Acid-Base Regulation and Physiological Responses to Aquaculture and Global Change Stressors in Euryhaline Crustaceans

by

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A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Decapod crustaceans regularly face intrinsic and extrinsic stressors that challenge pH homeostasis, which is compensated through the process of acid-base regulation. Prior research on acid-base regulation in crustaceans has focused on stressors commonly experienced during day-to-day life like exercise or that experienced in estuarine environments such as changes in O_2, CO_2, and salinity. More recent work has centralized on ocean acidification. However, the effects of global change on freshwater crustaceans (chapter 2) and the effects of elevated CO_2 in aquaculture (chapter 3) have been largely ignored. In addition, the effects of feeding on acid-base regulation (chapter 4) have gone unstudied and may have direct implications on responses to global change and aquaculture. This thesis addressed this gap in the literature using the Chinese mitten crab *Eriocheir sinensis*, Whiteleg shrimp *Litopenaeus vannamei*, and Green crab *Carcinus maenas* as models. In chapter 2, I identified that freshwater acidification leads to a greater reliance on protein catabolism and an energetic trade-off allowing for compensation of pH homeostasis but impairment of other physiological processes like calcification and locomotory behaviour. In chapter 3, I determined that chronic growth of Whiteleg shrimp in CO_2 levels found in aquaculture facilