrate reserves during the initial starvation period; however, these are quickly exhausted (3–4 days), and after 30 days of food deprivation, structural proteins are catabolized, resulting in 50% loss of body protein (Regnault, 1981). Enzyme activities of adult Antarctic krill (*Euphausia superba*) indicate a catabolism shift from protein to lipid during seasonal starvation (Auerswald et al., 2009). In contrast, the juvenile spiny lobster (*Sagmariasus verreauxi*) utilizes hepatopancreas lipid as a primary source of energy (60% depletion within 3 days), even though abdominal muscle protein represented 74% to 90% of the total energy reserve (Simon et al., 2015).

Because various nutrient sources are exhausted during starvation, this can have secondary effects on a number of other physiological processes. Starvation in the white shrimp (*Litopenaeus vannamei*) induces a progressive increase in cellular immunity (hemolytic infiltration) and cell death (necrosis, apoptosis, and autophagy) in the hepatopancreas (Cervellione et al., 2017). Following 28-day food deprivation of spiny lobster juveniles (*Sagmariasus verreauxi*), hemolymph glucose decreases by 53%, total proteins by 63% and oxygenated Hc levels by 40% (Simon et al., 2015). As Hc acts as an oxygen transporter and glucose is utilized in anaerobiosis, these changes might further affect the ability of animals to deal with environmental stressors, specifically hypoxia (Bernatis et al., 2007; Curtis et al., 2010).

1.4 Potential interactive effects of hypoxia and food deprivation state

Environmental oxygen levels often change in line with other abiotic factors such as temperature and salinity; the responses of crustaceans to such multiple stressors have been covered elsewhere (Bozinovic and Pörtner, 2015; Pörtner et al., 2017; Sokolova et al., 2012). However, interactions between abiotic stressors and biotic conditions (e.g., starvation, competition, and predatory pressure) are also important and can alter the hypoxic tolerance as well as the aerobic performance of crustaceans.

In this study, I focus on the impact of the food deprivation state (starved, fasted, fed) on the physiological and behavioral performances of crabs during hypoxia. It is well known that starvation results in suppressed metabolic rates and spontaneous activity, as well as a reduction of total proteins and Hc in the hemolymph of crustaceans (Matozzo et al., 2011; Simon et al., 2015). As such, starved crustaceans may respond differently to environmental stressors. To date, only a few studies of crustaceans have combined the effects of food deprivation state and hypoxia (Baden et al., 1994; Legeay and Massabuau, 2000). This lack of information is surprising given that a. the reduced hemocyanin concentration during starvation would result in a decreased oxygen transport ability (Terwilliger, 2015), b. the limited energy budget of starved crabs may affect their stress response (Sokolova et al., 2012). At the other end of the scale, when postprandial crabs encounter hypoxia, they will be exhibiting an increased oxygen demand at a time when oxygen supplies are limiting (Carlos et al., 1998).

1.5 The Atlantic rock crab (*Cancer irroratus*)

The Atlantic rock crab (*Cancer irroratus* Say, 1817) is a common benthic brachyuran species on the east coast of North America. Their natural range is from Labrador, Canada, to Haiti (Drew, 2011). Recently, the rock crab has invaded and successfully settled the southwestern coast of Iceland (Gíslason et al., 2014). *Cancer irroratus* is distributed from the shallow subtidal zone down to depths of 390 m, with maximum abundances reported between 40–60 m depth (Haefner Jr, 1976; Hudon and Lamarche, 1989). This species usually occurs on rocky bottoms with macroalgae, where they can easily find shelter (Haefner Jr, 1976; Hudon and Lamarche, 1989). They inhabit areas with salinities 16–35‰ and temperatures 4–25°C (Breen and Metaxas, 2008; Williams, 1984). As an important prey and predator, *C. irroratus* occupy an essential ecological niche (Bigford, 1979). Adult crabs mainly prey on polychaetes, bivalves, echinoderms and small crustaceans, while juvenile *C. irroratus* is an important food source to some demersal fish species and large decapods, including the commercially important lobster (*Homarus americanus*) (DFO, 2014; Gendron et al., 2001; Scarratt and Lowe, 1972).

Under the changing climate scenario, hypoxic areas in coastal waters have expanded to the natural habitat of the rock crab (Levin et al., 2009). For example, the oxygen concentration in the bottom waters reached 65 µmol/L (20.7% saturation at 15°C) in the Gulf of St. Lawrence (Gilbert et al., 2005). Combined with warming temperature, loss of dissolved oxygen has altered the natural habitats of this species (Deutsch et al., 2015). However, few studies have addressed the interactions between food deprivation state,

digestion and hypoxia on behavioral and physiological responses of crustaceans (Penney et al., 2016; Wheatly, 1987). Thus, in this study, I will fill those gaps to better understand the impact of hypoxia on this species and understand how climate change could affect the population dynamics and marine ecosystem (Bozinovic and Pörtner, 2015).

1.6 Objectives

The overall goals of this project were to determine whether crustaceans can mediate the conflict between increasing oxygen requirement (SDA) and limited supply (hypoxia) and how poor nutritional state and increased foraging motivation (starvation) interact at the physiological and behavioral level. I used *Cancer irroratus* as a model organism. There are four separate chapters in the Ph.D. dissertation:

a) Effects of food deprivation state on critical oxygen partial pressure (P_{crit}) and characteristics of the specific dynamic action (SDA) during mild and severe hypoxic exposure

Rationale: Starved crabs have lower resting metabolic rates than fasted crabs in normoxia (Ansell, 1973). However, metabolic rates of crustaceans become elevated (2–3 fold) after feeding (Ansell, 1973; Secor, 2009). With this in mind, it might be expected that starved and fasted crabs would display different postprandial responses following feeding and that these might also be affected by the degree of hypoxic exposure (Mente et al., 2003).

Hypotheses: In rock crabs, the food deprivation state will directly influence hypoxia tolerance. Postprandial metabolic rates will also differ between fasted and starved crabs as a function of the severity of hypoxic exposure.

Objectives: Determine how the food deprivation state affects hypoxia tolerance. In order to accomplish this, the oxygen consumption rates of starved, fasted and fed crabs were measured during progressive hypoxia. In a subsequent series of experiments, the postprandial metabolism of starved and fasted crabs after feeding was compared during exposure to normoxia, mild and severe hypoxia (above and below P_{crit}).

b) Effects of food deprivation state on biochemical responses to hypoxia

Rationale: These experiments were carried out to determine possible underlying mechanisms influencing the differences in oxygen consumption during hypoxia as a function of food deprivation state. Starved crabs exhibit lower hemolymph protein and hemocyanin concentrations than fasted crabs (Simon et al., 2015), which may influence their ability to tolerate hypoxia. In addition, starved crabs with lower hemolymph glucose reserves may not be able to use anaerobic mechanisms as efficiently as fasted crabs. Differences in ventilatory performance and substrate use may also influence the acid-base status in fasted and starved animals (Bonvillain et al., 2012; McMahon et al., 1974).

Hypotheses: Both food deprivation state and the degree of hypoxia will influence the biochemical responses of postprandial rock crabs.

Objectives: Determine the biochemical parameters of the hemolymph of starved and fasted crabs following feeding and subsequent exposure to hypoxia. In these

experiments, the following biochemical parameters were measured in fasted and starved crabs in 100%, 50% and 20% oxygen saturation before and after feeding. Arterial P_{O2} and hemocyanin levels indicate oxygen transportation capacity; L-lactate was used to determine the degree of anaerobic metabolism; pH is for internal acid-base status.

c) Effects of food deprivation state on feeding behavior and gastric evacuation during hypoxia

Rationale: This chapter investigated whether the food deprivation state influenced the amount of food consumed as a function of the severity of hypoxia. Once an animal had fed, the second aim was to determine how the food deprivation state affects gastric processing (food passage through the digestive system) in response to the severity of hypoxia. There could be a trade-off between acquiring sufficient food or reducing the postprandial effects of digestion. Crustaceans may reduce food intake to avoid elevated postprandial metabolism or slow gastric processing during hypoxia (Bernatis et al., 2007; McGaw, 2008). However, starvation may increase their feeding motivation, and they may be more likely to forage and feed under increased risks, in this case, hypoxia (Moore and Howarth, 1996; Wang et al., 2006).

Hypothesis: The food deprivation state and the hypoxic level will affect the amount of food consumed and the time for food processing at the gastric level.

Objectives: 1. Determine how much food starved and fasted crabs will consume during exposure to hypoxia. 2. Find out whether gastric processes will be affected differentially in these two groups. The food intake of fasted and starved crabs was

measured in normoxia, mild and severe hypoxia (above and below P_{crit}). Gastric processing was measured by feeding crabs a radiopaque meal and subsequently taking an X-ray image as digesta traversed the gut. This was carried for fasted and starved crabs in normoxia, mild and severe hypoxia (above and below P_{crit}).

d) Effects of food deprivation state on avoidance and locomotor activity during hypoxia

Rationale: This chapter sought to determine whether rock crabs can detect different oxygen regimes and actively avoid these hypoxic conditions. Building upon this initial framework, I also sought to determine whether the food deprivation state and environmental oxygen levels influenced their locomotor activity. Crustaceans can use behavior to avoid hypoxia (Haselmair et al., 2010; Taylor et al., 1973). However, increased activity is associated with a substantial increase in oxygen demand, and this may be further compounded by the food deprivation state. Compared with fasted crabs, recently fed crabs may spend less energy on locomotion due to their higher oxygen demands associated with the SDA. In contrast, the starved crabs may not be able to maintain a high aerobic scope during locomotion due to poor nutritional conditions and lower hemocyanin levels, which would also affect oxygen transport.

Hypothesis: The crabs will be able to discern between different oxygen levels and exhibit a preference. The food deprivation state and the level of hypoxia will also alter activity rates and oxygen concentration preferences.

Objectives: Determine if activity levels and oxygen preferences are influenced by the food deprivation state. In order to accomplish this, I monitored the movement of starved, fasted and fed crabs in different oxygen combinations and for different durations. A Loligo® Shuttle Box system was used to create two different oxygen conditions connected with a narrow walkway allowing a crab to move freely and choose the preferred oxygen level. This system has been used previously with success to monitor the choice behaviors of finfish, but there are few studies for crustaceans that are negatively phototactic and positively thigmotaxic. Therefore, the secondary aim of this study was to assess the suitability of this newly designed equipment for use with crustaceans. The coordinates of the crab were recorded by a digital camera and converted to the real-time speed (cm/s), total distance traveled (cm) and the time (s) spent in each arena of the shuttle box.

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Chapter 2. Effects of food deprivation state on critical oxygen partial pressure (P_{crit}) and characteristics of the specific dynamic action (SDA) of rock crabs during mild and severe hypoxic exposure[1]

[1]A modified version of this chapter has been submitted to Comparative Biochemistry and Physiology part A.

2.1 Abstract

Climate change and anthropogenic activities have led to a substantial increase in both the spatial and temporal extent of hypoxia in marine waters in recent decades. Increasing attention has thus focused on the effects of ocean deoxygenation on marine organisms. However, few studies have addressed the effects of different food deprivation states on hypoxia tolerance. In the present study, the metabolic responses of Atlantic rock crabs, *Cancer irroratus,* in three food deprivation states (starved – 28 d, fasted – 3 d, recently fed) were investigated in response to different levels of oxygen saturation (100%, 50% and 20%). Starved crabs exhibited the lowest critical oxygen partial pressure (P_{crit}), while fed crabs had the highest P_{crit}. Elevated oxygen consumption of postprandial crabs below P_{crit} indicated a possible adaption to hypoxia with reserved aerobic scopes for critical activities. Following feeding, crabs exhibited the typical postprandial increase (specific dynamic action: SDA) in oxygen consumption (MO_2) characterized by a rapid increase, followed by a gradual decline to pre-feeding levels. Hypoxia retarded the SDA response resulting in lower peak MO_2 and longer duration that MO_2 remained elevated for both fasted and starved crabs. The food deprivation state had an interactive effect, with starved crabs exhibiting a lower peak MO_2, prolonged duration and higher energy expenditure than fasted crabs. The results suggest that the physiological condition of rock crabs affects their hypoxic tolerance. Starvation may trigger a cross-tolerance to hypoxia since both long-term starvation and severe hypoxia would elicit similar physiological responses of hypometabolism. Because crabs can undergo long periods of food

deprivation in their natural environment, future studies should take into consideration how this may affect their ability to deal with environmental perturbations.

Keywords: crab, hypoxia, oxygen, specific dynamic action, feeding, starvation

2.2 Introduction

Under the background of climate change, increasing attention has focused on ocean deoxygenation (Breitburg et al., 2018; Diaz and Rosenberg, 1995; Keeling et al., 2010). In coastal areas, global warming reduces oxygen solubility and enhances the stratification of seawater. In addition, anthropogenic increases in nutrient input (nitrogen and phosphate) and organic matter enhance primary and secondary production of the ocean, which further reduces the available oxygen for marine organisms (Gilbert et al., 2010; Schmidtko et al., 2017; Watson, 2016). As a result, oxygen dead zones (≤ 63 μmol/L, 20.1% oxygen saturation at 15°C) in global coastal oceans have increased by 90% since 1950 (Diaz and Rosenberg, 2008).

Crustaceans are among the most hypoxia-sensitive taxa in the oceans (Vaquer-Sunyer and Duarte, 2008). While behavioral avoidance of hypoxic water is usually the first line of defense, such regimes may be too widespread or severe for behavioral mechanisms to be effective. In such cases, crustaceans can display a suite of physiological responses in order to tolerate hypoxia (Brill et al., 2015; McMahon, 2001; Pörtner and Grieshaber, 1993; Taylor, 1976). The immediate response of most crustaceans is to elevate ventilation rates to increase the water flow through the branchial chambers and

gas exchange across the gills (Airriess and McMahon, 1994; Taylor, 1976). Hypoxia also induces a progressive bradycardia, while the cardiac output can be compensated by a concomitant increase in cardiac stroke volume (Reiber and McMahon, 1998; Wilkes and McMahon, 1982): this mechanism may serve to reduce energy expenditure (Guadagnoli et al., 2007; Taylor and Butler, 1973; Wilkens et al., 1996). Oxygen uptake can also be augmented by increasing branchial hemolymph flow (McMahon, 2001a) by diverting hemolymph from the viscera to the sternal circuit, which ultimately perfuses the gills (Airriess and McMahon, 1994). During long-term hypoxia, increases in or modification of the intrinsic properties of hemocyanin may also facilitate oxygen transport (Cheng et al., 2003; Defur et al., 1990; Giomi and Beltramini, 2007; Mangum, 1994; Senkbeil and Wriston, 1981). Most crustaceans are oxyregulators, but below a critical oxygen tension (P_{crit}), aerobic metabolism is generally depressed, and the deficit may be compensated by anaerobic metabolism (Brill et al., 2015; Seibel, 2011; Spicer et al., 2002; Yannicelli et al., 2013).

Traditionally the hypoxia tolerance of post-absorptive, undisturbed animals has been studied in order to reduce the interactive effect of any endogenous factors, which may alter their metabolism and even hypoxia tolerance (Brill et al., 2015; Herreid, 1980). Most commonly, crustaceans are fasted for several days prior to experimentation. This standard fasting period is important because postprandial crustaceans typically exhibit a sustained increase in metabolism (2–4 fold), associated with the ingestion, absorption, and assimilation of a meal; this is termed the specific dynamic action (SDA) (McCue, 2006; Secor, 2009). However, in nature, organisms do not always fast prior to experiencing

environmental perturbations: this might be pertinent for postprandial crabs' experiencing hypoxia as their demand for oxygen is higher at a time when oxygen supplies are limiting (Brill et al., 2015; Mente et al., 2003). When crabs experience hypoxia after feeding, it significantly diminishes the elevation of postprandial oxygen consumption by blocking protein synthesis (Mente et al., 2003; Wang et al., 2019). This reduction is caused by limited oxygen supply as well as blood diversion away from digestive structures (McGaw, 2005). When oxygen saturation decreases to 25%, both the peak and median metabolic rate are reduced by half, and the duration of SDA is significantly prolonged in the postprandial blue crab (*Callinectes sapidus*) (Brill et al., 2015). Similarly, the white shrimp (*Penaeus setiferus*) reduces both the resting metabolic rate (RMR) and peak MR by over 50% when exposed to 2 mg/L (34% oxygen at 28°C) dissolved oxygen, while both the time to reach peak MR and the duration of SDA increase (Carlos et al., 1998).

Crustaceans can often experience long periods without access to food; such periods of starvation may occur on a seasonal basis, during molting, or in response to changes in the environment (Moreira, 2015; Simon et al., 2015). Long-term food deprivation is associated with a decrease in metabolism by 40% to 60% (Regnault, 1981; Wallace, 1973), which results from the downregulation of protein synthesis (Wang et al., 2019). During food deprivation, crustaceans mobilize carbohydrate, lipid and protein reserves as an energy source (Sacristán et al., 2017; Sánchez-Paz et al., 2006). As a result, starved crustaceans have lowered plasma glucose and protein levels, and since over 50% of the plasma protein is hemocyanin (Hc), levels of this respiratory pigment can be reduced by up to 40% during long-term starvation (Chen and Cheng, 1993; Sánchez-Paz et al., 2007;

Simon et al., 2015). Because Hc acts as an oxygen transporter and glucose is utilized in anaerobic respiration, these changes might further affect the ability of animals to deal with environmental stressors (Bernatis et al., 2007; Curtis et al., 2010). Although the effects of some stressors (such as hypoxia, temperature and salinity) on feeding and digestive processes have received some attention, fewer studies address the interaction between starvation and these stressors (Bernatis et al., 2007; Brill et al., 2015; McGaw, 2008; Mente et al., 2003).

The Atlantic rock crab*, Cancer irroratus* is a common benthic crustacean along the Atlantic coast of North America. *Cancer irroratus* is distributed from the shallow subtidal zone down to depths of 390 m, with maximum abundances between 40–60 m (Haefner Jr, 1976). This species is most commonly found on rocky bottoms with macroalgae, where they can easily find shelter (Hudon and Lamarche, 1989). The rock crab is an important prey of some demersal fish species and large decapods, including the commercially important lobster (*Homarus americanus*) (DFO, 2014; Gendron et al., 2001). Under the changing climate, hypoxic areas in coastal waters have expanded to the natural habitats of the rock crab (Levin et al., 2009). For example, dissolved oxygen levels in the bottom waters in the Gulf of St. Lawrence have declined to 65 µmol/L (20.7% saturation at 15 °C) (Gilbert et al., 2005). Understanding the crabs' hypoxic tolerance in different physiological states will help inform about the potential impact of climate change on the marine ecosystem.

The present study aimed to determine whether the food deprivation state (starved – 28 days, fasted – 3 days, and recently fed) would affect the physiological responses of

C. irroratus to hypoxia. For example, postprandial crabs exhibit an increase in oxygen consumption, while starved crabs will have a significantly lowered metabolism, lower hemocyanin levels and limited energy reserves (Sacristán et al., 2017; Secor, 2009). However, it is unknown how these factors may interact and affect physiological responses and hypoxia tolerance. I first determined how the critical oxygen concentration (P_{crit}) was influenced by the food deprivation state. Secondly, the effect of food deprivation state on characteristics of SDA response during hypoxia was investigated.

2.3 Material and Methods

2.3.1 Crab collection and housing

Rock crabs (*Cancer irroratus*) were trapped in multiple locations along the coast of the northern Avalon Peninsula, Newfoundland, Canada. Adult crabs (80–160 g) that showed no obvious physical damage, weakness or parasite in the hard-intermolt stage, and females bearing no eggs were retained. A similar range of sizes was used throughout experimental trials to avoid any bias (see the effects of animal size on aerobic metabolism, Appendix A). The crabs were transferred to the Department of Ocean Sciences at Memorial University in St John's, NL, Canada and held in 400L sediment-free tanks with flow-through aerated seawater at ambient temperatures (4–10°C) and salinity (30–32‰). The crabs were fed chopped herring (*Clupea harengus*) to excess once a week, and any uneaten food was removed on the same day to avoid fouling the water. At least two weeks before the experiments started, crabs were acclimated in the laboratory tanks (300

L) in flow-through aerated seawater (salinity 30–32‰) at 12–13°C and constant dim red light. They were also fed herrings twice a week. A two-week period is the minimum time to effectively acclimate the crabs to experimental temperatures and to abolish any underlying photoperiod rhythms (Taylor et al., 1977). Initial experiments showed no differences in oxygen consumption rates between sexes; therefore, crabs of both sexes were chosen indiscriminately in each treatment at the approximate ratio of collection (male: female, 3:1).

In this study, crabs were classified into three food deprivation states, each with specific physiological attributes:

a) Starved crabs were held in individual perforated plastic chambers (25 × 15 × 7.5 cm) to prevent cannibalism at 12–13°C and starved for 28–35 days. Preliminary experiments showed that 1-month of constrained acclimation did not induce any significant changes in metabolism when compared with free-moving crabs (Appendix A). The mortality rate of starvation was 10–15%, which was similar to fasted crabs. The starved crabs have lower resting metabolic rates (Ansell, 1973; Wallace, 1973) and reduced blood protein levels (and hence hemocyanin levels), and utilize body proteins for energy (Sacristán et al., 2017).

b) Fasted crabs were deprived of food for 3–5 days before experiments began, which allowed them to evacuate all food from the digestive system. Studies provide evidence to suggest crabs used in the current study reached resting metabolic rates without the effects of prolonged starvation (Ansell, 1973; McGaw and Curtis, 2013; Wallace, 1973).

c) Fed crabs were fed shrimp muscle, 2% of their body mass, 1 h before experiments. They were held in individual chambers and ensured consuming all food. The postprandial crabs typically exhibit a 2–3 fold increase in metabolism associated with digestion, absorption and protein synthesis (Secor, 2009).

2.3.2 Experimental design

Rates of oxygen consumption were measured using an L-DAQ intermittent flow respirometry system (Loligo System, Viborg, Denmark). This fully automated system is equipped with two pumps. The first pump continually flushes seawater through a cylindrical chamber (20 cm diameter and 12 cm depth) containing the crab (the weight was measured before crab was put into the chamber). For measurements, the first pump is automatically turned off, the chamber is sealed, and the second pump recirculates the water through the chamber at a rate of 10 L/min, which prevents oxygen gradients from forming in the chamber. Oxygen consumption is calculated during this sealed period as the oxygen levels decline due to respiration of the crab, then the chamber is continuously flushed between readings. Data were recorded by a computer linked to a Loligo data acquisition system (Viborg, Denmark), which calculated MO_2 as $mgO_2/kg/h$ (Penney et al., 2016).

All experiments were conducted at 12–13°C in dim red light, and the entire apparatus was surrounded with black sheeting to avoid any visual disturbance to animals (Figure 2.1). Control experiments were conducted in empty respirometry chambers, and

the background oxygen consumption was found to be negligible. For reference, 100% oxygen saturation is approximately 21 kPa.

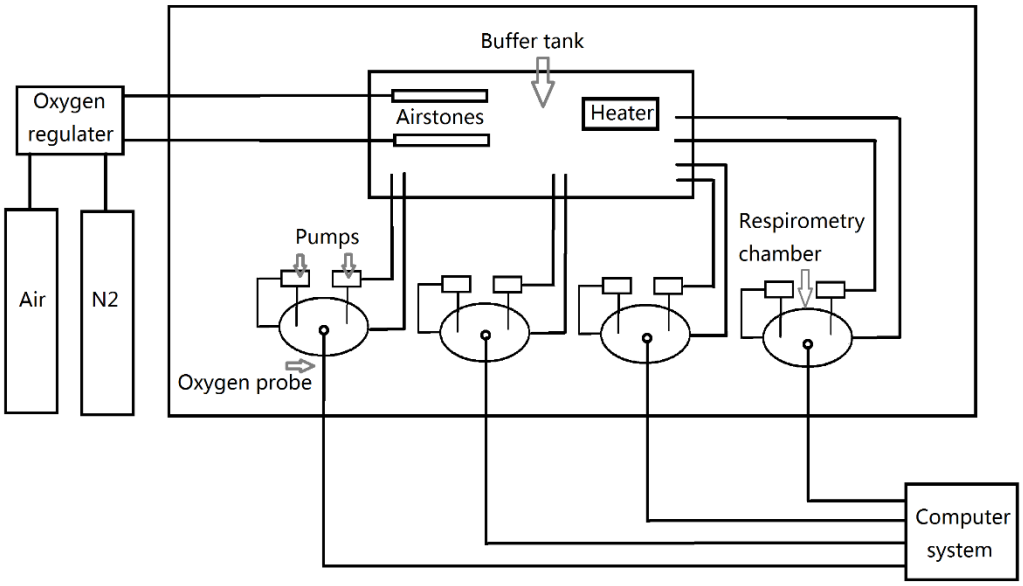

Figure 2.3 Experimental holding conditions and intermittent flow respirometry system. All experiments were conducted at 12–13°C in dim red light and the entire apparatus was surrounded with black sheeting to avoid any visual disturbance to animals. The oxygen saturation was maintained using an oxygen regulator connected to an air source and a nitrogen cylinder (Loligo System, Viborg, Denmark).

2.3.2.1 Critical oxygen partial pressure (P_{crit})

To determine the lowest oxygen levels at which the crabs could sustain their oxygen demand, the P_{crit} of crabs for the three food deprivation states (starved, fasted and fed, n = 8–9 for each group, total sample size = 26) were calculated as the oxygen saturation was progressively diminished (Brill et al., 2015). The oxygen saturation was controlled by an oxygen regulator (Loligo System, Viborg, Denmark) bubbling either air or nitrogen into the buffer tank. The rates of oxygen consumption were recorded for each crab during a stepwise reduction (8 oxygen levels) from 100% oxygen saturation to target levels of 75%, 50%, 40%, 30%, 20%, 10% and 5%. At each step, while the oxygen consumption rate was being recorded (when the chamber was sealed), the oxygen level in the buffer tank was decreased so that when the chamber reopened, it was flushed with the new lower test oxygen level for 10 min before being sealed for 20 min for the next reading. This step was repeated until the lowest test oxygen of 5% was achieved. Following the 5% oxygen saturation treatment, the oxygen level was raised to 100% saturation, and oxygen consumption was recorded during a one-hour recovery period. Using this timing, the entire experiment lasted 5 h, which ensured the fed crabs still maintained an elevated postprandial oxygen consumption (Penney et al., 2016).

2.3.2.2 Resting metabolic rates in different oxygen

In a second experimental trial, the resting metabolic rate (RMR, $mgO_2/kg/h$) of unfed fasted and starved crabs were determined in oxygen levels of 100%, 50% and 20% (n = 9–11, total sample size = 60). The crabs were allowed to settle overnight for 12 h in these

respective oxygen levels, and the RMR was then calculated over 5 h of consecutive recordings. This experiment was carried out to determine the baseline MO_2 of unfed fasted and starved crabs in different hypoxic conditions. It also enabled the calculation of the duration of the SDA response (detailed below).

2.3.2.3 Specific dynamic action (SDA)

In the final experimental series, the rates of postprandial oxygen consumption of fasted and starved crabs were recorded at three oxygen saturations (100%, 50% and 20%, n = 9–10, total sample size = 56). An oxygen saturation level of 100% was used as a normoxic control. An oxygen saturation level of 50% was chosen as mild hypoxia since it was above the P_{crit} of starved and fasted crabs, while severe hypoxia of 20% oxygen saturation was below the P_{crit} of the two groups. The crabs were left in the apparatus to settle overnight, after which resting metabolic rates were recorded in 100% O_2 for 5 h. They were then fed with shrimp muscle of approximately 2% of body mass; those not finishing all the meal within 30 min were excluded from the analysis. After feeding, the oxygen saturation was changed to the target level (100%, 50% or 20%) over a period of 30 min and the saturation was maintained at this level using an oxygen regulator (Loligo System, Viborg, Denmark) connected to an air source and a nitrogen cylinder. The experiment continued until the oxygen consumption returned to the pre-feeding levels or remained constant for a period of more than 12 h.

The following parameters of SDA were calculated (Secor, 2009):

a) Peak metabolic rate ($mgO_2/kg/h$), the highest oxygen consumption during postprandial metabolism.

b) Time to peak (h), the time to reach peak metabolic rate following food intake.

c) Duration (h), the time from feeding to when the metabolic rate dropped back to the pre-feeding RMR in hypoxia. As detailed above, all crabs, regardless of treatments, were initially maintained and fed in normoxia (to ensure they consumed all of the meal) before the oxygen level was dropped. In order to determine when postprandial MO_2 had declined to basal (pre-fed) levels in hypoxia, an estimate of RMR of another group of crabs in respective oxygen levels was carried out (section 2.4.2). If metabolic rate did not reach pre-feeding levels after 72 h, the endpoint was considered as the point at which no further change was observed for a period of 12 h.

d) Factorial scope, peak metabolic rate divided by the pre-feeding oxygen consumption levels.

e) Equivalent energy consumption (kJ/kg), the total energy cost of SDA. This was calculated from the accumulated amount of O_2 consumption above baseline during SDA and converted to an energy equivalent [14 kJ energy consume 1 g O_2 from Secor (2009)]. It includes the energetic cost of nutrient breakdown, transport, and intracellular protein synthesis.

2.3.3 Data analysis

All statistical tests were conducted in R (Team, 2018). The normality of data was checked by a Shapiro-Wilks test. For these data that did not pass the normality test, a

Box-Cox transformation was applied (λ value was reported). A Bartlett test was performed to check the homoscedasticity of data. In order to deal with heterogeneity, variance structures were added in model fitting, and the best variance structure was achieved by likelihood ratio tests with Akaike's information criterion (AIC). Tukey-adjusted post hoc tests were carried out using the 'emmeans' package. Statistical significance was accepted at $p < 0.05$. All data in the figures and tables are shown as the mean ± standard error of the mean (SEM).

The oxygen consumption rates during progressive hypoxia (P_{crit} experiment) were analyzed using a linear mixed-effects model in R package 'nlme': the fixed factors were the oxygen treatment and food deprivation state. Individual crabs were treated as a random factor. The P_{crit} of each crab was determined using two different methods: piecewise linear regression (Brill et al., 2015) and nonlinear regression (Marshall et al., 2013) fitted to the oxygen consumption–ambient oxygen data. For nonlinear regression, the data followed the Michaelis-Menten equation: $\dot{V}O_2 = a \cdot PO_2 / (b+PO_2)$, where $\dot{V}O_2$ is standardized oxygen consumption and PO_2 is oxygen partial pressure. The PO_2 was defined as P_{crit} where the derivative of the equation is 0.065. Additionally, a regulation index (RI) was calculated (Mueller and Seymour, 2011). The RI is a relative measure of oxygen regulatory ability assessed by calculating the area under the oxygen consumption data versus the actual PO_2 curve that is greater than a linear trend (Figure 2.2). The index could vary from 0.0 (perfect conformity) to 1.0 (perfect regulation). The P_{crit} and RI were then analyzed using a generalized least square fitted linear model (gls function in R package 'nlme'), and the food deprivation state was the factor to be tested. Differences

in the parameters of SDA were also analyzed by generalized least square fitted linear models, and the oxygen level and food deprivation state were two factors to be tested.

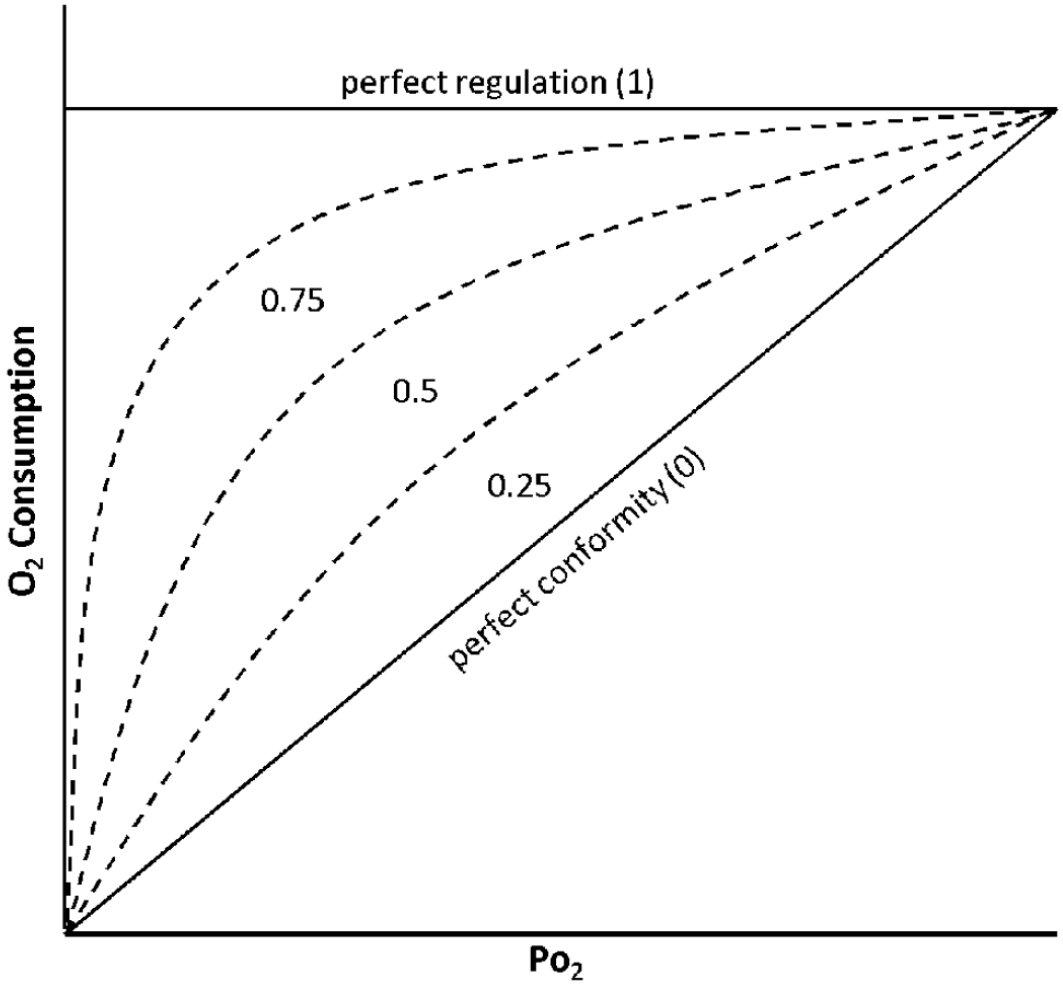

Figure 2.4 Examples of the relationship between oxygen consumption and P_{O2} adapted from Mueller and Seymour (2011). The area under the curve above diagonal line is used to calculate the regulation index (RI), with a larger area indicating a better regulation. Perfect regulation is not realistically possible but represents a definite endpoint of the RI scale.

2.4 Results

2.4.1 Effects of the food deprivation state on hypoxia tolerance

2.4.1.1 The oxygen consumption rate (MO_2) during graded hypoxia and recovery

The food deprivation state of rock crabs had a significant effect on MO_2 during a decrease in ambient oxygen levels (Figure 2.3, linear mixed-effects model, $F_{2,23} = 56.2$, $p < 0.001$). The fed crabs had the highest MO_2 with mean levels of 60.6 ± 4.2 $mgO_2/kg/h$ in 100% oxygen saturation, and they maintained these levels between 100% and 40% oxygen saturation. The MO_2 of fasted crabs was significantly lower than the fed crabs (Tukey's HSD, $t = -5.1$, $p < 0.001$). They maintained stable MO_2 levels between 38.2 ± 4.6 and 33.9 ± 1.3 $mgO_2/kg/h$ until the oxygen saturation dropped to 30%. The starved crabs had the lowest MO_2 (between 22.6 ± 1.2 and 18.0 ± 1.4 $mgO_2/kg/h$) in oxygen saturations of 100% to 20%; these levels were lower than both the fed and fasted crabs (Tukey's HSD, $t = -10.0$, $p < 0.001$ and $t = -4.6$, $p < 0.001$ respectively).

It was notable that, at the lowest oxygen saturation (5%), the fasted crabs maintained MO_2 (16.2 ± 0.6 $mgO_2/kg/h$) which was 2.3 times higher than the fed crabs (Tukey's HSD, $t = 2.2$, $p = 0.08$). A pronounced decrease in MO_2 of starved crabs also occurred in 5% oxygen saturation, although this apparent decline turned out to be statistically insignificant (5% vs. 30% oxygen: Tukey's HSD, $t = -3.1$, $p = 0.07$).

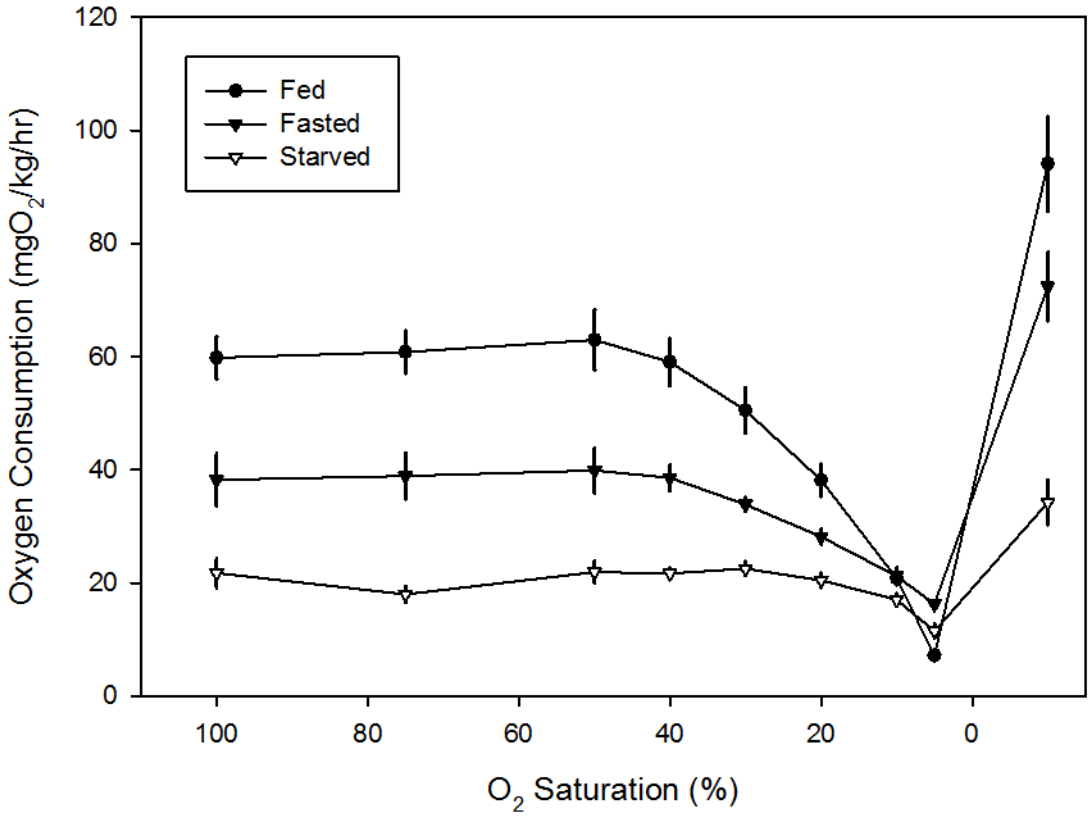

Figure 2.5 Oxygen consumption rates of fasted, fed and starved rock crabs during a progressive decrease in oxygen levels from 100% oxygen saturation to target levels of 75%, 50%, 40%, 30%, 20%, 10%, and 5%. followed by a 1 h recovery period at 100% oxygen saturation. Data are shown as the mean ± SEM (N = 8).

There were significant differences in MO_2 during the recovery phase (GLS, $F_{2,23}$ = 20.50, $p < 0.001$). In line with the initial MO_2 of the three food deprivation states in 100% oxygen, the fed crabs had the highest recovery MO_2 of 96.1 ± 7.5 $mgO_2/kg/h$, while the starved crabs exhibited the lowest recovery MO_2 (37.6 ± 3.9 $mgO_2/kg/h$). It was notable that when the recovery MO_2 was divided by the initial MO_2 of each crab (measured in 100% oxygen saturation), the fasted crabs had the highest factorial scopes (1.7 ± 0.2), and the fed crabs had the lowest (1.4 ± 0.1); there was, however, no statistically significant differences among these values (GLS, $F_{2,23}$ = 2.58, p = 0.378).

2.4.1.2 P_{crit} using piecewise linear and nonlinear regression

Analyses from both the piecewise linear regression and nonlinear regression showed that the food deprivation state significantly affected the P_{crit} (GLS: piecewise linear regression, $F_{2,23}$ = 10.7, $p < 0.001$; nonlinear regression, $F_{2,23}$ = 21.7, $p < 0.001$), and there was a significant positive correlation between the two results (Pearson correlation, r = 0.57, df = 26, p = 0.002). Using the piecewise linear regression (Figure 2.4), the starved crabs had the lowest P_{crit} of 20.7 ± 2.3%, which was significantly lower than 27.7 ± 1.7% of fasted crabs and the 34.6 ± 2.3% of fed crabs (Tukey's HSD, t = -2.2, p = 0.04 and t = -4.6, $p < 0.001$, respectively). The P_{crit} values for the fasted and fed crabs were not significantly different from one another (Tukey's HSD, t = -2.3, p = 0.06).

The nonlinear regression method produced the same trend. There was a significant difference among each food deprivation state (Tukey's HSD tests, $p < 0.05$). However, the estimated P_{crit} values were consistently lower than those produced by the piecewise

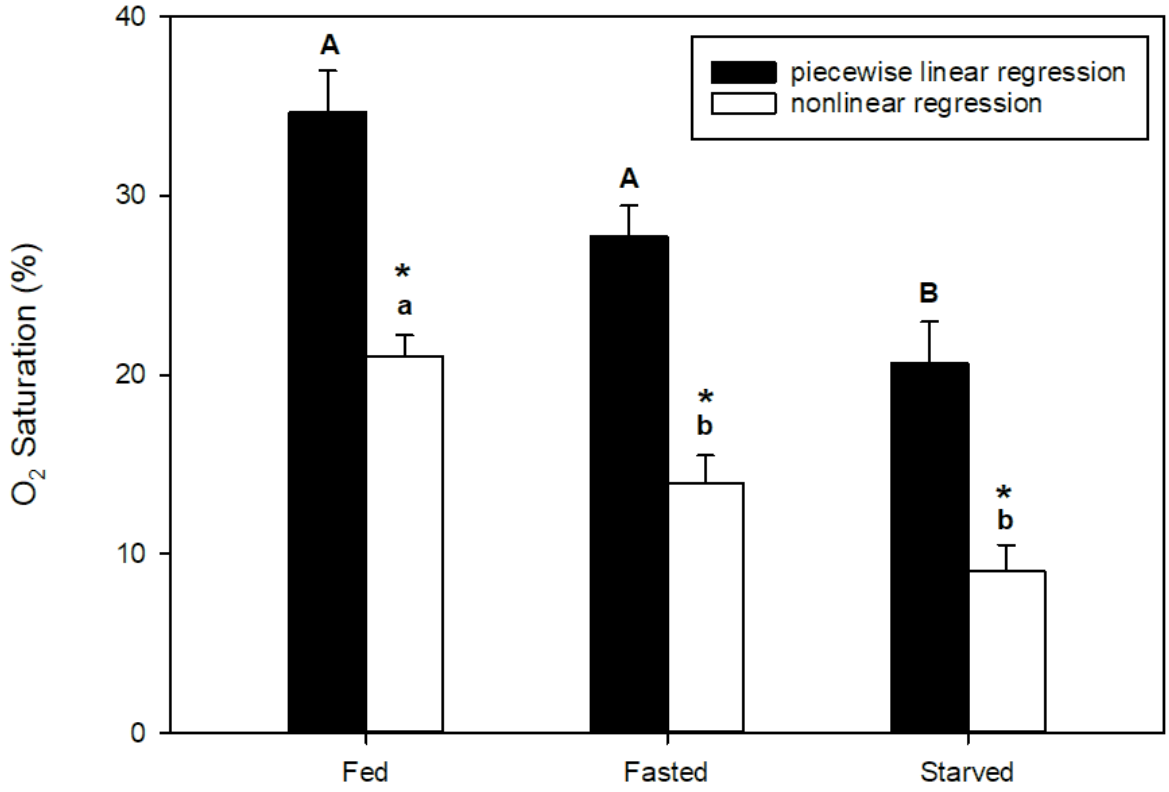

Figure 2.6 The critical oxygen saturation (P_{crit}) value of fasted, fed and starved crabs, calculated by piecewise linear regression and nonlinear regression. Data are shown as the mean ± SEM (N = 8–10). Asterisk denotes significant differences between the two regression methods and different letters show significant differences for oxygen levels (capital letters for piecewise linear regression and lower case for nonlinear regression).

linear regression (paired t-test, t = 9.3, df = 25, $p < 0.001$). For example, the P_{crit} of fasted crabs was 13.9 ± 1.5% determined from the nonlinear regression, which was about 50% lower than 27.7 ± 1.7% from the piecewise linear regression (Figure 2.4).

2.4.1.3 The regulation indexes

The regulation index (RI) is another parameter that can be used to determine the ability of animals to deal with declining oxygen levels (Mueller and Seymour, 2011). A similar trend was evident as seen for the P_{crit}; the fasted crabs had the best oxyregulation ability (55.3 ± 3.5%), followed by the starved crabs (50.2 ± 5.8%), while the fed crabs had the lowest RI (41.0 ± 3.5%). However, despite the apparent trend, the effect of the food deprivation state did not have a significant effect (GLS, $F_{2,23} = 2.9$, $p = 0.08$).

2.4.2 Resting metabolic rate in different oxygen levels

The food deprivation state (fasted, starved) significantly affected the resting metabolic rate (Figure 2.5; GLS, $F_{1,54} = 18.4$, $p < 0.001$). The fasted crabs maintained a significantly higher RMR than the starved crabs: (38.9 ± 2.9 vs. 25.4 ± 2.1 $mgO_2/kg/h$) in 100% oxygen saturation (Tukey's HSD, t = 3.9, $p < 0.001$) and (36.9 ± 2.4 vs. 26.5 ± 1.7 $mgO_2/kg/h$) in 50% oxygen saturation (Tukey's HSD, t = 3.2, $p = 0.03$). In 20% oxygen, there was no significant difference in RMR between the fasted and starved crabs, with a mean level of 27.6 ± 1.7 $mgO_2/kg/h$.

Although no significant effects of oxygen level (hypoxia) were found (GLS, $F_{2,54} = 1.5$, $p = 0.24$), the RMR of the fasted crabs dropped more rapidly than the starved crabs with

the decreasing oxygen saturation (Figure 2.5). For fasted crabs, it decreased from 38.9 ± 2.9 mgO_2/kg/h in 100% oxygen saturation to 29.1 ± 1.5 mgO_2/kg/h in 20% oxygen (Tukey's HSD, t = 2.7, p = 0.03). In contrast, the starved crabs maintained their RMR between mean levels of 25.4 ± 2.8 and 26.2 ± 3.0 mgO_2/kg/h in all three oxygen saturations.

Table 2.1 Results of the generalized least squares model (GLS) for the resting metabolic rate (RMR). The two explanatory variables (oxygen level and food deprivation state) and their interaction were tested sequentially. Bold font indicates statistical significance ($p < 0.05$).

Variables	Factors	$df_{num,den}$	*F*	*P* value
RMR	Oxygen level (100, 50, 20%)	2,54	1.47	0.240
	Food deprivation state (fasted, starved)	**1,54**	**18.41**	**<0.001**
	Interaction	2,54	2.05	0.140

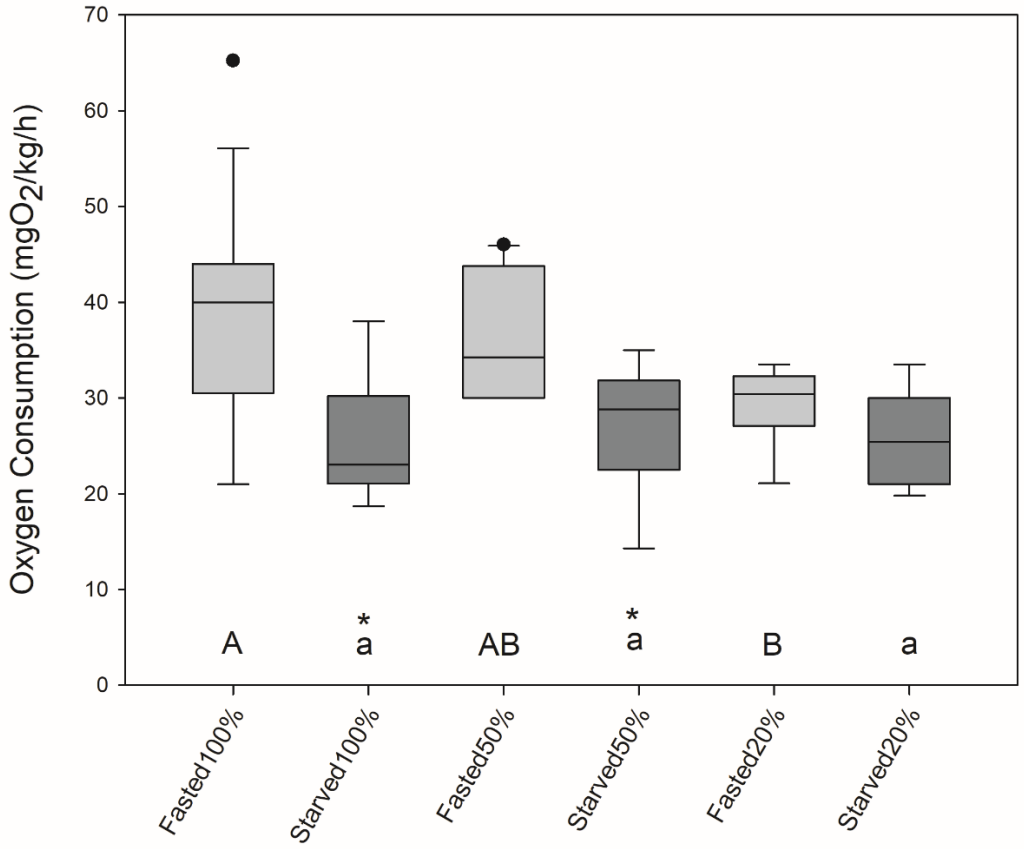

Figure 2.7 Resting metabolic rates of fasted and starved crabs in 100%, 50% and 20% oxygen saturations (N = 9–11). These data were used to calculate the baseline of SDA in Figure 2.6 and to determine the parameters of SDA in Figure 2.7. Data are shown as vertical boxes with error bars (the horizontal lines are median, the boxes represent 25th and 75th percentiles, the whiskers are 10th and 90th and outliers are presented by the circle). Asterisk denotes significant differences between starved and fasted crabs, and different letters denote significant differences between oxygen treatments (capital letters within fasted group and lower case within starved group). All significant differences were accepted at the $p < 0.05$ level.

2.4.3 Effects of food deprivation state and oxygen level on SDA

In general, the typical curve of SDA was found in all treatments (Figure 2.6), represented by a sharp increase in MO_2, followed by a more gradual decline back to pre-feeding levels. Overall, the significant effects of starvation were a lower peak MO_2, prolonged duration and higher energy expenditure of the SDA. Meanwhile, hypoxia retarded the SDA response with a lower peak MO_2 and longer duration for both fasted and starved crabs.

2.4.3.1 Peak metabolic rate

All crabs exhibited an increase in oxygen consumption following feeding (Figure 2.6). The peak MO_2 was significantly affected by both the food deprivation state (GLS, $F_{1,50}$ = 10.9, p = 0.002) and the oxygen level (GLS, $F_{2,50}$ = 20.7, p < 0.001). On average, the fasted crabs exhibited a peak MO_2 that was approximately 10–40% higher than the starved crabs (Figure 2.7A).

The peak MO2 of the fasted crabs dropped significantly from 84.0 ± 2.9 mgO2/kg/h in 100% oxygen to 67.6 ± 3.9 mgO2/kg/h in 50% oxygen saturation (Tukey's HSD, t = -3.2, p = 0.03). There was a further decline in the peak MO2 in 20% oxygen saturation, decreasing by approximately 45% relative to levels measured in 100% oxygen saturation (Tukey's HSD, t = -6.3, p < 0.001). In contrast, no significant change in peak MO2 was observed for starved crabs between 100% and 50% oxygen saturation (61.5 ± 4.6 mgO2/kg/h to 40.4 ± 4.9 mgO2/kg/h). There was a significant decline in 20% oxygen,

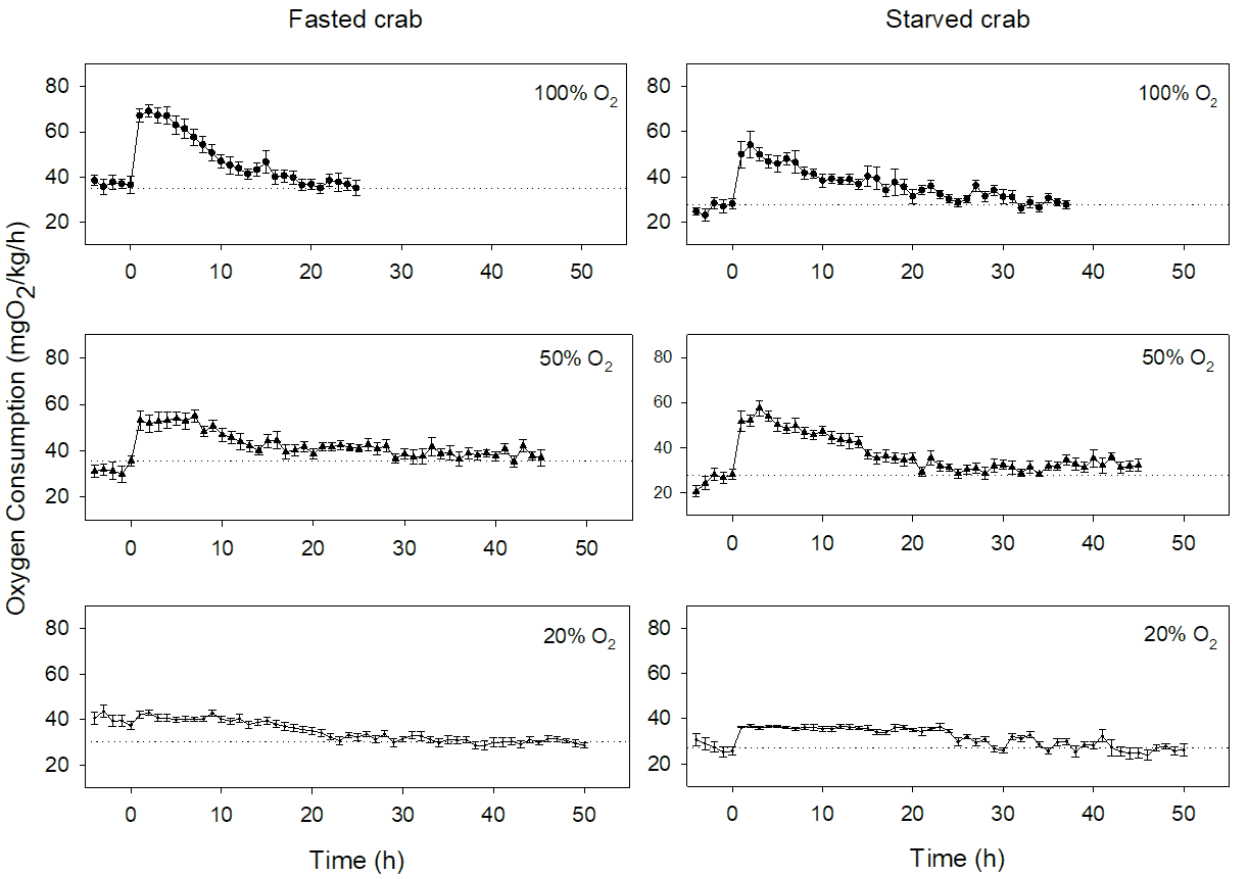

Figure 2.8 Changes in oxygen consumption rates (mgO_2/kg) for fasted and starved rock crabs. The crabs were fed shrimp muscle of 2% of their body mass in 100% oxygen at 0 h. Once the meal had been consumed (within 30 min), oxygen levels were changed to either 100%, 50% or 20% oxygen saturations and MO_2 was followed until it declined to baseline levels. The dashed line indicates the baseline of SDA or pre-feeding levels (estimated in a separate group of crabs Fig. 2.5). Data are shown as the mean ± SEM (N = 9–10).

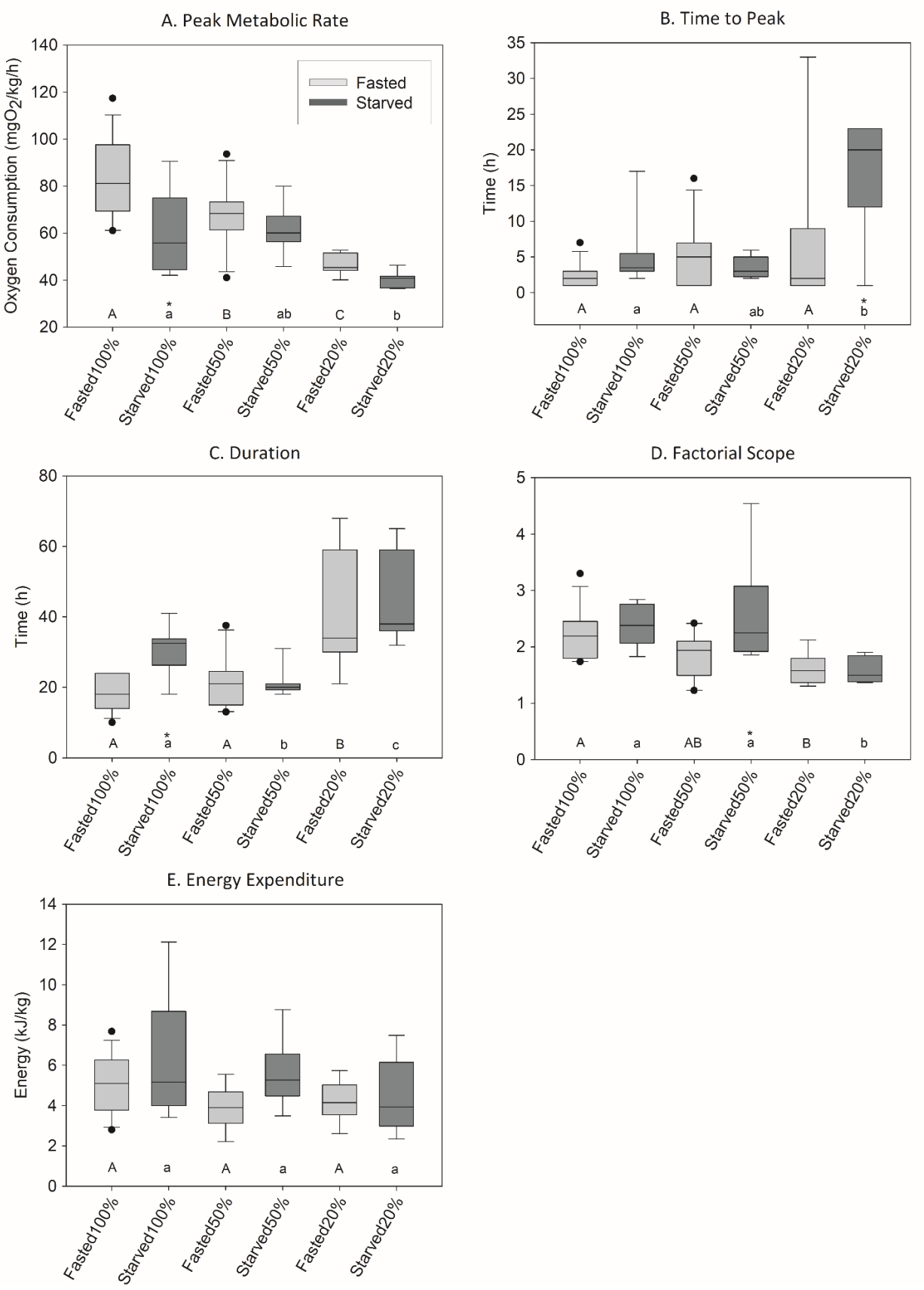
A. Peak Metabolic Rate
Oxygen Consumption (mgO2/kg/h)
Fasted
Starved
A
a*
B
ab
C
b
B. Time to Peak
Time (h)
A
a
A
ab
A
b*
C. Duration
Time (h)
A
a*
A
b
B
c
D. Factorial Scope
A
a
AB
a*
B
b
E. Energy Expenditure
Energy (kJ/kg)
A
a
A
a
A
a
Fasted100%
Starved100%
Fasted50%
Starved50%
Fasted20%
Starved20%

Figure 2.9 SDA parameters of fasted and starved rock crabs after consuming shrimp muscle 2% of their body mass (N = 9–10): A. peak MR of SDA, B. time to reach the peak value, C. duration of SDA, D. Factorial scope of SDA, E. energy expenditure of SDA. Data are shown as vertical boxes with error bars (the horizontal lines are median, the boxes represent 25th and 75th percentiles, the whiskers are 10th and 90th and outliers are presented by the circle). Asterisk denotes significant differences between starved and fasted crabs, and different letters denote significant differences between oxygen treatments (capital letters within fasted group and lower case within starved group). All significant differences were accepted at the $p < 0.05$ level.

whereby the peak MO_2 of starved crabs was approximately 33% lower than the levels measured in 100% oxygen saturation (Tukey's HSD, t = -2.9, p = 0.01).

2.4.3.2 Time to peak MO_2

Both the food deprivation state and the oxygen level had significant effects on the time to reach the peak MO_2 (GLS: food deprivation state, $F_{1,50} = 7.1$, $p = 0.01$; oxygen level, $F_{2,50} = 4.7$, $p = 0.013$). The times in 100% and 50% oxygen saturations were similar for both the fasted and starved crabs and ranged between 2.5 ± 1.5 h and 5.3 ± 2.0 h (Figure 2.7B). However, the fasted crabs reached peak MO_2 in a significantly shorter time than the starved crabs in 20% oxygen saturation (7.3 ± 2.2 h vs. 16.7 ± 2.1 h, Tukey's HSD, t = 3.1, p = 0.003).

2.4.3.3 Duration of SDA

The duration of SDA was significantly affected by both the food deprivation state (GLS, $F_{1,50} = 5.7$, $p = 0.02$) and the oxygen level (GLS, $F_{2,50} = 26.5$, $p < 0.001$). Overall, the trend was that the fasted crabs exhibited shorter durations compared with the starved crabs (Figure 2.7C); however, these differences were only significant in 100% oxygen saturation with levels of 18.4 ± 2.4 h vs. 30.8 ± 3.2 h for the fasted and starved crabs, respectively (Tukey's HSD, t = 3.1, p = 0.003). There was no significant difference in duration between 100% and 50% oxygen saturations for the fasted crabs, but there was a significant decrease from 30.7 ± 3.3 h to 21.3 ± 3.2 h for the starved crabs (Tukey's HSD, t = -2.1, p = 0.04). Meanwhile, when exposed to severe hypoxia (20% oxygen), the

duration for both the fasted (40.6 ± 16.8 h) and starved (46.1 ± 13.4 h) crabs almost doubled compared with those measured in 100% oxygen.

2.4.3.4 Factorial scope

The factorial scope (peak MO_2/baseline MO_2) was influenced by the oxygen level (GLS, $F_{2,50} = 12.5$, $p < 0.001$), but there was no significant effect of food deprivation state (GLS, $F_{1,50} = 3.9$, $p = 0.05$). Both the fasted and starved crabs exhibited a 2 to 3-fold increase in MO_2 in 100% and 50% oxygen saturations (Figure 2.7D). In severe hypoxia (20% oxygen), the scope was significantly lower than that measured in 100% oxygen: the scope of fasted crabs dropped from 2.2 ± 0.4 to 1.6 ± 0.3 (Tukey's HSD, $t = -2.8$, $p = 0.02$), while that of the starved crabs dropped from 2.3 ± 0.4 to 1.6 ± 0.3 (Tukey's HSD, $t = -3.1$, $p < 0.001$).

2.4.3.5 Equivalent energy consumption

The equivalent energy expenditure was significantly affected by the food deprivation state (GLS, $F_{1,50} = 4.2$, $p = 0.04$), but not the oxygen level (GLS, $F_{2,50} = 1.9$, $p = 0.17$). On average, the starved crabs expended between 8% to 43% more energy than fasted crabs, but no significant difference was found between the two groups in each oxygen level (Figure 2.7E). Although no significant effect of oxygen level was found, there was an overall trend for a decrease in energy expenditure (for both fasted and starved crabs) as the oxygen saturation declined. The individual crabs with higher factorial scopes tended to have higher energy expenditures (Pearson correlation, $r = -0.31$, $df = 53$, $p = 0.024$).

Table 2.2 Summary of generalized least squares model (GLS) for the five parameters of SDA. The two explanatory variables (oxygen level and food deprivation state) and their interaction were tested sequentially. λ value is presented if Box-cox transformation was applied to the data. Bold font indicates statistical significance ($p < 0.05$).

Variables	Factors	$df_{num,den}$	*F*	*P* value
Peak MR	**Oxygen level (100, 50, 20%)**	**2,50**	**20.66**	**<0.001**
	Food deprivation state (fasted, starved)	**1,50**	**10.90**	**0.002**
	Interaction	2,50	2.98	0.060
Time to Peak (λ = 0)	**Oxygen level (100, 50, 20%)**	**2,50**	**4.73**	**0.013**
	Food deprivation state (fasted, starved)	**1,50**	**7.16**	**0.010**
	Interaction	2,50	2.63	0.082
Duration	**Oxygen level (100, 50, 20%)**	**2,50**	**26.46**	**<0.001**
	Food deprivation state (fasted, starved)	**1,50**	**5.68**	**0.021**
	Interaction	2,50	2.19	0.123
Factorial scope (λ = 0)	**Oxygen level (100, 50, 20%)**	**2,50**	**12.54**	**<0.001**
	Food deprivation state (fasted, starved)	1,50	3.85	0.051
	Interaction	2,50	2.90	0.064
Energy expenditure (λ = 0)	Oxygen level (100, 50, 20%)	2,50	1.86	0.168
	Food deprivation state (fasted, starved)	**1,50**	**4.22**	**0.044**
	Interaction	2,50	1.00	0.375

2.5 Discussion

In this study, I found that the food deprivation state altered the physiological responses of crabs to hypoxic exposure. Long-term starvation resulted in a decrease in resting metabolic rate, and these crabs also had a lower P_{crit} than fasted crabs. In contrast, the crabs that had recently ingested food had a higher metabolic rate coupled with a higher P_{crit}. Following feeding, hypoxia had a more pronounced effect on the characteristics of the specific dynamic action (SDA) of fasted crabs compared with starved crabs.

2.5.1 Critical oxygen partial pressure (P_{crit})

The fasted rock crabs had a relatively high P_{crit} of 5.8 ± 0.4 kPa (27.7 ± 1.7%) at 13°C, which is above 80% of the published P_{crit} values for other crustaceans (Wishner et al., 2018). The interspecific differences in hypoxia tolerance may reflect evolutionary physiological adaptations to cope with low oxygen conditions (Spicer, 2014; Vaquer-Sunyer and Duarte, 2008). For example, Dungeness crabs (*Cancer magister*), which frequently encounter hypoxia in their muddy estuarine habitats, have a P_{crit} below 3 kPa (Airriess and McMahon, 1994). In contrast, the ventilatory performance of the brown crab (*Cancer pagurus*) starts to decline at 5 kPa, but given that their rocky subtidal habitat is less prone to hypoxia, such physiological tolerance mechanisms are largely unnecessary (Bradford and Taylor, 1982). A similar effect is likely for rock crabs here, since this species primarily resides in rocky bottoms and cold waters, where it is less likely to experience severe low oxygen conditions (Gíslason et al., 2014; Haefner Jr, 1976). However, given the

predicted changes in ocean temperature and anthropogenic eutrophication, it is more likely that they will encounter hypoxia in their natural environment in the future. The high P_{crit} measured here for rock crabs suggests these hypoxic events may have a significant impact on these species in the future (Gilbert et al., 2005; Levin et al., 2009).

Given the poor physiological condition (depleted energy reserves and lower MO_2) of the starved rock crabs, it was expected that they might be less tolerant of hypoxia (Matozzo et al., 2011; McCue, 2010). However, the P_{crit} and regulation index (RI) did not reflect this assumption: starved crabs had a lower P_{crit} compared with fasted crabs. This lower P_{crit} level was likely associated with lower resting MO_2 of starved crabs in normoxia, which is consistent with other studies that a lower P_{crit} is generally correlated with lower aerobic metabolism and vice versa (Brill et al., 2015; Crear and Forteath, 2000). Hypometabolism or metabolic depression can be triggered by both starvation and hypoxia (McCue, 2010). The metabolic depression during starvation is limited by energy reserves (Sacristán et al., 2017), while that observed during hypoxia is limited by oxygen supply (Storey, 2015). In this case, the starved rock crabs may have developed a cross-tolerance to hypoxia because both long-term starvation and hypoxia are associated with a lowered metabolic rate (Seibel, 2011; Storey, 2015). Cross-tolerance describes how adaptation to a particular environmental stressor will increase resistance to a different environmental stressor because both adaptations share similar regulatory mechanisms (Kalra et al., 2017; Steinberg, 2012). A number of articles have shown that the protein synthesis rates of starved crabs are extremely low (Wang et al., 2019; Wang et al., 2006), and a similar downregulation of protein assimilation occurs during severe hypoxia (Carter

and Mente, 2014; Mente et al., 2003; Storey, 2015). Additionally, the lower MO_2 of starved crabs is associated with a decline in heart rate and locomotor activities, which are common responses to hypoxia (Ansell, 1973; Simon et al., 2015). At the cellular level, both hypoxia and starvation would severely constrain the rate of ATP production, and metabolically expensive processes (such as ion pumping, protein turnover and mitochondrial proton leak) could potentially be downregulated to balance between ATP production and consumption (Hand and Hardewig, 1996; McCue, 2010; Staples and Buck, 2009). Thus, it is likely that the metabolic depression of starved crabs reduced some of the potential oxygen demand when encountering hypoxia.

In contrast to both fasted and starved crabs, those that had recently fed exhibited a typical elevated postprandial metabolism (SDA) and had a higher P_{crit} than fasted or starved crabs. This high P_{crit} suggests that the fed animals were not able to take up enough oxygen to maintain the increased energy demand associated with digestion and absorption of the meal. This result is in concordance with other studies where crustaceans have a higher P_{crit} when they are burdened with extra oxygen demands. They will unload these metabolic demands such as high locomotor activities or SDA at a higher oxygen level (a higher P_{crit}) by retarding or stopping these activities (Brill et al., 2015; Crear and Forteath, 2000).

It was noteworthy that even below their P_{crit} level, the fed crabs still had higher MO_2 compared with the fasted crabs, indicating a possible aerobic scope of SDA below P_{crit}. This is also supported by the fact that both the fasted and starved crabs were still able to elevate their metabolism above resting levels (scope of 1.6 ± 0.3) after consumption of a

meal, even in severe hypoxia (20% oxygen). Thus, it is reasonable to assume that the digestion process would stimulate the cardiorespiratory system of rock crabs to enable an increase in MO_2 below P_{crit}, (McGaw, 2005). Similar aerobic scope of activity at or below P_{crit} has been found in the blue crab (*Callinectes sapidus*) and the southern rock lobster (*Jasus edwardsii*) (Brill et al., 2015; Crear and Forteath, 2000). It indicates that crustaceans may be able to reduce the demands of some physiological processes or alter behavioral activities below P_{crit} to cope with hypoxia while they still reserve the aerobic scope to deal with critical activities such as feeding or escape from predators.

In this study, the resting metabolic rate (RMR), rather than the standard metabolic rate (SMR), was used to determine the P_{crit}. Standard metabolic rate is defined as the minimum energy expenditure of a post-absorptive organism, exhibiting no functional activity (Chabot et al., 2016; Hulbert and Else, 2004). However, it was not feasible to completely immobilize the rock crabs during the experiments. In such cases, where there is a limited activity, "low routine" or "resting" metabolic rates are more appropriate approximations of SMR (Chabot et al., 2016; Hulbert and Else, 2004). Thus, another possible explanation of crabs being able to elevate their aerobic scope below P_{crit} is that during the decline in oxygen, the fasted and starved crabs had a voluntary shift and became less active, which resulted in a downregulation of RMR to SMR before the P_{crit} was reached. Certainly, decapods do tend to cease activity during severe hypoxia exposure in order to reduce any unnecessary energy expenditure (Bell et al., 2003; Bernatis et al., 2007; Brante and Hughes, 2001; Mistri, 2004). The mechanisms whereby these crabs can increase their metabolic scopes in low environmental oxygen levels may

therefore have something to do with the way we measure or interpret the switch over from oxyregulation to oxyconformity (P_{crit}).

Despite some criticism, P_{crit} has been and continues to be a widely accepted measurement of hypoxia tolerance (Mueller and Seymour, 2011; Wood, 2018). The fact that both starved and fasted crabs exhibited an increase in MO_2 following feeding in hypoxia levels that were below their measured P_{crit} is contradictory to its conventional definition. In the conventional definition, P_{crit} stands for the critical oxygen partial pressure where physiological mechanisms (ventilatory and cardiac systems) can no longer maintain a constant MO_2 and delivery, which gives the presumption that MO_2 only starts to fall at the ambient oxygen level where the respiratory limitation is reached (Pörtner and Grieshaber, 1993; Wang et al., 2009). It means animals cannot further increase MO_2 (aerobic scope = 1) below P_{crit}, and the oxygen deficit must be either paid by anaerobic metabolism or reduced by depressing metabolism (Hochachka, 1986; Seibel, 2011; Wood, 2018). However, the present study showed that rock crabs could increase MO_2 below P_{crit} when they consume and digest a meal (aerobic scope > 1). Possible explanations were firstly presented in oxygen conformers by Pörtner and Grieshaber (1993), who state that oxyconformity without anaerobiosis (aerobic conformer) results from the inability of an animal to maintain a constant rate of MO_2 with falling ambient PO_2. Only at very low oxygen tensions must the animal rely on anaerobic metabolism. They defined two P_{crit} measures for oxygen conformers: a higher P_{crit}R (R for a respiratory change), characterized by the transition from oxyregulation towards oxyconformity and a P_{crit}M (M for a metabolic change), which indicates the shift to anaerobic metabolism. This may also occur

here for rock crabs, which are oxyregulators. The $P_{crit}R$ may represent a voluntary shift to aerobic depression rather than the inability of the physiological mechanisms to maintain a constant MO_2. Thus, the $P_{crit}M$ may be a more reliable index of hypoxia tolerance, where animals switch from aerobic to anaerobic metabolism and cannot further increase MO_2 (aerobic scope=1). To determine the most reliable P_{crit} (where an animal initiates anaerobic metabolism), it requires the measurement of aerobic scopes and the metabolites of anaerobic metabolism, e.g., L-lactate (Alter et al., 2015; Ocampo et al., 2003). Although the $P_{crit}R$ revealed an oxygen tension of 27.7 ± 1.7% for fasted crabs, there was no significant accumulation of L-lactate in fasted crabs at 20% oxygen (Jiang, Chapter Three), indicating the $P_{crit}M$ of fasted rock crabs lies below 20% oxygen. Thus, care should be taken when relying on P_{crit} as the definitive measure of hypoxia tolerance, especially when comparing with other studies.

2.5.2 SDA

2.5.2.1 Effects of hypoxia on SDA

Under low ambient oxygen levels, especially below the $P_{crit}R$, the rock crabs exhibited a decrease in the magnitude of the SDA response (peak MO_2 and time to reach peak MO_2), which appeared to be compensated by an increase in SDA duration. This result conforms with the SDA of blue crabs (*Callinectes sapidus*) and juvenile white shrimp (*Penaeus setiferus*) in hypoxia, where the scope of the postprandial MO_2 is suppressed, while the duration is prolonged (Brill et al., 2015; Carlos et al., 1998). In the red rock crab (*Cancer productus*), exposure to hypoxia is associated with a decrease in foregut contraction rate,

resulting in a longer time for digesta to pass through the digestive tract (McGaw, 2008). This may be coupled with a diversion of hemolymph away from digestive organs and towards the muscles and gills (McGaw, 2005). A similar pattern of prolonged gastric processing was also observed here for rock crabs in 20% oxygen (Jiang, Chapter Four). Mechanical digestion may account for 25–30% of the overall postprandial increase in MO_2 (McGaw and Van Leeuwen, 2017), and because it is slowed during hypoxia, this would also be reflected by a prolonged duration of the SDA. Although increased gastric activity accounts for a portion of the SDA, by far the largest proportion (60% to 80%) of the postprandial increase in MO_2 is due to intracellular protein synthesis (Fraser and Rogers, 2007; Wang et al., 2019; Whiteley et al., 2001). Thus the reduction of postprandial MO_2 in hypoxia is likely associated with reduced protein synthesis (Carter and Mente, 2014; Mente et al., 2003). Certainly, during hypoxic exposure (P_{O2} = 3 kPa), postprandial green crabs (*Carcinus maenas*) exhibit a reduction in the magnitude of protein synthesis in the heart, skeletal muscle, and hepatopancreas (Mente et al., 2003). This control of protein synthesis may be mediated by a translational arrest in mRNA, although at present, the exact mechanisms are not fully understood (Bonvillain et al., 2012; Hochachka et al., 1996; Staples and Buck, 2009; Wei et al., 2016). Either way, both the reduction in gastric activity (and associated hemolymph diversion) and reduced protein synthesis can be assumed to be an energy-saving strategy to deal with reduced metabolic scope (Carter and Mente, 2014), and these alterations will be paralleled by changes in MO_2.

2.5.2.2 Effects of food deprivation state on SDA

During normoxia (100% oxygen), starved crabs had a significantly lower RMR compared with the fasted crabs. Following feeding in 100% oxygen, the peak MO_2 of the starved crabs was lower, and the time MO_2 remained elevated above pre-feeding levels was also longer (SDA duration) compared with the fasted crabs. When the ambient oxygen supply is sufficient, the lower MO_2 of starved crabs may be explained by their physiological condition (Simon et al., 2015). During starvation, downregulation of digestive enzyme synthesis and activity (such as proteases in hepatopancreas) may lead to a longer duration of SDA, as the starved crabs would require increased energy (and time) to synthesize and to upregulate previously dormant enzymes and hormones for digestion (Muhlia-Almazan and García-Carreño, 2002; Sánchez-Paz et al., 2003). The prolonged SDA of starved rock crabs may also be explained by the plasticity of the gastrointestinal organs, which is well studied in other animals (Wang et al., 2006). The gastrointestinal organs of amphibians and reptiles may undergo a marked structural and functional reduction during long-term fasting, which may contribute to the reduction in the basal metabolism of starved animals (Cant et al., 1996; McCue, 2007; Piersma and Lindström, 1997). However, when the animals start feeding again, their gastrointestinal organs will undergo drastic changes in order to recover digestive functions (Wang et al., 2006). For example, starved snakes increase the mass of the small intestine (particularly intestinal mucosa and length of microvilli) within the first 12–24 h after ingestion in order to increase the nutrient transport capacities for various amino acids and glucose (Lignot et al., 2005; Secor and Diamond, 2000; Secor et al., 1994). The process of re-feeding leads to partial and gradual regeneration of organ structure and digestive ability (Cervellione et

al., 2017; Comoglio et al., 2004; Silva-Castiglioni et al., 2016; Włodarczyk et al., 2017). Thus, when the crabs first consumed a meal after long-term starvation, they may have a more prolonged SDA response compared with fasted crabs.

When the oxygen levels declined, the difference in SDA characteristics between fasted and starved rock crabs also started to diminish. In 20% oxygen, the only significant difference was that starved crabs took a longer time to reach peak MO_2 compared with the fasted crabs. The initial hypothesis was that starved crabs would show a reduced SDA response compared with fasted crabs because starved crustaceans have lower hemocyanin concentrations in the hemolymph, which may adversely affect oxygen transport ability. For example, starved green crabs (*Carcinus maenas*) have a 30–50% higher perfusion index (cardiac output/MO_2) than fasted ones during both normoxia and hypoxia, which means starvation may reduce the oxygen-carrying capacity of hemolymph (Depledge, 1985). However, in the present study, the lower oxygen-carrying capacity of starved rock crabs appeared to be compensated by their lower overall oxygen demand (lower RMR). As a result, the starved crabs maintained their factorial scope of SDA during severe hypoxia.

2.5.3 Conclusion

The P_{crit} measured for the different physiological states suggests that the transition ($P_{crit}M$) from an oxygen-independent to an oxygen-dependent pattern of MO_2 may represent a shift to hypometabolism, which indicates an adaption to hypoxia with reserved aerobic scopes for critical activities. However, the possible role of anaerobic

metabolism in determining the $P_{crit}R$ is yet to be studied in crustaceans. The food deprivation state of rock crabs did affect the aerobic metabolism of SDA in normoxia, as starved crabs exhibited a reduced scope and prolonged duration of SDA. However, food deprivation did not adversely affect hypoxia tolerance because starvation may trigger a cross-tolerance to hypoxia since both long-term starvation and severe hypoxia would elicit similar physiological responses of hypometabolism. Further studies are required to determine whether the hypometabolism results from the reversible depression of SMR (basal somatic maintenance, energy-consuming cellular processes such as ion pumping and macromolecular synthesis) or the downregulation of RMR (activities such as locomotion, growth and reproduction/development) (Kooijman, 2010; Sokolova et al., 2012). Based on the results of this study, prolonged hypoxia may have adverse effects on the growth and reproduction of crustaceans (Galic et al., 2019), while the effects of long-term food deprivation may be more limited.

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Chapter 3. Effects of food deprivation state on hemolymph biochemical responses to hypoxia in rock crabs[1]

[1]A modified version of this chapter has been submitted to Comparative Biochemistry and Physiology part A.

3.1 Abstract

Climate change and anthropogenic activities have caused a substantial increase in both the spatial and temporal extent of hypoxia in nearshore marine environments over the past half-century. Decapod crustaceans are reported to be particularly sensitive to hypoxia, and their physiological and behavioral responses to low environmental oxygen are well documented. That being said, we are only starting to understand interactive effects such as changes in food availability and feeding on hypoxia tolerance. In this study, changes in hemolymph oxygen partial pressure (P_{O2}), pH, hemocyanin (Hc) and L-lactate concentrations of fasted (3-5 d) and starved (28-35 d) rock crabs (*Cancer irroratus*) were examined in different levels of oxygen (100%, 50% and 20%), before and after feeding. The P_{O2} dropped as a function of decreasing ambient oxygen levels in both fasted and starved crabs, and this decline was most pronounced below the critical partial pressure of oxygen (P_{crit}) of the crabs. Compared with starved crabs, the higher Hc concentration of fasted crabs suggested they have better oxygen transport capacity. Although the experimental period (72 h) may have been too short to observe significant increases in Hc concentration, hypoxia-induced alkalosis and elevated L-lactate could have improved the Hc-oxygen affinity. Following feeding, the fasted crabs exhibited a significant increase in L-lactate concentration in severe hypoxia. In contrast, anaerobic metabolism was not observed in postprandial starved crabs: the lower oxygen demand in this treatment group was likely associated with the slower recovery of dormant digestive processes during refeeding. Because crustaceans undergo periods of food deprivation in their natural environment it is important to take these into account when determining physiological responses to hypoxia.

Keywords: crab, hypoxia, P_{O2}, hemocyanin, pH, L-lactate, starvation, feeding

3.2 Introduction

Periodic hypoxia has become more prevalent in the marine environment during the past several decades, especially in shallow coastal zones (Diaz and Rosenberg, 2008; Watson, 2016). In addition to upwelling events, coastal hypoxia is associated with anthropogenic eutrophication from estuarine influx (Breitburg et al., 2018; Grantham et al., 2004). These widespread increases in hypoxia may reduce the habitat and diversity of benthic communities (Deutsch et al., 2015; Diaz and Rosenberg, 1995; Spicer, 2014). This might be especially important for crustaceans, which often comprise a significant proportion of the biomass in benthic communities and are sensitive to changes in oxygen availability (Vaquer-Sunyer and Duarte, 2008).

Crustaceans living in fluctuating oxygen regimes often use physiological mechanisms to maintain aerobic metabolism (MO_2). This typically involves an increase in the rate and depth of ventilation of the branchial chambers in order to maintain adequate oxygen consumption rates (Airriess and McMahon, 1994; Taylor, 1976). The maintenance of MO_2 may be paired with an increase in cardiac output to ensure adequate oxygen delivery to the tissues (McGaw and Reiber, 2015; Reiber and McMahon, 1998). These cardiorespiratory responses of crustaceans to hypoxia are associated with concomitant biochemical responses to enable efficient oxygen transport and delivery.

In decapod crustaceans, over 50% oxygen is carried in combination with the respiratory pigment, hemocyanin (Hc), and the arterial partial pressure of oxygen (P_{aO2}) influences the Hc O_2 saturation (Decker and Van Holde, 2010; Terwilliger, 2015; Truchot, 1992). The P_{aO2} of decapod crustaceans is reported to range from approximately 2 to 10 kPa in normoxia (21kPa oxygen)

(Forgue et al., 1992; Lehtonen and Burnett, 2016). Although some crustaceans have evolved a "low blood oxygenation strategy" and maintain an extremely low arterial P_{aO2} (1–3 kPa) (Forgue et al., 1992; Massabuau, 2001; Mente et al., 2003), the postbranchial P_{aO2} of most crustaceans supports 70–90% Hc O_2 saturation (Terwilliger, 2015; Truchot, 1992). However, during severe hypoxia, the P_{aO2} of decapod crustaceans typically declines in line with that of the ambient oxygen content, which may result in a significant reduction in Hc O_2 saturation (Lehtonen and Burnett, 2016; McMahon, 1988).

In hypoxic conditions, crustaceans may enhance oxygen delivery by augmenting hemocyanin concentration or increasing its oxygen affinity (Giomi and Beltramini, 2007; Hagerman, 1986). During severe hypoxic exposure (19% to 31% oxygen for 2–3 days), the American lobster (*Homarus americanus*) exhibits an increase in the hemocyanin biosynthetic rate (Senkbeil and Wriston, 1981). Similar increases in Hc concentration during hypoxia are found in the giant river prawn (*Macrobrachium rosenbergii*) (Cheng et al., 2003), brown shrimp (*Crangon crangon*) (Hagerman, 1986) and blue crab (*Callinectes sapidus*) (Defur et al., 1990). However, there are some contradictory findings: in the white shrimp (*Litopenaeus vannamei*), no significant changes in hemocyanin occur during 12 h of hypoxic exposure (Wei et al., 2016), while juvenile king crabs (*Lithodes santolla*) exhibit a decrease in Hc concentrations only after 10 days exposure to hypoxia (Paschke et al., 2009).

In addition to increasing the concentration of Hc, the oxygen-binding affinity of the Hc molecule can be enhanced by extrinsic inorganic and organic allosteric modulators. Hyperventilation induced by hypoxia may result in an alkalosis, and the decreasing H^+ concentration enhances the oxygen affinity of Hc within a certain pH interval (negative Bohr

effect) (Bohr et al., 1904; Truchot, 1992). Meanwhile, if the pH is well buffered (no Bohr shift), the accumulation of L-lactate during hypoxia acts as an allosteric modulator, which facilitates an increase in oxygen affinity of Hc (Morris, 1990; Truchot, 1980). Decapod crustaceans may also improve the "quality" of Hc during hypoxia. The basic Hc structure consists of six subunits (75 kDa each with an oxygen-binding site) assembled into a hexamer, and the changes in Hc structure and subunits can affect Hc oxygen affinity (Markl and Decker, 1992; Terwilliger, 1998). During hypoxia, the blue crab (*Callinectes sapidus*) has a higher ratio of the 1 × 6-meric oligomer (hexameric structure) of Hc, which has higher oxygen affinity than the 2 × 6-meric oligomer (dodecameric structure), which is predominant during normoxia (Defur et al., 1990; Mangum, 1994; Mangum, 1997). Moreover, changes in the frequency of the six subunits of hexamers can also alter the Hc oxygen affinity. For example, blue crabs (*Callinectes sapidus*) with higher hypoxia tolerance have higher ratios of the subunit 1 and lower ratios of subunit 4, 5 and 6, resulting in a higher Hc oxygen affinity (Bell et al., 2010; Defur et al., 1990).

As the oxygen content of the water declines, a point is usually reached where modulation of cardiac, ventilatory and Hc parameters is no longer sufficient to maintain oxygen homeostasis. Below this level (P_{crit}), crustaceans have to suppress their metabolic rate or undergo anaerobic metabolism in order to produce ATP (Lehtonen and Burnett, 2016; Spicer et al., 2002; Yannicelli et al., 2013). Although glycolysis rapidly produces ATP (independent of oxygen support), the end product, L-lactate acid, can build up in the body and disrupt acid-base balance (Bonvillain et al., 2012; Hochachka and Mommsen, 1983; Hochachka and Somero, 2002). Crustaceans can buffer the acidosis by mobilizing calcium carbonate from the calcified cuticle, liberating HCO_3^- and Ca^{2+} ions (Henry and Wheatly, 1992; Wheatly and Henry, 1992; Whiteley and Taylor, 2015). In addition

to increasing buffering capacity, crustaceans can maintain acid-base balance by excreting acid or base equivalents (Na^+ for NH_4^+/H^+ and Cl^- for HCO_3^-), which is achieved by ionic regulation through the gill epithelia, especially chloride cells (Fehsenfeld and Weihrauch, 2017; Freire et al., 2008; Whiteley, 1999). During reoxygenation, L-lactate is converted into glucose via gluconeogenesis or catabolized as a fuel for aerobic metabolism through the tricarboxylic acid cycle (Henry et al., 1994; Hui et al., 2017; Maciel et al., 2008). Although L-lactate is typically considered as an endpoint of anaerobic metabolism, it may play a number of other roles. For example, the accumulated L-lactate may act as a behavioral and metabolic signaling molecule during hypoxic conditions. Green crabs (*Carcinus maenas*) move to colder environments when injected with an iso-osmotic L-lactate solution in normoxia, which may help the crabs benefit from lowered metabolism in hypoxic conditions (De Wachter et al., 1997).

In the previous chapter, I found that the MO_2 of the rock crab (*Cancer irroratus*) during exposure to hypoxia was not only influenced by the severity of the hypoxia but was also significantly impacted by their food deprivation state (Jiang, Chapter Two). Postprandial rock crabs exhibited a sustained increase in metabolism and had a higher P_{crit} than fasted (3–5 d food deprivation) or starved crabs (28–30 d food deprivation). Although the resting metabolic rates of starved crabs in normoxia were lower than those of fasted crabs, the P_{crit} of starved crabs and fasted crabs were similar to one another. Following feeding, the characteristics of the specific dynamic action (SDA) were also affected by hypoxic levels and the food deprivation state. Overall, hypoxia reduced the magnitude of the SDA response but prolonged its duration, which was reflected in a slowing of gastric processing (Jiang, Chapter Four). This suggests that the crabs cannot maintain their normal digestive processes, as oxygen is diverted to other vital metabolic

processes (Airriess and McMahon, 1994; McGaw, 2005). In addition, starvation also reduced the magnitude of the SDA response and prolonged its duration. However, this effect of food deprivation state became less pronounced in severe hypoxia.

Given the differences in MO_2 as a function of both the level of hypoxia and the food deprivation state, the goals of the present study were to determine how these differences may affect downstream processes of postprandial fasted and starved crabs exposed to mild or severe hypoxia. Oxygen transport in the hemolymph was assessed by measuring arterial P_{O2} and Hc concentration, while pH and L-lactate levels were used as indicators of anaerobic metabolism.

3.3 Material and Methods

3.3.1 Crab collection and housing

Male and female rock crabs (*Cancer irroratus*) were trapped along the coast of the northern Avalon Peninsula, Newfoundland, Canada. Crabs (80–160 g) in the hard-intermolt stage were retained, which showed no obvious physical damage or weakness. A similar range of sizes was used to avoid any bias (the effects of animal size on aerobic metabolism were tested in Appendix A). The crabs were transferred to the Department of Ocean Sciences at Memorial University in St John's, NL, Canada and held in three 400L sediment-free tanks (20–30 crabs per tank) with flow-through aerated seawater (21 kPa oxygen) at ambient temperatures (4–10°C) and salinity (30–32‰). The crabs were fed chopped herring (*Clupea harengus*) to satisfaction once a week, and any food remain was removed on the same day to avoid fouling the water. At least two weeks before the experiments started, crabs were acclimated in the laboratory tanks (300 L) in flow-

through aerated seawater (salinity 30–32‰) at 12–13°C and constant dim red light to reduce diurnal rhythms. A two-week period is the minimum time to effectively acclimate the crabs to experimental temperatures and to abolish any underlying locomotor rhythms (Taylor et al., 1977). Initial experiments showed no different oxygen consumption rates between sexes; therefore, crabs of both sexes were chosen indiscriminately in each treatment at the approximate ratio of collection (male: female, 3: 1).

In this study, crabs were classified into three food deprivation states, each with specific physiological attributes:

a) Starved crabs were held in individual perforated plastic chambers (25 × 15 × 7.5 cm) to prevent cannibalism at 12–13°C and starved for 4–5 weeks. The mortality rate of starvation was about 10–15%, which was not different from fasted crabs. Preliminary experiments showed that 1-month of constrained acclimation did not induce any significant changes in metabolism when compared with free-moving crabs (Appendix A). The starved crabs have lower resting metabolic rates (Ansell, 1973; Wallace, 1973) and reduced blood protein levels (and hence hemocyanin levels), and utilize body proteins for energy (Sacristán et al., 2017).

b) Fasted crabs were deprived of food for 3–5 days before experiments began, which allowed them to evacuate all food from the digestive system. Studies suggest crabs used in the study had metabolism without the effects of prolonged starvation (Ansell, 1973; McGaw and Curtis, 2013; Wallace, 1973)

c) Fed crabs were fed shrimp muscle, 2% of their body mass. Fasted or starved crabs were held in individual chambers and ensu–red consuming all food within 1 h before experiments. The

postprandial crabs typically exhibit a 2–3 fold increase in metabolism associated with digestion, absorption and protein synthesis (Secor, 2009).

3.3.2 Experimental design

The previous chapter showed that food deprivation states affected the P_{crit} and postprandial oxygen uptake during hypoxia. In this chapter, I determined how hemolymph parameters (P_{O2}, hemocyanin, L-lactate and pH levels) varied before and after feeding in 3 different hypoxia regimes: 100% (21 kPa), 50% and 20% oxygen. An oxygen saturation level of 100% was used as a normoxic control. Fifty percent oxygen was chosen as mild hypoxia since it was above the P_{crit} of starved and fasted crabs, while severe hypoxia of 20% oxygen was below the P_{crit} of the two groups. For reference, 100% oxygen is approximately 21 kPa.

The experiments were conducted in a flow-through tank (150 L) at 12–13°C in dim red light and the entire apparatus was surrounded by black sheeting to avoid any visual disturbance to crabs. For each treatment, 9–10 fasted or starved crabs (55 crabs in total) were individually kept in perforated plastic chambers (25 × 15 × 7.5 cm) (free water flow through chambers was tested) at 100% oxygen overnight. Following this settling period, shrimp muscle (2% of crab body mass) was offered to each crab, and they were allowed to eat for 20 min. Within 30 min after feeding, the oxygen saturation was changed to the target value (100%, 50% or 20%) and maintained at this level using an oxygen regulator (Loligo System, Viborg, Denmark) connected to an air source and a nitrogen cylinder. Both arterial (post-branchial) and venous (pre-branchial) hemolymph samples were repeatedly collected from each crab after 1, 6, 12, 24, 36, 48, and 72 h exposure to hypoxia (385 hemolymph samples in total). Control experiments (unfed treatment) were

carried out in 100%, 50%, and 20% oxygen for a separate group of fasted and starved crabs (n = 9–10, with extra 57 crabs and 399 hemolymph samples in total). As I used repeated sampling in the experiments, several assumptions were made. First, it was assumed that in 100% oxygen, the hemolymph parameters of unfed fasted crabs remained stable without repeated sampling, and any change of unfed fasted crabs over experimental time was caused by repeated sampling. Second, there is no interactive effect between the repeated sampling and the treatments (the oxygen level, food deprivation state or feeding condition). Thus, the effects of different treatments over time can only be compared with the control group. For example, the effect of feeding was estimated by comparing the fed group and unfed group, rather than looking at the change over time in the fed group alone.

Post-branchial hemolymph was sampled from the pericardial sinus via a prepared sampling port on the dorsal surface of the carapace (Johnson et al., 2011; Lehtonen and Burnett, 2016). The area of the carapace directly above the heart was sanitized by wiping with isopropanol solution (70%). A port (1 mm diameter) was drilled above the pericardial sinus, and a thin piece of sterile latex dam (3 × 3 mm^2) was glued over the port to prevent hemolymph loss. Animals were then allowed to recover for 12 h before experimentation. Pre-branchial hemolymph was sampled from the infrabranchial sinus via the arthrodial membrane at the base of pereiopods.

Both post-branchial (400 µL) and pre-branchial (200 µL) samples were withdrawn with chilled 1-mL airtight Hamilton syringes and 20-gauge needles. The post-branchial hemolymph was immediately used for P_{O2} (200 µL) and pH (200 µL) measurements. Meanwhile, 200 µL of the

pre-branchial hemolymph was stored in 1.5-mL Eppendorf tubes at -80°C for later hemocyanin and L-lactate analyses.

3.3.2.1 Post-branchial P_{O2} and pH

Post-branchial P_{O2} was measured via a Fibox-3 O_2 analyzer (PreSens, Regensburg, Germany). It was calibrated using fully aerated seawater (100 % saturation) and deoxygenated seawater saturated with sodium sulfite (Na_2SO_3). Immediately following collection, 200 µL of the post-branchial sample was injected below a layer of mineral oil in an Eppendorf and placed in a water bath controlled at 13 ± 0.5°C. An oxygen dipping probe (DP-PSt3, PreSens, Regensburg, Germany) was inserted into the sample, and readings were taken once P_{O2} had stabilized (after 5 min) using OxyView software (PreSens, Regensburg, Germany).

The remaining 200 µL sample was used to measure post-branchial pH via a pH-1 Mini v2 analyzer (PreSens, Regensburg, Germany). The sample was injected below a layer of mineral oil in an Eppendorf tube which was maintained at 13 ± 0.5°C in a water bath. A fiber optic pH dipping probe (DP-HP5, PreSens, Regensburg, Germany) was inserted into the sample, and readings were taken once pH had stabilized (after 5 min) using pH 1-view software (PreSens, Regensburg, Germany). The probe had been previously calibrated using colorless pH reference buffers (Ricca Chemical Company, Arlington, Texas, USA).

3.3.2.2 Pre-branchial hemocyanin and L-lactate

Hemocyanin concentration of the hemolymph was used as an indicator of oxygen transport capability (Nickerson and Van Holde, 1971). The pre-branchial samples were thawed at room

temperature, then mixed for 10 seconds to distribute the protein evenly; then a 50 µL aliquot of hemolymph was diluted with 950 µL of distilled water. A 350µL sample of the diluted hemolymph was injected into a 96-well microplate (Corning, C3595), and the absorbance was measured at 336 nm with a Spectramax M5 multimode microplate reader (Molecular Devices, California, USA). Seawater was used as a blank. All samples and controls were duplicated in the microplates to reduce measurement error. Hemocyanin concentration was calculated with the functional subunit of 74 kDa, using an extinction coefficient of ε = 17.20 mmol/L/cm based on the $E_{1cm}^{1\%}$ value of 2.29 reported for *Cancer borealis* (Nickerson and Van Holde, 1971):

Concentration (mM) = ε (17.20 mmol/L/cm) × Pass Length (1 cm) × Absorbance

The hemolymph L-lactate levels were used as an indicator of anaerobic metabolism and were determined using a spectrophotometric assay (Clow et al., 2016). The thawed pre-branchial samples were deproteinized using 6% perchloric acid with a dilution ratio of 1:10 and then centrifuged at 15,000 g for 10 min. A 25 µL sample of the supernatant was then added to 200 µL of assay medium (pH = 9.0) containing glycine buffer (Sigma, G5418) and 2.5 mmol/L NAD+. Absorbances were measured at 340 nm using a DTX 880 microplate reader (Beckman Coulter, Ontario, Canada) before and 90 min after the addition of 10 IU/mL L-lactic dehydrogenase (Sigma, L2500). The difference value of absorbance before and after was used to calculate L-lactate concentrations, based on a standard curve which was made of 8 standard solutions of L-lactic acid (Sigma, L6402): 20 mM, 15 mM, 10 mM, 7.5 mM, 5 mM, 2.5 mM and 0 mM (seawater was used as a blank) and fitted in linear regression. All samples and standards were duplicated in the microplates (Corning, C3595) to reduce measurement error.

3.3.3 Data analysis

All statistical tests were conducted in R 3.4.2 (Team, 2018). The normality of data was checked by a Shapiro-Wilks test. For these data that did not pass the normality test, a Box-Cox transformation was applied (λ value was reported). A Bartlett test was performed to check the homoscedasticity of data. In order to deal with heterogeneity, variance structures were added in model fitting, and the best model was achieved by likelihood ratio tests with Akaike's information criterion (AIC). Tukey-adjusted *post hoc* tests were carried out using the 'emmeans' package. Statistical significance was accepted when $p < 0.05$. All data in the figures and tables were shown in means ± standard error of the mean (SEM).

Linear mixed-effect models (in R package 'nlme') were applied to deal with repeated measures per crab. To assess the effect of repeated sampling (sampling time), the dependent variables (P_{O2}, hemocyanin, L-lactate and pH) of unfed fasted and starved crabs in 100% oxygen were analyzed separately as a function of sampling time (0–72 h) and food deprivation state (fasted, starved). Separately, four dependent variables (P_{O2}, hemocyanin, L-lactate and pH) were analyzed as a function of four independent variables; feeding (unfed, fed), food deprivation state (fasted, starved), oxygen level (100%, 50% and 20%) and time (0–72 h). For random effects, individual crab was identified as the main factor on the random intercept.

3.4 Results

3.4.1 Effects of repeated sampling

The fasted and starved crabs in the unfed control group (100% oxygen) were used to assess any effect of repeated sampling on hemolymph biochemical parameters. There were some slight but significant changes in the biochemical parameters over the sampling period (Table 3.1). The postbranchial hemolymph P_{O2} of the fasted crabs (Figure 3.1A) was 7.7 ± 0.7 kPa at the start of the experiment (1 h), and this level was maintained until 48 h. By 72 h, the P_{O2} (10.6 ± 0.8 kPa) was significantly higher than that measured at 1 h (Tukey's HSD, t = 3.3, p = 0.02). In contrast, there was no significant change in P_{O2} of the starved crabs, which was maintained at a mean level of 9.5 ± 0.7 kPa.

The hemocyanin concentration of the fasted crabs (Figure 3.1B) was maintained at mean levels of 0.47 ± 0.05 mmol/L until 48 h but had decreased to 0.39 ± 0.04 mmol/L by 72 h (Tukey's HSD, t = 3.2, p = 0.026). In contrast, there was no significant change in the hemocyanin concentration of the starved crabs during the 72 h experimental period (mean levels of 0.33 ± 0.05 mmol/L).

A decline in the L-lactate concentration occurred in both fasted and starved crabs during the 72-h experimental period (Figure 3.1C). The L-lactate concentration of the fasted crabs decreased from initial levels of 0.36 ± 0.16 to 0.14 ± 0.07 mmol/L by 48 h (Tukey's HSD, t = 3.1, p = 0.038) and did not change significantly thereafter. A similar pattern was observed for starved crabs, where L-lactate concentrations dropped from initial levels of 0.75 ± 0.19 to 0.51 ± 0.09 mmol/L

after 24 h (Tukey's HSD, $t = 3.5$, $p = 0.011$). Although the L-lactate concentration appeared to continue to decline after 24 h, this change was not statistically significant.

The hemolymph pH of the fasted crabs remained fairly stable over the sampling period, with a mean value of 8.01 ± 0.02 (Figure 3.1D). The pH of the starved crabs also remained stable during the first 48 h but increased to 8.03 ± 0.02 by 72 h; these levels were significantly higher than the pH of 7.96 ± 0.02 measured at the start of the experiment (Tukey's HSD, $t = 3.2$, $p = 0.028$).

Table 3.3 The summary of linear mixed-effects models to assess the effect of repeated sampling on the biochemical parameters of the fasted and starved crabs in the unfed control group in 100% oxygen saturation. Bold font indicates statistical significance ($p < 0.05$).

Variables	Factors	$df_{num,den}$	*F*	*P* value
P_{O2}	Food deprivation state (fasted, starved)	1,18	0.09	0.770
	Time (0-72 h)	**6,101**	**3.00**	**0.010**
	Food deprivation state: Time	6,101	1.41	0.219
Hemocyanin	**Food deprivation state (fasted, starved)**	**1,18**	**8.54**	**0.008**
	Time (0-72 h)	**6,101**	**6.43**	**<0.001**
	Food deprivation state: Time	6,101	1.40	0.255
L-lactate	Food deprivation state (fasted, starved)	1,18	3.3	0.085
	Time (0-72 h)	**6,101**	**8.4**	**<0.001**
	Food deprivation state: Time	6,101	0.4	0.880
pH	Food deprivation state (fasted, starved)	1,18	2.23	0.157
	Time (0-72 h)	**6,101**	**5.31**	**<0.001**
	Food deprivation state: Time	6,101	1.65	0.166

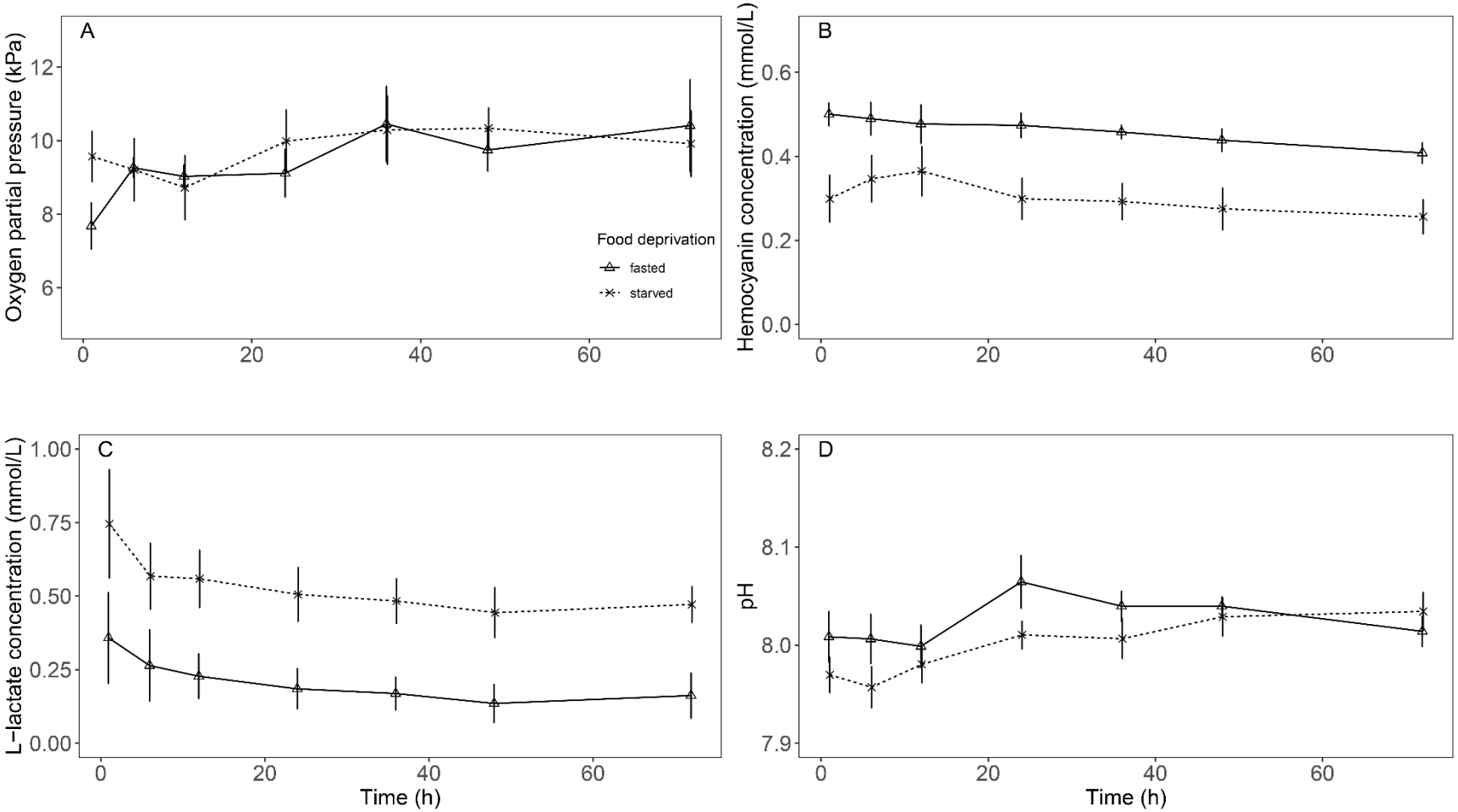

Figure 3.10 The A. post-branchial oxygen partial pressure (kPa), B. hemocyanin concentration (mmol/L), C. L-lactate concentration (mmol/L) and D. pH of the fasted crabs and starved crabs before feeding when exposed to 100% oxygen saturations. Data are shown as mean with SEM (N = 8–10).

3.4.2 Effects of the food deprivation state, oxygen level and food consumption

Numerous comparisons of each biochemical parameter can be made as a function of the oxygen level (100%, 50% and 20%), the food deprivation state (fasted, starved), the non-fed (control) vs. fed animals and the sampling period (time). Initial comparisons were made for the control group (non-fed fasted and non-fed starved crabs) to determine any effects of the oxygen level and food deprivation state over time. Following this, the same comparisons were made for the fasted and starved crabs in the fed group. Finally, the effect of feeding was estimated between pre- and postprandial groups for both fasted and starved crabs.

3.4.2.1 Post-branchial oxygen partial pressure (P_{O2})

Unfed fasted and starved crabs **(effect of oxygen level and food deprivation)**

The ambient oxygen levels significantly influenced the hemolymph P_{O2} (linear mixed-effects model, $F_{2,101} = 850.8$, $p < 0.001$). For the unfed control groups, in 100% oxygen, the arterial P_{O2} of the fasted crabs (Figure 3.2A) was maintained between 7.7 ± 0.6 kPa and 10.5 ± 1.0 kPa for the duration of the experiment. In 50% oxygen, the arterial P_{O2} of fasted crabs was approximately 14-18% lower than that measured in 100% oxygen (Tukey's HSD, $t = 4.8$, $p < 0.0001$) and was maintained between 6.4 ± 0.6 kPa and 8.3 ± 0.3 kPa. The arterial P_{O2} of fasted crabs in 20% oxygen was significantly reduced by 60-85% when compared with that measured in 100% oxygen (Tukey's HSD, $t = 16.4$, $p < 0.0001$) and was maintained between mean levels of 1.5 ± 0.3 kPa and 3.1 ± 0.2 kPa. The arterial P_{O2} of the unfed starved crabs followed a similar pattern (Figure 3.2B), and there was no significant difference between the unfed fasted and unfed starved crabs (linear mixed-effects model, $F_{1,101} = 0.9$, $p = 0.35$).

Fasted and starved crabs post-feeding **(effect of oxygen level and food deprivation)**

After feeding, the P_{O2} of the fasted (Figure 3.2C) and starved crabs (Figure 3.2D) also decreased as a function of ambient oxygen level (linear mixed-effects model, $F_{2,101} = 850.8$, $p < 0.001$). In 100% oxygen, the arterial P_{O2} of the fed fasted crabs was maintained between 10.0 ± 1.6 kPa and 13.2 ± 1.3 kPa during the 72 h experimental period. In 50% oxygen, the arterial P_{O2} of fasted crabs was approximately 15-40% lower than that measured in 100% oxygen (Tukey's HSD, $t = 6.7$, $p < 0.0001$) and was maintained between mean levels of 7.8 ± 0.3 kPa and 8.5 ± 0.3 kPa. The lowest P_{O2} levels of fasted crabs (3.0 ± 0.2 kPa and 3.3 ± 0.4 kPa) were measured during exposure to 20% oxygen, where the arterial P_{O2} was 67-77% lower than that measured in 100% oxygen (Tukey's HSD, $t = 16.1$, $p < 0.0001$). The arterial P_{O2} of the unfed starved crabs followed a similar pattern, and there was no significant difference between the unfed fasted and starved crabs (linear mixed-effects model, $F_{1,101} = 0.9$, $p = 0.35$).

Unfed versus fed animals **(effect of feeding)**

When factoring in the effect of food consumption, the fed crabs (Figure 3.2C&D) had significantly elevated P_{O2} levels compared with unfed animals (linear mixed-effects model, $F_{1,101} = 20.6$, $p < 0.001$), and there was a strong interaction of feeding and oxygen level (linear mixed-effects model, $F_{2,101} = 3.9$, $p = 0.02$). Both the fed fasted and fed starved crabs had significantly higher mean arterial P_{O2}'s in 100% oxygen saturation compared with the respective unfed animals (fasted crabs: 11.1 ± 0.3 kPa vs. 9.4 ± 0.2 kPa; starved crabs: 11.2 ± 0.3 kPa vs. 9.5 ± 0.2 kPa). In contrast, there was no significant difference in the P_{O2} between the unfed and feeding groups for both the fasted and starved crabs in 50% and 20% oxygen (Tukey's HSD, $P > 0.05$).

3.4.2.2 Pre-branchial hemocyanin concentration

Unfed fasted and starved crabs (effect of oxygen level and food deprivation)

The oxygen level did not significantly affect the hemocyanin concentration (Hc) for both fasted and starved crabs (linear mixed-effects model, $F_{2,101} = 0.1$, $p = 0.92$); however, there was a strong interaction between the oxygen level and time (linear mixed-effects model, $F_{12,599} = 2.4$, $p = 0.006$). For the unfed fasted crabs (Figure 3.3A), although no statistically significant differences in Hc occurred at each sampling period among the three oxygen levels, Hc concentration dropped more rapidly in severe hypoxia (20% oxygen), compared with those in normoxia. In 100% oxygen, Hc of the fasted crabs declined slightly, but significantly, from initial levels of 0.50 ± 0.03 down to 0.41 ± 0.03 mmol/L at 72 h (Tukey's HSD, $t = 4.2$, $p = 0.0008$). In 50% oxygen, the Hc of the fasted crabs was significantly lower than initial levels (0.45 ± 0.06 vs. 0.37 ± 0.04 mmol/L) after 48 h (Tukey's HSD, $t = 3.5$, $p = 0.01$). In 20% oxygen, a significant decrease of Hc had occurred (0.47 ± 0.05 vs. 0.39 ± 0.05 mmol/L) by 12 h (Tukey's HSD, $t = 3.6$, $p = 0.006$), and it reached the lowest levels of 0.29 ± 0.03 mmol/L after 72 h (Tukey's HSD, $t = 7.4$, $p < 0.0001$).

Although the food deprivation state had no significant effect on the Hc concentration (linear mixed-effects model, $F_{1,101} = 3.3$, $p = 0.07$), there was an interaction between food deprivation state and sampling time (linear mixed-effects model, $F_{6,599} = 6.0$, $p < 0.001$). This was due to the unfed starved crabs exhibiting a smaller decline in Hc concentration compared with the fasted crabs (Figure 3.3B). In 100% oxygen, the Hc of unfed starved crabs was 0.30 ± 0.07 mmol/L at 1 h, which was significantly lower than 0.50 ± 0.03 mmol/L of fasted crabs (Tukey's HSD, $t = 2.7$, $p = 0.009$). At 72 h, the Hc of unfed starved crabs dropped by 13% to 0.26 ± 0.05 mmol/L (18% for the fasted crabs); thus, the Hc of unfed starved crabs was lower than that of unfed fasted crabs, except for the 72h period. Similarly, in 50% oxygen, for the unfed starved crabs, Hc

concentrations decreased by 14% from 0.42 ± 0.07 to 0.36 ± 0.05 mmol/L (compared with 20% for fasted crabs), whereas in 20% oxygen, the Hc of unfed starved crabs declined by 26% from 0.43 ± 0.06 to 0.32 ± 0.05 mmol/L (compared with 38% for fasted crabs).

Fasted and starved crabs post-feeding **(effect of oxygen level and food deprivation)**

When fasted and starved crabs were fed, the digestion of the meal did not have a significant effect on the Hc concentrations (linear mixed-effects model, $F_{1,101} = 0.2$, $p = 0.68$). Thus, the changes in Hc for both fed groups followed a similar pattern to that of the unfed crabs. There was, however, a significant interaction between oxygen level and time (linear mixed-effects model, $F_{12,599} = 2.4$, $p = 0.006$). Hc concentrations of the fed fasted crabs in 100% oxygen declined significantly from initial mean levels of 0.47 ± 0.06 to 0.30 ± 0.03 mmol/L at 72 h (Tukey's HSD, $t = 4.2$, $p = 0.0008$). In 50% oxygen, Hc of the fasted crabs was maintained at stable levels between 0.42 ± 0.08 and 0.36 ± 0.06 mmol/L; while in 20% oxygen, a significant decrease in Hc occurred after 24 h (from 0.55 ± 0.09 to 0.45 ± 0.08 mmol/L, Tukey's HSD, $t = 3.7$, $p = 0.005$), but remained stable thereafter. In contrast, no effect of oxygen level or interaction between oxygen level and time was found in fed starved crabs (Figure 3.3D).

There was also a significant interactive effect of the food deprivation state (following feeding) with sampling time on Hc concentrations (linear mixed-effects model, $F_{6,599} = 6.0$, $p < 0.001$). When compared with the fasted crabs, the starved crabs had a lower mean Hc in all three oxygen levels after 6 h (0.34 ± 0.07 vs. 0.44 ± 0.09 mmol/L, Tukey's HSD, $t = 2.1$, $p = 0.044$), and there was no significant difference thereafter. When comparing the food deprivation states at

each oxygen level, the only significant difference was found in 20% oxygen at 1 h (starved vs. fasted crabs: 0.36 ± 0.06 vs. 0.55 ± 0.09 mmol/L, Tukey's HSD, t = 2.3, p = 0.029).

3.4.2.3 Pre-branchial L-lactate concentration

Unfed fasted and starved crabs **(effect of oxygen level and food deprivation)**

The L-lactate concentration was significantly affected by the oxygen level (linear mixed-effects model, $F_{2,101}$ = 8.1, p < 0.001). For the unfed fasted crabs, the mean L-lactate concentration was 0.31 ± 0.09 mmol/L in 100% oxygen (Figure 3.4A). This was significantly lower than the mean concentration of 0.86 ± 0.13 mmol/L measured in 50% oxygen (Tukey's HSD; t = 2.8, p = 0.019). Although L-lactate concentration in 100% oxygen also appeared lower compared to that measured in 20% oxygen (0.70 ± 0.11 mmol/L), this fell just shy of significance (Tukey's HSD, t = 2.1, p = 0.054). In contrast to the fasted crabs, there was no significant difference in the L-lactate concentration of unfed starved crabs among the three oxygen levels (Figure 3.4B). This difference between the unfed fasted and starved crabs resulted in a strong interaction between the oxygen level and the food deprivation state (linear mixed-effects model, $F_{2,101}$ = 9.0, p < 0.001). In 100% oxygen, the L-lactate of the unfed starved crabs was significantly higher than the fasted crabs (Tukey's HSD, t = 2.2, p = 0.032). In 50% oxygen, the L-lactate of unfed starved crabs was significantly lower than the fasted crabs (Tukey's HSD, t = 3.0, p = 0.004), and L-lactate concentrations were similar to one another in 20% oxygen.

Fasted and fed crabs post-feeding **(effect of oxygen level and food deprivation)**

After feeding, there was no significant difference in L-lactate concentration of the fasted crabs between 100% and 50% oxygen, where concentrations were maintained between 1.17 ±

0.25 and 0.50 ± 0.07 mmol/L (Figure 3.4C). However, there was a large and noticeable rise in L-lactate concentrations (compared with 100% oxygen) in 20% oxygen, reaching maximal levels of 3.45 ± 2.53 vs. 0.51 ± 0.04 mmol/L after 72 h (Tukey's HSD, t = 4.0, p = 0.0007). In contrast, the fed starved crabs exhibited no significant differences in L-lactate concentration among the three oxygen levels, with L-lactate concentrations remaining below 1 mmol/L during the course of the experiment. Because the fed starved crabs did not exhibit the large increase in L-lactate concentration in 20% oxygen that was observed in the fed fasted crabs, this resulted in a significant difference (starting at 24 h) between the two food deprivation groups with mean L-lactate concentrations of 0.50 ± 0.11 vs. 1.97 ± 0.78 mmol/L (Tukey's HSD, t = 2.8, p = 0.009).

Unfed versus fed animals **(effect of feeding)**

The large increase in L-lactate concentrations in fasted crabs following feeding in 20% oxygen resulted in a strong interaction between the oxygen level and feeding (linear mixed-effects model, $F_{2,101} = 7.5$, $p < 0.001$). This interaction was driven by differences between the unfed and fed fasted crabs in 20% oxygen that occurred after 24 h (Figure 3.4A&C). No significant differences in L-lactate concentration were found in any other of the pairwise comparisons for fasted or starved crabs.

3.4.2.4 Post-branchial pH

Unfed fasted and starved crabs **(effect of oxygen level and food deprivation)**

Overall, the hemolymph pH increased significantly as the ambient oxygen levels declined (linear mixed-effects model, $F_{2,101} = 41.0$, $p < 0.001$), and there was a strong interaction between oxygen level and the sampling time (linear mixed-effects model, $F_{12,599} = 2.3$, $p = 0.006$). The pH

of the unfed fasted crabs was maintained between 8.00 ± 0.03 and 8.06 ± 0.03 in 100% oxygen and 7.98 ± 0.02 and 8.08 ± 0.03 in 50% oxygen (Figure 3.5A); these levels were similar to one another. However, in 20% oxygen, the pH of the unfed fasted crabs increased to 8.15 ± 0.02 after 36 h, and this was significantly higher than levels measured in 100% oxygen (Tukey's HSD, $t = 4.7$, $p < 0.0001$). A similar pattern was found in the starved crabs, where the pH in 20% oxygen was significantly higher than that measured in 50% or 100% oxygen concentrations.

The food deprivation state did not have a significant effect on the hemolymph pH (linear mixed-effects model, $F_{1,101} = 2.5$, $p = 0.15$); however, there was a significant interaction between food deprivation state and time (linear mixed-effects model, $F_{6,599} = 9.2$, $p < 0.001$). This interaction occurred because the hemolymph pH of the unfed fasted crabs was more variable over time (Figure 3.5B). In 100% and 50% oxygen, the pH of the unfed fasted and starved crabs were similar. However, in 20% oxygen, the pH of the fasted crabs was significantly lower during the first 12 h of the experiment (by 0.06 to 0.13 pH units) (Tukey's HSD, $t = 4.7$, $p < 0.0001$), but significantly higher than the unfed starved crabs between 36 and 72 h (Tukey's HSD tests, $p < 0.005$).

Fasted and starved crabs post-feeding **(effect of oxygen level and food deprivation)**

After feeding, differences in hemolymph pH levels among the three oxygen treatments became more apparent. Overall, the fasted crabs exhibited significantly higher hemolymph pH levels in 20% and 50% oxygen, compared with crabs maintained in 100% oxygen (Tukey's HSD tests: $t = 10.6$, $p < 0.0001$; $t = 5.2$, $p < 0.0001$). The magnitude of these differences tended to decline over the duration of the experiment and became insignificant by 72 h (Tukey's HSD, $t =$

2.4, P > 0.05). This pattern was not as pronounced for the fed starved crabs, where the hemolymph pH was only significantly higher in 20% oxygen (Tukey's HSD tests: 20% vs. 100% oxygen, t = 6.0, p < 0.0001; 20% vs. 100% oxygen, t = 5.5, p < 0.0001).

A number of differences were noted between the hemolymph pH of fed fasted and fed starved crabs. The starved animals had a higher pH (0.16 ± 0.02 units) during the first 12 h in 100% oxygen (Tukey's HSD, t = 7.0, p < 0.0001). This pattern was reversed in 50% and 20% oxygen, whereby the starved crabs exhibited lower pH levels at some time points: In 50% oxygen, significant differences occurred at 6 h and 48 h (Tukey's HSD tests, p < 0.01); while in 20% oxygen, slightly lower pH levels for fed starved crabs were found at 6 h, 12 h, 48 h and 72 h (Tukey's HSD tests, p < 0.05).

Unfed versus fed animals

The fed crabs had significantly lower pH levels compared with the unfed animals (linear mixed-effects model, $F_{1,101} = 10.6$, p = 0.002), and there were strong interactions with the oxygen level (linear mixed-effects model, feeding vs. oxygen level: $F_{2,101} = 6.2$, p = 0.003) and time (linear mixed-effects model, feeding vs. time: $F_{6,599} = 3.5$, p = 0.02). During the first 24 h in 100% oxygen, the fed fasted crabs had a significantly lower hemolymph pH (by 0.11 to 0.17 units) compared with the unfed crabs (Figure 3.5C). In 50% oxygen, the pH of the fed fasted crabs did not significantly differ from the unfed fasted group, while in 20% oxygen, the only significant difference was found during the first 12 h; here, the fed fasted crabs had higher pH of 0.09 to 0.15 units than unfed crabs. There was a slightly different pattern when comparing the unfed and fed starved crabs (Figure 3.5D). Here the fed crabs exhibited significantly lower pH levels (by 0.07

to 0.10 pH units) than the unfed group between 6 and 12 h in all three oxygen levels (Figure 3.5D).

Table 3.4 The summary of linear mixed-effects model analysis for the four biochemical parameters of the fasted and starved crabs in oxygen levels of 100%, 50% or 20% for both unfed control crabs and those after being offered a shrimp meal of 2% body mass. Only the interactions with significant effects were presented. If Box-cox transformation was applied to the data, λ value was presented. Bold font indicates statistical significance ($p < 0.05$).

Variables	Factors	$df_{num,den}$	*F*	*P* value
P_{O2} (λ=0.4)	**Feeding (unfed, fed)**	**1,101**	**20.64**	**<0.001**
	Food deprivation state (fasted, starved)	1,101	0.88	0.350
	Oxygen level (100, 50, 20%)	**2,101**	**850.75**	**<0.001**
	Time (0-72 h)	**6,599**	**12.14**	**<0.001**
	Feeding: Oxygen level	**2,101**	**3.92**	**0.023**
	Feeding: Time	**6,599**	**3.94**	**<0.001**
	Feeding: Food deprivation state: Time	**6,599**	**2.97**	**0.007**
Hemocyanin (λ=0.8)	Feeding (unfed, fed)	1,101	0.170	0.681
	Food deprivation state (fasted, starved)	1,101	3.30	0.072
	Oxygen level (100, 50, 20%)	2,101	0.089	0.915
	Time (0-72 h)	**6,599**	**49.49**	**<0.001**
	Oxygen level: Time	**12,599**	**2.37**	**0.006**
	Food deprivation state: Time	**6,599**	**5.99**	**<0.001**
L-lactate (λ=0)	Feeding (unfed, fed)	1,101	0.90	0.345
	Food deprivation state (fasted, starved)	**1,101**	**5.63**	**0.020**
	Oxygen level (100, 50, 20%)	**2,101**	**8.05**	**<0.001**
	Time (0-72 h)	**6,599**	**9.72**	**<0.001**
	Oxygen level: Feeding	**2,101**	**7.48**	**<0.001**
	Oxygen level: Food deprivation state	**2,101**	**8.99**	**<0.001**
	Feeding: Food deprivation state	**1,101**	**8.79**	**0.004**
pH	**Feeding (unfed, fed)**	**1,101**	**10.57**	**0.002**
	Food deprivation state (fasted, starved)	1,101	2.48	0.153
	Oxygen level (100, 50, 20%)	**2,101**	**41.01**	**<0.001**
	Time (0-72 h)	**6,599**	**73.96**	**<0.001**
	Oxygen level: Time	**12,599**	**2.26**	**0.006**
	Oxygen level: Feeding	**2,101**	**6.22**	**0.003**
	Feeding: Time	**6,599**	**3.54**	**0.015**
	Food deprivation state: Time	**6,599**	**9.18**	**<0.001**
	Oxygen level: Feeding: Time	**12,599**	**6.02**	**<0.001**

Table 3.5 Post hoc pairwise comparisons (Tukey's HSD) between fasted and starved crabs (unfed and fed groups) during exposure to 100%, 50% and 20% oxygen levels (time = 72 h). This summarizes the paired comparisons between food deprivation states, i.e., Figure A vs. B and C vs. D only. Asterisk indicates statistical significance between fasted and starved crabs ($p < 0.05$).

Variables	Feeding	Oxygen Level	Time (Fasted vs. Starved)						
			1h	6h	12h	24h	36h	48h	72h
P_{O2} (Figure 3.2)	unfed	100%	*	-	-	-	-	-	-
		50%	-	-	-	-	-	-	-
		20%	-	-	-	-	-	-	-
	fed	100%	-	-	-	-	-	-	-
		50%	-	-	-	-	-	-	-
		20%	-	-	-	-	-	-	-
Hc (Figure 3.3)	unfed	100%	*	-	-	*	*	*	-
		50%	-	-	-	-	-	-	-
		20%	-	-	-	-	-	-	-
	fed	100%	-	-	-	-	-	-	-
		50%	-	-	-	-	-	-	-
		20%	*	-	-	-	-	-	-
L-lactate (Figure 3.4)	unfed	100%	*	-	-	-	-	*	*
		50%	*	*	*	*	*	*	*
		20%	-	-	*	-	-	-	-
	fed	100%	-	-	-	-	-	-	-
		50%	-	-	-	-	-	-	-
		20%	-	-	-	*	*	-	*
pH (Figure 3.5)	unfed	100%	-	-	-	-	-	-	-
		50%	-	-	-	-	-	*	-
		20%	*	*	*	-	*	*	*
	fed	100%	*	*	*	-	-	-	-
		50%	-	*	*	-	-	*	-
		20%	-	*	*	-	-	*	*

Table 3.6 Post hoc pairwise comparisons (Tukey's HSD) between unfed and fed groups (fasted and starved crabs) during exposure of 100%, 50% and 20% oxygen levels (time = 72 h). The data summarizes comparisons as a function of whether a crab had fed or not, i.e., Figure A vs. C and B vs. D only. Asterisk indicates statistical significance between unfed and fed crabs ($p < 0.05$).

Variables	Food Deprivation	Oxygen Level	Time (Unfed vs. Fed)						
			1h	6h	12h	24h	36h	48h	72h
P_{O2} (Figure 3.2)	fasted	100%	*	*	*	-	-	*	-
		50%	*	-	-	-	-	-	-
		20%	*	-	-	-	-	-	-
	starved	100%	-	*	*	-	*	*	-
		50%	*	-	-	-	-	-	-
		20%	-	-	-	-	-	-	-
Hc (Figure 3.3)	fasted	100%	-	-	-	-	-	-	-
		50%	-	-	-	-	-	-	-
		20%	-	-	-	-	-	-	-
	starved	100%	-	-	-	-	-	-	-
		50%	-	-	-	-	-	-	-
		20%	-	-	-	-	-	-	-
L-lactate (Figure 3.4)	fasted	100%	-	-	-	-	-	-	-
		50%	-	-	-	-	-	-	-
		20%	-	-	-	*	*	-	*
	starved	100%	-	-	-	-	-	-	-
		50%	-	-	-	-	-	-	-
		20%	-	-	-	-	-	-	-
pH (Figure 3.5)	fasted	100%	*	*	*	*	-	*	-
		50%	-	-	-	-	-	-	-
		20%	*	*	*	-	-	-	-
	starved	100%	*	-	-	-	-	-	-
		50%	-	*	-	-	-	-	-
		20%	-	*	*	-	-	-	-

Table 3.7 Post hoc pairwise comparisons (Tukey's HSD) between 100%, 50% and 20% oxygen levels for fasted and starved crabs in unfed and fed groups (time = 72 h). Different letters indicate statistical significance in three oxygen levels ($p < 0.05$) within a treatment (i.e., within Figure A, B, C, or D). For example, in row one P_{O2}, for unfed crabs a-a-b, denotes no difference between 100% and 50% O_2, but a difference between 20% O_2 and the other in Figure 3.2A.

Variables	Food Deprivation	Feeding	Time (100%, 50% and 20% oxygen)						
			1h	6h	12h	24h	36h	48h	72h
P_{O2} (Figure 3.2)	fasted	unfed	a-a-b	a-a-b	a-a-b	a-a-b	a-b-c	a-a-b	a-b-c
		fed	a-b-c	a-b-c	a-b-c	a-a-b	a-b-c	a-b-c	a-b-c
	starved	unfed	a-b-c	a-a-b	a-a-b	a-b-c	a-b-c	a-b-c	a-a-b
		fed	a-b-c	a-b-c	a-b-c	a-b-c	a-b-c	a-b-c	a-b-c
Hc (Figure 3.3)	fasted	unfed	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a
		fed	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a
	starved	unfed	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a
		fed	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a
L-lactate (Figure 3.4)	fasted	unfed	a-b-b	a-b-b	a-b-b	a-b-b	a-b-b	a-b-b	a-b-ab
		fed	a-a-a	a-a-a	a-a-a	a-a-b	a-a-b	a-a-a	a-a-b
	starved	unfed	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a
		fed	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a	a-a-a
pH (Figure 3.5)	fasted	unfed	a-a-a	a-a-a	a-a-a	a-a-a	a-a-b	a-a-b	a-a-b
		fed	a-b-c	a-b-c	a-b-c	a-b-b	a-b-b	a-b-b	ab-a-b
	starved	unfed	a-ab-b	a-a-b	a-a-b	a-a-a	a-ab-b	a-a-a	a-a-a
		fed	a-a-b	a-b-c	a-b-c	a-a-b	a-a-b	a-a-b	a-a-a

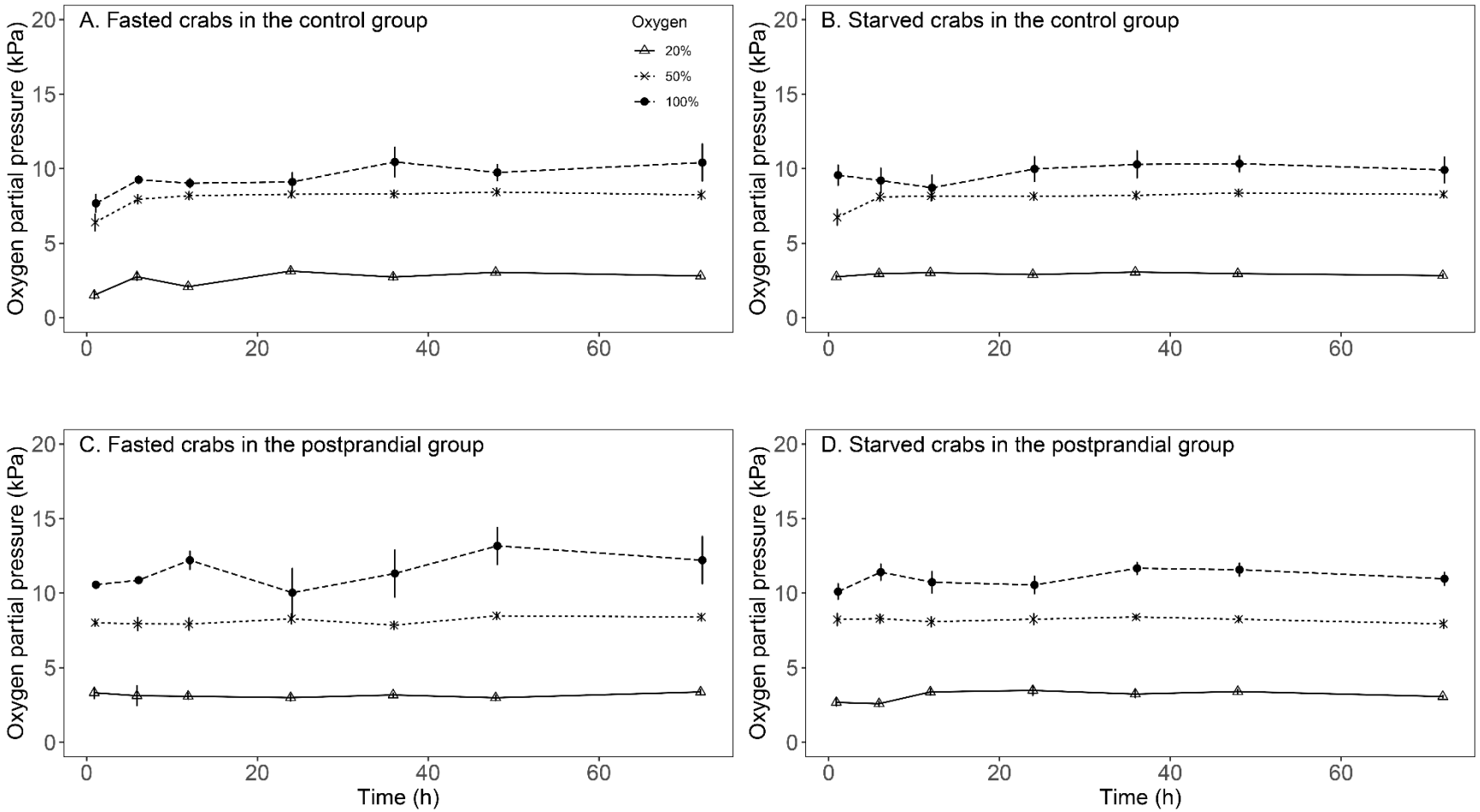

Figure 3.11 The post-branchial oxygen partial pressure (kPa) of A. unfed fasted crabs, B. unfed starved crabs, C. fed fasted crabs and D. fed starved crabs when exposed to 100%, 50% and 20% oxygen saturations. Data are shown as mean with SEM (N = 8–10).

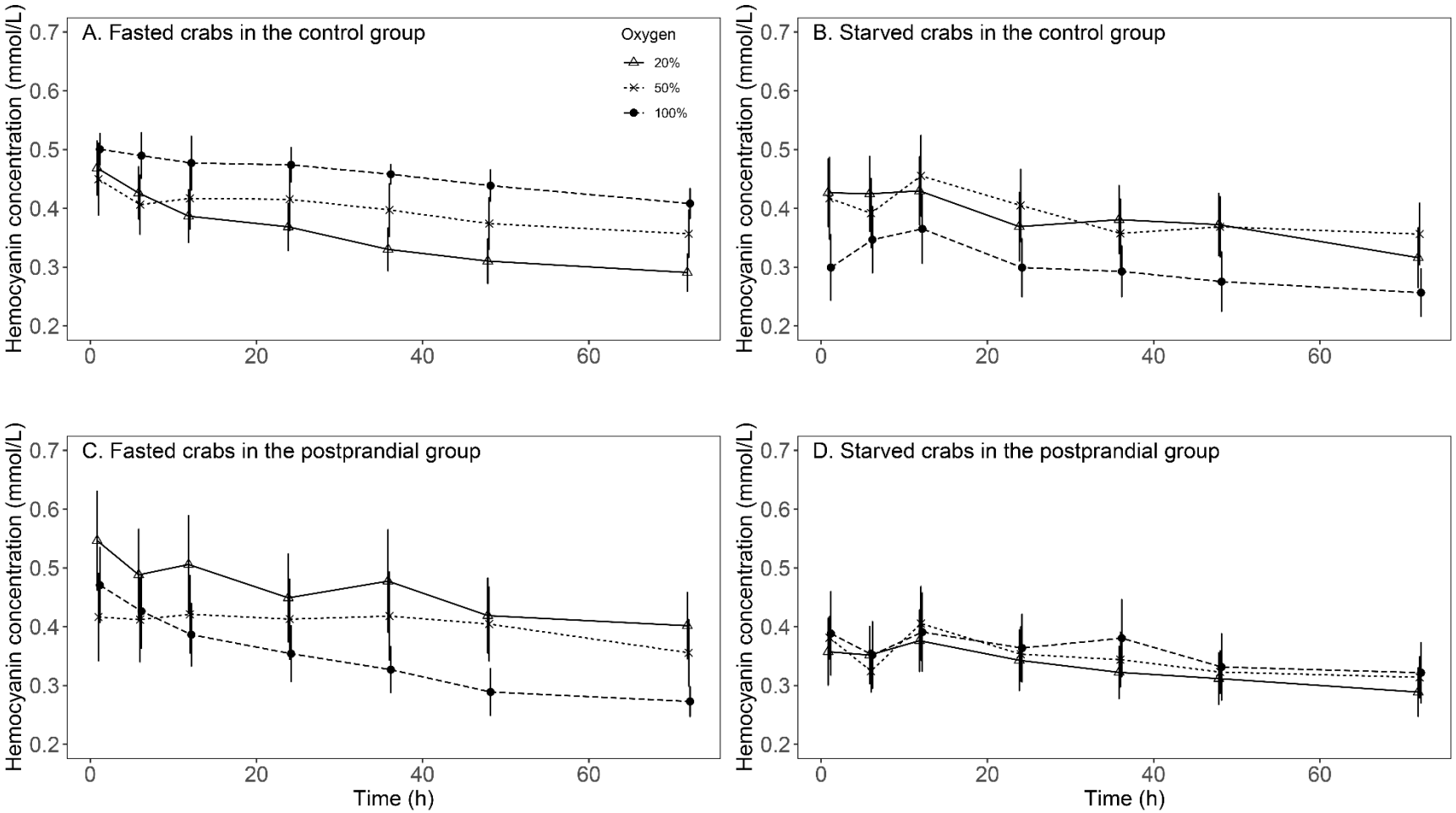

Figure 3.12 The pre-branchial hemocyanin concentration (mmol/L) of A. unfed fasted crabs, B. unfed starved crabs, C. fed fasted crabs and D. fed starved crabs when exposed to 100%, 50% and 20% oxygen saturations. Data are shown as mean with SEM (N = 8–10).

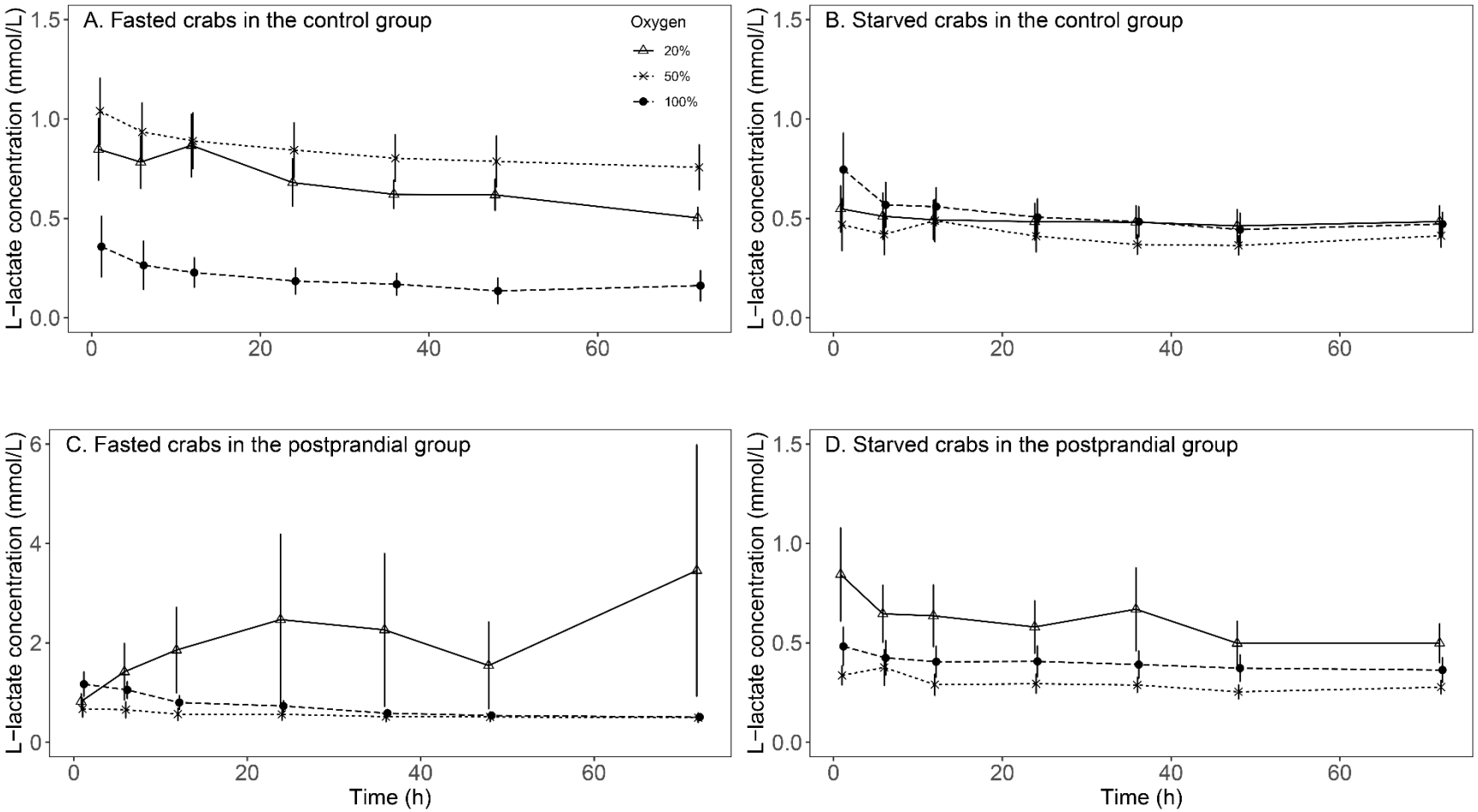

Figure 3.13 The pre-branchial L-lactate concentration (mmol/L) of A. unfed fasted crabs, B. unfed starved crabs, C. fed fasted crabs and D. fed starved crabs when exposed to 100%, 50% and 20% oxygen saturations. Data are shown as mean with SEM (N = 8–10). The scale is different in Figure C.

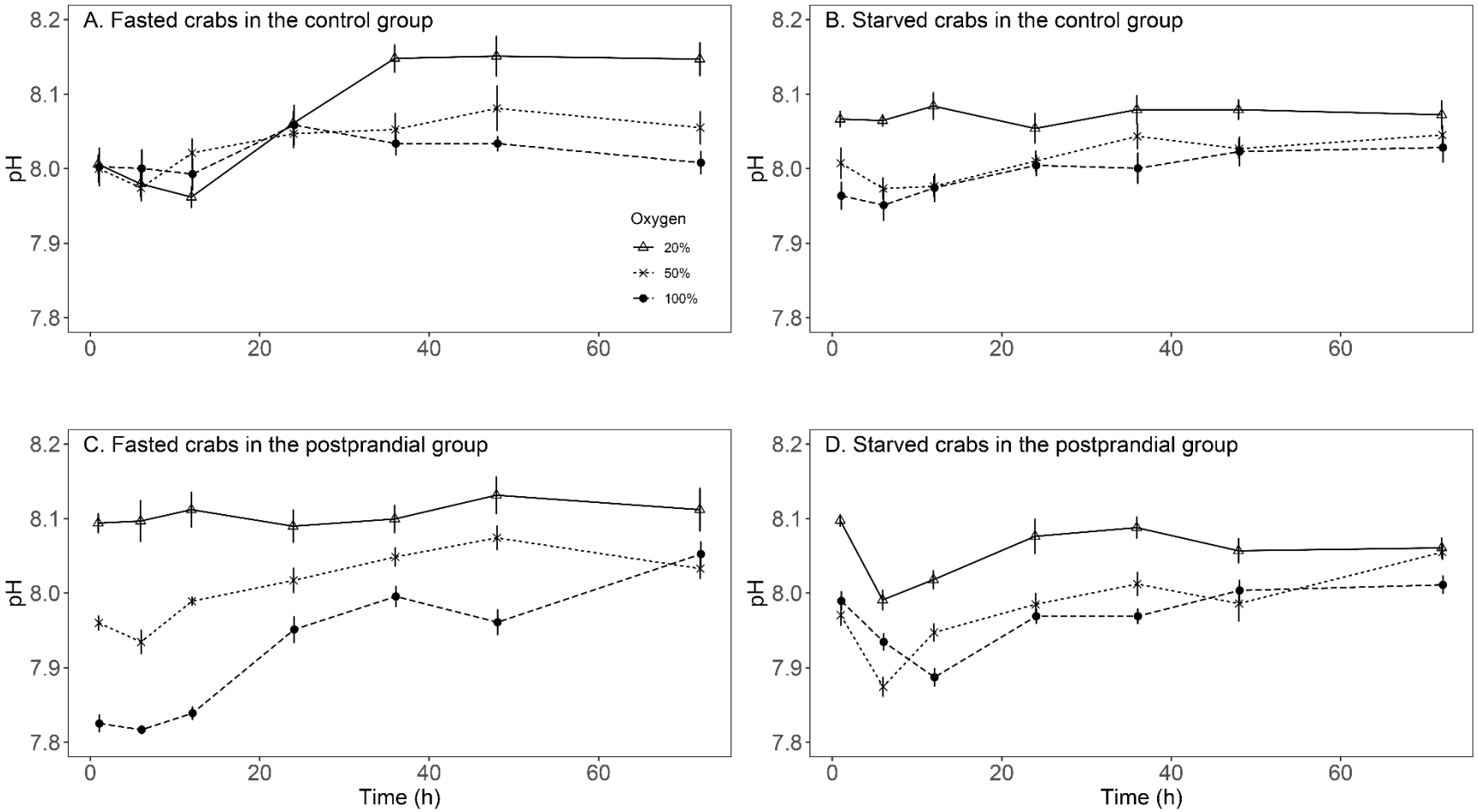

Figure 3.14 The post-branchial pH of the fasted and starved rock crabs in A. unfed fasted crabs, B. unfed starved crabs, C. fed fasted crabs and D. fed starved crabs when exposed to 100%, 50% and 20% oxygen saturations. Data are shown as mean with SEM (N = 8–10).

3.5 Discussion

The present study showed that the level of hypoxic exposure had the largest influence on oxygen transport, anaerobic metabolism and acid-base balance, but there was also a strong interaction with food deprivation and feeding state. Overall, the starved crabs appeared to be less well adapted to tolerate hypoxic exposure.

3.5.1 Effects of repeated sampling

Hemolymph samples were repeatedly withdrawn from each crab (7 in total) during the 72 h experimental period. This repeated experimental procedure was partly dictated by logistics. If individual animals were used for each time point, the experiment would have required the use of over 800 crabs of similar size and condition. This was complicated by the fact that long-term starvation (> 4 weeks) necessitated individual storage to prevent cannibalism and thus limited the number that could be stored at any one time. If different animals were used (70 per treatment), it would spread experiments over many months, complicating results with factors such as reproductive status, molt stage, thermal history or endogenous seasonal changes (Chen and Cheng, 1993; Terwilliger and Brown, 1993). For large crustaceans (> 500g), it is possible to sample individuals repeatedly (Racotta and Palacios, 1998; Webster, 1996). The hemolymph volume of crustaceans comprises approximately 25% to 30% of their wet body mass (Gleeson and Paul, 1977; Hurton et al., 2005; McMahon and Wilkens, 1977). In this study, approximately 2% of the total hemolymph volume was removed during each sampling

period. Although decreases in both hemocyanin and L-lactate concentrations were found, likely reflecting extracellular volume adjustments (Taylor and Anstiss, 1999), these decreases were only significantly different from initial levels after 48 h, indicating rock crabs may undergo 5 or 6 repeated sampling events without ill effects. In addition, anemia induced by repeated hemolymph withdrawals may be compensated by hematopoiesis (Söderhäll, 2016). For example, the prawn (*Macrobrachium rosenbergii*) can increase hemocyanin concentration by 7.4% within 24 h and 22.5% after 8 days (Chen and Kou, 1998; Cheng et al., 2003). There is the possibility that repeated handling may have impacted biochemical parameters, as hyperglycemia and elevation of L-lactate occur within 2 h of repeated sampling in penaeid shrimps and green crabs (Mercier et al., 2006; Racotta and Palacios, 1998). However, these variables usually decline within 4-6 h (Wilson et al., 2021). In this study, sampling events were separated by a minimum period of 6 h, which would limit such impacts.

3.5.2 Arterial P_{O2}

The arterial P_{O2} of rock crabs was affected by both the ambient oxygen level and feeding, but there were no apparent effects caused by the food deprivation state. The decrease in arterial P_{O2} as a function of the ambient oxygen level was consistent with other studies: in blue crabs (*Callinectes sapidus*), exposure to 50% oxygen results in a 36% decrease in the arterial P_{O2} (8 to 5.1 kPa), while P_{O2} decreases by 74% (1.2 kPa) in 20% oxygen (Lehtonen and Burnett, 2016). The results are consistent with that below P_{crit} (20% oxygen), the limited oxygen supply led to a significant reduction in MO_2 (Jiang, Chapter

Two). The actual amount of oxygen bound with hemocyanin (Hc oxygen saturation) is a function of arterial P_{O2} (Hc-oxygen equilibrium curve) (McMahon, 2001; Truchot, 1992). The arterial P_{O2} of rock crabs in 100% and 50% oxygen was above the inflection point (5 kPa) of a typical decapod Hc-oxygen equilibrium curve (Figure 3.6) (McMahon and Hankinson, 1993; Wilkes and McMahon, 1982). As a result, it would be likely that oxygen delivery to the tissues would be maintained. A significant drop of arterial P_{O2} occurred below the P_{crit} (20% oxygen), indicating that hyperventilation was not able to maintain oxygen content in the hemolymph (Wilkes and McMahon, 1982) and would likely lead to a significant reduction in Hc-bound oxygen. This assumption is supported by the study of blue crab (*Callinectes sapidus*); when the Hc oxygen saturation is significantly reduced in 20% oxygen, and delivery of oxygen to the tissues is substantially compromised (Lehtonen and Burnett, 2016).

Following feeding, a significant elevation of arterial P_{O2} (approximately 18% increase) was observed in normoxia, suggesting that hyperventilation may help raise oxygen content in the hemolymph to support the elevated postprandial metabolism (McGaw, 2005). This has also been recorded for the green crab (*Carcinus maenas*), where increases in arterial P_{O2} by 45% occur within 4 hours after feeding in normoxia (Mente et al., 2003). In contrast, no noticeable increase of arterial P_{O2} was found in postprandial rock crabs in 50% or 20% oxygen. Despite the fact that an elevation of MO_2 occurs in rock crabs following feeding in hypoxia (Jiang, Chapter Two), this increase in postprandial oxygen consumption must come from other mechanisms: the lower P_{O2} in postprandial venous hemolymph would extract more oxygen from the "venous reserve" (Legeay and

Massabuau, 1999; Lehtonen and Burnett, 2016; Mangum and Weiland, 1975). It could also result from the increased cardiac output, which may accelerate hemolymph circulation and oxygen exchange between hemolymph and tissue cells (Legeay and Massabuau, 1999; McGaw and Reiber, 2000; McMahon, 1988). Increasing Hc oxygen concentration or affinity is another common mechanism to facilitate oxygen transport efficiency (Bridges, 2001; Terwilliger, 2015; Zeis et al., 1992). This is discussed below.

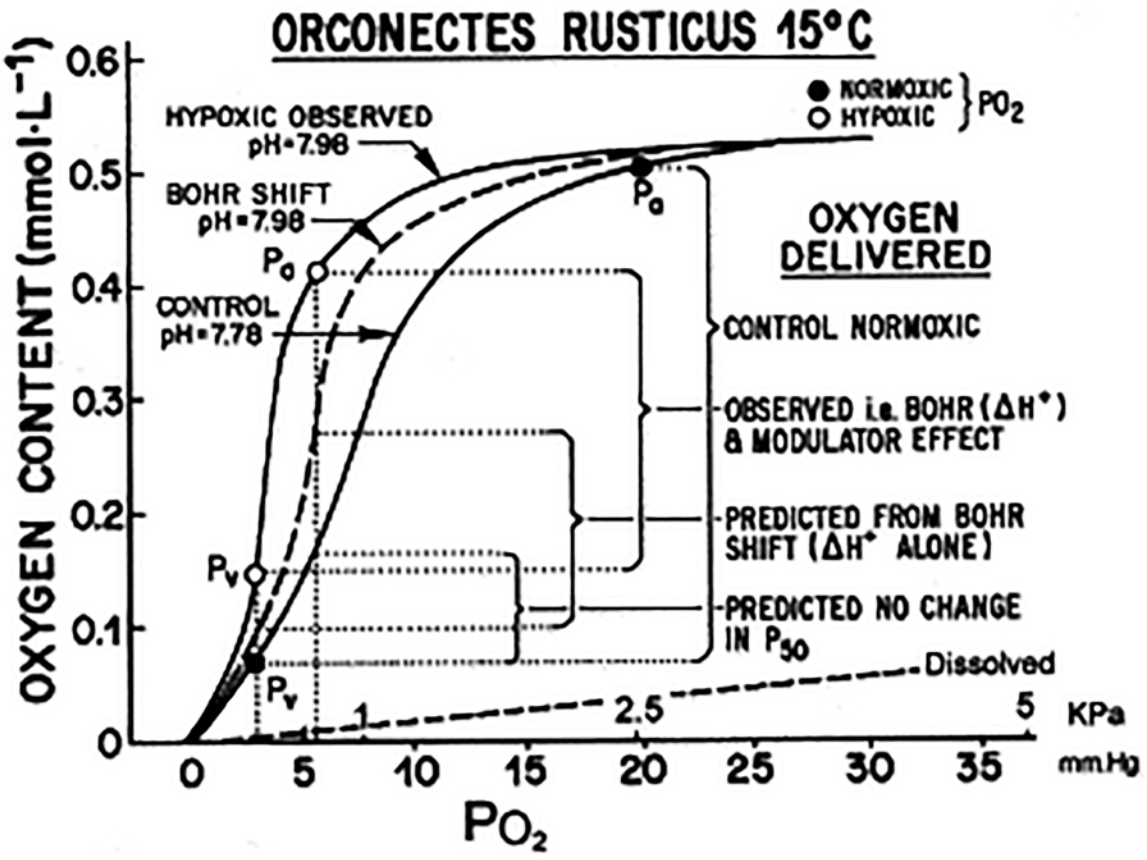

Figure 3.15 The example of typical hemocyanin-oxygen equilibrium curves. Potential factors affect Hc-oxygen binding in *Orconectes rusticus* hemolymph during maintained hypoxic exposure. P_a, partial pressure of O_2 in post-branchial hemolymph; Pv, partial pressure of O_2 in pre-branchial hemolymph. The curve for the Bohr effect was presented to demonstrate the changes of Hc oxygen affinity. The figure was adapted from McMahon (2001).

3.5.3 Hemocyanin concentration

Contrary to the original hypothesis, no significant effect of hypoxia or feeding was found on Hc concentration. This is surprising because a number of studies show hypoxia induces an increase in Hc concentration in crustaceans (Chen and Kou, 1998; Cheng et al., 2003; Defur et al., 1990; Hagerman, 1986; Senkbeil and Wriston, 1981; Tommerdahl et al., 2015). However, these changes may be limited in magnitude and time. For example, in the giant river prawn (*Macrobrachium rosenbergii*), Hc only increases by 7% during 48 h exposure to 20% oxygen (Cheng et al., 2003), and after 8-day hypoxic exposure, Hc is elevated by just 19% (Chen and Kou, 1998). In this study, the 72 h experimental period, coupled with the effects of repeated sampling after 48 h, may simply have been too short of a period to see a significant elevation of Hc.

Although an increase in Hc concentration may aid in oxygen transport, the oxygen carrying capacity of Hc can be improved independently of concentration by allosteric modification or subunit composition in Hc, which further enhances Hc oxygen affinity (Coates and Costa-Paiva, 2020; Terwilliger, 2015). Although we did not characterize the subunit type, the changes of several allosteric modulators during severe hypoxia (20% oxygen) were observed in this study, such as increased pH and L-lactate concentration. Hypoxia-induced alkalosis may result from hyperventilation, which flushes out CO_2 in hemolymph (McMahon, 2001); the subsequent decrease in H^+ concentration enhances the oxygen affinity of Hc within a certain pH interval (Bohr effect) (Bohr et al., 1904; Truchot, 1992). L-lactate also enhances Hc oxygen affinity (Morris, 1990; Truchot, 1980).

In the European lobster (*Homarus vulgaris*), a rise in L-lactate from 0.3 to 4.5 mmol/L causes the P_{50} (hemolymph P_{O2} where Hc is 50% oxygen saturated) to decrease from 0.89 to 0.65 kPa at pH of 7.99 ± 0.03 (Zeis et al., 1992). In this study, the highest L-lactate (3.5 ± 2.5 mmol/L) was observed in the postprandial fasted crabs in 20% oxygen. Although this high level of L-lactate reflected anaerobic respiration, it would also help increase the efficiency of oxygen transport independent of Hc concentration.

The mean initial Hc concentration of starved crabs was 19% lower than that of fasted crabs. This suggests that Hc, the main blood protein reserve, is mobilized as an energy source (Hagerman, 1983; Sacristán et al., 2017). While the decline in Hc here was not as pronounced as the 50% decline in Hc measured in juvenile spiny lobster (*Sagmariasus verreauxi*) after 28 d starvation, it still likely impacted oxygen transport (Simon et al., 2015). This lower Hc concentration of starved crabs probably contributed to the less pronounced increase of MO_2 after feeding compared with postprandial fasted crabs (Figure 2.7, Jiang Chapter Two).

3.5.4 Anaerobic metabolism

Anaerobic metabolism can offset the energy deficit of aerobic metabolism below the P_{crit} (Spicer et al., 2002; Yannicelli et al., 2013). However, unfed rock crabs did not exhibit an increase in L-lactate concentration, even in oxygen levels below their P_{crit} (20% oxygen; Figure 3.4A&C). This result backs up the hypothesis proposed in Chapter Two that there are two different P_{crit} for the rock crab (Pörtner and Grieshaber, 1993): P_{crit}R, where the crabs can downregulate resting metabolic rate by decreasing activity and reserve aerobic

scope without the onset of anaerobic metabolism, and $P_{crit}M$ (true P_{crit}), where the maximum MO_2 is below standard MO_2, and the deficit of aerobic metabolism is compensated by anaerobic metabolism. Here for unfed crabs, 20% oxygen was likely below $P_{crit}R$ but above $P_{crit}M$ (Jiang, Chapter Two). The $P_{crit}R$ likely represents a voluntary shift to aerobic depression, possibly by a decrease in locomotor activity, rather than the inability of the physiological mechanisms to maintain a constant MO_2. In contrast, the $P_{crit}M$ indicates where animals switch from aerobic to anaerobic metabolism and cannot further increase MO_2 (aerobic scope = 1).

Following feeding in 20% oxygen fasted rock crabs resorted to anaerobic respiration as seen by a gradual, but substantial increase in L-lactate acid. By 1 h after feeding, there was no elevation of L-lactate concentration. During this time, mechanical digestion in the foregut would be underway, but very little digesta would have entered the midgut (and hepatopancreas). This indicates that rock crabs were able to balance the oxygen demand of mechanical digestion alone in response to severe hypoxia. This is not unexpected because mechanical activity in the foregut only accounts for between 5% and 25% of the postprandial increase in MO_2 (Carefoot, 1990; Clemens et al., 1998; McGaw, 2008; McGaw and Van Leeuwen, 2017). Thereafter, the L-lactate concentration increased over 24 h; this time period corresponds to the movement of digesta into the midgut and hepatopancreas, where intracellular digestion begins (Jiang, Chapter Four). Intracellular protein synthesis is also well underway during this time (Carter and Mente, 2014; Mente et al., 2003). Since protein synthesis is thought to account for the majority of the increase in MO_2, it suggests that the fed crabs cannot supply enough oxygen to the tissues and

therefore have undergone anaerobic respiration accounting for L-lactate accumulation. Although rock crabs may be able to delay mechanical digestion during hypoxia, once intracellular protein synthesis begins, they may be committed to these processes (McGaw et al., 2009).

Interestingly, the elevated L-lactate concentration of postprandial rock crabs did not cause metabolic acidosis. This indicates that a mild acidosis can be buffered in the hemolymph by modulating their hemolymph bicarbonate levels (Fehsenfeld and Weihrauch, 2017; Wheatly and Henry, 1992; Whiteley, 1999). In addition, without metabolic acidosis, the elevated L-lactate concentration would increase Hc oxygen affinity and assist oxygen transport in severe hypoxia (Morris, 1990; Truchot, 1980).

In contrast to the fed fasted crabs, the fed starved crabs did not exhibit an increase in L-lactate concentration in 20% oxygen. It is possible that starved rock crabs may lack the energy reserves to support fermentation. In crustaceans, mobilization of glycogen from the muscles and hepatopancreas supports anaerobic respiration during hypoxia (da Silva-Castiglioni et al., 2011; Ocampo et al., 2003). However, during long-term starvation, these glycogen stores are significantly reduced (Baden et al., 1994; Sacristán et al., 2017); thus, it could be assumed the starved rock crabs simply do not have enough glucose to fuel anaerobic metabolism (Rich, 2003; Schmidt-Rohr, 2020). An alternative explanation would be that the starved crabs are not processing the food as rapidly, and thus downstream processes such as intracellular digestion and protein synthesis are proceeding at a slower rate, thereby reducing overall oxygen demand (Cervellione et al., 2017). In support of this assumption, starved rock crabs took a longer time to pass food

into and fill the midgut (Jiang, Chapter Four). This was associated with a lower magnitude of postprandial increases in MO_2 (Jiang, Chapter Two). The slower processing of nutrients suggests that starved crabs were able to supply oxygen at a rate that did not require them to resort to anaerobic production of ATP.

3.5.5 Acid-base balance

The hemolymph pH of unfed fasted rock crabs (8.25 ± 0.03 in normoxia) was within the range previously reported for this species and other crustaceans (Fehsenfeld and Weihrauch, 2017; Lehtonen and Burnett, 2016; Wheatly, 1987). In severe hypoxia (20% oxygen), the typical respiratory alkalosis was found in both fasted and starved crabs. This would be associated with hyperventilation resulting in a decrease in P_{CO2} and thus free H^+ ions (Wheatly, 1987; Wheatly and Henry, 1992).

After feeding, there was an immediate acidosis in the hemolymph of rock crabs. This SDA-induced acidosis also occurs in postprandial green crab (*Carcinus maenas*) in normoxia (Legeay and Massabuau, 1999). This is the result of an elevated P_{CO2} associated with increased postprandial oxygen consumption after feeding (Legeay and Massabuau, 1999). The magnitude of this postprandial acidosis was lower in starved crabs compared with fasted crabs, especially in normoxia. This was reflected by the higher postprandial MO_2 (and thus CO_2 production) of fasted crabs in normoxia and hypoxia (Jiang, Chapter Two).

The postprandial acidosis seems contradictory to the commonly reported postprandial “alkaline tide” of vertebrates (Wang et al., 2001; Wood et al., 2005). In

vertebrate digestion, acid secretion in the stomach causes a reciprocal increase in plasma HCO_3^- concentration (Wood, 2019). One rare example of postprandial "acidic tide" is the agastric killifish (*Fundulus heteroclitus*), which lacks a gastric H^+/K^+ ATPase and operates an alkaline digestive system (Babkin and Bowie, 1928; Wood et al., 2010). Although crustaceans employ acid digestion, the pH of the gastric fluid is around 5 to 6, much higher than vertebrates (< 2.5) (Bañuelos-Vargas et al., 2018; Figueiredo et al., 2001; Navarrete del Toro et al., 2011; Saborowski et al., 2004). Thus, for postprandial rock crabs, food stimulates less acid secretion and causes lower hemolymph alkaline tide, which is counteracted by respiratory acidosis (an increased P_{CO2}) and results in a mild postprandial "acidic tide."

The hemolymph pH of starved crabs was more conserved than that of fasted crabs in response to hypoxia and feeding, although starved crabs were expected to have poor acid-base regulation. Although few studies address the effect of starvation on acid-base regulation, the limited energy reserves and depressed MO_2 of starved crabs may indicate the active acid-base regulation is depressed in starved crabs (Whiteley and Taylor, 2015). The active acid-base regulation is an energy-expensive process involving Na^+/K^+-ATPase, V-type H^+-ATPase, Rhesus-like protein and the microtubule network (Fehsenfeld and Weihrauch, 2013; Pörtner et al., 2004; Wang et al., 2018). The acid-base regulation of other aquatic animals can cost over above 50% of the baseline metabolic rate (Wood et al., 2002). Thus, the starved crabs may not have enough energy to utilize active acid-base regulation. Moreover, the starved crabs have low hemolymph proteins, which are important passive hemolymph buffers (Fehsenfeld and Weihrauch, 2017; Henry and

Wheatly, 1992). Therefore, the lower pH fluctuation of starved crabs may result from lowered metabolic activities (postprandial SDA and hypoxia-induced hyperventilation) compared with fasted crabs. Further studies are needed to investigate the effect of food deprivation on acid-base regulation such as the role of hyperglycemic hormone (Chen et al., 2020) and pH buffers like bicarbonate and ammonia (Fehsenfeld and Weihrauch, 2017; Weihrauch and Allen, 2018).

3.5.6 Conclusion

The ambient oxygen level and a lesser degree food deprivation state affected oxygen transport ability, anaerobic metabolism as well as acid-base balance before and after feeding in the rock crab (*Cancer irroratus*). The higher Hc concentration of fasted crabs suggested a better oxygen transport capacity than starved crabs. Although the experimental period (72 h) may be too short to observe a significant increase in hemocyanin concentration, hypoxia-induced alkalosis and elevated lactate improved the Hc oxygen affinity. These changes, along with anaerobic metabolism, support the elevated oxygen demand of SDA of fasted crabs in severe hypoxia (Jiang, Chapter Two). In contrast, postprandial starved crabs did not undergo anaerobic metabolism in 20% oxygen, suggesting a lower postprandial oxygen demand. This likely occurred because digestive processes were regained more slowly during upregulation of the previously dormant digestive system (Jiang, Chapter Four).

3.6 References

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Chapter 4. Effects of food deprivation state on feeding behavior and gastric evacuation of rock crabs during hypoxia[1]

[1]A modified version of this manuscript has been submitted to Marine and Freshwater Behaviour and Physiology.

4.1 Abstract

Climate change and an increase in anthropological activities have led to an expansion of hypoxia into the natural habitat of Atlantic rock crabs (*Cancer irroratus*). In this study, we examined the effects of hypoxia (100%, 50% and 20% oxygen) and food deprivation state (starved – 28 days, fasted – 3 days) on food intake and the subsequent gastric processing of the meal. Three different techniques were used to measure food intake. The gravimetric analysis of dry food was the most accurate method, while the gravimetric analysis of wet food overestimated food intake and counting radiopaque beads in the food using a fluoroscope underestimated food intake. During exposure to severe hypoxia (20% oxygen), rock crabs reduced food intake, and a higher percentage of crabs refused to eat. Although food deprivation state did not significantly affect food intake, more starved crabs fed in the lowest oxygen regime. The subsequent digestion of the meal was also affected by the oxygen level; here, prolonged gastric emptying times paralleled previously measured changes in postprandial oxygen consumption in hypoxia. Starved crabs also exhibited slightly longer transit times of digesta compared with fasted crabs. This was likely due to the lower energy stores and a gradual upregulation of dormant digestive organs and enzymes. These results suggest that although a trade-off may occur in starved rock crabs between the need to procure nutrients and deal with hypoxic stress, impaired digestive processing may still have a deleterious effect on these animals.

Keywords: crab, hypoxia, starvation, behavior, feeding, gastric processing

4.2 Introduction

Marine hypoxia has become much more common during the last few decades (Diaz and Rosenberg, 2008; Riedel et al., 2012). The adverse effects of global warming (reduced oxygen solubility and enhanced stratification) contribute to deoxygenation in marine environments (Watson, 2016). Additionally, both natural fluctuation (upwelling) and anthropologic eutrophication exacerbate the current scenario of ocean deoxygenation in coastal waters (Breitburg et al., 2018; Helly and Levin, 2004; Watson, 2016). As a result, both the frequency and duration of hypoxic zones have been and will continue to expand (Conley et al., 2009; Keeling et al., 2010). Thus, it becomes imperative to study the possible impacts of hypoxic events on marine animals (Ekau et al., 2010; Gray et al., 2002; Spicer, 2014; Wu, 2002). Oxygen is essential for the energy-costly activities of aerobic organisms (Kooijman, 2010) as such frequent exposure to hypoxia could retard the growth, reproduction and overall fitness of marine organisms (Galic et al., 2019; Wang et al., 2009). These effects generally result from reduced food intake associated with impaired foraging and feeding behaviors in response to hypoxia (Brante and Hughes, 2001; Carlos et al., 1998; Das and Stickle, 1993; Seidman and Lawrence, 1985; Wang et al., 2009). Decapod crustaceans are reported to be among some of the most sensitive of organisms to changing oxygen levels (Vaquer-Sunyer and Duarte, 2008) and therefore make good indicator species for the possible effects of changes in environmental hypoxia.

Although crustaceans may be able to sense oxygen levels and, in some cases, use behavioral avoidance, when hypoxia is widespread, orientation in response to or

avoidance of these areas is not possible (Bell et al., 2003a; de Lima et al., 2021; Froehlich et al., 2014). Depending on the magnitude and time of the hypoxic event, this may impact foraging and feeding behaviors. The process of feeding (in normoxia) is associated with an increase in foraging activity and food handling, which can elevate MO_2 over two-fold (McGaw and Van Leeuwen, 2017; Weissburg and Zimmer-Faust, 1994). Thus, the mismatch between the increased oxygen requirement for feeding and reduced oxygen availability in hypoxia will likely impact foraging activities. For example, in hypoxic conditions, prey-handling time (breaking bivalve shells) of shore crabs (*Carcinus maenas* and *C. aestuarii*) increases 2–3 fold, resulting in reduced prey-handling efficiency (Brante and Hughes, 2001; Mistri, 2004). This leads to a decrease in actual feeding time and food consumption rates (Bell et al., 2003b; Bernatis et al., 2007; Das and Stickle, 1993).

In crustaceans, the subsequent digestion and assimilation of a meal are also energetically costly, and postprandial crustaceans typically exhibit a 2–4-fold increase in metabolism, termed the specific dynamic action (McCue, 2006; Secor, 2009). In the event that digestion occurs during hypoxia, crustaceans will need to balance the extra demand for oxygen consumption at a time when the external oxygen supply is limited. Postprandial crustaceans may attempt to maximize digestive efficiency during hypoxia by reducing locomotor activity, thereby optimizing oxygen delivery to the digestive system or settling in areas with higher oxygen levels (Bell et al., 2003b; Bernatis et al., 2007). However, this is not always possible, and ultimately, the inability to balance the demands of physiological systems during hypoxia may lead to a reduction in gastric processing (McGaw, 2008). Briefly, ingested food is processed in three functional areas (Johnston,

2007; McGaw and Curtis, 2013b). The foregut houses the gastric mill, which further masticates the food. Digesta then passes from the foregut into the midgut entering the hepatopancreas via the pyloric caeca, where intracellular digestion begins. Finally, the waste is encapsulated in a peritrophic membrane, and rhythmic peristaltic movements of the hindgut expel the feces (Mercier and Lee, 2002; Musolf et al., 2009). The gastric system is innervated by the stomatogastric nervous system, and during hypoxia, low arterial oxygen levels act in a neuromodulatory fashion on the stomatogastric nervous system, which leads to a slowing and uncoupling of the pyloric and gastric pattern generator rhythms (Clemens et al., 1998; McGaw, 2008; McGaw and Curtis, 2013b). This results in an increase in the transit time of digesta through the foregut and midgut regions and ultimately slowing of delivery of processed nutrients to the cells.

Although foraging activities and food ingestion are curtailed during hypoxia, long-term starvation may impart an interactive effect. Hungry crabs are more persistent and spend more time trying to access their prey, such as clams (Hughes and Seed, 1995; Sun et al., 2015). They may also become more aggressive for food, showing intraguild predation (killing and sometimes eating a potential competitor of a different species) during foraging (Hazlett, 1966; Liu et al., 2019; Liu et al., 2017). Moreover, a trade-off behavior occurs where starved crustaceans are more likely to move into areas that are physiologically stressful in order in order to access food (Curtis and McGaw, 2012; McKillup and McKillup, 1994). For example, the likelihood that Dungeness crabs (*Cancer magister*) will feed in low salinity increases with starvation (Curtis et al., 2010). This suggests that the aversion to food uptake in physiologically stressful conditions may be

overridden by the need to procure nutrients (Curtis et al., 2010). In addition, degradation of the gastric system occurs during long-term starvation, which may impair the digestive function during refeeding (Cervellione et al., 2017; Sonakowska et al., 2016). In other starved aquatic ectotherms, the degraded gastric system leads to prolonged digestive processes during refeeding (Bar and Volkoff, 2012; Talbot et al., 1984). However, it is still unclear how long-term starvation may influence feeding and the subsequent gastric processes of crustaceans in hypoxic conditions.

The Atlantic rock crab *Cancer irroratus* is a common benthic crustacean along the Atlantic coast of North America and is distributed from the shallow subtidal zone down to depths of 390 m (Haefner Jr, 1976). *Cancer irroratus* usually occurs on rocky bottoms with macroalgae, where they can easily find shelter (Haefner Jr, 1976; Hudon and Lamarche, 1989). This species is an important predator on polychaetes, bivalves, echinoderms and small crustaceans (DFO, 2014; Gendron et al., 2001; Scarratt and Lowe, 1972). Under the background of global climate change, hypoxic areas in coastal waters have expanded to the natural habitat of rock crabs (Levin et al., 2009). For example, the oxygen concentration in the bottom waters in the Gulf of St. Lawrence declined to 65 μmol/L (20.7% saturation at 15°C) (Gilbert et al., 2005). As these hypoxic events have become more common, they may impact both the feeding behavior and digestive physiology of this shallow water decapod.

My recent work showed that the level of hypoxia affected the postprandial metabolism and oxygen delivery in rock crabs (Jiang, Chapter Two & Three). Overall, hypoxia reduced the magnitude but prolonged the duration that MO_2 remained elevated.

In response to severe hypoxia (20% oxygen), the respiratory alkalosis and elevated L-lactate in hemolymph may facilitate hemocyanin-oxygen affinity of postprandial crabs, enabling more efficient oxygen delivery to the tissues. These responses were further influenced by the food deprivation state and tended to be less pronounced in starved crabs. Given that both the levels of hypoxia and food deprivation state influence metabolic responses, the possible effects of hypoxia and food deprivation state on feeding behaviors and gastric processing were studied in this chapter. To achieve these goals, the food intake of fasted and starved rock crabs was measured using different methods in oxygen concentrations above and below the P_{crit}. The subsequent processing of the meal through the entire digestive system was measured in the same oxygen levels using a fluoroscope.

4.3 Material and Methods

4.3.1 Crab collection and housing

Male and female rock crabs (*Cancer irroratus*) were trapped in multiple locations along the coast of the northern Avalon Peninsula, Newfoundland, Canada. Crabs (100 to 180 g) in the hard intermolt stage that showed no obvious physical damage or weakness, and females bearing no eggs were retained. The crabs were transferred to the Department of Ocean Sciences at Memorial University, St John's, NL, Canada, and held in 400 L sediment-free tanks with flow-through aerated seawater at ambient temperatures (4–10°C) and salinity (30–32‰). The crabs were fed chopped herring (*Clupea harengus*) to excess once a week, and any uneaten food was removed on the same day to avoid

fouling the water. At least two weeks before the experiments started, crabs were acclimated in the laboratory tanks (300 L) in flow-through aerated seawater (salinity 30–32‰) at 12–13°C and constant dim red light. A two-week period is the minimum time to effectively acclimate the crabs to experimental temperatures and to abolish any underlying locomotor rhythms (Taylor et al., 1977). Crabs of both sexes were chosen indiscriminately in each treatment at the approximate ratio of collection (male: female, 3:1).

In this study, crabs in two food deprivation states were used, each with specific physiological attributes:

a) Starved crabs were held in individual perforated plastic chambers (25 × 15 × 7.5 cm) to prevent cannibalism at 12–13°C and starved for 4–5 weeks. The starved crabs have lower resting metabolic rates (Ansell, 1973; Wallace, 1973) and reduced blood protein levels (and hence hemocyanin levels), and utilize body proteins for energy (Sacristán et al., 2017).

b) Fasted crabs were deprived of food for 3–5 days before experiments began, which allowed them to evacuate all food from the digestive system. Studies provide evidence to suggest fasted crabs in the current study without the effects of prolonged starvation or digestion (Ansell, 1973; McGaw and Curtis, 2013; Wallace, 1973).

4.3.2 Experimental design

All experiments were conducted in a small tank (150 L) at 12–13°C in dim red light. During experiments, the crabs were individually housed in plastic chambers (25 × 15 × 7.5

cm) with 1 mm mesh screens on the sides. Three levels of hypoxia were used in experiments: an oxygen saturation level of 100% was used as a normoxic control; 50% was chosen as mild hypoxia since it was above the P_{crit} of starved and fasted crabs, while severe hypoxia of 20% oxygen saturation was below the P_{crit} of the two groups (Jiang, Chapter Two). The desired oxygen level was maintained using an oxygen regulator (Loligo System, Viborg, Denmark) connected to an air source and a nitrogen cylinder. For reference, 100% oxygen saturation is approximately 21 kPa.

4.3.2.1 Feeding behaviors

Three independent measurements, gravimetric analyses (wet shrimp muscle and dry commercial food pellets) and radiographic analysis (food with radiopaque ballotini beads), were used to determine food intake of fasted and starved crabs in three oxygen levels (100%, 50% and 20%). Shrimp muscle was used as the wet food, and it was soaked in seawater at 4°C overnight to reduce any changes associated with water loss or gain. For dry food, the crabs were fed sinking commercial food pellets (3.5 g, KYORIN ltd., Himeji, Japan). For gravimetric analyses of wet and dry food, following feeding on a known mass, the uneaten food remains were weighed in order to calculate the mass consumed. The radiopaque food was made of 90% (by mass) ground shrimp muscle and 9.6% gelatin solution evenly mixed with 0.4% glass ballotini beads (Barium Titanate, 200 um diameter) which acted as the radiopaque marker. Before being offered to the crabs, five 1-g subsamples were removed from the food, and the number of individual ballotini beads in each sample was counted using a LIXI PS500 OS X-ray fluoroscope. The mean number of beads per gram of food was used to calculate the amount of food consumed (Rayner and

McGaw, 2019). For X-ray analysis, an opaque plastic box (15 × 10 × 8 cm) was submerged in the chamber, and the crab was coaxed into the box. The box was sealed and placed in the X-ray fluoroscope, and a still image was captured. The number of individual ballotini beads was counted, allowing calculation of the consumed food mass.

In each food intake measurement method, 11–13 fasted and starved crabs (total sample size = 73 for wet food, 68 for dry food and 67 for X-ray food) were acclimated overnight in each oxygen level (100%, 50% or 20%). Then, they were offered an excessive amount of pre-weighed food (wet, dry or radiopaque food) and were allowed to eat for 1 h. Once they finished eating, the uneaten food was collected by pouring the remains through filter paper. Any crabs that did not feed in each treatment were noted. The collected food remains were patted dry with paper towels to remove excess water and then weighed. Control samples of food were put into an empty chamber in the same tank, they were weighed before and after the experiment, and any change in mass was utilized as a correction factor when calculating the final mass eaten. Because the different food types had different water content, the samples were dried to a constant weight at 60°C, before being reweighed. The consumed food mass was then expressed as a percentage of dry food mass as a function of the wet body mass of crabs.

In order to check the accuracy of the above methods, the gastric system of each crab was excised, and the gut contents were collected. To carry out this procedure, the crabs were immediately removed from the tank following feeding and were immersed in iced seawater for 5 min to induce a cold shock coma before being frozen and stored at -20°C

for later analysis. The frozen animals were thawed at room temperature, and the foregut was dissected out and opened. The contents were removed, rinsed with distilled water, and then dried to constant weight at 60°C. The calculated amount of wet, dry and radiopaque food eaten was then plotted against the actual mass (gut contents). In each method, the amount of food consumed was expressed as dry mass eaten as a percentage of the wet mass of the crab.

4.3.2.2 Gastric processing

The gastric evacuation rates of fasted and starved crabs were followed in real-time. The crabs were fed a radiopaque meal consisting of 75% (by mass) of ground shrimp muscle and 10% gelatin solution with 15% barium sulfate as a radiopaque marker (McGaw, 2006). Ballotini-labeled food and iron powder were also tested as candidates for radiopaque markers in preliminary experiments. Compared with the other two radiopaque foods, barium meals had no apparent adverse influence on the appetite of rock crabs (McGaw, 2007). The use of ballotini-labeled to track gastric processing was less efficient as some of the beads became trapped by the pyloric setal filter of the foregut. The iron powder was also tested but rapidly oxidized in seawater and thus may have interacted with the digestive system.

During the experiments, the animals (n = 8–10 per treatment, total sample size = 53) were individually housed in perforated plastic chambers (25 × 15 × 7.5 cm) and were left to settle overnight in 100% oxygen (approximately 21 kPa) saturation at 12–13°C and constant dim light. The crabs were then offered the radiopaque meal of 2% of their body

mass (in 100% oxygen); those that did not consume the entire meal were excluded from the analysis. Once the crab had finished the meal (approximately 20 min), the oxygen concentration was changed to the target value (100%, 50% or 20%). For X-ray readings, a black plastic box was submerged in the chamber, and the crab was gently coaxed into the box. The box was sealed and placed in a LIXI PS500 OS X-ray machine. Technical specifications for the X-ray were 35 kV tube voltage and 155µA tube current with a 5-cm focal window (McGaw 2006). A still X-ray image of the digestive system was captured at hourly intervals for the first 8 h, at 2-h intervals between 8 h and 12 h, then at 4-h intervals between 12 h and 24 h, and at 8-h intervals thereafter, until all digesta evacuated from the system. At each interval, the amount of digesta in the foregut, midgut, and hindgut regions was estimated by outlining the boundaries of each gut region and determining the percent fullness. The emptying time of the different gut regions was also noted (McGaw, 2006).

4.3.3 Data analysis

All statistical tests were conducted in R (Team, 2018). The normality of data was checked by a Shapiro-Wilks test. For these data that did not pass the normality test, a Box-Cox transformation was applied (λ value was reported). A Bartlett test was performed to check the homoscedasticity of data. In order to deal with heterogeneity, variance structures were added in model fitting, and the best model was achieved by likelihood ratio tests with Akaike's information criterion (AIC). Tukey-adjusted *post hoc* tests were carried out using the 'emmeans' package. Statistical significance was accepted when $p <$

0.05. All data in the figures and tables were shown in means ± standard error of the mean (SEM).

For feeding behaviors, because a large proportion of the crabs did not consume any food in the severe hypoxia treatments (Table 4.1), the zero-inflated negative binomial model (in R package 'pscl') with logit link (Zuur et al., 2009) was used to analyze the effects of the oxygen level and food deprivation state on the food intake, for the three food types independently. This model could deal with overdispersion due to zero inflation. It contains two sub-models: a logistic model and a count model. The logistic model handles the probability of excessive zeros, and the count model deals with the observed variable (food intake dry mass ratios were transformed to integers as parts per ten thousand) in the function of explanatory variables (the oxygen level and food deprivation state) with the consideration of the excessive zeros estimated by the logistic model. The significance of each explanatory factor was estimated by comparing the full model with the model dropping each factor using the likelihood ratio test (in R package 'lmtest'). Then the accuracy of three food measurement methods was assessed by comparing the calculated values and the gut contents of each crab with two-tailed paired t-tests.

For the gastric evacuation experiments, differences in the fullness of each gut section over time were analyzed using linear mixed-effects models (in R package 'nlme'). The fixed factors were the oxygen level, food deprivation state and time. Each individual crab was treated as a random factor. The emptying times of each section of the gut were

analyzed using generalized least square fitted linear models (in R package 'nlme'), and oxygen level and food deprivation state were analyzed as independent variables.

4.4 Results

4.4.1 Feeding behaviors

4.4.1.1 Wet food

When examining the amount of wet food (shrimp muscle) consumed, the count model showed no significant effects of the food deprivation state or the oxygen level on the amount of food consumed. The amount of food consumed (consumed dry food mass as a percent wet body mass) by the fasted and starved crabs was 0.36 ± 0.05% BM and 0.39 ± 0.03% respectively in 100% oxygen. During exposure to 50% oxygen, fasted and starved crabs consumed 0.40 ± 0.03% and 0.31 ± 0.05%; these levels were not significantly different from those measured in 100% oxygen. In 20% oxygen, the fasted crabs consumed a slightly lower amount of shrimp (0.24 ± 0.04% BM), while the starved crabs maintained their consumption amount (0.31 ± 0.06% BM) in 20% oxygen (Figure 4.1A). Although it appeared that a higher number of the fasted crabs refused to feed (Table 4.1), the logistic model showed that the number of crabs that initiate feeding (both fasted and starved) was only significantly affected by the oxygen level (likelihood ratio test, df = 9, X^2 = 10.9, p = 0.03). More crabs refused the wet food in 20% oxygen compared with the two higher oxygen levels (Table 4.1).

4.4.1.2 Dry food

The amount of dry food consumed by the crabs was significantly affected the oxygen level (Figure 4.1B) (likelihood ratio test, df = 9, X^2 = 9.9, p = 0.04). The fasted crabs consumed 0.63 ± 0.11% BM in 100% oxygen. The amount consumed was similar in 50% oxygen, while in 20% oxygen, their food intake dropped significantly to 0.25 ± 0.09% BM (Tukey's HSD, t = 3.0, p = 0.007). A similar pattern was found for the starved crabs: the food intake was 0.73 ± 0.11% in 100% oxygen; despite an apparent increase to 0.93 ± 0.07% in 50% oxygen, this proved to be statistically insignificant. There was a significant decrease in food intake (0.43 ± 0.09%) in 20% oxygen (Tukey's HSD, t = 4.3, p = 0.0001). Although the starved crabs had a slightly higher food intake than the fasted crabs in all three oxygen levels (Figure 4.1B), these differences proved to be statistically insignificant. The logistic model showed that the oxygen level significantly affected the percentage of the crabs that refused to feed (likelihood ratio test, df = 9, X^2 = 16.7, p = 0.002). The highest number of crabs refused to feed in 20% (Table 4.1). Although there was a trend for a greater number of fasted crabs to refuse food in 20% oxygen (65% for the fasted crabs and 33% for the starved crabs), the overall effect of food deprivation state was not statistically significant (likelihood ratio test, df=10, X^2 = 5.7, p = 0.13).

4.4.1.3 Radiopaque food

There were significant effects of both the oxygen level and food deprivation state when food intake was measured using radiopaque food (likelihood ratio test, oxygen level: df = 9, X^2 = 16.1, p = 0.003; food deprivation state: df = 10, X^2 = 27.8, p < 0.001). The fasted crabs maintained the amount of food eaten between 0.25 ± 0.03% to 0.27 ± 0.03% in 100% and 50% oxygen, while the amount consumed dropped to 0.15 ± 0.04% in 20% oxygen

(Tukey's HSD, t = 2.3, p = 0.049). In contrast, the food intake of the starved crabs was maintained between 0.15 ± 0.02% and 0.21 ± 0.04% in all three oxygen levels. The amount of food consumed by the fasted crabs was significantly higher than the starved crabs in 100% and 50% oxygen (Tukey's HSD tests: 100% oxygen, t = 3.2, p = 0.002; 50% oxygen, t = 2.1, p = 0.03). This difference disappeared in 20%, which created a significant interaction between the oxygen level and food deprivation state (likelihood ratio test, df = 11, X^2 = 7.0, p = 0.03).

Despite the fact that slightly more fasted crabs (50%) than the starved crabs (20%) refused to eat the radiopaque food in 20% oxygen (Table 4.1), the logistic model showed that neither oxygen level nor food deprivation state had any statistically significant effect.

Table 4.8 The percentage of the fasted and starved crabs that refused to eat the three food types in three oxygen saturations.

Food Deprivation	Oxygen Level	Wet food	Dry food	Radiopaque food
Fasted	**100%**	10%	20%	21%
	50%	0%	10%	10%
	20%	37%	65%	50%
Starved	**100%**	0%	10%	11%
	50%	22%	0%	20%
	20%	25%	33%	20%

Table 4.9 The likelihood ratio tests and Akaike information criterions (AICs), when each factor was removed from the original zero-inflated negative binomial model for the wet food consuming amount. Bold font indicates statistical significance ($p < 0.05$).

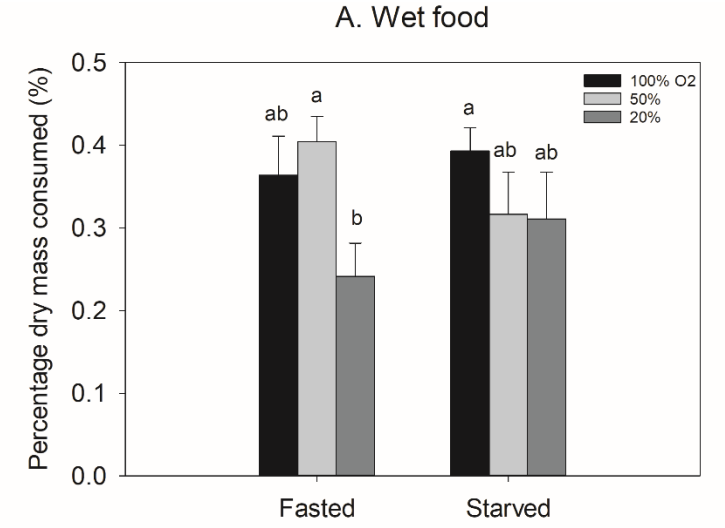
	Dropped term	**df**	**AIC**	**Likelihood ratio test**	
	None	13	514.8		
Count Model	Oxygen	9	509.1	X^2=2.27	p=0.686
	Food deprivation	10	511.0	X^2=2.16	p=0.540
	Oxygen: Food deprivation	11	512.8	X^2=1.96	p=0.376
Logistic Model	**Oxygen**	**9**	**513.1**	**X^2=10.94**	**p=0.027**
	Food deprivation	10	517.8	X^2=4.27	p=0.234
	Oxygen: Food deprivation	11	515.1	X^2=4.24	p=0.120

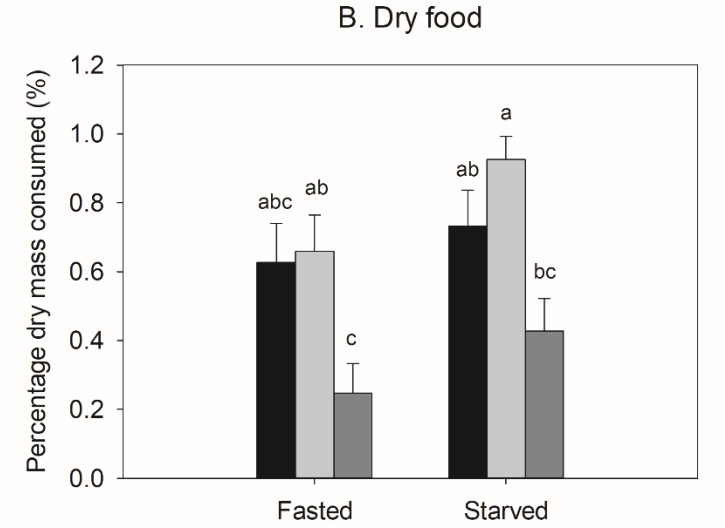
Table 4.10 The likelihood ratio tests and Akaike information criterions (AICs), when each factor was removed from the original zero-inflated negative binomial model for the dry food consuming amount. Bold font indicates statistical significance ($p < 0.05$).

	Dropped term	**df**	**AIC**	**Likelihood ratio test**	
	None	13	493.4		
Count Model	**Oxygen level**	**9**	**495.2**	**X^2=9.89**	**p=0.04**
	Food deprivation	10	492.9	X^2=5.50	p=0.14
	Oxygen: Food deprivation	11	494.7	X^2=5.30	p=0.07
Logistic Model	**Oxygen level**	**9**	**502.1**	**X^2=16.72**	**p=0.002**
	Food deprivation	10	493.0	X^2=5.69	p=0.13
	Oxygen: Food deprivation	11	490.2	X^2=0.86	p=0.65

Table 4.11 The likelihood ratio tests and Akaike information criterions (AICs), when each factor was removed from the original zero-inflated negative binomial model for the radiopaque food consuming amount. Bold font indicates statistical significance ($p < 0.05$).

	Dropped term	**df**	**AIC**	**Likelihood ratio test**	
	None	13	410.0		
Count Model	**Oxygen level**	**9**	**418,0**	**X^2=16.07**	**p=0.003**
	Food deprivation	**10**	**431.8**	**X^2=27.81**	**p<0.001**
	Oxygen: Food deprivation	**11**	**413.0**	**X^2=7.02**	**p=0.03**
Logistic Model	Oxygen level	9	410.6	X^2=8.60	p=0.07
	Food deprivation	10	406.9	X^2=2.90	p=0.41
	Oxygen: Food deprivation	11	408.3	X^2=2.42	p=0.31


A. Wet food
Percentage dry mass consumed (%)
0.5
0.4
0.3
0.2
0.1
0.0
100% O2
50%
20%
ab
a
b
a
ab
ab
Fasted
Starved


B. Dry food
Percentage dry mass consumed (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
abc
ab
c
ab
a
bc
Fasted
Starved

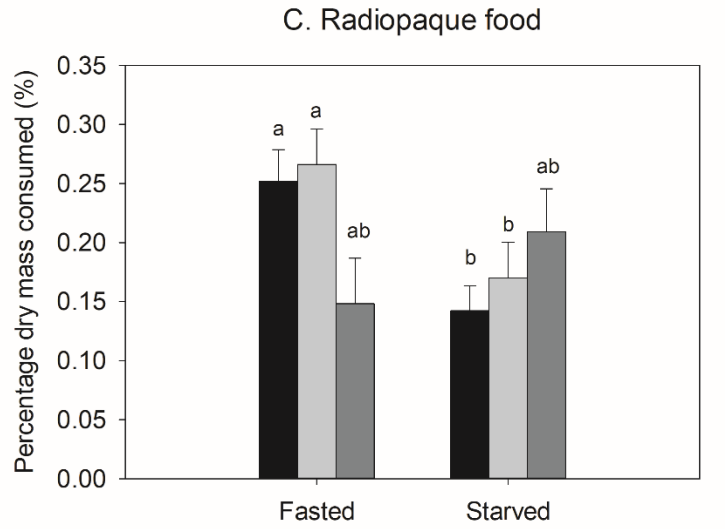

C. Radiopaque food
Percentage dry mass consumed (%)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
a
ab
b
b
ab
Fasted
Starved

Figure 4.16 The food intake of fasted and starved crabs in three oxygen levels (100%, 50%, and 20%): A. wet food, B. dry food and C. radiopaque food. Data represents the mean ± SEM (N = 11–13). Different letters indicate significant differences ($p < 0.05$) between treatments.

4.4.2 Accuracy and comparison of the three methods

The accuracy of the three different methods for measuring the amount of food consumed was tested by dissecting the foregut of the postprandial crabs and weighing the actual amount of food consumed (Figure 4.2). The food consumption calculated by weighing the remains of uneaten wet shrimp muscle produced significantly higher values than weighing the gut contents (two-tailed paired t-test, $t = 4.9$, $p < 0.001$). The actual mass of food consumed (gut contents) was approximately 14.0% lower than the calculated amount. For the dry food, no significant difference was found between the calculated mass of food consumed and the actual gut contents (two-tailed paired t-test, $t = 0.9$, $p = 0.35$). In contrast, the radiopaque food method significantly underestimated the amount of food consumed when compared with the gut contents (two-tailed paired t-test, $t = -2.4$, $p = 0.02$). The radiopaque calculation method produced values that were 7.5% lower than the actual mass of the gut contents. When comparing the amount of food consumed using the three food types (using gut contents), the crabs consumed significantly more dry food (0.57 ± 0.06%) than the other two food types (generalized least squares model, $F_{2,101} = 25.38$, $p < 0.001$); there was no significant difference in the amount of food consumed between the wet and radiopaque food.

Table 4.12 Accuracy of three measuring methods of food intake compared with dissection. Bold font indicates statistical significance of paired t-test ($p < 0.05$).

Method	*df*	*t*	*P*-value
wet	**60**	**4.93**	**<0.001**
Dry	50	0.94	0.354
Radiopaque	**51**	**-2.39**	**0.021**

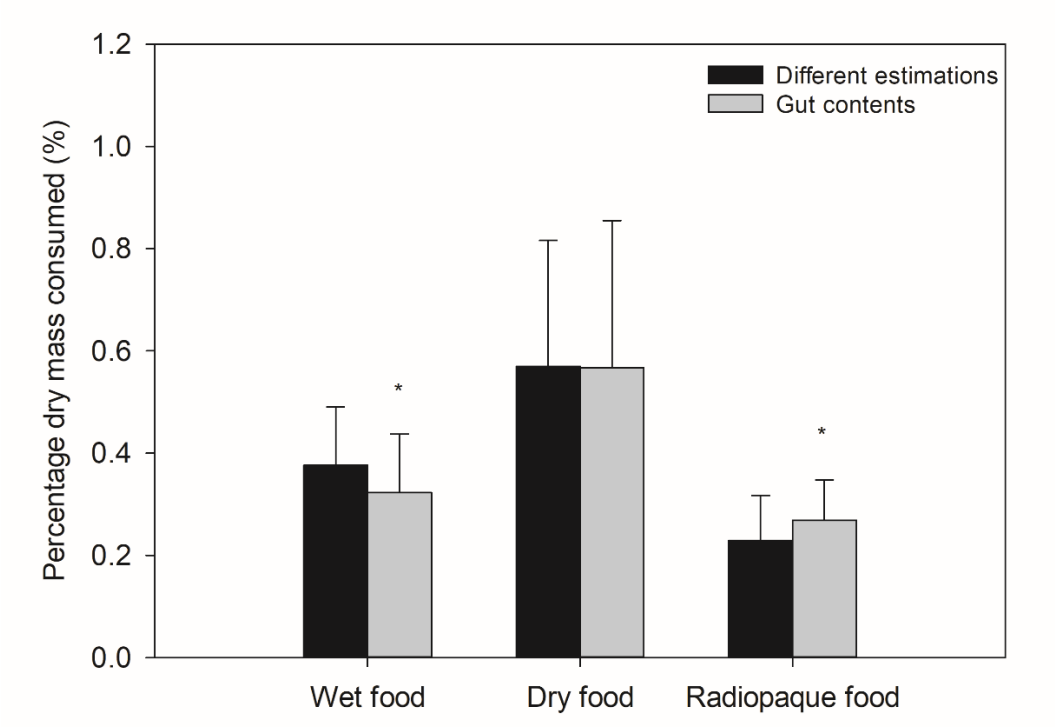


Figure 4.17 Comparison of accuracy of the food intake estimated by three different food types with subsequent analysis of gut contents. Data represent the mean ± SEM (N = 67–73). Asterisk indicates significant differences within a food type ($p < 0.05$).

4.4.3 Gastric processing

4.4.3.1 Foregut

The digesta started to exit the foregut within 1 h after feeding (Figure 4.3) and continuously moved through the midgut over time (linear mixed-effects models, $F_{9,439}$ = 435.0, $p < 0.001$). There were significant differences in the foregut fullness as a function of the oxygen level (linear mixed-effects models, $F_{2,49}$=14.7, $p<0.001$). For the fasted crabs in 100% oxygen, over half of the digesta had moved out of the foregut after 4 h. In contrast, a slower rate of foregut clearance occurred in 20% oxygen, with 66.4 ± 8.0% of the digesta remaining in the foregut at this time (Tukey's HSD, t = 3.3, p = 0.004). After 6 h, the fasted crabs in 100% oxygen had almost cleared the foregut, while a significant divergence in foregut fullness in 50% and 20% oxygen also occurred at this time (Tukey's HSD, t = 2.7, p = 0.02).

A similar pattern was observed for the starved crabs. There was no significant difference in the foregut fullness between 100% and 50% oxygen during the entire experimental period. In contrast, evacuation of digesta from foregut was slower in 20% oxygen, and differences between crabs maintained in 100% and 20% oxygen became apparent after 5 h (Tukey's HSD, t = 3.17, p = 0.007). After 8 h, differences of foregut fullness between crabs maintained in 50% and 20% oxygen also became significant (Tukey's HSD, t = 2.7, p = 0.03).

The fasted crabs processed digesta in foregut more rapidly than the starved crabs in both 100% and 50% oxygen, with significant differences observed after 4 h in 100% oxygen (Tukey's HSD, t = 2.4 p = 0.02) and in 50% oxygen (Tukey's HSD, t = 2.2, p = 0.04).

No significant differences were found in the percentage of digesta remaining in the foregut of the fasted and starved crabs in 20% oxygen.

Hypoxic exposure also increased the time for the foregut to completely empty (generalized least squares model, $F_{2,47} = 13.6$, $p < 0.001$, Figure 4.4A). For the fasted crabs, in 100% oxygen, it took 6.6 ± 0.5 h to empty the foregut. There was an apparent, but statistically insignificant increase (9.0 ± 1.2 h) in emptying time in 50% oxygen. Meanwhile, the emptying time significantly increased to 14.4 ± 3.0 h in 20% oxygen (Tukey's HSD, $t = 3.8$, $p < 0.001$). A similar pattern was found in the starved crabs, with foregut emptying time increased significantly from 9.3 ± 1.3 h in 100% oxygen to 16.3 ± 1.6 h in 20% oxygen (Tukey's HSD, $t = 3.5$, $p = 0.003$). However, the food deprivation state did not significantly affect the foregut emptying time (Figure 4.4A).

4.4.3.2 Midgut

Hypoxic exposure prolonged the processing of digesta through the midgut section, with significant differences observed among the three oxygen levels (linear mixed-effects model, $F_{20,500} = 6.5$, $p < 0.0001$, Figure 4.3). For the fasted crabs in 100% oxygen, it took 3 h to fill the midgut, which was maintained at full capacity until 6 h. Although the filling rate of the midgut in 50% oxygen was similar to that measured in 100% oxygen, the digesta remained in the midgut for a longer time (Tukey's HSD, $t = 3.3$, $p = 0.005$).

The movement of digesta into and through the midgut was slower in 20% oxygen. It took 4 h to fill the midgut, and the amount of digesta declined slowly thereafter, with significant differences apparent after 10 h (20% vs. 50% oxygen: 52.1 ± 17.0% vs. 39.4 ± 14.3%, Tukey's HSD, $t = 2.7$, $p = 0.028$). A similar pattern was observed for the starved

crabs. There was no significant difference in the midgut processing times between 100% and 50% oxygen over the entire experimental period, while a slower processing rate was found in 20% oxygen (Figure 4.3).

In general, the fasted crabs processed digesta through the midgut at a faster rate at all oxygen levels. Although the digesta moved into the midgut at similar rates, the subsequent movement into the hindgut was slower for the starved crabs. These differences between the food deprivation states resulted in a significant interaction with the time (linear mixed-effects model, $F_{10,500} = 5.3$, $p < 0.0001$).

There was also a significant effect of the oxygen level on the midgut emptying time (generalized least squares model, $F_{2,47} = 16.4$, $p < 0.001$, Figure 4.4). For the fasted crabs, it took 10.8 ± 0.5 h to completely empty the midgut in 100% oxygen, and this was similar to 12.9 ± 1.3 h in 50% oxygen but significantly shorter than 17.7 ± 2.4 h recorded in 20% oxygen (Tukey's HSD, $t = 5.5$, $p < 0.001$). A similar pattern was found for the starved crabs. The midgut emptying time in 20% oxygen (21.3 ± 1.5 h) was significantly longer than that in 50% (13.1 ± 1.4 h) or 100% oxygen (13.8 ± 1.2 h). However, the emptying time of midgut was not significantly different between fasted and starved crabs (generalized least squares model, $F_{1,47} = 4.0$, $p = 0.05$).

4.4.3.3 Hindgut

Evacuation of feces from the hindgut occurred in a series of pulses which led to intermittent filling and emptying of this region. As such, this variance resulted in only the interaction between the oxygen level and time being statistically significant (linear mixed-effects model, $F_{20,500} = 6.4$, $p < 0.0001$). For the fasted crabs, in 100% oxygen, the maximal

hindgut fullness was reached by 5 h, declining slowly thereafter. There was no significant difference between 100% and 50% oxygen. In 20% oxygen, filling of the hindgut was significantly slower with maximal levels reached by 8 h. By 12 h, and the hindgut remained over 75% full in 20% oxygen, while only approximately 10% of digesta remained in 100% oxygen at this time (Tukey's HSD, $t = 3.4$, $p = 0.004$). Likewise, for the starved crabs, there was no significant difference in filling rates between 100% and 50% oxygen, while exposure to 20% oxygen significantly delayed the movement of digesta into the hindgut. After 12 h, the hindgut remained 62.2 ± 8.5% full in 20% oxygen, while it had dropped to 36.0 ± 10.0% in 100% oxygen (Tukey's HSD, $t = 4.8$, $p < 0.0001$). There was no significant effect of food deprivation state on the movement of digesta through the hindgut section (linear mixed-effects model, $F_{1,49} = 3.2$, $p = 0.08$).

The time for complete emptying of the hindgut was also significantly affected by the oxygen level (generalized least squares model, $F_{2,47} = 21.0$, $p < 0.001$, Figure 4.4). In fasted crabs, the emptying time increased significantly from 13.0 ± 0.9 h in 100% oxygen to 16.4 ± 1.2 h in 50% oxygen (Tukey's HSD, $t = 2.2$, $p = 0.03$). Meanwhile, the emptying time significantly increased (relative to that in 100% oxygen) to 24.9 ± 3.0 h in 20% (Tukey's HSD, $t = 4.6$, $p < 0.001$). A similar increase in emptying times was observed for the starved crabs increasing from 16.2 ± 1.1 h 100% oxygen to 22.9 ± 1.2 h in 20% oxygen (Tukey's HSD, $t = 4.3$, $p < 0.001$). There was no significant effect of food deprivation state on hindgut emptying times (generalized least squares model, $F_{1,47} = 3.3$, $p = 0.08$).

Table 4.13 The results of the linear mixed-effects model analysis for the gastric evacuation process as a function of the oxygen level, feeding state, time and their interactions. Bold font indicates statistical significance ($p < 0.05$). Only significant interactions were presented.

Gut section	Factor	$df_{num,den}$	*F*	*P*-value
Foregut	**Oxygen Level**	**2, 49**	**14.71**	**<0.0001**
	Food Deprivation	**1, 49**	**4.60**	**0.037**
	Time	**9, 439**	**434.95**	**<0.0001**
	Time: Oxygen	**18, 439**	**7.49**	**<0.0001**
	Time: Food Deprivation	**9, 439**	**2.86**	**0.003**
Midgut	Oxygen Level	2, 49	2.71	0.08
	Food Deprivation	1, 49	3.73	0.06
	Time	**10, 500**	**88.32**	**<0.0001**
	Time: Oxygen	**20, 500**	**6.50**	**<0.0001**
	Time: Food Deprivation	**10, 500**	**5.34**	**<0.0001**
Hindgut	Oxygen Level	2, 49	0.76	0.47
	Food Deprivation	1, 49	3.21	0.08
	Time	**10, 500**	**41.22**	**<0.0001**
	Time: Oxygen	**20, 500**	**6.40**	**<0.0001**

Table 4.14 The results generalized least squares models (GLS) for the gastric emptying time as a function of the oxygen level, feeding state and their interactions. Bold font indicates statistical significance ($p < 0.05$).

Gut section	Factor	$df_{num,den}$	*F*	*P*-value
Foregut	**Oxygen Level**	**2, 47**	**13.61**	**<0.001**
	Food Deprivation	1, 47	2.50	0.12
	Interaction	2, 47	0.15	0.86
Midgut	**Oxygen Level**	**2, 47**	**16.35**	**<0.001**
	Food Deprivation	1, 47	3.96	0.05
	Interaction	2, 47	0.46	0.63
Hindgut	**Oxygen Level**	**2, 47**	**21.02**	**<0.001**
	Food Deprivation	1, 47	3.31	0.08
	Interaction	2, 47	1.43	0.37

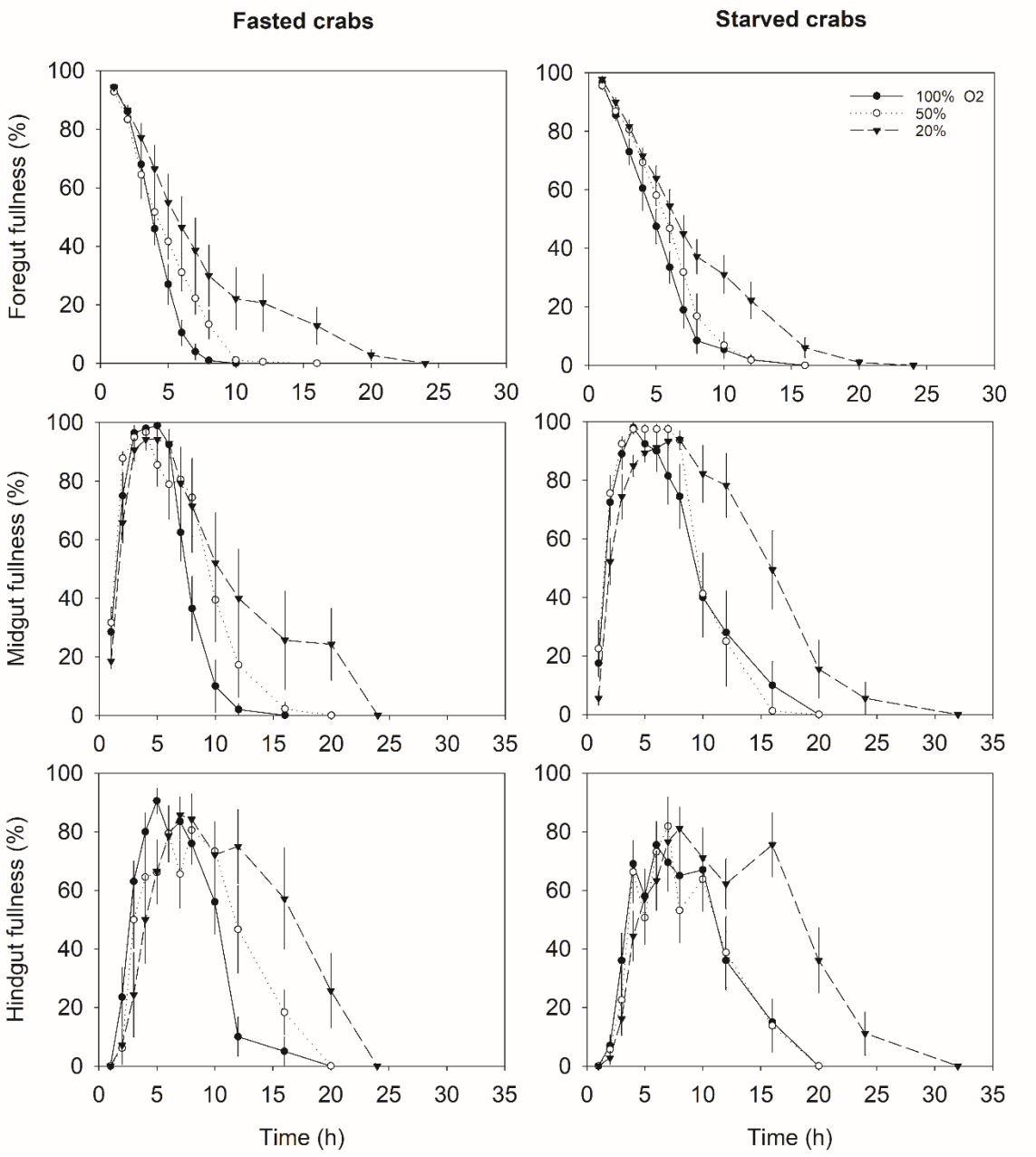


Figure 4.18 The passage of digesta through the foregut, midgut and hindgut regions of the fasted and starved rock crabs in 100%, 50%, and 20% oxygen. Data represent the mean ± SEM (N = 8–10).

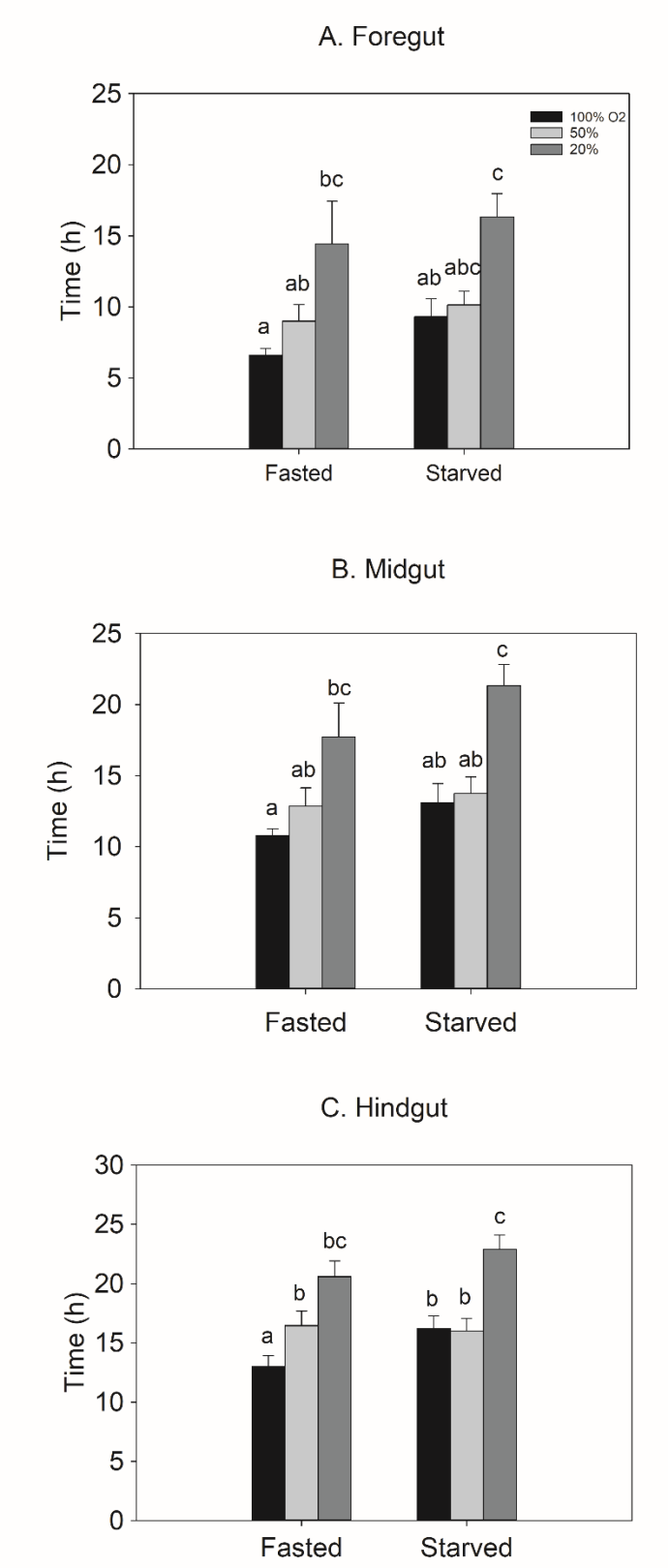

A. Foregut
Time (h)
25
20
15
10
5
0
100% O2
50%
20%
a
ab
bc
ab
abc
c
Fasted
Starved
B. Midgut
Time (h)
25
20
15
10
5
0
a
ab
bc
ab
ab
c
Fasted
Starved
C. Hindgut
Time (h)
30
25
20
15
10
5
0
a
b
bc
b
b
c
Fasted
Starved

Figure 4.19 The mean times (± SEM, N = 8–10) for emptying of the A. foregut, B. midgut and C. hindgut of the fasted and starved rock crabs in 100%, 50% and 20% oxygen saturations after they consumed the radiopaque meals 2% of body mass. Different letters indicate significant differences ($p < 0.05$) among oxygen levels.

4.5 Discussion

4.5.1 Accuracy of different food intake measurements

In this study, I estimated the accuracy of three different techniques for the measurement of food intake. The gravimetric analysis of dry food was the most accurate method, while the gravimetric analysis of wet food consumption overestimated food intake and counting ballotini beads in the radiolabelled food underestimated food intake, respectively.

Gravimetric analysis of wet food remains the most common non-invasive measurement of food intake for individual animals. It likely overestimates consumption rates because crustaceans are "messy feeders" tearing food into small pieces before mastication, which may create a considerable number of small particles (Barker and Gibson, 1977; Thomas et al., 2002). Although the seawater and all its contents in the container were collected on filter paper, it was still hard to collect every small piece of uneaten food. In addition, some of the substances in the shrimp muscle could have dissolved in the seawater. Finally, prior to weighing, the remaining food was blotted dry with a paper towel, but it was still difficult to standardize the moisture content in shrimp muscle, and there may be changes in the mass of the shrimp muscle associated with water absorption or loss during the experiment.

In contrast, dry food pellets were homogeneous, and the loss of dissolvable substances appeared negligible based on preliminary experiments (dry pellets soaked in

seawater were collected and dried to measure possible weight changes). As a result, the gravimetric analysis of commercial dry food estimated the food intake more accurately than shrimp muscle. However, the problem of using dried food for many crustaceans is palatability and thus their acceptance of artificial pellet food. Many aquaculture studies focus on the effect of the ingredients, texture and size of artificial food on the attractability and palatability in order to maximize consumption rates in crustaceans (Holland and Borski, 1993; Lee and Meyers, 1996; Nunes et al., 2006; Smith et al., 2005; Suresh and Nates, 2011). This acceptance of artificial food is coupled with the higher costs, and the ability to obtain the correct formulation is likely to preclude its widespread use in laboratory experiments.

The use of ballotini beads in radiopaque food underestimated food intake. This may also be caused by the messy feeding of rock crabs, whereby the crabs broke apart the food, and some of the inert markers would be lost. As a result, fewer beads were found in the gut. This underestimation of food intake (by 6 to 7%) is also found in other radiography analyses (Ahvenharju and Ruohonen, 2005; McCarthy et al., 1992; Thomas et al., 2002). Improvements in the accuracy of radiographic analysis could be achieved by changing the properties of the food, such as the moisture content and binding agent, which may hinder the loss of the radiographic markers (Thomas et al., 2002).

The advantage of inert marker techniques (over the two gravimetric methods) is that it allows one to monitor the food intake of multiple animals at any one time and to obtain feeding hierarchies when feeding in a group (Leavitt, 1985; McCarthy et al., 1992; Smith and Tabrett, 2004). Although radiographic analysis requires specialized equipment,

another advantage of the passage of digesta can be followed *in situ* through the entire digestive system, allowing the calculation of gastric clearance rates (McGaw, 2008; McGaw and Curtis, 2013b).

4.5.2 Feeding behavior

4.5.2.1 Effects of hypoxia

Hypoxia caused both a reduction in food intake, and a refusal to feed. In contrast, the food deprivation state did not appear to have a pronounced effect on food intake. In normoxia and mild hypoxia, both fasted and starved rock crabs consumed a similar amount of food, and there was no difference in the number of crabs refusing to feed. However, in 20% oxygen, the number of animals that refused the food was dependent on food type – more (fasted and starved) crabs refused to feed on the dry food or radiopaque food, while no significant difference was observed for crabs offered the wet food. The amount of food the crabs consumed in 20% oxygen (only feeding crabs) was also significantly lower compared with the crabs in 100% and 50% oxygen. Suppressed feeding activities in response to hypoxia appears to be a common response in decapod crustaceans, having been reported for *Carcinus maenas* (Brante and Hughes, 2001), *Callinectes sapidus* (Bell et al., 2003b), *Cancer magister* (Bernatis et al., 2007), *Carcinus aestuarii* and *Musculista senhousia* (Mistri, 2004). The actual process of food handling and mechanical mastication can elevate MO_2 over two-fold from resting levels (Hughes and Seed, 1995; McGaw and Van Leeuwen, 2017). It suggests that severe hypoxia (below P_{crit}) would cause crustaceans to depress feeding activities because physiological

compensation by ventilation or cardiac systems would not meet the increased oxygen demand associated with these feeding processes (Diaz and Rosenberg, 1995; McMahon, 2001). This imbalance may result in a longer feeding time or reduced food intake (Bell et al., 2003b; Bernatis et al., 2007). This reduction in food intake would affect downstream processes resulting in a reduced postprandial metabolism (Jiang, Chapter Two) since the magnitude of SDA characteristics are a function of the meal size (McCue, 2006; McGaw and Curtis, 2013a).

The sinus glands in the eyestalks produce neurohormones that might regulate the underlying feeding behaviors (neuronal circuit appetite control). Eyestalk ablation usually induces hyperphagia (Sears et al., 1991; Vijayakumaran and Radhakrishnan, 1984). For example, in intact Dungeness crab (*Cancer magister*), feeding behavior and food consumption are inhibited during exposure to acute low salinity, while eyestalk ablation removes this inhibition (Curtis and McGaw, 2011). A recent study shows that during hypoxic exposure, the river prawn (*Macrobrachium nipponense*) upregulates a short neuropeptide F (sNPF), which is localized in neuroendocrine cells of the eyestalk (Sun et al., 2020). In other arthropods, sNPF plays multiple roles in regulating feeding, growth and metabolic homeostasis (Fadda et al., 2019). For example, in the desert locust (*Schistocerca gregaria*), sNPF injection inhibits food intake, while knockdown of sNPF precursor significantly increases food intake (Dillen et al., 2014; Dillen et al., 2013). Meanwhile, hypoxia-inducible factor-1 (HIF-1) regulates the fish leptin gene expression (an anti-obesity hormone that suppresses food intake) during hypoxia (Chu et al., 2010;

Copeland et al., 2011; Meister, 2007). Therefore, it is possible that the synthesis of crustaceans sNPF and thus appetite may also be regulated by HIF.

4.5.2.2 Effects of food deprivation state

The food deprivation state did not have a significant effect on the amount of food ingested. Contrary to my original hypothesis, the starved crabs consumed no more food than fasted animals. In 100% and 50% oxygen, both fasted and starved crabs fed until the gut was full. This likely occurred because appetite is a graded function based on satiation, and feeding is inhibited by mechanical stimuli resulting from filling the gut with food (Elliott and Susswein, 2002). In severe hypoxia (20% oxygen), the physiological capacity to support oxygen delivery likely determined the amount of food consumed. Although there was no significant difference in the amount of food consumed between fasted and starved crabs, the starved crabs were more likely to hold the remaining food under their thorax after they ceased feeding. This behavior was observed in both normoxic and hypoxic conditions. The similar food-holding behavior is observed in *Cancer magister* (Bernatis et al., 2007) and *Carcinus maenas* (Brante and Hughes, 2001). This suggests that the starved crabs did not give up the food even when their foregut was full or were unable to continue feeding in severe hypoxia. This behavior is in accordance with other studies that starved crustaceans are more aggressive for food and/or feed for longer (Hughes and Seed, 1995; Liu et al., 2019; Sun et al., 2015). However, additional experiments are required to determine whether they would continue to “guard” the food and initiate second feeding once physiologically possible.

Although fewer starved crabs refused to feed in 20% oxygen compared with the fasted crabs, this trend was not statistically significant. Nevertheless, it does suggest that starvation would increase the urgency for procuring a meal, whereby the food deprivation state would alter the trade-off between stress tolerance and feeding behaviors (Curtis et al., 2010; Liu et al., 2019; Sun et al., 2015; Wang et al., 2006). In classical foraging theories (Stephens and Krebs, 1987), a hungry animal is more likely to forage, and this need to obtain food will override normal predator avoidance behaviors (Bonter et al., 2013; Cerri and Fraser, 1983; Lima et al., 1985; McKillup and McKillup, 1994; Morton and Chan, 1999). Likewise, starved Dungeness crabs (*Cancer magister*) tend to initiate feeding in low salinity, and they spend more time searching for sustenance compared with fasted crabs (Curtis and McGaw, 2012; Curtis et al., 2010). Thus, a new take on classical foraging theories could describe the interplay between hypoxia and feeding propensity here as the "physiological theory of foraging."

4.5.3 Gastric processing

4.5.3.1 Effects of hypoxia

The overall effect of hypoxia was to slow gastric processing and increase time for passage through and clearance of regions of the gut. Similar patterns are described for other crustaceans (McGaw, 2008; McGaw and Curtis, 2013b). For example, in *Cancer magister*, exposure to severe hypoxia (5 kPa oxygen) results in a decrease in pyloric contraction rates by about 25% and a subsequent two-fold increase in time for gastric evacuation (McGaw, 2008). The hemolymph P_{O2} may act as a neuromodulator on the

stomatogastric nervous system, which controls the crustacean foregut (Clemens et al., 1998; McGaw, 2008). The lower hemolymph P_{O2} induced by hypoxia (Jiang, Chapter Three) may result in decreased contraction rates of the pyloric region of the foregut which would reduce the pyloric filtering and transfer of digesta into the hepatopancreas and midgut (Bargmann, 2012; Clemens et al., 2001; Marder and Bucher, 2007).

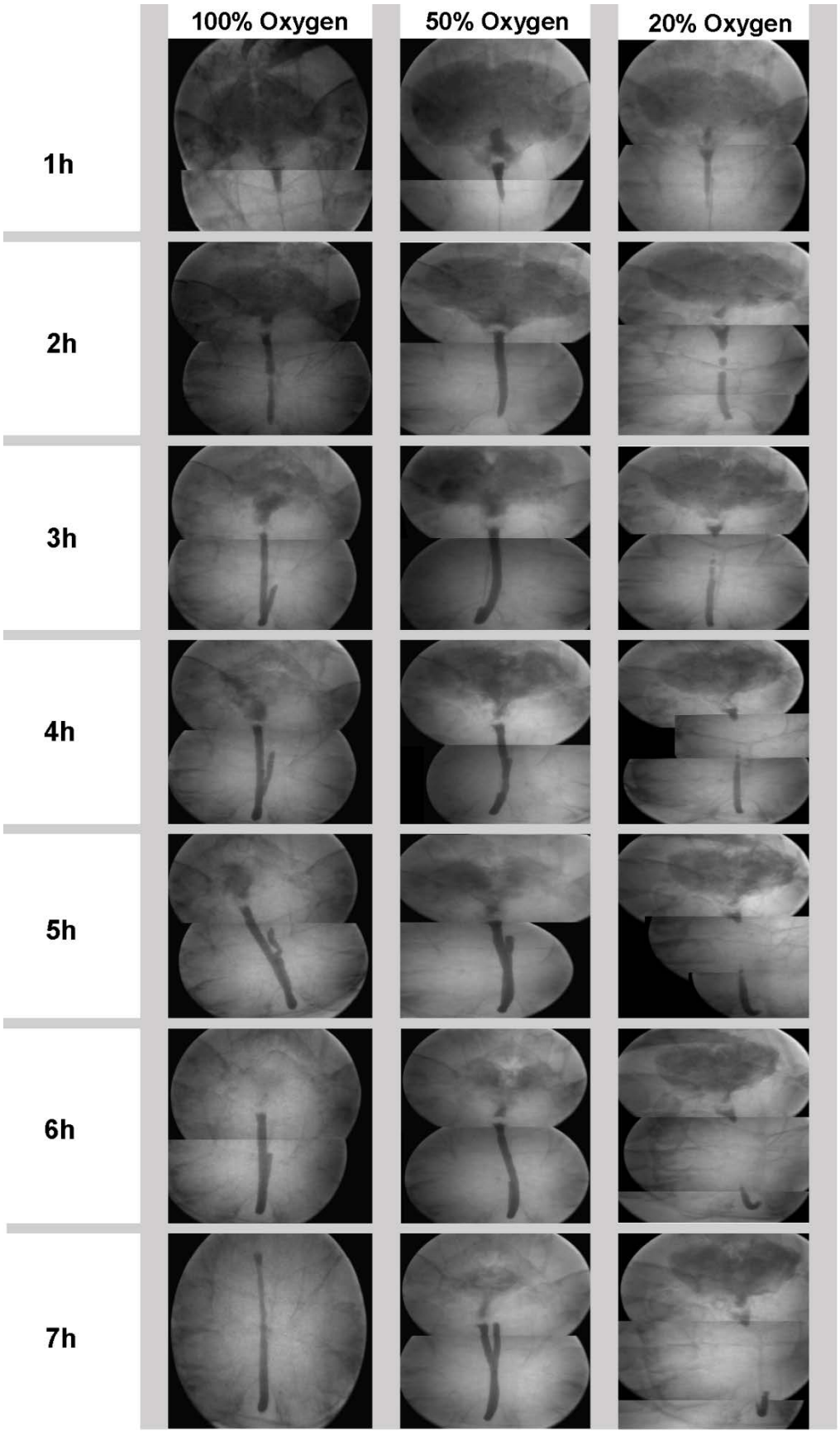
As the pyloric contractions of the foregut control the rate at which digesta enters the midgut, hypoxia also prolonged entry of digesta into and subsequent processing in the midgut and hepatopancreas. Two distinct patterns of midgut processing were observed in severe hypoxia. In the first pattern (observed in about 20% of the crabs, Figure 4.5), digesta entered the midgut in a series of pulses, and the entire midgut was cleared before another pulse of digesta entered, suggesting that pyloric contractions were halted for several hours at a time. In the second pattern (Figure 4.6), digesta continuously moved through the midgut but in smaller amounts than that observed in normoxic conditions. This may indicate pyloric contractions were less powerful, moving less digesta into the midgut. Either way, both suggest a reduced (slowing) but not a cessation of food processing in response to severe hypoxia. A limitation of this study is that processing rates of digesta within the hepatopancreas could not be followed. The radiopaque barium particles were filtered from the digesta by the setae of the midgut caeca (Hopkin and Nott, 1980; McGaw and Curtis, 2013b). Nevertheless, it can be assumed that at the same time material passes into the midgut, the liquid digesta passes into the hepatopancreas, where the majority of intracellular digestion occurs (Holliday et al., 1980; Lovett and Felder,

1990), and that slowing of the movement of digesta into the midgut represents a reduction of the actual digestive process.

Evacuation rates of the hindgut region were also measured, but these may not be a good indicator of the actual digestive processes *per se* (compared with foregut and midgut). The primary function of the hindgut of crustaceans is expelling feces and ion and water uptake (McGaw and Curtis, 2013b; Mykles, 1979). In this study, digesta remained in hindgut for several hours, especially when foregut and midgut were empty. In other crustaceans, digesta can remain in the hindgut for several weeks and is not expelled until another meal is consumed (McGaw and Curtis, 2013b; Mercier and Lee, 2002; Musolf et al., 2009).

The increase in MO_2 that occurs after feeding (SDA) is a composite of a number of different processes such as mechanical digestion, intracellular digestion and absorption and the subsequent intracellular protein synthesis (McGaw and Curtis, 2013b; McGaw and Van Leeuwen, 2017; Wang et al., 2019). However, protein synthesis is thought to account for the majority of this postprandial increase in MO_2. The reduced rates of food processing in both the foregut and midgut would have downstream effects on intracellular protein synthesis as less nutrient is delivered to the cells. Indeed, lower rates of intracellular protein synthesis in the hepatopancreas and other tissues have been reported for the crabs *Callinectes sapidus* and *Carcinus maenas*, in hypoxia (Brouwer et al., 2004; Carter and Mente, 2014; Mente et al., 2003; Wheaton and Chandel, 2011). As all these processes are slowed but not halted in hypoxia, it would effectively reduce the magnitude of the SDA response and prolong the duration because the food was processed

more slowly – the exact postprandial MO_2 response observed in hypoxia (Jiang, Chapter Two).


100% Oxygen
50% Oxygen
20% Oxygen
1h
2h
3h
4h
5h
6h
7h

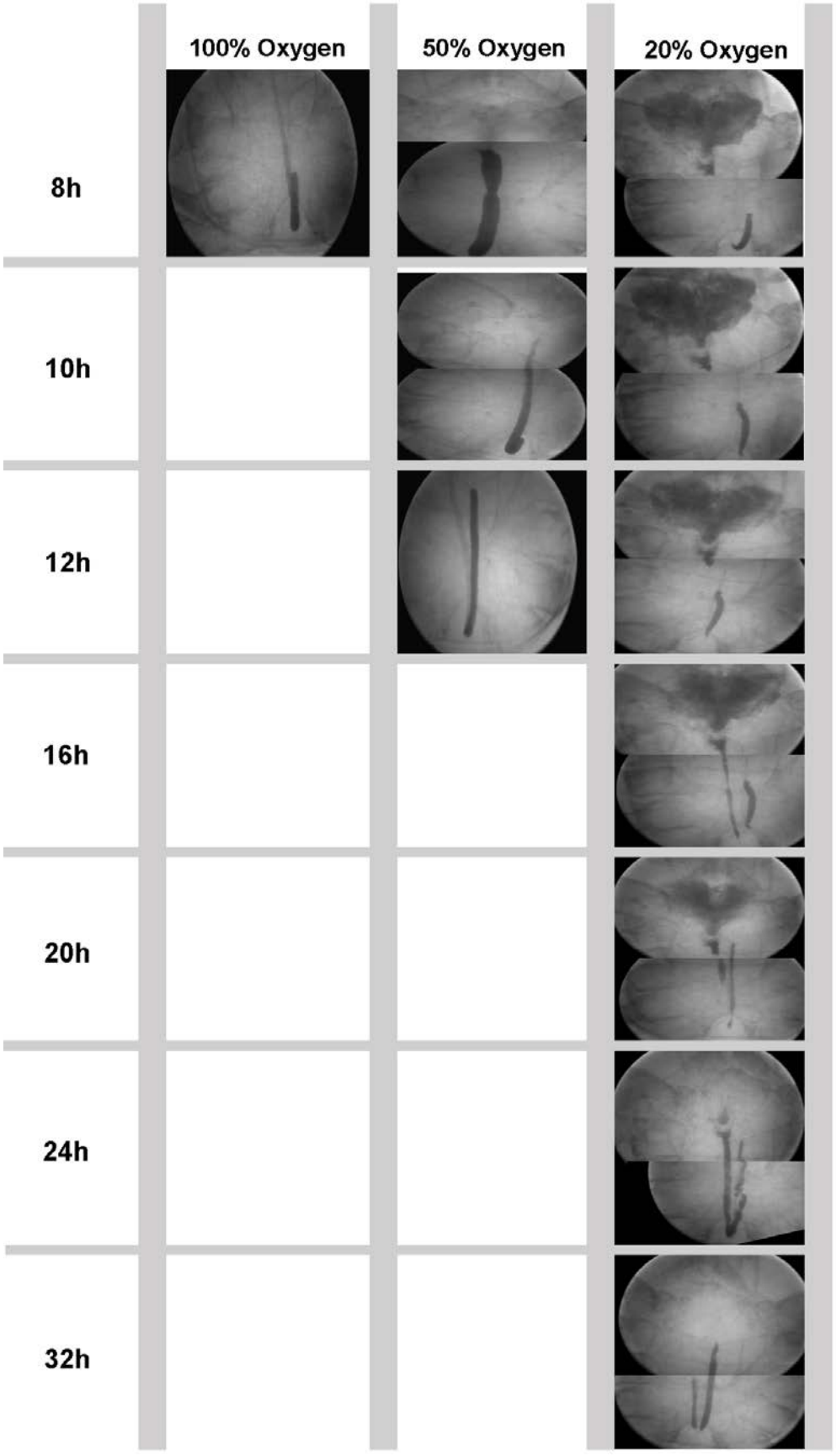
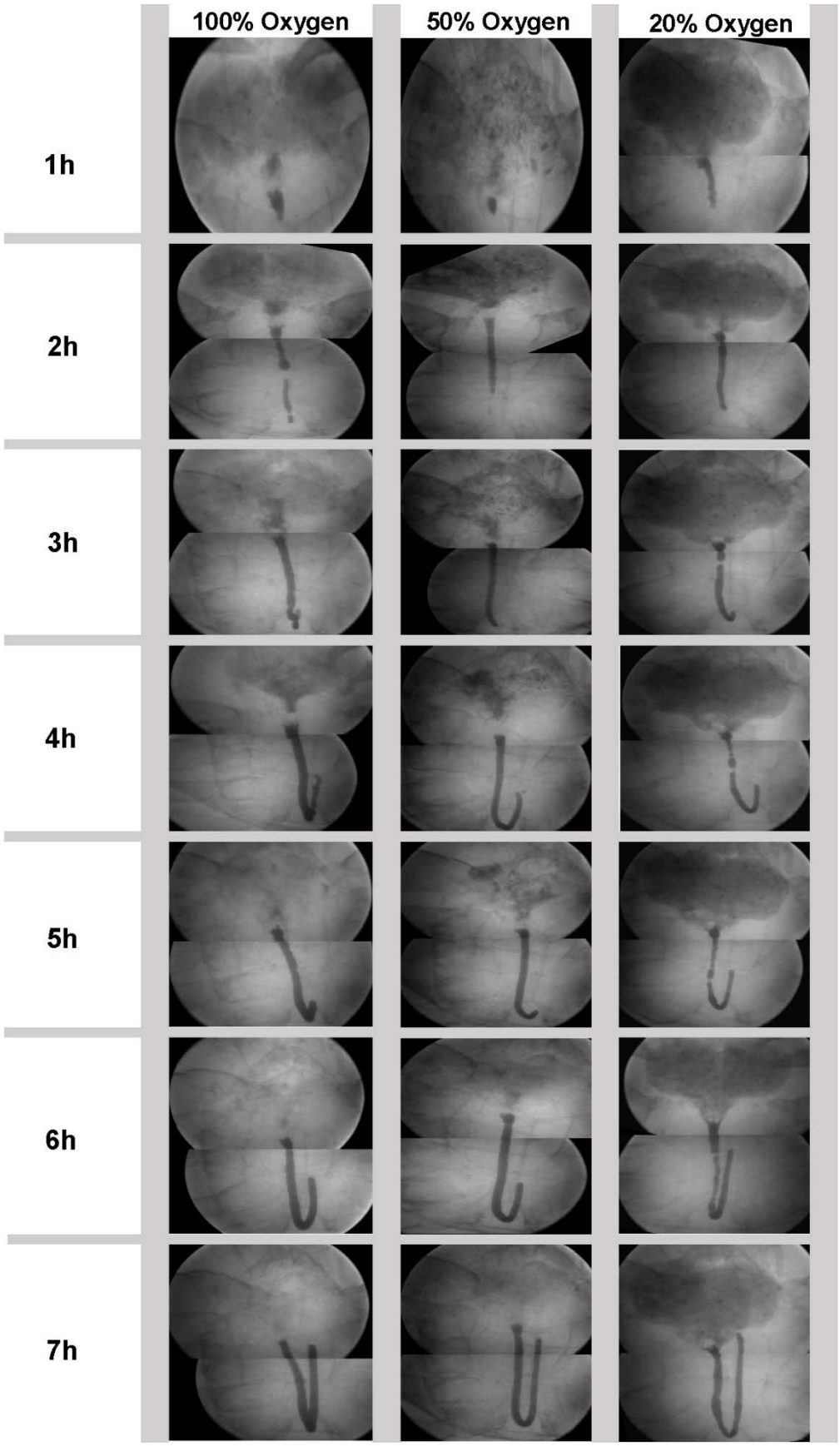

100% Oxygen
50% Oxygen
20% Oxygen
8h
10h
12h
16h
20h
24h
32h

Figure 4.20 X-ray images showing the transit of a radiopaque meal through the digestive system of the individual fasted rock crab in 100%, 50% and 20% oxygen levels.


100% Oxygen
50% Oxygen
20% Oxygen
1h
2h
3h
4h
5h
6h
7h

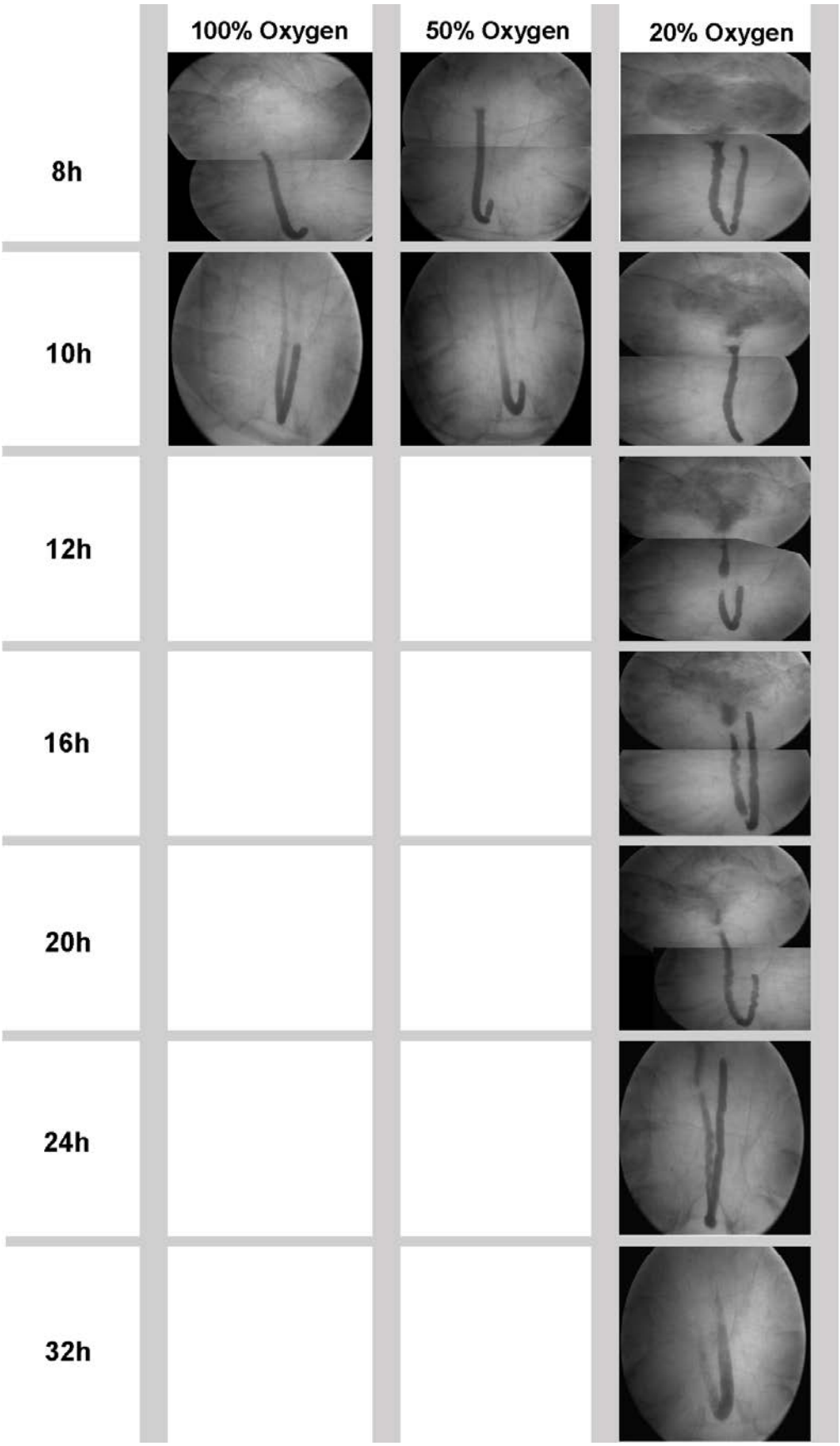

100% Oxygen
50% Oxygen
20% Oxygen
8h
10h
12h
16h
20h
24h
32h

Figure 4.21 X-ray images showing the transit of a radiopaque meal through the digestive system of the individual starved rock crab in 100%, 50% and 20% oxygen levels.

4.5.3.2 Effects of food deprivation state

Both the movement of digesta through each gut section over time (percent fullness) and gastric emptying times (complete emptying of each gut region) were used to follow the digestive processes of the rock crabs. In most other studies, only emptying times are used (Elliott, 1972; Loya-Javellana et al., 1995; McGaw and Curtis, 2013b). After the initial food processing phase (first 6 h), X-ray images were taken at intermittent intervals (every 4–12 h) to reduce handling and thus stress on the crabs. However, when X-ray images were taken at, for example, 12, 16 or 20 h, any crabs that cleared their guts within this 4 h interval would be considered to have the same emptying time. As a result, using emptying times alone may not have allowed the detection of more subtle differences between fasted and starved crabs. Therefore, the passage of food over time through each section of the gut provided a more reliable indicator of the rate of gastric processing.

Overall, long-term starvation slowed gastric processing, with the most obvious effects in normoxia and 50% oxygen (Figure 4.3). This could account for the shallower drawn-out SDA curve of starved crabs (Jiang, Chapter Two). The slower gastric processing of starved crabs cannot be caused by lower internal oxygen levels acting on the stomatogastric nervous system, as no difference of arterial P_{O2} was found between fasted and starved rock crabs in all three oxygen levels tested (Jiang, Chapter Three). More likely, because these patterns were most prevalent in normoxia, this slowing gastric processing may be caused by limited energy supply due to poor nutritional conditions. Digestion and the subsequent absorption of nutrients are energetically expensive, resulting in a 2-3 fold increase in MO_2 (McGaw and Curtis, 2013b; McGaw and Van Leeuwen, 2017; Wang et al., 2019). The starved crabs would have much lower energy stores and

thus be unable to process food as rapidly as the fasted crabs (Vinagre and Chung, 2016; Wang et al., 2019).

An alternative explanation is that rather than a lack of energy, the slower gastric processing of starved crabs indicates they need more time and energy to mobilize the digestive system during refeeding, as long-term starvation would cause degradation of both structural and functional capacities. During long-term starvation in the freshwater shrimp (*Neocaridina davidi*), the degeneration of epithelial cells (D-cells in the intestine, B- and F- cells in the hepatopancreas) at the ultrastructural level causes an increase of cells with depolarized (non-active) mitochondria (Włodarczyk et al., 2017). Histological analysis of starved crayfish (*Cherax quadricarinatus*) gut tissue also shows structural disorganization and loss of the digestive gland tubules, with concomitant changes in R, B and F cells of the hepatopancreas (Sacristán et al., 2016). In addition, starvation may progressively induce death processes (apoptosis, necrosis and autophagy) in the cells of the midgut intestine and hepatopancreas (Cervellione et al., 2017; Sonakowska et al., 2016). The cellular degradation results in functional impairment of synthesis and secretion of digestive enzymes (Comoglio et al., 2004; Johnston et al., 2004; Muhlia-Almazan and García-Carreño, 2002). Thus, starved crabs may have lower extracellular enzyme activity in foregut and reduced ability for intracellular digestion in the hepatopancreas. The process of refeeding leads to a gradual regeneration of dormant digestive organ structures; this could be responsible for the slower processing rates observed here (Cervellione et al., 2017; Comoglio et al., 2004; Silva-Castiglioni et al., 2016; Włodarczyk et al., 2017). This hypothesis is consistent with the results in Chapter Two that starved crabs generally had higher energy expenditure of SDA than fasted crabs,

indicating the energy cost summation of the regeneration of the digestive system and digestion processes.

4.5.4 Conclusion

When comparing three different methods of measuring food intake, although dry commercial food is best for estimated food intake, it is harder to procure, more expensive, and the crabs may be less inclined to eat it compared with natural food. Using radiopaque food (while underestimating food intake) is useful for assessing the amount eaten in multiple animals, as well as investigating digestive physiology. However, the use of specialized techniques and equipment will put it out of reach for most behavioral ecologists. Most people will continue to use wet food and differences in mass before and after eating as an estimate of the amount of food consumed – but should be aware that this does tend to overestimate the actual amount consumed.

The present study has shown that hypoxia adversely affected feeding behaviors and gastric processing of rock crabs. The reduced energy intake and slower processing rates in hypoxia may further impair their growth and reproduction in the long term (Galic et al., 2019). Compared with hypoxia, the food deprivation state had less pronounced effects on feeding behaviors and gastric processing. Although the amount of food ingested did not differ, slower gastric processing of starved crabs indicates that when this is coupled with hypoxia, it may have a more deleterious effect on these animals.

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Chapter 5. Do lab-based experiments accurately depict the behavioral responses of rock crabs to exposure to hypoxia?[1]

[1]A modified version of this manuscript has been submitted to the Thalassas.

5.1 Abstract

When encountering hypoxic waters, marine animals, including crustaceans, often exhibit an increase in activity and, if able, migrate to higher oxygen waters. We have previously shown that the food deprivation state of rock crabs affects their physiological responses to hypoxia. In this study, we examined the behavioral responses of rock crabs in different food deprivation states and their ability to detect and avoid specific levels of hypoxia. Individual crabs were monitored in a Loligo® Shuttle Box system. This advanced system tracked the movement of each crab between two adjoining arenas, each with a different oxygen level. The rock crabs did not show any significant preference for a specific oxygen regime. However, rock crabs increased moving speed in the lower oxygen level (20% vs. 50% saturation), especially for fed crabs. In addition, fasted and fed crabs were monitored for 12 h in static hypoxia (same oxygen level in both arenas). The fed crabs exhibited similar activity rates in 30% oxygen compared to 100% oxygen, even though this level was below their critical oxygen partial pressure (P_{crit}). However, fed crabs were less active than their fasted conspecifics in 20% oxygen, likely because they could not balance the simultaneous demands of digestion and increased activity. Overall, there was a considerable variation in activity rates among individuals within the same treatment; these masked some of the behavioral responses. The current study highlights some of the problems associated with lab-based studies. We suggest that in the future, it will be important to conduct *in situ* field studies to observe realistic behaviors of freely moving decapods in response to environmental perturbations.

Keywords: crab, hypoxia, locomotion, avoidance, food deprivation, activity, oxygen, Shuttle Box system

5.2 Introduction

Marine hypoxia has become much more common in coastal environments during the last few decades (Breitburg et al., 2018; Diaz and Rosenberg, 2008; Riedel et al., 2012). The adverse effects of global warming (reduced oxygen solubility and enhanced stratification) contribute to deoxygenation in marine environments (Watson, 2016). Additionally, both natural fluctuation and anthropologic eutrophication exacerbate the current scenario of ocean deoxygenation in coastal waters (Breitburg et al., 2018; Helly and Levin, 2004; Watson, 2016). Since 1950, oxygen dead zones (≤ 2 mgO_2/L) in the global coastal oceans have increased by 90% (Diaz and Rosenberg, 2008). The published thresholds of hypoxia tolerance span a broad range from 0.29 to 4 mgO_2/L (approximately 3 to 44% oxygen saturation) for marine invertebrates and fishes (Conley et al., 2009; Vaquer-Sunyer and Duarte, 2008). The spatial and temporal extent of hypoxic water below these thresholds can modify both the distribution and population dynamics of fish and large invertebrates (Ekau et al., 2010; Gray et al., 2002; Spicer, 2014; Wu, 2002). Crustaceans are among the most sensitive taxa to hypoxia, making them a good indicator species for environmental change (Deutsch et al., 2015; Vaquer-Sunyer and Duarte, 2008). Hypoxic expansion may affect their movement and distribution; this may be especially important for commercially fished species (Craig, 2012; Purcell et al., 2017; Zimmerman and Nance, 2001). Thus, it is becoming increasingly important to understand the behavioral responses of decapod crustaceans to hypoxia.

Avoidance behavior is often the first response to hypoxia when marine fish and large invertebrates cannot maintain their routine oxygen consumption (Jones, 1952; Pihl et al., 1991).

Crustaceans are also able to sense and avoid hypoxic environments (Bell et al., 2003a; Craig, 2012; Das and Stickle, 1994; Pihl et al., 1991; Purcell et al., 2017; Wannamaker and Rice, 2000). Many factors affect the success of avoidance behaviors. In general, crustaceans can avoid hypoxic or anoxic water in deep areas by moving towards inshore waters with higher oxygen levels (Bell et al., 2003a; Craig, 2012; Froehlich et al., 2014; Pihl et al., 1991; Purcell et al., 2017). Orientation mechanisms are critical for successful hypoxia avoidance. Blue crabs (*Callinectes sapidus*) may use the current direction as a cue to move down current and orient away from hypoxic upwelling water (Bell et al., 2003a). Moreover, blue crabs are more sensitive to rapid oxygen changes in seawater; crabs tend to become more active and are more effective at avoiding areas where a rapid oxygen decline occurs (Bell et al., 2009; Haselmair et al., 2010). However, in the absence of directional currents, crustaceans may be less effective at avoiding hypoxia, especially if the hypoxic zone is widespread (Bernatis et al., 2007; Das and Stickle, 1994; Froehlich et al., 2014).

The avoidance behavior of crustaceans to hypoxia involves an increase in locomotor activity (Haselmair et al., 2010; McGaw, 2007), which may result in a two- to four-fold increase in oxygen consumption (Booth et al., 1982; Henry et al., 1994; McGaw, 2007). This increase in oxygen consumption comes at a time when oxygen in the environment is limiting, and if this initial increase in locomotion does not extirpate the animal, the reduced oxygen supply may impair subsequent escape attempts (Das and Stickle, 1994). During chronic and/or severe hypoxic exposure, crustaceans may become quiescent in order to reduce oxygen consumption (Wu 2002; Eriksson and Baden, 1997). In addition, they may exhibit atypical activities such as loss of intraspecific aggression and habitat segregation (Haselmair et al., 2010; Riedel et al., 2014). Predator-prey activities are also altered by hypoxia: predators may become less active, while prey

may leave their shelter and seek residence in areas with more oxygen (Johansson, 1997; Sandberg and Bonsdorff, 1996).

In nature, organisms do not always stop feeding before experiencing environmental perturbations. However, only a few studies have addressed the effect of the feeding state on the behavioral responses of crustaceans to hypoxia (Bell et al., 2009; Bernatis et al., 2007). Postprandial crustaceans typically exhibit a two- to four-fold increase in metabolism, associated with the energetic cost of the ingestion, absorption, and assimilation of a meal (McCue, 2006; Secor, 2009). Thus, if digestion occurs during hypoxia, it places an additional demand on oxygen requirements when environmental oxygen is limiting. In such circumstances, postprandial crustaceans exhibit a trade-off between locomotion and digestion, reducing locomotor activity or settling in areas of higher oxygen concentrations (Bell et al., 2003b; Bernatis et al., 2007). For example, free-ranging Dungeness crabs (*Cancer magister*) become quiescent after feeding, which allows them to divert energy from the skeletal muscles to the digestive organs; in contrast, unfed crabs remain active (Bernatis et al., 2007; McGaw, 2005).

Conversely, the metabolic processes of crustaceans may be adversely affected by long-term starvation. During food deprivation, crustaceans mobilize carbohydrate, lipid, and protein reserves as an energy source (Sacristán et al., 2017; Sánchez-Paz et al., 2006). As a result, starved crustaceans have lowered plasma glucose and protein levels (Chen and Cheng, 1993; Sánchez-Paz et al., 2007; Simon et al., 2015). In order to reduce the use of limited energy reserves, starved crustaceans may decrease locomotory, ventilatory, and respiratory rates, resulting in a depression of metabolism by 40% to 60% (Hervant et al., 1997; Regnault, 1981; Wallace, 1973; Wang et al., 2019). However, it remains unknown whether the downregulation of metabolism

observed in starved crustaceans will affect how they respond to hypoxia compared with fasted animals.

The Atlantic rock crab (*Cancer irroratus*) is a common benthic decapod along the Atlantic coast of North America, ranging from Labrador to South Carolina. They are distributed from the shallow subtidal zone down to 390 m (Haefner Jr, 1976). *Cancer irroratus* usually occurs on rocky bottoms with macroalgae, where they can easily find shelter (Haefner Jr, 1976; Hudon and Lamarche, 1989). The rock crab is an important predator on polychaetes, bivalves, echinoderms and small crustaceans, and it is also an important prey of some demersal fish species and large decapods, including the commercially important lobster (*Homarus americanus*) (DFO, 2014; Gendron et al., 2001; Scarratt and Lowe, 1972). Under the background of global climate change, hypoxic areas in coastal waters have expanded to their natural habitat. For example, in 2003, the oxygen concentration in the bottom waters of the Gulf of St. Lawrence declined to 65 μmol/L (21% saturation) (Gilbert et al., 2005; Levin et al., 2009). As these hypoxic events are increasing, it becomes more important to study the impact of hypoxia on this shallow water decapod.

The previous chapters highlighted the interactive effects of hypoxia and food deprivation state on oxygen consumption of rock crabs. Fed crabs had a higher MO_2, while starved crabs exhibited a lower MO_2, than 2–4 d fasted animals. In general, fed crabs appeared to be less tolerant of hypoxia than starved and fasted crabs. Therefore, the primary aim of the present study was to determine if the different food deprivation states may also affect the activity and behavioral responses of rock crabs to hypoxia. It was hypothesized that postprandial crabs with higher oxygen demand would avoid hypoxia or be less active than fasted crabs. In addition, the starved crabs with a lower metabolism and hemocyanin levels might lack the energy reserves for

an active avoidance response. In order to achieve these goals, a Loligo® Shuttle Box system was used to create two different oxygen saturations in two connected arenas, allowing a crab to move freely and choose the preferred oxygen level (Figure 5.1). A camera logged the position of the crab allowing calculation of time spent in each oxygen tension and activity rates and patterns. This system has been used previously with success to monitor the choice behaviors of finfish (Barker et al., 2018; Christensen et al., 2021; Kates et al., 2012; Nay et al., 2020; Stich et al., 2016; Tucker and Suski, 2019). However, only few studies have used this system with crustaceans (Fredricks et al., 2020; Nielsen and McGaw, 2016). Therefore, the secondary aim of this study was to assess the suitability of this newly designed equipment for use with crustaceans.

5.3 Material and Methods

5.3.1 Crab collection and housing

Male and female rock crabs (*Cancer irroratus*) were trapped at multiple locations along the coast of the northern Avalon Peninsula, Newfoundland, Canada. Crabs (100–180 g) that showed no obvious physical damage or weakness in the hard-intermolt stage, and females bearing no eggs were retained. The crabs were transferred to the Department of Ocean Sciences at Memorial University in St John's, NL, Canada and held in 400-L sediment-free tanks with flow-through aerated seawater at ambient temperature (4 – 10°C) and salinity (30 – 32‰) for a maximum time of 2 months. The crabs were fed chopped herring (*Clupea harengus*) to excess once a week. Any uneaten food was removed on the same day to avoid fouling the water. At least two weeks before the experiments began, crabs were acclimated in the laboratory tanks

(300 L) in flow-through aerated seawater (salinity 30–32‰) at 12–13°C and constant dim red light. A two-week period is the minimum time to effectively acclimate the crabs to experimental temperatures and abolish any underlying locomotor rhythms (Taylor et al., 1977). Preliminary experiments showed no difference in oxygen consumption and feeding rates between sexes, and crabs of both sexes were chosen indiscriminately in each treatment at the same ratio as collection 3: 1 (males: females).

In this study, crabs were classified into three food deprivation states, each with specific physiological attributes:

a) Starved crabs were held in individual perforated plastic chambers (25 × 15 × 7.5 cm^3) to prevent cannibalism at 12–13°C and starved for 4–5 weeks. The death rate of starvation was about 10–15%, similar to fasted crabs. The starved crabs have lower resting metabolic rates (Ansell, 1973; Wallace, 1973) and reduced blood protein levels (and hence hemocyanin levels), and utilize body proteins for energy (Sacristán et al., 2017).

b) Fasted crabs were deprived of food for 3–5 days before experiments began, which allowed them to evacuate all food from the digestive system. Studies suggest fasted crabs in this study had no effects of prolonged starvation (Ansell, 1973; McGaw and Curtis, 2013; Wallace, 1973).

c) Fed crabs were fed shrimp muscle, 2% of their body mass, one hour before experiments. The crabs were held in individual chambers and ensured consuming all food. The postprandial crabs typically exhibit a 2–3 fold increase in metabolism associated with digestion, absorption and protein synthesis (Secor, 2009).

5.3.2 Experimental design

A Shuttle Box system (Loligo® System, Viborg, Denmark) was used to investigate the behavior of rock crabs of three food deprivation states in response to hypoxia. This system (Figure 5.1) consisted of two circular arenas (100 cm diameter × 50 cm deep). The arenas were connected by a narrow, rectangular walkway (25 cm wide × 50 cm long), through which the animals could move freely between the two arenas. The depth of seawater was kept 20–30 cm during the experiments. The computer-controlled system (ShuttleSoft 2.6.4, Loligo System, Viborg, Denmark) independently maintained two different dissolved oxygen levels of seawater in the two arenas. This was accomplished by water being drawn continuously from each arena by a pump and delivered into an external oxygen buffer tank (dimensions: 20 cm wide × 20 cm long × 60 cm deep). An oxygen regulator (Loligo® System, Viborg, Denmark) was connected to an air pump and a compressed nitrogen cylinder, which was used to maintain a preprogrammed oxygen tension in the buffer tank. The seawater in the buffer tank was delivered back to the arena via gravitational flow (2–2.5 L/min). This created a slow circular current, which prevented mixing between the two arenas. In each trial, the oxygen level (in a single arena) was controlled within a range of 10% variation, and the temperature was maintained at 12–13°C by coolers placed in the buffer tanks.

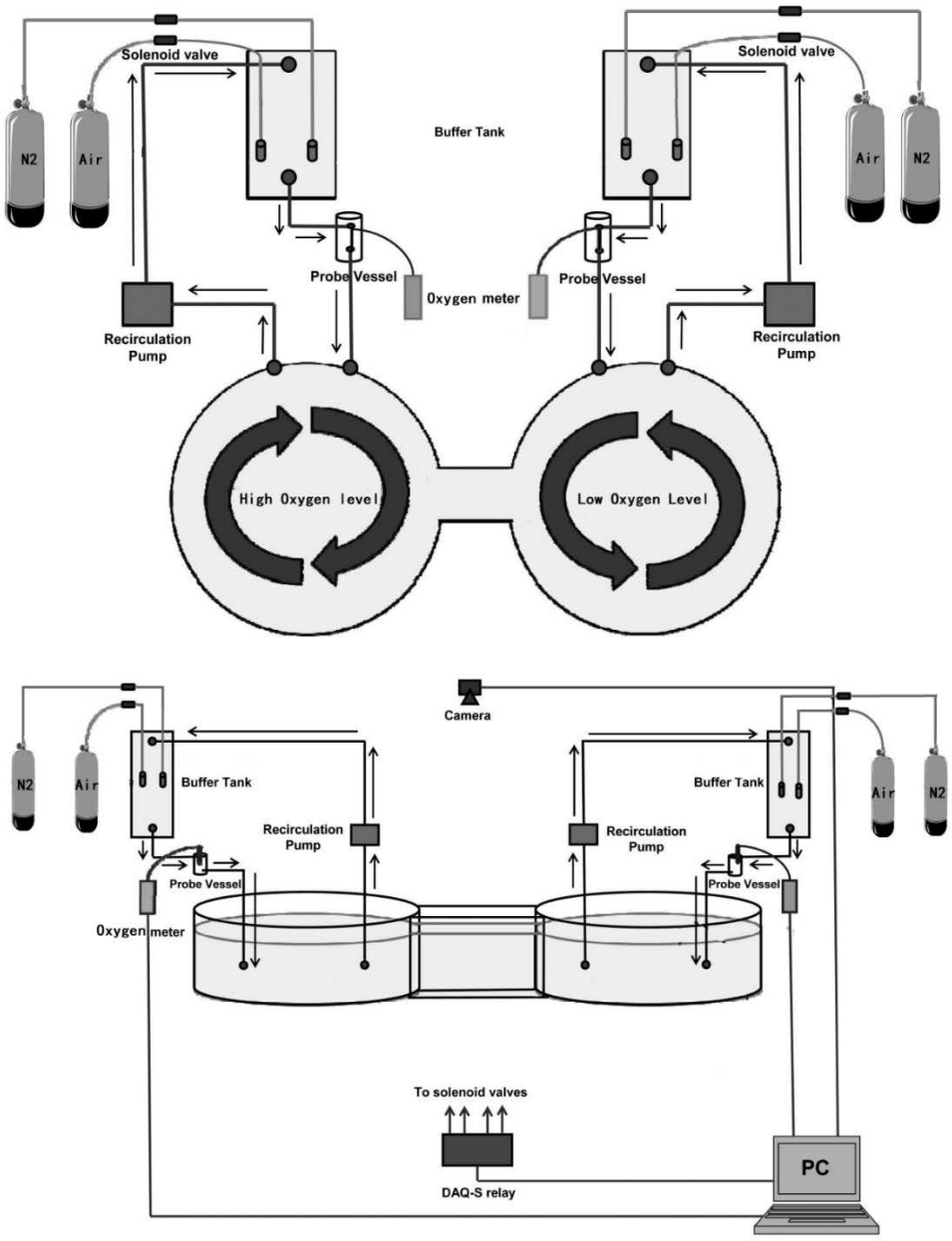


Figure 5.22 Schematic line drawing of the Loligo® Shuttle Box system modified from (Kates et al., 2012), showing major components. An overhead view and side view of the system. Arrows depict the flow of water. The oxygen level was controlled in the buffer with the addition of air and N_2 via solenoid valves.

A computer-controlled digital camera (USB uEye SE, Viborg, Denmark) was positioned 3 m above the apparatus, allowing a full view of both arenas (Figure 5.1). The Loligo software used the contrast between the organism and the arena to track the crab's behavior. The system recorded the real-time oxygen level in each arena and the coordinates recorded from the center of the contrasting image (the crab) every second.

5.3.2.1 Behavioral responses to paired oxygen treatments

Paired oxygen choices with different levels in each of the two areas were used to determine whether rock crabs could sense different oxygen levels and exhibit avoidance of, or preference for, a certain oxygen regime (i.e., one of the arenas). The choice of oxygen combinations was partly dictated by the maximum difference that the system could consistently maintain over several hours. Thus, three different combinations of oxygen saturations were created: 100% vs. 100% (control), 100% vs. 50%, and 50% vs. 20%. Fifty percent oxygen was chosen as mild hypoxia (above the P_{crit} of crabs for all food deprivation states), and 20% oxygen was chosen as severe hypoxia below the P_{crit} (Jiang, Chapter Two). Starved, fasted and fed crabs were used in each oxygen combination (n = 7–11 for each treatment, total sample size = 79). Each crab was randomly chosen from the acclimation tank and placed into either arena of the Shuttle Box system to avoid any bias. In order to reduce any handling-related stress, the crabs were allowed to settle in the apparatus for 45 min before recording was started. Each trial lasted for 4 h to ensure that the fed crabs still exhibited elevated postprandial metabolic rates. In this experiment, the total time (h) spent in each arena was used to indicate whether crabs showed a preference for, or avoidance of, a certain oxygen regime. Further, specific responses (detailed in the data analysis section) were analyzed in each arena to determine if the behavior was differentially

modulated in response to the oxygen level. In a separate analysis, the activity levels (during movement back and forth between the two arenas) were analyzed to determine the effects of food deprivation state and oxygen treatment during the 4 h experimental period.

5.3.2.2 Behavioral responses to static hypoxia (single oxygen treatments)

The results of the paired-oxygen experiments (above) showed no evidence that rock crabs in three food deprivation states preferred a certain arena. However, they did modulate their activity levels during short-term hypoxic exposure, and this was especially noticeable for fed crabs. The paired-oxygen treatment provided crabs with two different oxygen regimes; it was hypothesized that movement to the higher regime could allow crabs to recover from oxygen deficiency and subsequently return to the lower oxygen arena. Alternatively, the change between the two regimes may have been stressful, resulting in continuous high activity. Thus, in a separate series of experiments, the effect of static hypoxia (same oxygen concentration in each arena) on locomotor activity was assessed. Because the crabs were active throughout the 4 h duration of the paired oxygen experiments, the time for monitoring responses was extended to 12 h to test whether the crabs were still stressed during the first 4 h due to handling (Nielsen and McGaw, 2016; Wilson et al., 2021). A time period of 12 h also allowed us to monitor the fed crabs during different stages of the digestive/gastric process. Only fed and fasted animals were used here due to the logistics required to prepare starved crabs and the fact that starved and fasted crabs showed similar responses in the paired oxygen experiments.

As before, crabs were introduced randomly into one of the arenas and allowed to settle for 45 minutes before the experiment. One hundred percent oxygen was chosen as the control; 30%

oxygen was above the P_{crit} of the fasted crabs (28 ± 2%) but below the P_{crit} of the fed crabs (35 ± 2%), and 20% oxygen was below the P_{crit} of the fasted and fed crabs (n = 7–11 for each treatment, total sample size = 54). These hypoxic levels were chosen to test the effect of P_{crit} on locomotion and possible interactions with digestive processes.

5.3.3 Data analysis

All statistical tests were conducted in R (Team, 2018). The normality of data was checked by a Shapiro-Wilks test. For these data that did not pass the normality test, a Box-Cox transformation was applied (λ value was reported). A Bartlett test was performed to check the homoscedasticity of data. In order to deal with heterogeneity, variance structures were added in model fitting, and the best model was achieved by likelihood ratio tests with Akaike's information criterion (AIC). Tukey-adjusted *post hoc* tests were carried out using the 'emmeans' package. Statistical significance was accepted at $P < 0.05$. All data in the figures and tables are shown as the mean ± standard error of the mean (SEM).

5.3.3.1 Behavioral responses to paired oxygen treatments

For each paired oxygen choice and food deprivation state, the total time spent and the average moving speed in each arena were first analyzed by two-tailed paired t-tests, respectively. The time spent in each arena was used as an indicator of preference/avoidance of an oxygen regime (time the crabs spent in the walkway, about 10% of the total time, connecting the two arenas was excluded from the analysis). The changes in average moving speed determine whether the crabs could detect different oxygen regimes and if they exhibited behavioral modulation in response to oxygen changes. In order to increase the power of statistical analysis,

the ratio of time spent in two arenas (T_{low}/T_{high}) and the ratio of average speed in two arenas (V_{low}/V_{high}) were calculated for each crab as new parameters of avoidance behaviors. The two parameters from different paired-oxygen treatments and food deprivation states were pooled and analyzed using a generalized least square fitted linear model (gls function in R).

5.3.3.2 Behavioral responses to static hypoxia (single oxygen treatments)

Based on the initial analysis of the data (Figure 5.2), two primary behavioral phases were observed: a resting phase and an active phase. In the resting phase, the crabs were stationary or moving very slowly (< 0.5 cm/s). In the active phase, the crabs were moving around the arena at a relatively high speed (> 0.5 cm/s). Thus, to differentiate these patterns of individual crabs in response to the different oxygen levels, the following parameters were calculated (in each arena) from the raw data (real-time speed and position coordinates):

a) The average moving speed (cm/s): the mean speed of both the resting and active phases.

b) The active moving speed (cm/s): the mean speed of the active phase (speed > 0.5 cm/s).

c) The activity level (%): the percentage of time when a crab was active (speed > 0.5 cm/s).

In the static hypoxia experiments conducted over 12 h, the average moving speed, active moving speed and activity level were calculated in 3-h intervals during the 12-h experimental period to quantify the behavioral responses of crabs over time. Three-hour intervals were chosen to observe the effect of a longer period and to avoid too many *post hoc* tests among time intervals (increase the power of each *post hoc* test). A linear mixed-effects model in R package 'nlme' was used with the fixed factors: the time, oxygen treatment and food deprivation state. Individual crabs were treated as a random factor.

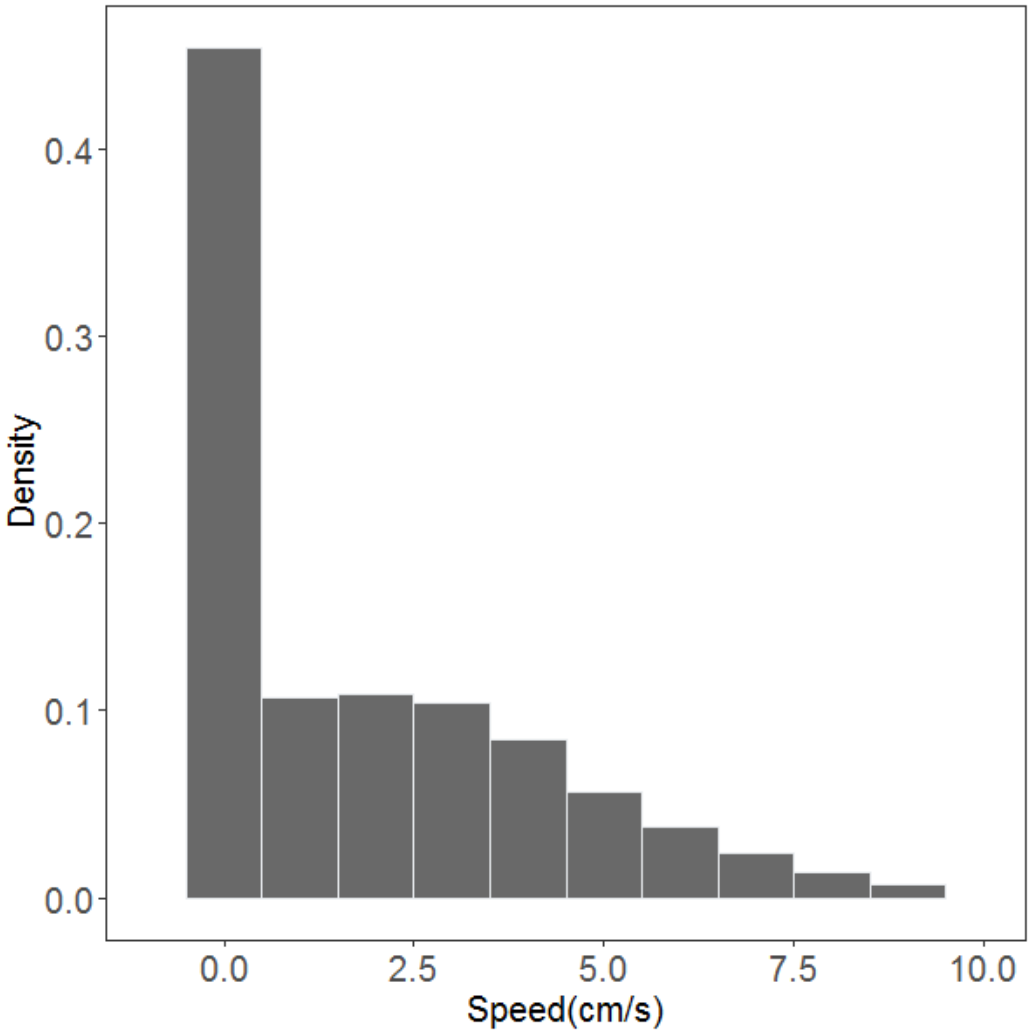


Figure 5.23 A typical frequency distribution of moving speed of fasted rock crabs in 100% vs. 100% oxygen treatment over four hours.

5.4 Results

5.4.1 Behavioral responses to paired oxygen treatments

There were no significant differences in time spent in each arena as a function of the oxygen level for starved, fed and fasted crabs (Table 5.1, two-tailed paired t-tests, $p > 0.05$). The data for the average moving speed were very variable (Table 5.2). Consequently, there were also very few statistically significant differences. There were some obvious trends in the 50% vs. 20% oxygen treatment. The fed crabs spent a slightly longer time in the 50% than 20% oxygen arena (Table 5.1, two-tailed paired t-test, $t = 2.3$, $df = 6$, $p = 0.07$). The starved crabs had a slightly higher average speed in 20% oxygen than 50% oxygen (Table 5.2, two-tailed paired t-tests, $t = 2.1$, $df = 7$, $p = 0.08$).

The generalized least squares model (GLS) of time ratio showed no significant changes in time ratios as a function of oxygen treatment or food deprivation state (Table 5.3). However, the GLS of average speed showed that moving speed significantly increased in the lower oxygen arena (GLS, $F_{2,69} = 3.6$, $p = 0.03$). Post hoc tests showed fasted and starved crabs had slightly higher speed ratio (V_{low}/V_{high}) in the 20% vs. 50% oxygen treatment (1.3 ± 0.2 and 1.2 ± 0.1 respectively), and only fed crabs had a significantly higher speed ratio (1.5 ± 0.2) in 20% vs. 50% oxygen, compared with 0.9 ± 0.1 in 100% vs. 50% oxygen (Tukey's HSD, $t = 2.4$, $p = 0.048$).

Table 5.15 The total time (h) spent in each arena of the Shuttle Box for the crabs in the three food deprivation states in the three oxygen treatments during 4-h trials (time spent in two arenas may not add up to 4h because the time they spent in the walkway connecting arenas was excluded). Two-tailed paired t-test values are given for each experiment. Bold font indicates statistical significance ($p < 0.05$).

Food Deprivation State	Treatment (oxygen pairs)	Time in High O_2 (h)	Time in Low O_2 (h)	*df*	*T*	*P-value*
Starved	100% vs. 100%	1.7 ± 0.1	1.9 ± 0.2	8	-0.46	0.66
	100% vs. 50%	1.8 ± 0.3	1.9 ± 0.2	8	-0.22	0.83
	50% vs. 20%	2.0 ± 0.2	1.5 ± 0.2	7	1.53	0.18
Fasted	100% vs. 100%	1.9 ± 0.2	1.9 ± 0.2	10	0.02	0.98
	100% vs. 50%	1.6 ± 0.1	2.0 ± 0.2	7	-1.04	0.33
	50% vs. 20%	1.8 ± 0.2	1.9 ± 0.3	7	-0.23	0.83
Fed	100% vs. 100%	1.8 ± 0.2	2.0 ± 0.2	8	-0.61	0.69
	100% vs. 50%	1.6 ± 0.2	2.0 ± 0.2	9	-1.10	0.39
	50% vs. 20%	2.3 ± 0.3	1.3 ± 0.2	6	2.25	0.07

Table 5.16 The average moving speed (cm/s) in each arena of the Shuttle Box for the crabs of the three food deprivation states in the three oxygen treatments during 4-h trials. Two-tailed paired t-test values are given for each experiment. Bold font indicates statistical significance ($p < 0.05$).

Food Deprivation State	Treatment (oxygen pairs)	Speed in High O_2 (cm/s)	Speed in Low O_2 (cm/s)	*df*	*T*	*P-value*
Starved	100% vs. 100%	2.4 ± 0.5	2.3 ± 0.4	8	0.67	0.52
	100% vs. 50%	1.7 ± 0.3	1.8 ± 0.3	8	-0.11	0.92
	50% vs. 20%	1.8 ± 0.3	2.1 ± 0.1	7	-2.09	0.08
Fasted	100% vs. 100%	1.6 ± 0.3	1.5 ± 0.2	10	0.85	0.42
	100% vs. 50%	1.7 ± 0.3	1.7 ± 0.3	7	0.42	0.69
	50% vs. 20%	1.3 ± 0.4	2.1 ± 0.6	7	-1.09	0.32
Fed	100% vs. 100%	1.7 ± 0.3	1.6 ± 0.4	8	0.67	0.52
	100% vs. 50%	2.1 ± 0.2	1.9 ± 0.4	9	0.96	0.36
	50% vs. 20%	0.7 ± 0.1	0.9 ± 0.1	6	-1.19	0.29

Table 5.17 Summary of generalized least squares model (GLS) for investigating differences in time ratio and average moving speed ratio in the 4-h paired oxygen treatments. The two explanatory variables (oxygen level and food deprivation state) and their interaction were tested sequentially. λ value is presented if Box-cox transformation was applied to the data. Bold font indicates statistical significance ($P < 0.05$).

Parameter	Factor	$df_{num,den}$	*F*	*P*-value
Time Ratio (λ=0)	Oxygen Level	2, 69	1.75	0.182
	Food Deprivation State	2, 69	0.46	0.629
	Interaction	4, 69	1.50	0.211
Average Moving Speed Ratio (λ=0.3)	**Oxygen Level**	**2, 69**	**3.58**	**0.034**
	Food Deprivation State	2, 69	0.26	0.775
	Interaction	4, 69	0.42	0.800

5.4.2 Behavioral responses to static hypoxia

5.4.2.1 The average moving speed

The average moving speed was significantly affected by oxygen level (linear mixed-effects model, $F_{2,48} = 14.0$, $p < 0.0001$). There was also a significant interaction between oxygen level and time (linear mixed-effects model, $F_{6,144} = 2.9$, $p = 0.011$). There was no significant effect of food deprivation state (linear mixed-effects model, $F_{1,48} = 4.0$, $p = 0.050$).

For the fasted crabs, no significant difference in average speed was found between 100% and 30% oxygen or between 30% and 20% oxygen. In the 20% oxygen level, the fasted crabs had a significantly lower speed (0.7 ± 0.1 cm/s vs. 1.7 ± 0.2 cm/s) compared with the 100% oxygen level during the 12 h experimental period (Tukey's HSD tests, $p < 0.01$).

For the fed crabs, there was no significant difference in the speed between 100% and 30% oxygen (Figure 5.6). In 100% oxygen, the speed dropped from 1.3 ± 0.2 cm/s at 6 h to 0.6 ± 0.1 cm/s at 9 h (Tukey's HSD, $t = 5.5$, $p < 0.0001$) and was maintained at similar levels thereafter. In 30% oxygen, the speed was maintained at mean levels of 1.1 ± 0.2 cm/s during the 12 h experimental period. The speed in 20% oxygen was significantly lower than that measured in 100% oxygen level, between 3 h and 6 h (Tukey's HSD tests, $p < 0.001$). The speed in 20% oxygen was also significantly lower than that measured in 30% oxygen level during the 12 h experimental period (Tukey's HSD tests, $p < 0.01$).

The only post hoc difference in average moving speed between fasted and fed crabs was found in 100% oxygen: the fed crabs had significantly lower speeds than fasted crabs after 9 h (Tukey's HSD tests, $p < 0.05$).

5.4.2.2 The active moving speed

The active moving speed (Figure 5.7) was significantly affected by oxygen level (linear mixed-effects model, $F_{2,48} = 21.4$, $p < 0.0001$), and oxygen level also significantly interacted with time (linear mixed-effects model, $F_{6,144} = 2.9$, $p = 0.011$). No significant effect of food deprivation state was found (linear mixed-effects model, $F_{1,48} = 0.9$, $p = 0.36$).

For the fasted crabs, the active speed in 100% oxygen was higher than that measured in 30% oxygen between 3 h and 6 h with mean levels of 2.9 ± 0.2 cm/s vs. 1.9 ± 0.2 cm/s (Tukey's HSD tests, $p < 0.05$). The active speed of fasted crabs in 20% oxygen (1.5 ± 0.1 cm/s) was significantly lower than the 2.8 ± 0.2 cm/s in 100% oxygen during 12 h experimental period (Tukey's HSD tests, $p < 0.001$). For the fed crabs, there was no significant difference in active speed between 100% and 30% oxygen. The active speed of 1.0 ± 0.1 cm/s for fed crabs in 20% oxygen level was significantly lower than that measured in both 100% (2.6 ± 0.1 cm/s) and 30% oxygen (1.9 ± 0.2 cm/s) after 3 h (Tukey's HSD tests, $p < 0.05$).

5.4.2.3 The activity level

The activity level (Figure 5.8) was significantly affected by oxygen level (linear mixed-effects model, $F_{2,48} = 6.5$, $p = 0.003$) and time (linear mixed-effects model, $F_{3,144} = 2.8$, $p = 0.044$) and oxygen level significantly interacted with time (linear mixed-effects model, $F_{6,144} = 2.9$, $p = 0.010$). The effect of food deprivation state fell just shy of significance (linear mixed-effects model, $F_{1,48} = 4.0$, $p = 0.052$).

There was no change in activity levels among the three oxygen levels for the fasted crabs (Figure 5.8). The fed crabs had similar activity levels in 100% and 30% oxygen. In 20% oxygen, the

activity level of fed crabs was significantly lower than that in both 100% and 30% oxygen levels. The difference between 20% and 100% oxygen levels was only apparent for the first 6 h of the experiment (7.3 ± 2.2% vs. 43.8 ± 4.4%, Tukey's HSD tests, $p < 0.01$). In contrast, differences between 30% and 20% oxygen (46.2 ± 7.0% vs. 8.2 ± 2.6%) were maintained during 12 h experimental period (Tukey's HSD tests, $p < 0.01$).

Post hoc differences in activity levels between fed and fasted crabs were found only in 20% oxygen between 3 and 9 h. The activity levels of fed crabs ranged between 4.7 ± 1.4% and 10.3 ± 2.6% compared with 42.5 ± 5.7% and 43.9 ± 5.8% for the fasted crabs (Tukey's HSD tests, $p < 0.05$).

Table 5.18 The summary of linear mixed-effects models of the average moving speed (cm/s), the active moving speed (cm/s) and the active time (h) for the crabs of the two food deprivation states (fasted and fed) in the three oxygen treatments (100%, 30% and 20%) over 12-h trials. λ value was presented if Box-cox transformation was applied to the data. Bold font indicates the statistical significance of each factor ($p < 0.05$).

Parameter	Factor	$df_{num,den}$	*F*	*P*-value
Average Moving Speed (λ=0.4)	**Oxygen Level**	**2, 48**	**14.04**	**<0.0001**
	Food Deprivation State	1, 48	4.04	0.050
	Time	3, 144	1.47	0.226
	Oxygen: State	2, 48	2.21	0.121
	Time: Oxygen	**6, 144**	**2.86**	**0.011**
	Time: State	3, 144	0.48	0.695
	Oxygen: State: Time	6, 144	1.14	0.340
Active Moving Speed	**Oxygen Level**	**2, 48**	**21.42**	**<0.0001**
	Food Deprivation State	1, 48	0.86	0.357
	Time	3, 144	0.46	0.710
	Oxygen: State	2, 48	0.91	0.408
	Time: Oxygen	**6, 144**	**2.91**	**0.011**
	Time: State	3, 144	2.12	0.100
	Oxygen: State: Time	6, 144	1.94	0.078
Activity Level	**Oxygen Level**	**2, 48**	**6.47**	**0.003**
	Food Deprivation State	1, 48	3.97	0.052
	Time	**3, 144**	**2.76**	**0.044**
	Oxygen: State	2, 48	2.32	0.110
	Time: Oxygen	**6, 144**	**2.92**	**0.010**
	Time: State	3, 144	0.30	0.824
	Oxygen: State: Time	6, 144	0.960	0.455

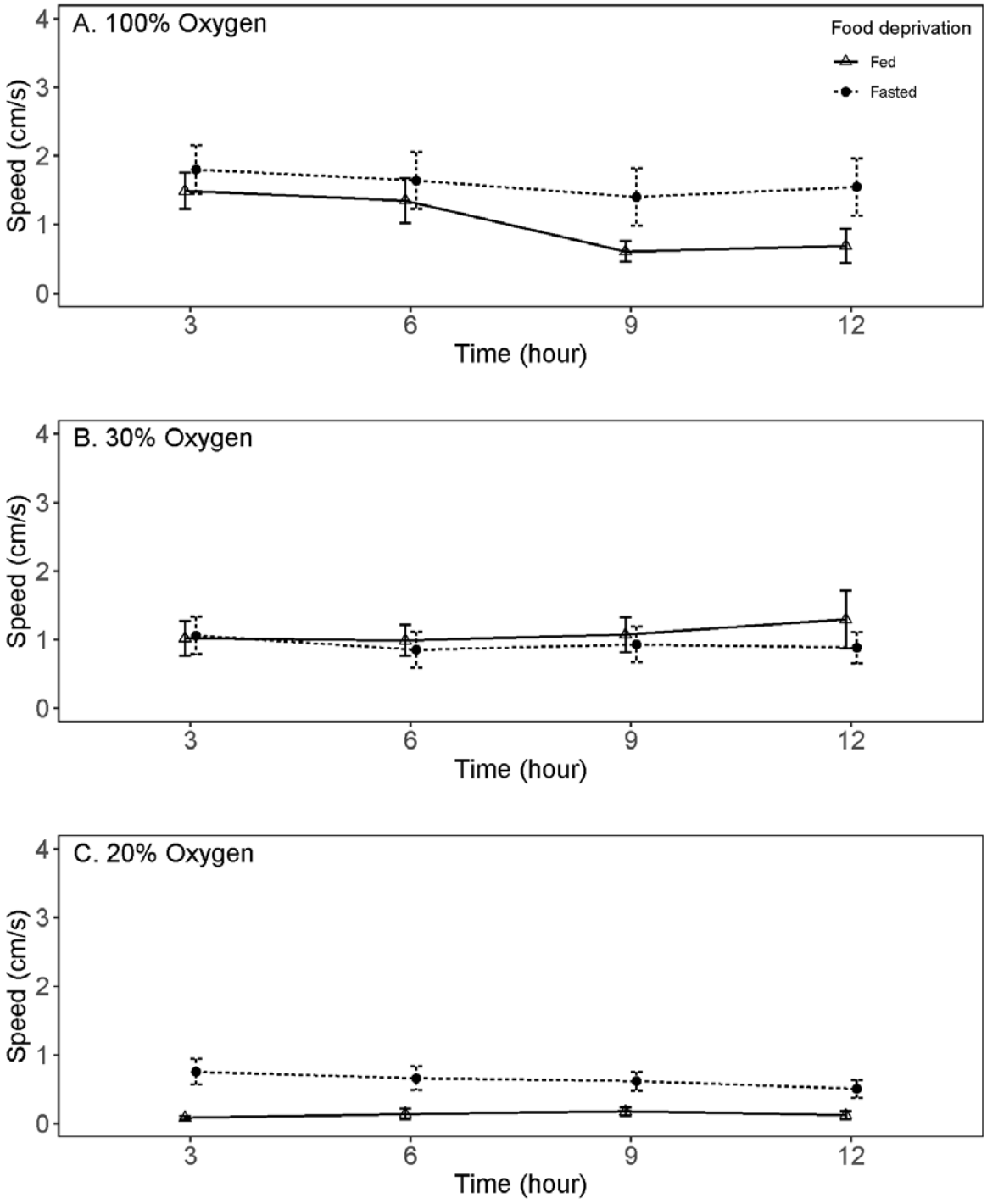


Figure 5.24 The average moving speed (cm/s) in the Shuttle Box system for fed and fasted rock crabs in the three static oxygen treatments during the 12 h experimental period. Data are shown as mean with standard error of mean (N = 7–11).

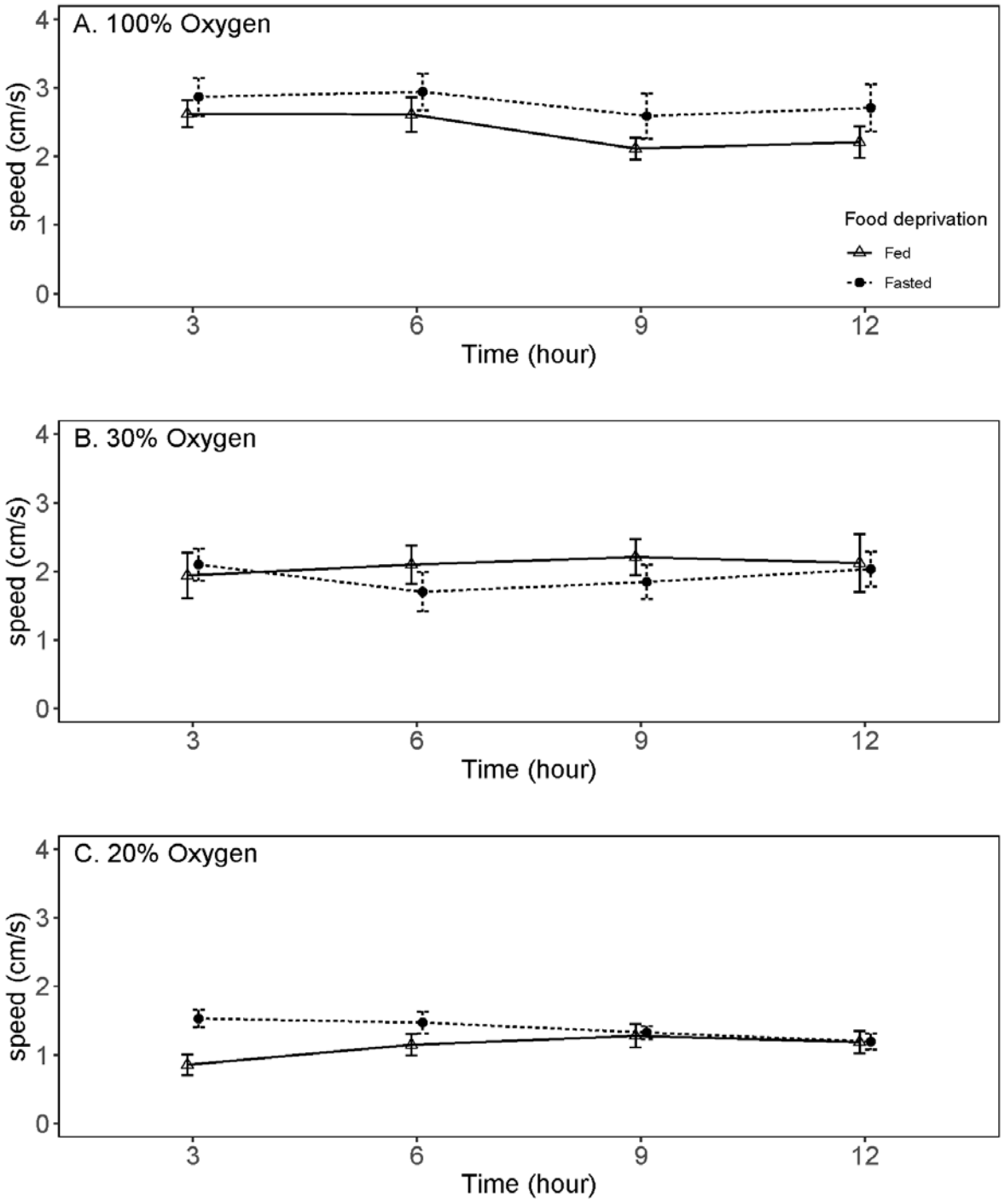


Figure 5.25 The active moving speed (cm/s) in the Shuttle Box system for fed and fasted rock crabs in the three static oxygen treatments during the 12 h experimental period. Data are shown as mean with standard error of mean (N = 7–11).

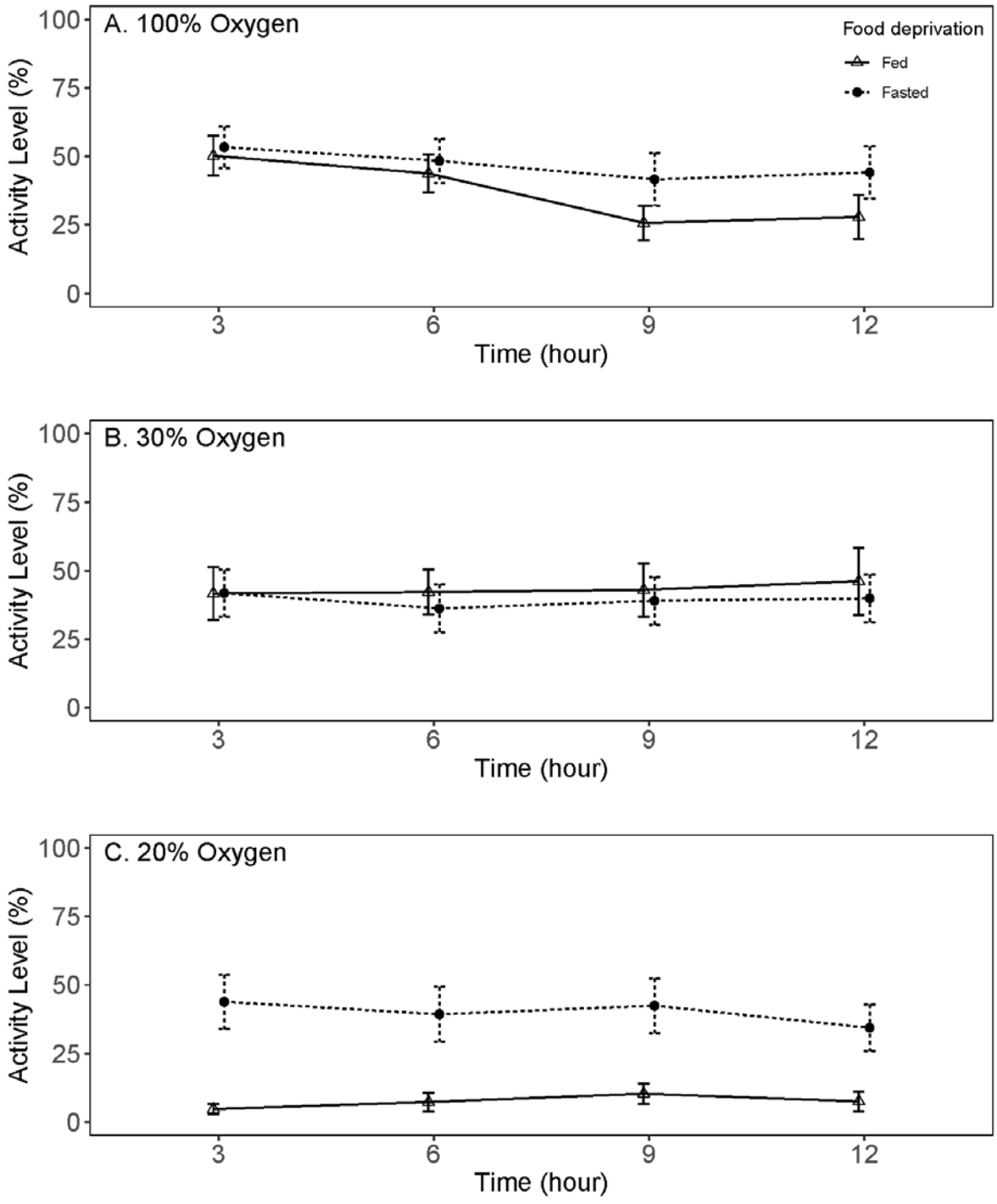


Figure 5.26 The activity level (%) in the Shuttle Box for fed and fasted rock crabs 100%, 30% and 20% oxygen treatments during the 12 h experimental period. Data are shown as mean with standard error of mean (N = 7–11).

5.5 Discussion

Rock crabs that were offered a choice of two different oxygen levels showed a trend towards increasing their moving speed in lower oxygen arenas. However, there was considerable inter-animal variability that may have obscured definitive patterns. In contrast, the crabs exhibited a significant decrease in locomotor activity as a function of the oxygen level during long-term exposure (12 h) to stable oxygen regimes. Fed rock crabs were more likely than fasted or starved crabs to avoid hypoxia and exhibited lower activity levels during long-term hypoxic exposure. Overall, the crabs remained relatively active in the Shuttle Box apparatus. The nature of the apparatus was such that it may have induced the wide variation in crab activity, which obscured more subtle patterns of avoidance behavior.

5.5.1 Behavioral response to paired oxygen treatments

Avoidance behavior is usually the first defense for mobile animals when encountering hypoxic regimes (Jones, 1952; Pihl et al., 1991; Wannamaker and Rice, 2000). However, the starved, fasted and fed rock crabs did not appear to avoid lower oxygen arenas in the paired-oxygen treatments. It is unlikely that rock crabs were unable to sense the different oxygen regimes because they did show a behavioral modification in the static hypoxia regimes (Figure 5.3–5.5). A recent study reviews the possible mechanisms of oxygen sensing in crustaceans (de Lima et al., 2021). Oxygen chemoreceptors of crustaceans are tentatively identified as sensory neurons located on the gill spines (Laverack and Saier, 1993). The orthologues of atypical soluble guanylyl cyclase (sGCs), hypoxia-inducible factors (HIFs), and prolyl hydroxylases (PHs) are

identified in the chemosensory organs and neurons of crustaceans, and the sGCs, HIFs and PHs have been proved to play important roles in oxygen sensing pathways of nematodes and insects (de Lima et al., 2021; Derby et al., 2016; Gorr et al., 2006).

It is possible that the oxygen levels used in the present study were not low enough to induce clear-cut responses. Even though 20% oxygen was below the P_{crit} of rock crabs for all food deprivation states (Jiang, Chapter Two), it may not have been low enough to initiate an escape response. In support of this assumption, the freshwater crayfish (*Paranephrops zealandicus*) does not display an avoidance behavior at a P_{O2} of 4 kPa (below their P_{crit} of 6 kPa), and they only emerge into air when oxygen levels are very low – 0.6 kPa (Broughton et al., 2017). Although I attempted to maintain lower oxygen concentrations in the Shuttle Box, the system could not maintain constant differences below 20% oxygen saturation for longer than an hour.

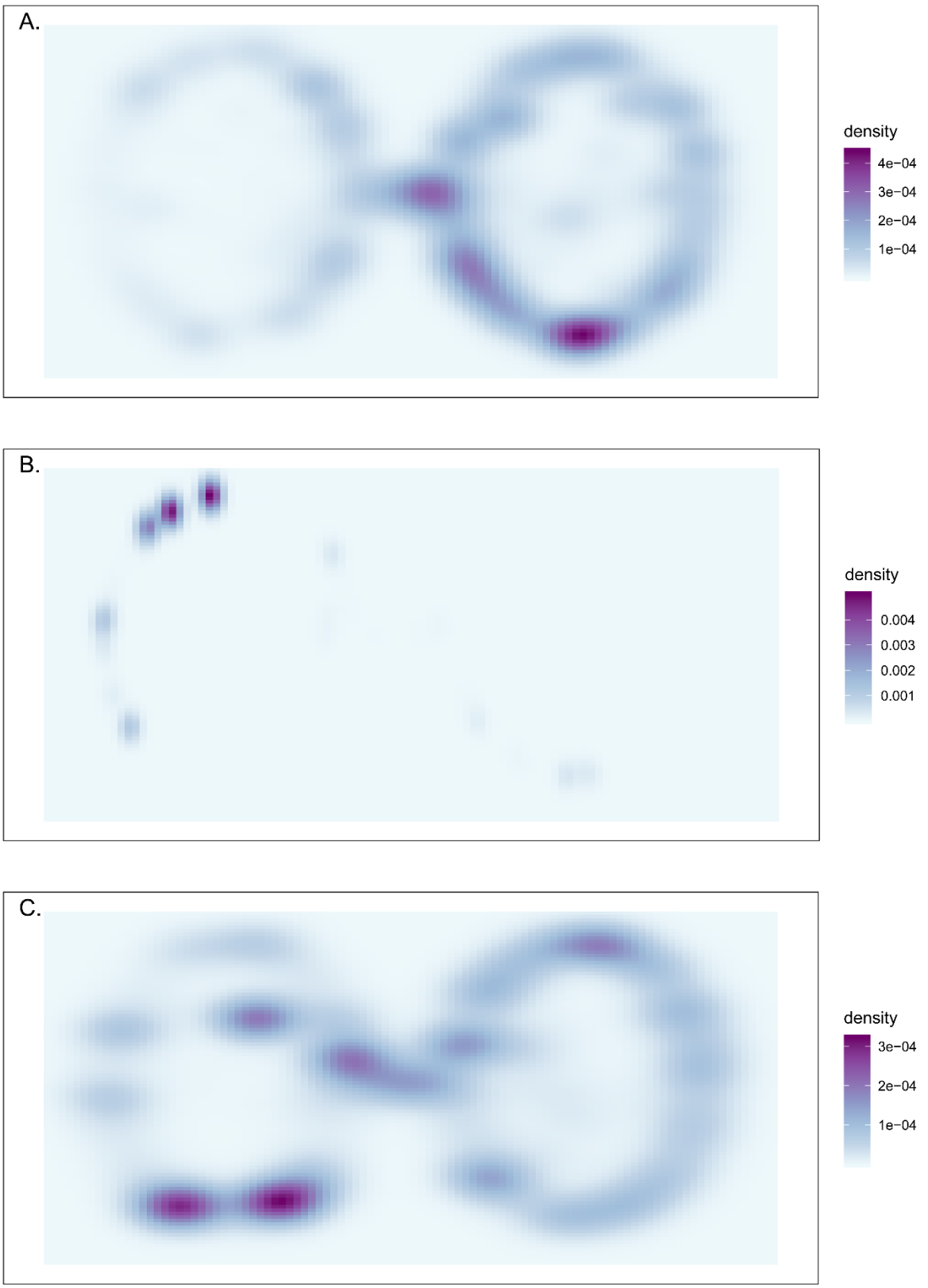
Starving the crabs did not appear to have any adverse effects on locomotor activity: the starved crabs were as active as their fasted conspecifics during the 4 h experimental period. This is contradictory to my original hypothesis that starved crabs with low energy reserves may not have been able to support the energetic cost of locomotion to the same level as the fasted crabs. This study suggests starved rock crabs have sufficient energy to perform a routine locomotory activity, at least within the confines of the Shuttle Box apparatus. However, it is unclear whether the animals could orientate and make longer migrations to avoid widespread hypoxic regimes in the natural environment. Further studies are needed to examine the aerobic scope and stamina of starved and fasted crabs.

The fed crabs exhibited more pronounced responses in the lowest oxygen treatment compared with starved and fasted crabs, with an increasing ratio in moving speed when switching between the 50% and 20% oxygen arenas. Fed crabs have a higher MO_2 and P_{crit} than their starved and fasted conspecifics, associated with the digestion and absorption of the meal (Jiang, Chapter Two). The greater oxygen demand would mean that they would avoid low oxygen areas more rapidly than fasted and starved crabs (van Raaij et al., 1996). This behavior has been observed in other species: postprandial Dungeness crabs (*Cancer magister*) are more active in low oxygen tensions and frequently try to break the water surface, presumably attempting to gain aerial oxygen (Bernatis et al., 2007; Taylor and Butler, 1973). Although the fed crabs exhibited an increase in their moving speed and were more active in the 20% oxygen arena than that in the 50% oxygen arena with a ratio of 1.5 ± 0.2, as a whole, they did not spend more time in the higher oxygen regime, i.e., no successful avoidance of hypoxia.

There was a considerable variation in locomotor activity among individual fed crabs within the 50% vs. 20% oxygen treatment. Three responses were evident (Figure 5.6): A. three crabs spent over 80% of the time in 50% oxygen while making numerous short-term excursions into 20% oxygen. B. two crabs spent over 90% of the time in 20% oxygen and remained quiescent (activity level < 10%). C. two crabs remained active and moved back and forth between the two arenas, showing no preference for either arena. This variation in activity even occurred in the control treatment (100% vs. 100% oxygen) when no hypoxia was present. For example, the fasted crabs showed a great variety of locomotion. Three crabs were largely “inactive” (average moving speed < 0.5 cm/s) with an activity level < 25%. In contrast, four crabs maintained active (speed > 0.5 cm/s) over 70% of the experimental period with average moving speed > 2.0 cm/s. This

variation in activity patterns among individual animals undoubtedly contributed to the lack of discernable avoidance behavior in the experimental treatments.

These intraspecific behavioral differences may be related to animal personality, which describes the pattern of repeatable individual differences in behavior over time (Careau and Garland, 2012; Dall et al., 2004; Gherardi et al., 2012; Wolf et al., 2007). Recent studies have shown crustaceans display personality differences whereby individual crustaceans exhibit different behavioral responses, which persist in repeated experiments (Gherardi et al., 2012; Réale et al., 2010; Sih et al., 2004). For example, there is a behavioral syndrome of bold and shy crabs; bold crabs are more active in exploration and more aggressive in agonistic behaviors than shy crabs (Belgrad et al., 2017; Courtene-Jones and Briffa, 2014; Mowles et al., 2012; Su et al., 2019). Although the behavior of the same individual was not repeatedly measured in different oxygen treatments here, locomotor activities were continuously recorded for each animal during the experimental period. The consistent difference in activity rates among individuals agrees with the personality theory. The shy rock crabs may remain quiescent regardless of hypoxic exposure, while bold crabs may actively explore both arenas. This bold-shy hypothesis warrants further investigation.


A.
density
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B.
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density
3e−04
2e−04
1e−04
plotting scale (1m)

Figure 5.27 Density distribution of the position of individual fed rock crabs during the 4-h 50% vs. 20% oxygen treatments (low oxygen arena on the left) to show the variation in behaviors among individual animals. The darker color means the longer time a crab remained in that area (i.e., density = 0.004, the crab spent 0.004 × 4h = 1min in that 1 cm^2 raster). A. the crab spent more time in 50% oxygen; B. the crab was quiescent and spent more time in 20% oxygen; C. the crab was relatively active and moved back and forth between the arenas.

5.5.2 The effects of the oxygen level and the food deprivation state on the locomotion responses to 12-h single-oxygen treatments

The fasted crabs exhibited a significant reduction in locomotor activity in the lowest oxygen regime (20% oxygen). This oxygen level is below the calculated P_{crit} of fasted crabs (Jiang, Chapter Two) and at a level where the routine activities of the crabs became impaired: fasted crabs consume less food in 20% oxygen or refuse to feed altogether (Jiang, Chapter Four). For those that do feed, the gastric processing of the meal takes longer (Jiang, Chapter Four), which is paralleled by a prolonged postprandial metabolism (Jiang, Chapter Two). Interestingly (and in contrast to fed crabs), the fasted crabs did not become entirely quiescent in 20% oxygen; they maintained an average speed of approximately 40% of that measured in 100% oxygen. This is unexpected because the P_{crit} (28 ± 2% oxygen for fasted crabs) represents the oxygen tension at which the crabs can no longer maintain adequate oxygen uptake (Pörtner and Grieshaber, 1993; Seibel et al., 2021; Wang et al., 2009). This leads to the question how they can still maintain locomotor activity (albeit at a lower rate) when oxygen is limiting. As previously discussed (Jiang, Chapter Two), care is advised when interpreting the P_{crit} (Wood, 2018); these findings back up the theory of two different critical partial pressures (Pörtner and Grieshaber, 1993; Jiang, Chapter Two). The calculated $P_{crit}R$ of 28 ± 2% oxygen likely represents a voluntary reduction in MO_2, which would be accomplished by the animals becoming quiescent (Jiang, Chapter Two). This reserves a small aerobic scope for critical activities such as foraging or escape from predators. A much lower $P_{crit}M$ (anaerobic metabolism, not measured in this study) would represent the true P_{crit} and would be achieved by the crabs becoming completely quiescent, with a switch from

aerobic to anaerobic metabolism, where animals cannot further increase MO_2 (aerobic scope = 1). Thus, although the physiological and behavioral responses of fasted crabs may decrease according to limited oxygen supply in hypoxia, the ability to increase oxygen consumption below calculated $P_{crit}R$ (respiratory) may help them avoid (increased locomotor activity) hypoxic conditions in their natural environment.

The P_{crit} of fed crabs (35 ± 2% oxygen) is slightly higher than fasted crabs (Jiang, Chapter Two). Despite this fact, their locomotor activity was not compromised in 30% oxygen, suggesting that the fed crabs can balance the concurrent oxygen demands associated with digestion and locomotion at oxygen levels below their P_{crit}. This would likely be achieved by slowing the passage of digesta/digestion (Jiang, Chapter Four), allowing diversion of oxygen to the skeletal muscles (Houlihan and Mathers, 1985; McGaw, 2007). However, when comparing the locomotor activity of fed and fasted crabs in 20% oxygen, the fed crabs were noticeably less active and became mostly quiescent (activity level < 10%). This indicates that fed animals, even when substantially slowing digestive processes (Jiang, Chapter Four), cannot balance the simultaneous oxygen demand associated with digestion and locomotion in 20% oxygen. The continued passage of digesta (Jiang, Chapter Four), albeit very slow, and the elevated postprandial metabolism (Jiang, Chapter Two) show digestive processes cannot be halted entirely in 20% oxygen. Once digestion has started, particularly intracellular protein synthesis, the crabs may be committed to these processes (Mente et al., 2003; Penney et al., 2016). Thus, fed crabs may have to submit to the oxygen needs of digestion and exclude locomotor activity. In nature, it would suggest that recently fed animals would not be able to mount an effective escape response from prolonged and/or widespread hypoxia compared with animals that had not recently fed. Since all animals

have to feed, the current findings have important implications when determining the effects of widespread hypoxia on mobile benthic communities.

5.5.3 Use of the Shuttle Box system to determine behavioral responses of decapod crustaceans

The second aim of our study was to assess the suitability of this apparatus to study the behavioral responses of decapod crustaceans. The Shuttle Box system uses advanced technology to maintain a relatively constant and large difference in oxygen levels between two arenas while allowing animals free access to each area. This system can also record fine-scale movements (every 1 sec) of each animal as they move between different regimes, which allows us to analyze the behavior of each individual in detail (Kates et al., 2012; Nielsen and McGaw, 2016).

However, despite these advantages, this study highlights potential problems from inferring responses of crustaceans in the field from those in laboratory-based experiments. We showed that this particular set of apparatus does have some limitations. Other authors too suggest that an animals' behavioral performance may differ in the laboratory and field, and one should take care when drawing inferences between the two (Andrews and Enstipp, 2016; Bernatis et al., 2007; Costa and Sinervo, 2004; Curtis and McGaw, 2012; Ngcamphalala et al., 2021; Williams and Hindle, 2021). The design of the Shuttle Box was such that a constant circling current is set up in each arena: this helps maintain the oxygen differences between the two arenas (Figure 5.1). However, this current may have influenced the rock crabs' behavior, as water current can be an orientation cue for crustaceans. For example, foraging crustaceans may move upstream (rheotaxis) when the current carries food odor (Koehl, 2006; Weissburg and Dusenbery, 2002).

Alternatively, crabs may orient down-current (shoreward) to avoid hypoxia during upwelling events (Bell et al., 2009; Bell et al., 2003a). In the present study, the current may have simply kept the crabs moving in circles as they were observed to constantly circle the perimeter of the chamber (Figure 5.6 and 5.7). In other behavioral studies, animals monitored in a tube or rectangular chamber with a directional water flow were able to orient and avoid hypoxia more readily (Bell et al., 2009; Bernatis et al., 2007; Das and Stickle, 1994; Wannamaker and Rice, 2000).

The experimental light setting in this study may have also affected the locomotor activity of rock crabs. Red lights were initially used to illuminate the apparatus because crustaceans are not sensitive to this wavelength (Cronin and Forward, 1988; Nguyen et al., 2017). However, the light level was not high enough for the camera to detect the contrast difference between the crab and apparatus. Subsequently, the whole apparatus was surrounded by a flexible white LED light, which provided enough contrast for the camera system to track the crab. Although the light was not excessively bright (approximately 100 lux), it may have disturbed the crabs. Demersal crustaceans are usually negatively phototactic. Rock crabs show a distinct diurnal rhythm: they are inactive during the daytime, usually hiding under rocks or macroalgae (Haefner Jr, 1976; Hudon and Lamarche, 1989), while they exhibit the greatest activity during the dark with peaks at dawn and dusk (Rebach, 1985). Thus, in constant light, the rock crabs were likely stressed and actively searching for a place to hide from the light.

Additionally, many crustaceans, including rock crabs, are positively thigmotaxic and actively seek shelter under rocks and seagrass (Alberstadt et al., 1995; Fišer et al., 2019). Here, the rock crabs moved along the perimeter of each arena (Figure 5.7) and kept reinvading the hypoxic

arena. This is likely because they would be stressed in the open and constantly searching for a shelter. Once in a shelter, animals do tend to become quiescent (McGaw, 2001; Nielsen and McGaw, 2016). I did try and introduce a shelter into the apparatus, but it disrupted the water flow within an arena and interfered with the camera tracking system.

Finally, although innovative, the system was not very efficient at maintaining differences between arenas at lower oxygen concentrations. As the oxygen was lowered in one arena, it did affect the oxygen concentration of the other arena, probably due to some mixing through the narrow walkway and oxygen exchange across the water surface. Therefore, while I was able to maintain a 50% difference in oxygen levels when using 50% and 100% oxygen, this difference declined to 30% at the lower oxygen range (50% vs. 20%). I was unable to maintain two different oxygen levels for prolonged periods using oxygen concentrations below 20% oxygen, and it could be that behavioral responses of rock crabs would be more evident at lower oxygen levels (Broughton et al., 2017; Wannamaker and Rice, 2000).

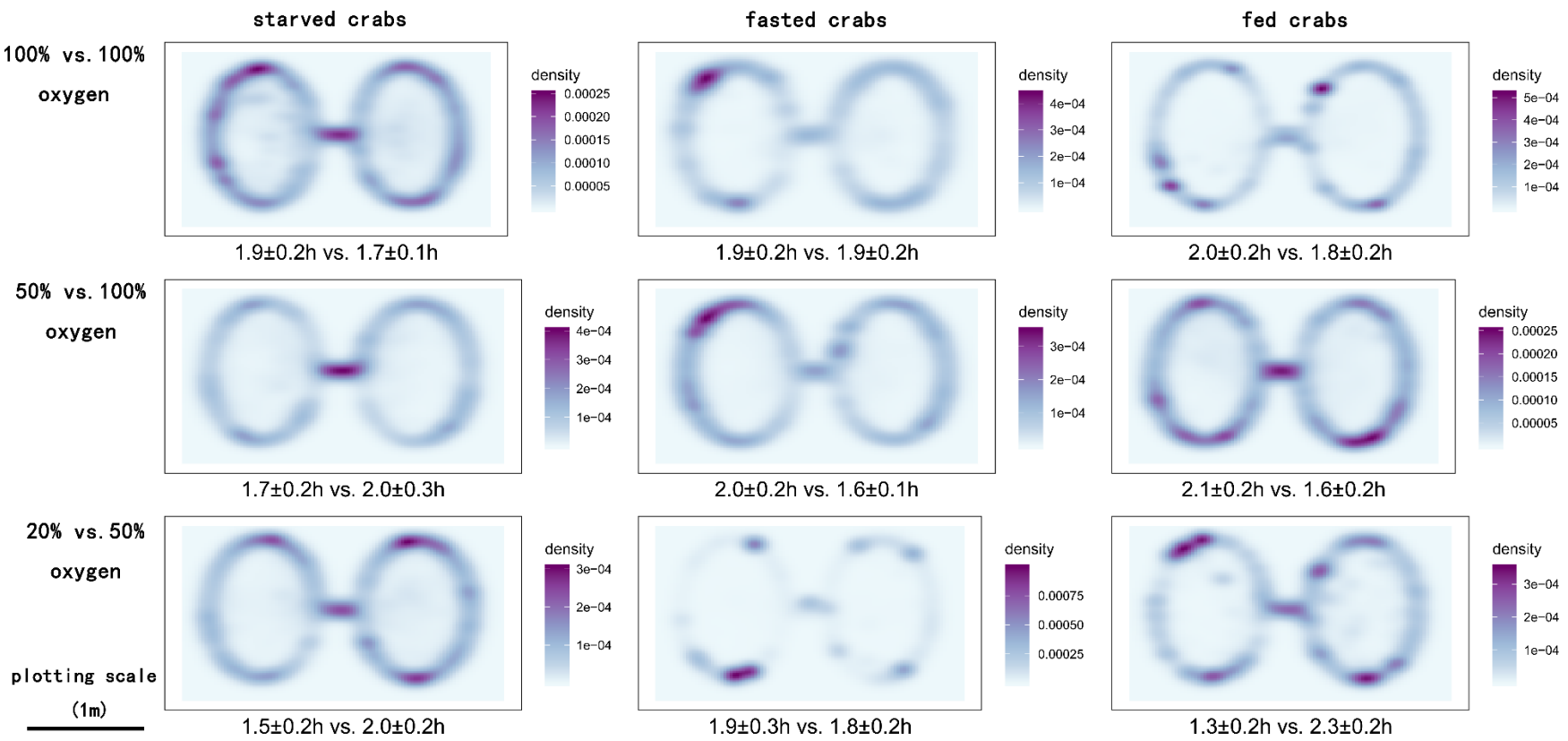

Figure 5.28 The accumulated position density of rock crabs in 100% vs. 100%, 50% vs. 100% and 50% vs. 20% oxygen treatments (low oxygen arena on the left). The time below each graph shows the mean time (± SEM, N = 7–11) the crabs spent in each arena. The darker the color, the greater amount of time an animal spent in the area.

5.5.4 Conclusion

The findings of this study do suggest that the feeding state will alter responses of rock crabs in the lowest oxygen regimes tested. That being said, for the most part, obvious avoidance of hypoxia, or preference for an oxygen regime, was not observed. The number of animals used in the present study was constrained by logistics. It is possible that significant patterns could have been detected if a larger sample size was used (see Appendix B for power analysis). Here I suggest possible modifications for the paired oxygen treatment if additional experiments were to be conducted in the future.

While the rock crabs may not be sensitive to the oxygen levels tested, there is a growing understanding of potential differences between laboratory-based studies and what actually may occur in nature (Bell et al., 2009; Bell et al., 2003a; Bernatis et al., 2007; Curtis and McGaw, 2008; Curtis and McGaw, 2012). This has led to a renewed interest in conducting experiments in the field (Williams and Hindle, 2021). New technologies and miniaturization of data-logging equipment have progressed to a stage where it will soon be feasible to measure fine-scale behavioral and physiological responses of large invertebrates (Williams and Hindle, 2021). In the future, it will be important to observe the behaviors of freely moving decapods in the field in order to gather more realistic data on their responses to environmental perturbations.

5.6 References

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Chapter 6. General Conclusions

6.1 Summary of findings

This thesis investigated the integrated physiological and behavioral responses of rock crabs (*Cancer irroratus*) to hypoxia and with respect to different food deprivation states. Hypoxia is becoming more widespread in shallow coastal environments and will be an important consideration when assessing the future impacts of climate change. In addition, marine animals may experience natural variations of food abundance and adaptation of their physiological condition from feast to famine (Wang et al., 2006). Such different food deprivation states are associated with changes in energy reserves, oxygen consumption, and behaviors. This thesis fills gaps of current understanding in the hypoxia tolerance of marine invertebrates.

In Chapter Two, the critical oxygen partial pressure (P_{crit}) was determined for starved, fasted and fed crabs. The value of P_{crit} obtained was related to the food deprivation state of the crabs. Starved crabs exhibited the lowest (P_{crit}), while fed crabs had the highest P_{crit}. Below the P_{crit} level, the higher oxygen consumption (MO_2) of fed crabs (compared with fasted crabs) indicated that the P_{crit} measured here may represent a shift to hypometabolism as a possible adaption to hypoxia – which allows some aerobic scope reserved for critical activities. This hypothesis was further supported by the results in Chapter Five – that fasted crabs, rather than becoming totally quiescent in 20% oxygen, still exhibited some locomotor activity (albeit somewhat lower) below P_{crit}. The specific dynamic action (SDA) of starved and fasted crabs was also determined in response to different oxygen levels. Following feeding, rock crabs exhibited the typical postprandial

increase in MO_2 characterized by a rapid increase, followed by a gradual decline to pre-feeding levels. Hypoxia retarded the SDA response of both fasted and starved crabs, resulting in lower peak MO_2 and more prolonged duration that MO_2 remained elevated. The food deprivation state had an interactive effect, with starved crabs exhibiting a lower peak MO_2, prolonged duration and higher energy expenditure than fasted crabs. Starvation may also trigger a cross-tolerance to hypoxia since both long-term starvation and severe hypoxia elicited similar physiological responses to hypometabolism.

In Chapter Three, biochemical mechanisms (hemolymph oxygen partial pressure (P_{O2}), pH, hemocyanin (Hc) and L-lactate concentrations) of fasted and starved rock crabs were investigated in response to hypoxia, both before and after feeding. The arterial P_{O2} dropped as a function of decreasing ambient oxygen levels in both fasted and starved crabs, and this decline was most pronounced below the P_{crit}. Compared with starved crabs, the higher Hc concentration of fasted crabs suggested they have better oxygen transport capacity. Although the experimental period (72 h) may have been too short to observe significant increases in Hc concentration, hypoxia-induced alkalosis and elevated L-lactate may have improved the Hc-oxygen affinity. Following feeding, the fasted crabs exhibited a significant increase in L-lactate concentration below P_{crit}. In contrast, anaerobic metabolism was not observed in postprandial starved crabs: the lower oxygen demand in this treatment group was likely associated with the slower recovery of dormant digestive processes during refeeding.

In Chapter Four, the feeding behaviors and subsequent gastric processing of starved and fasted rock crabs were examined in response to different oxygen levels. Three different techniques were used to measure food intake. The gravimetric analysis of dry commercial food pellets was the most accurate method, while the gravimetric analysis of wet shrimp muscle overestimated food intake and counting radiopaque beads in homogenized shrimp muscle (using a fluoroscope), underestimated food intake. During exposure to severe hypoxia (20% oxygen), rock crabs reduced food intake, and a higher percentage of crabs refused to eat. Although food deprivation state did not significantly affect food intake, more starved crabs fed in 20% oxygen. After feeding, the subsequent digestion of the meal was also affected by the oxygen level; prolonged gastric emptying times paralleled previously measured changes in postprandial oxygen consumption in hypoxia (Jiang, Chapter Two). Starved crabs exhibited slightly longer transit times for digesta compared with fasted crabs. This was likely due to the lower energy stores and a gradual upregulation of dormant digestive organs and enzymes. These results suggest that although a trade-off may occur in starved rock crabs between the need to procure nutrients and deal with hypoxic stress, impaired digestive processing may still have a deleterious effect on these animals.

In Chapter Five, the locomotor activity and behavioral responses to hypoxia were investigated for starved, fasted and fed rock crabs using a Loligo® Shuttle Box system. The ability to detect and avoid specific levels of hypoxia was tested by observing the movement of each crab between two arenas with different oxygen levels. The rock crabs

did not appear to show any preference for a specific oxygen regime or spend more time in the arena with higher oxygen. A high degree of variability in locomotor activity was observed among the individuals within the same treatment, which may be caused by the nature of the apparatus and undoubtedly contributed to the lack of discernable avoidance behavior among oxygen treatments. Nevertheless, despite a lack of clarity when offered a choice of oxygen levels, hypoxia played an important role in regulating the moving speed of rock crabs with respect to food deprivation state. The fed crabs were less active than their fasted conspecifics in 20% oxygen (below P_{crit} of both fed and fasted crabs), likely because they could not balance the simultaneous demands of digestion and increased activity.

In summary, during exposure to severe hypoxia (below the P_{crit}), rock crabs were unable to maintain their routine activities. They reduced food intake or refused to feed; for those that had already fed, gastric processes were slowed but not halted. The slowing of gastric processes was associated with a reduced scope and prolonged duration of SDA. Biochemical mechanisms facilitated oxygen transport in severe hypoxia: respiratory alkalosis and anaerobic metabolism may increase the hemocyanin oxygen affinity and compensate for the deficit of aerobic metabolism. In 20% oxygen, recently fed animals were unable to maintain oxygen consumption despite a slowing of gastric processes and halting locomotor activity. The fed crabs capitulated to the oxygen demand of digestion and became immobile. A 4-week starvation period induced limited effects on hypoxia tolerance compared with 5-day fasted crabs. Starved crabs had a similar P_{crit} to fasted

crabs. However, the dormant digestive system of starved crabs likely affected attributes of their SDA in hypoxia. Starved crabs exhibited a lower peak MO_2, prolonged duration and higher energy expenditure than fasted crabs.

6.2 Future directions

Based on the finding of this thesis, I highlight several directions here for future research:

The P_{crit} is a well-known concept that concerns the relationship between aerobic metabolism and hypoxia tolerance. However, there is increasing discussion on the meaning and significance of this concept (Pörtner and Grieshaber, 1993; Seibel et al., 2021; Ultsch and Regan, 2019; Wood, 2018). Wood (2018) criticized the use of P_{crit} because it carries minimal information content and is not a reliable index of hypoxia tolerance. Rather than hypoxia tolerance, a recent study suggests that P_{crit} represents the oxygen partial pressure at which physiological oxygen supply reaches its maximum capacity (MMR) and is species- and temperature-specific (Seibel et al., 2021). This thesis provides new insights to a more comprehensive understanding of P_{crit}. Here I found that rock crabs can elevate oxygen consumption slightly (during digestion and increased locomotor activity) below their measured P_{crit} (Jiang, Chapter Two and Five). This begs the question as to how they are able to do this when oxygen is limiting. These findings back up the theory of two different critical partial pressures, which need to be tested in marine animals (Pörtner and Grieshaber, 1993; Jiang, Chapter Two). The traditional P_{crit}R (respiratory, measured in this study) where MO_2 starts to decline as a function of ambient

P_{O2} likely represents a voluntary shift of MO_2 rather than the limitation of MMR. This reduced MO_2 may be achieved by a voluntary reduction in activity, essentially the downregulation of RMR to SMR. This reserves a small aerobic scope for critical activities such as foraging or escaping behaviors. A lower $P_{crit}M$ (metabolism, not measured in this study) may exist, representing the limitation of MMR (aerobic scope = 1) and initiation of anaerobic metabolism. Thus, my work suggests that hypoxia tolerance is better represented by the aerobic scope and anaerobic metabolism rather than RMR or SMR alone in hypoxia. To test this hypothesis, further studies can focus on measuring $P_{crit}M$: the changes of aerobic scope (difference between RMR and MMR) and the level of anaerobic metabolites in different oxygen levels.

It was initially hypothesized that starvation would impart significant effects on the physiological and behavioral responses of the crabs. However, responses associated with starvation were somewhat limited. Starved crabs did not have a higher P_{crit} than fasted crabs (Jiang, Chapter Two), nor did they exhibit lower feeding levels or locomotor activity (Jiang, Chapter Four and Five). The limited effects of starvation on hypoxia tolerance may be explained by the cross-tolerance theory, which describes how adaptation to a particular stressor will increase resistance to another different stressor (Kalra et al., 2017; Steinberg, 2012; Todgham and Stillman, 2013). On the other hand, the extreme energy deficiency of starved animals may affect their stress response (Sokolova et al., 2012). It has been proved that starvation can cause cross-tolerance of desiccation (Bubliy et al., 2012) but have adverse effects on thermal tolerance (Lee et al., 2016; McLean and

Todgham, 2015) and osmoregulation (Haller et al., 2015). Further studies may explore the possible cross-tolerance between starvation and hypoxia, such as the similarity in changes of metabolites and related metabolism pathways in response to starvation and hypoxia stressors. It would also be interesting to test the effects of starvation on the aerobic scope because increased energy consumption may be required to cope with the environmental stressors, such as ocean acidification and thermal stress.

In Chapter Five, a considerable variation in locomotor activity was found among individuals within the same treatment, which masked some behavioral responses. Future studies of personality and behavioral syndrome of rock crabs may explain this consistent locomotor difference among individuals (Gherardi et al., 2012; Réale et al., 2010; Sih et al., 2004). For example, there is a behavioral syndrome of bold and shy crabs; bold crabs are more active in exploration and more aggressive in agonistic behaviors than shy crabs (Belgrad et al., 2017; Courtene-Jones and Briffa, 2014; Mowles et al., 2012; Su et al., 2019). Future research can be conducted on the effects of personality on behavioral responses to hypoxia. It can be hypothesized that the shy rock crabs may remain quiescent regardless of hypoxic exposure, while bold crabs would actively explore and seek out high oxygen refuges. Future studies may also investigate if physiological conditions are related to different personalities. For example, bold crabs have a higher metabolism and higher energy cost than shy crabs (Belgrad et al., 2017; Su et al., 2019). It can be hypothesized that bold crabs need a higher aerobic scope and energy reserve to support their higher activities than shy crabs. I also suggest increasing sample sizes (see Appendix B) as this

increase the power of statistical tests or shed further light on possible behavioral syndromes.

In addition, Chapter Five highlighted some of the problems associated with lab-based studies. Animals' behavioral performance may differ in the laboratory and field (Andrews and Enstipp, 2016; Costa and Sinervo, 2004; Williams and Hindle, 2021). The present work suggested that the water current may keep rock crabs moving along the chamber's perimeter. As water current can be an orientation cue for crustaceans (Bell et al., 2009; Bell et al., 2003; Koehl, 2006; Weissburg and Dusenbery, 2002), future studies can explore the effects of the combined effects of water current direction and hypoxia on avoidance behavior. Moreover, there is a growing understanding of potential differences between laboratory-based studies and what actually may occur in nature (Spicer, 2014). New technologies and miniaturization of data-logging equipment have progressed to a stage where it will soon be feasible to measure fine-scale behavioral and physiological responses of large invertebrates (Williams and Hindle, 2021). For future studies, it will be important to conduct *in situ* field studies to observe realistic behaviors of freely moving decapods on their responses to environmental perturbations. For example, future studies may be allowed to monitor the aerobic metabolism with heart rates of crustaceans and the environmental oxygen level in their natural habitats (McGaw et al., 2018).

6.3 Ecological implications

This thesis provides us more detailed knowledge of what marine animals could encounter during hypoxic exposure. Moreover, marine animals are under strong selection for hypoxia tolerance (Deutsch et al., 2015; Deutsch et al., 2020). Large variance in the behavioral response to hypoxia reflects different survival strategies adapting to environmental stressors, which may accelerate the selection process during climate change (Dall et al., 2004; Sih et al., 2004; Wolf and Weissing, 2012).

Under the changing climate scenario, hypoxic areas in coastal waters have expanded to the natural habitat of the rock crab, such as the Gulf of St. Lawrence (Gilbert et al., 2005; Levin et al., 2009). It can be expected that hypoxia in their natural habitat would impair their routine feeding behaviors, digestive processes and locomotion. It indicates that although marine animals can survive in severe hypoxia (by suppressing their aerobic metabolism with reduced feeding behaviors, digestive processes and locomotion), the suppression of critical activities may hamper growth rates and reproductive rates in the long term (Deutsch et al., 2020; Penn et al., 2018). As a result, hypoxia could impart effects at the population level for benthic marine animals. The projected warmer and lower oxygenated oceans will force poleward and vertical contraction of metabolically viable habitats of marine animals (Deutsch et al., 2015). Failure of these animals to migrate away from such areas or to cope physiologically with such constraints of climate change may lead to mass mortality or even extinction of populations in the future (Penn et al., 2018).

Marine invertebrates play important roles in the marine ecosystem (Galic et al., 2019; Riedel et al., 2014). The high diversity of marine invertebrates contributes to nutrient cycling and supports the stability of the food web and the resistance to disturbance (Cardinale et al., 2012; Prather et al., 2013). The impact of hypoxia on one marine invertebrate may have cascading effects on the whole marine ecosystem (Conley et al., 2009; Vaquer-Sunyer and Duarte, 2008). For instance, interspecific differences in hypoxia tolerance may change predator-prey interactions in favor of hypoxia-tolerant species. These may alter the population size structure and biomass of the prey and predator. Juvenile rock crabs are important prey for lobster (DFO, 2014; Gendron et al., 2001; Scarratt and Lowe, 1972); a reduction in suitable habitats or a direct reduction of a rock crab population may lead to the collapse of localized lobster fisheries. Moreover, the loss of complex interactions among species may open ecological niches for invasive species, e.g., *Carcinus maenas*. Thus, knowledge of the effects of hypoxia, food availability about the individual animal and population level may be especially important for decision-makers to manage many harvestable marine species.

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Appendix

Appendix A. Effects of size and constrained acclimation on aerobic metabolism of rock crabs

During the experiments in Chapter One, the crabs were held in the laboratory for several months, and starved crabs were individually constrained in plastic boxes for 4–5 weeks to ensure they did not try to attack and consume one another. It was important to determine whether constraining the crabs for long periods in the lab had any effect on metabolism. In addition, the crabs varied in size between 80 and 160 g, so we also determined if this size variation had any significant effect on metabolic reactions.

1. Material and Methods

Freshly collected crabs (held in the laboratory less than one week) were divided into 3 groups (n = 8–10 crabs per group): free-moving group (174 ± 20 g), constrained group (167 ± 17 g), and small-size group (84 ± 13 g). Crabs of the free-moving and small-size groups were held in a 300 L tank where they could freely move and interact with each other, while the crabs in the constrained group were held individually in perforated plastic chambers (25 × 15 × 7.5 cm).

The crabs were held in the tank (300 L) in flow-through aerated seawater at a temperature of 12–13°C in constant dim red light. They were fed herring weekly but were isolated from food supplies for 4 days prior to oxygen consumption measurements. For each treatment, the resting and maximum metabolic rates were measured on the first day of collection, then 30, 60 and 90 days later (data for the small-size group on day 60 were found to be corrupted). The resting metabolic rates were calculated as the average of the 3 lowest readings after the crabs had settled in the respirometry chamber for 12 h. The maximum metabolic rates (MMR) were measured during exhaustive activity: An elastic band was glued onto the top of the crab's carapace into which small lead bars (total mass of 200–300 g) were inserted, the crab was then inverted; this caused each crab to struggle vigorously and try to right themselves (McGaw, 2007). The average of the 2 highest readings during 6 hours of this struggling behavior was taken as MMR.

The data for different holding methods and sizes were analyzed using a linear mixed-effects model conducted in R 3.4.2 (Team, 2018). The fixed factors were the treatment (free, constrained or size) and time. The random factor was the crab identity because I took repeated measures from the same crab over time. Statistical significance was accepted when $P < 0.05$. All data in figures and tables were shown in means ± standard error of the mean (SEM).

2. Results

2.1 Effects of holding conditions and acclimation time

2.1.1 Holding condition

The constrained holding condition had no significant effect on the standard metabolic rate (SMR), resting (RMR) or maximum (MMR) over the 90-day experimental period (Figure A.1, Table A.1). The SMR of both groups was similar for all time periods except the first day where it was lower in the free-moving crabs (30.3 ± 2.4 mgO_2/kg/h) than the constrained crabs (36.8 ± 1.8 mgO_2/kg/h) (Tukey's HSD, t = -2.4, p = 0.03). The only difference was found in MMR at day 60, when the free-moving crabs had MMR of 120.0 ± 6.5 mgO_2/kg/h, and the constrained crabs had 98.9 ± 7.0 mgO_2/kg/h (Tukey's HSD, t = 2.6, p = 0.02). Meanwhile, the free-moving crabs had a significantly higher scope than the constrained crabs (linear mixed-effects model, $F_{1,22}$ = 9.6, p = 0.02). Although the scope appeared to be higher over most experimental time periods, the only statistically significant difference occurred at day 30, where the scope of the free-moving crabs reached 7.0 ± 0.5 and 5.5 ± 0.2 for the constrained crabs (Tukey's HSD, t = 2.94, p = 0.008).

2.1.2 Acclimation time

The acclimation time had a significant effect on the SMR (linear mixed-effects model, $F_{3,64}$ = 28.4, p < 0.001), RMR (linear mixed-effects model, $F_{3,64}$ = 7.4, p < 0.001), MMR (linear mixed-effects model, $F_{3,64}$ = 18.8, p < 0.001) and scope (linear mixed-effects model, $F_{3,64}$ = 28.4, p < 0.001) for both holding conditions (Table A.1 and Figure A.1). The most

marked changes happened in the first 30 days. The SMR decreased 38% from 30.3 ± 2.4 mgO_2/kg/h for free-moving crabs (Tukey's HSD, t = 4.4, p = 0.0002) and 38% from 36.8 ± 1.8 mgO_2/kg/h for constrained crabs (Tukey's HSD, t = 5.3, p < 0.0001) while the RMR similarly decreased 29% from 36.4 ± 2.4 mgO_2/kg/h for free-moving crabs (Tukey's HSD, t = 3.3, p = 0.008) and 25% from 40.0 ± 1.8 mgO_2/kg/h for constrained crabs (Tukey's HSD, t = 3.3, p = 0.009). Meanwhile, by day 30, the MMR increased about 15% from 110.9 ± 3.5 mgO_2/kg/h for free-moving crabs (Tukey's HSD, t = -3.0, p = 0.02) and less than 10% from 111.7 ± 3.4 mgO_2/kg/h for constrained crabs (Tukey's HSD, t = -1.9, p = 0.23). These changes in SMR and MMR resulted in an approximate 2-fold elevation in the scope for both treatment groups between 1 and 30 days (Tukey's HSD tests: free-moving crabs, t = -6.8, p < 0.0001; constrained crabs: t = -5.3, p < 0.0001).

After day 30, the SMR and RMR tended to increase while MMR and scope decreased. Changing trends of metabolism reversed for both treatment groups. The SMR diverged between the two holding conditions from 30 to 90 days (linear mixed-effects model, $F_{3,64}$ = 2.8, p = 0.05). The SMR of the free-moving crabs significantly increased by approximately 50% (Tukey's HSD, t = -3.4, p = 0.006), while the concomitant increase of constrained crabs was less than 10% (Tukey's HSD, t = -0.6, p = 0.93). The MMR started to decline between 30 days to 90 days. The MMR of constrained crabs dropped 28% (Tukey's HSD, t = 6.1, p < 0.0001), while that of free-moving crabs declined 21% (Tukey's HSD, t = 4.5, p = 0.0002), resulting in a significant interaction of the holding condition and acclimation time (linear mixed-effects model, $F_{3,64}$ = 2.9, p = 0.04).

2.2 Effects of crab sizes and acclimation time

2.2.1 Size

During the 90-day acclimation period, the crab size significantly affected the SMR (linear mixed-effects model, $F_{1,18} = 5.2$, $p = 0.04$), MMR (linear mixed-effects model, $F_{1,18} = 5.9$, $p = 0.03$) and the scope (linear mixed-effects model, $F_{1,18} = 6.7$, $p = 0.02$) except for the RMR (Figure A.2 and Table A.2). The small crabs had a significantly lower SMR compared with the large crabs (19.6 ± 0.6 vs. 30.4 ± 2.4 $mgO_2/kg/h$) only on day 1 (Figure 2A, Tukey's HSD, $t = 3.5$, $p = 0.002$). Meanwhile, during the 90-day acclimation, the small crabs MMR ranged between 124.3 ± 9.1 and 144.1 ± 10.3 $mgO_2/kg/h$, which was approximately 13−20% higher than the large crabs (Figure A.2C). These differences in MMR and SMR resulted in a significantly higher scope for the small crabs at day 1 (Tukey's HSD, $t = -3.4$, $p = 0.004$) and after 90 days (Tukey's HSD, $t = -3.0$, $p = 0.008$) (Figure A.2C).

2.2.2 Acclimation time

The acclimation duration also significantly affected the SMR (linear mixed-effects model, $F_{2,34} = 4.7$, $p = 0.02$), RMR (linear mixed-effects model, $F_{2,34} = 3.8$, $p = 0.03$), MMR (linear mixed-effects model, $F_{2,34} = 6.1$, $p = 0.005$), and the scope (linear mixed-effects model, $F_{2,34} = 12.1$, $p < 0.001$). The SMR and RMR of large crabs exhibited the same trend in the holding-condition trial, which significantly decreased in the first 30 days and then increased thereafter. Meanwhile, the SMR and RMR of small crabs remained stable ranged between 19.6 ± 0.6 to 22.5 ± 3.3 $mgO_2/kg/h$ and 30.5 ± 3.3 to 31.4 ± 3.6 $mgO_2/kg/h$

respectively during the 90 days (Figure A.2A&B). However, the diverged responses of crab size over acclimation durations only resulted in a significant interaction in SMR (linear mixed-effects model, $F_{2,34} = 5.7$, $p = 0.007$). On the other hand, the direction of changes MMR of both sizes showed a similar trend over the 30-day acclimation period, increasing during the first 30 days and decreasing thereafter. The only significant decrease in the MMR was found in the large crabs between 30 to 90 days, dropping from 127.4 ± 4.7 to 103.3 ± 5.3 $mgO_2/kg/h$ (Tukey's HSD, $t = -2.8$, $p = 0.02$). As a result, the large crabs also exhibited a more variable scope over the acclimation period compared with the small crabs, which created a significant interaction (linear mixed-effects model, $F_{2,34} = 6.3$, $p = 0.005$).

3. Conclusions

The crabs were kept in laboratory conditions during the 28-day starvation period. It is, therefore, crucial to understand the effects of laboratory captivity because starvation and laboratory captivity may have had an interactive effect. For example, lab captivity of green crab (*Carcinus maenas*) over 2 to 3 weeks without exercise, results in a loss of aerobic and anaerobic capacity in the locomotor muscles producing reduced walking performance (Houlihan and Mathers, 1985). In this study, crabs were kept in the individual chambers during starvation so that they were not able to eat each other (cannibalism). Small chambers limited the activity of rock crabs, which also decreased their maximum metabolic performance, especially after 60 days. The wild brown trout increases metabolic rates with increasing time spent in captivity (Závorka et al., 2019). Similar

increases of RMR were found in this study, but quite the opposite for MMR. The crabs had the best aerobic capacity after 30-day acclimation (lowest resting and standard metabolic rates; highest maximum metabolic rates and aerobic scope). However, RMR and SMR increased while MMR decreased in the following 60 days. The increasing RMR and SMR could be caused by the different food quality and quantity in the laboratory and the wild, as food intake affects the masses of viscera that contribute to whole organism SMR (Armstrong and Bond, 2013; Auer et al., 2016). On the other hand, the decreasing MMR may be explained by limited activity during acclimation, as athletic species have higher MMR correlated with higher total mitochondrial and capillary erythrocyte volumes compared to non-athletic species (Weibel et al., 2004).

In the present study, I tried to use a narrow range of crab sizes because the effect of body mass on metabolic rates is well known, which is described as the Kleiber's law or metabolic allometry (Kleiber, 1947). The minimal metabolic rate of organisms scales to the 2/3 to 3/4 power of body mass, which means smaller animals usually have higher mass-specific metabolic rates (Killen et al., 2007). The MMR of rock crabs follows this rule, as the doubled body size reduced the mass-specific MMR to 84%. However, RMR and SMR of smaller crabs were more stable to acclimation, which may indicate they are less sensitive to environmental change. Although the body size has little effect on hypoxia tolerance of rock crabs, reduction of aerobic scope may induce a trade-off among various oxygen-consuming physiological functions (Guderley and Pörtner, 2010; Penn et al., 2018).

In conclusion, it is appropriate to acclimate the crabs within 30 days, which maximized the aerobic scope while minimized the effects of constrained captivity and body size.

a) Since I was using starved crabs within 30-45 days of being confined, this should not have a noticeable effect compared with uncaged crabs

b) There was a difference in responses over the size range I used, therefore in experiments, I made sure equal size ranges were used across the different trials.

4. References

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5. Tables

Table A.1 Results of the two-way ANOVA of the linear mixed model for the measures of the fasted crabs in free and constrained acclimation. The two explanatory variables (holding condition and time) and their interaction were tested. Bold font indicates statistical significance ($p < 0.05$).

Variables	Factors	$df_{num,den}$	*F*	*P* value
	Free vs. Constrained	1,22	1.55	0.226
SMR	**Time**	**3,64**	**28.38**	**<0.001**
	Interaction	**3,64**	**2.80**	**0.047**
	Free vs. Constrained	1,22	0.21	0.651
RMR	**Time**	**3,64**	**7.38**	**<0.001**
	Interaction	3,64	1.38	0.256
	Free vs. Constrained	1,22	2.51	0.128
MMR	**Time**	**3,64**	**18.82**	**<0.001**
	Interaction	**3,64**	**2.88**	**0.043**
	Free vs. Constrained	**1,22**	**6.07**	**0.022**
Scope	**Time**	**3,64**	**28.38**	**<0.001**
	Interaction	3,64	2.36	0.080

Table A.2 Results of the two-way ANOVA of the linear mixed model for the measures of fasted crabs in two sizes during acclimation. The two explanatory variables (size and time) and their interaction were tested. Bold font indicates statistical significance ($p < 0.05$).

Variables	Factors	$df_{num,den}$	*F*	*P* value
	Size	**1,18**	**5.21**	**0.035**
SMR	**Time**	**2,34**	**4.68**	**0.016**
	Interaction	**2,34**	**5.71**	**0.007**
	Size	1,18	0.66	0.428
RMR	**Time**	**2,34**	**3.78**	**0.033**
	Interaction	2,34	2.09	0.140
	Size	**1,18**	**5.91**	**0.026**
MMR	**Time**	**2,34**	**6.11**	**0.005**
	Interaction	2,34	0.07	0.936
	Size	**1,18**	**6.71**	**0.019**
Scope	**Time**	**2,34**	**12.07**	**<0.001**
	Interaction	**2,34**	**6.32**	**0.005**

5. Figures

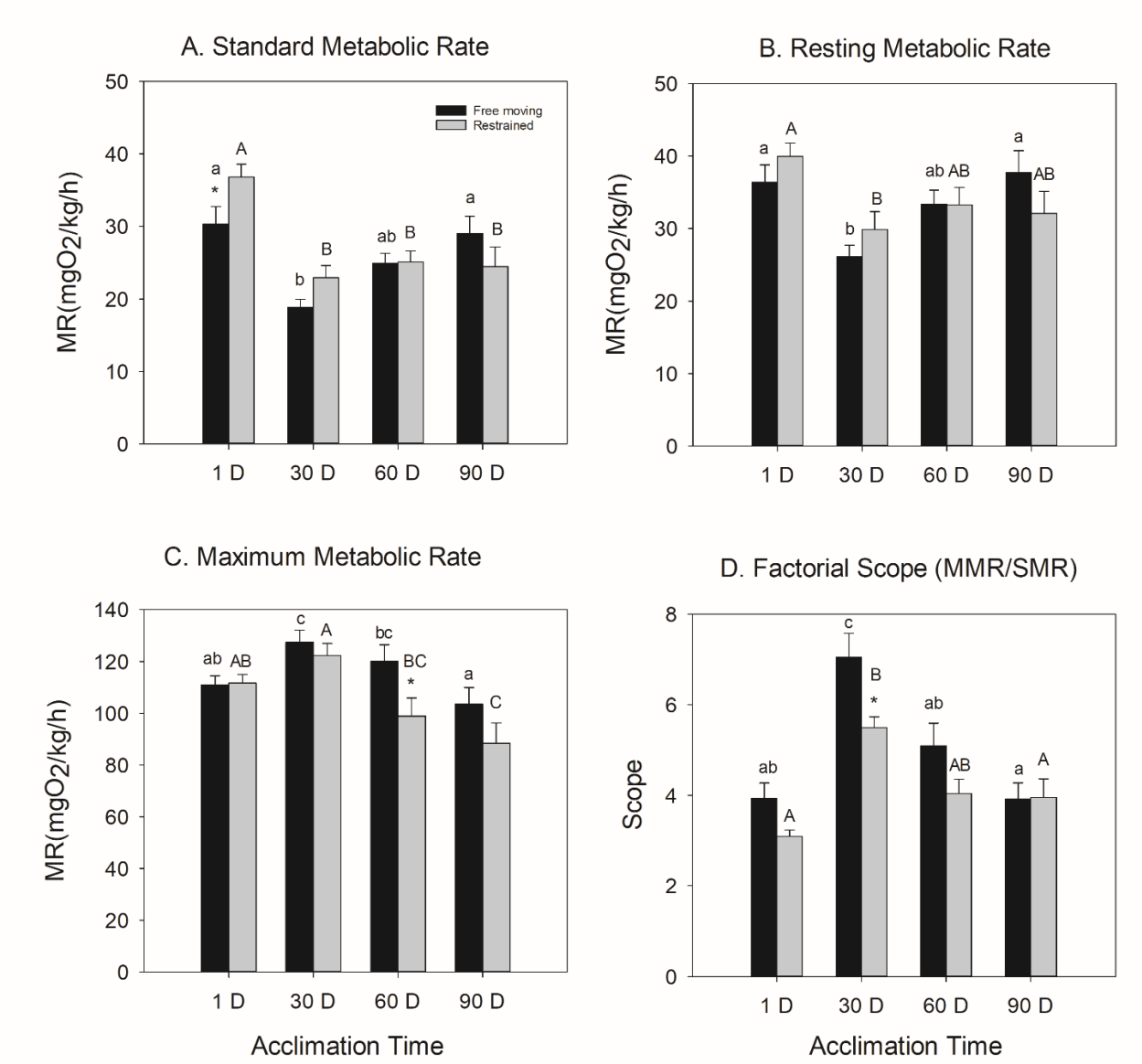

Figure A.1 The standard, resting, maximum metabolic rate, and the factorial scope of fasted crabs in free and constrained conditions during 90-day acclimation. Data are shown as mean with SEM. Asterisk denotes significant differences between free-moving and restrained crabs and letters for time periods (lower case for free-moving crabs and capital letters for constrained crabs).

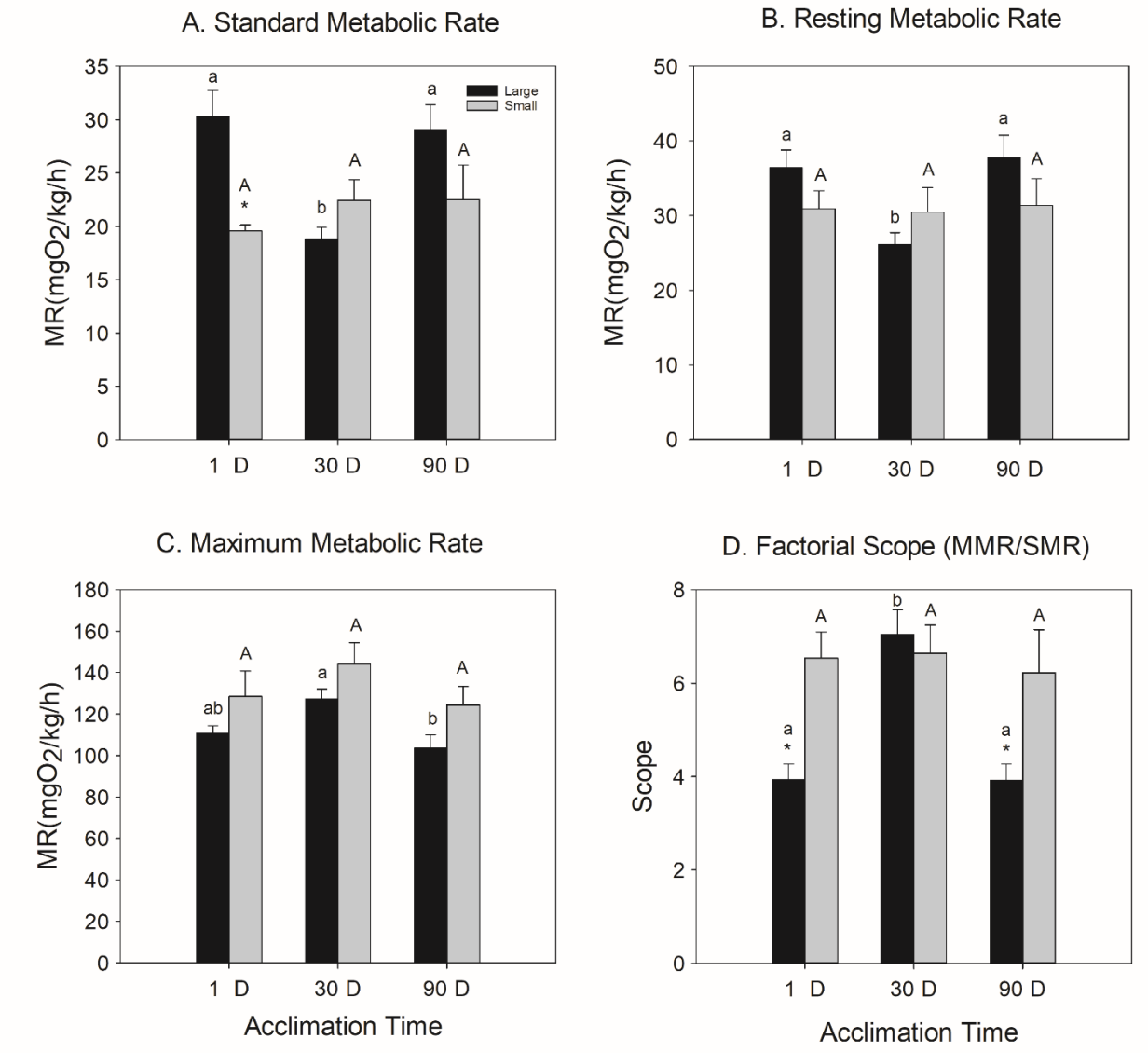

Figure A.2 The maximum metabolic rate, standard metabolic rate and the factorial scope of fasted crabs in large (174 ± 20 g) and small (84 ± 13 g) sizes during 90-day acclimation. Data are shown as mean with SEM. Asterisk denotes significant differences between the large and small crabs and letters for time periods (lower case for large crabs and capital letters for small crabs).

Appendix B. Power analysis of the paired oxygen behavior experiments in Chapter 5

In Chapter Five, paired t-tests did not show significant differences for most paired oxygen treatments (Table 5.1&5.2). Thus, a power analysis was conducted to estimate the sample size for future studies with similar effect sizes (Cohen's d). Cohen's d was calculated as the difference between two means divided by a standard deviation for the data. Then, given power = 0.8, the estimated sample size was calculated by the Pwr.t.test function in the pwr R-package. Given the small effect size and power value here, the sample size (Table B.1 & B.2) needs to be at least 13 for large effect size and over 30 for medium effect size for future behavior experiments of rock crabs using the Loligo shuttle box system.

Table B.1 The total time (h) spent in each arena of the Shuttle Box for the crabs in the three food deprivation states in the three oxygen treatments during 4-h trials (time spent in two arenas may not add up to 4h because the time they spent in the walkway connecting arenas was excluded). Two-tailed paired t-test values are given for each experiment. Bold font indicates statistical significance ($p < 0.05$). Based on the effect size (Cohen's d) of the current study, the estimated sample size to reach high power (0.8) for two-tailed paired t-tests was given for future studies.

Food Deprivation State	Treatment (oxygen pairs)	Speed in High O_2 (cm/s)	Speed in Low O_2 (cm/s)	*df*	*T*	*P-value*	Effect size (d)	Estimated sample size
Starved	100% vs. 100%	1.7 ± 0.1	1.9 ± 0.2	8	-0.46	0.66	-0.15	-
	100% vs. 50%	1.8 ± 0.3	1.9 ± 0.2	8	-0.22	0.83	-0.07	>100
	50% vs. 20%	2.0 ± 0.2	1.5 ± 0.2	7	1.53	0.18	0.58	26
Fasted	100% vs. 100%	1.9 ± 0.2	1.9 ± 0.2	10	0.02	0.98	<0.01	-
	100% vs. 50%	1.6 ± 0.1	2.0 ± 0.2	7	-1.04	0.33	-0.37	60
	50% vs. 20%	1.8 ± 0.2	1.9 ± 0.3	7	-0.23	0.83	0.09	>100
Fed	100% vs. 100%	1.8 ± 0.2	2.0 ± 0.2	8	-0.61	0.69	-0.14	-
	100% vs. 50%	1.6 ± 0.2	2.0 ± 0.2	9	-1.10	0.39	-0.29	98
	50% vs. 20%	2.3 ± 0.3	1.3 ± 0.2	6	2.25	0.07	0.88	13

Table B.2 The average moving speed (cm/s) in each arena of the Shuttle Box for the crabs of the three food deprivation states in the three oxygen treatments during 4-h trials. Two-tailed paired t-test values are given for each experiment. Bold font indicates statistical significance ($p < 0.05$). Based on the effect size (Cohen's d) of the current study, the estimated sample size to reach high power (0.8) for two-tailed paired t-tests was given for future studies.

Food Deprivation State	Treatment (oxygen pairs)	Speed in High O_2 (cm/s)	Speed in Low O_2 (cm/s)	*df*	*T*	*P-value*	Effect size (d)	Estimated sample size
Starved	100% vs. 100%	2.4 ± 0.5	2.3 ± 0.4	8	0.67	0.52	0.22	-
	100% vs. 50%	1.7 ± 0.3	1.8 ± 0.3	8	-0.11	0.92	-0.04	>100
	50% vs. 20%	1.8 ± 0.3	2.1 ± 0.1	7	-2.09	0.08	-0.79	15
Fasted	100% vs. 100%	1.6 ± 0.3	1.5 ± 0.2	10	0.85	0.42	0.26	-
	100% vs. 50%	1.7 ± 0.3	1.7 ± 0.3	7	0.42	0.69	0.15	>100
	50% vs. 20%	1.3 ± 0.4	2.1 ± 0.6	7	-1.09	0.32	-0.41	49
Fed	100% vs. 100%	1.7 ± 0.3	1.6 ± 0.4	8	0.67	0.52	0.22	-
	100% vs. 50%	2.1 ± 0.2	1.9 ± 0.4	9	0.96	0.36	0.30	88
	50% vs. 20%	0.7 ± 0.1	0.9 ± 0.1	6	-1.19	0.29	-0.48	36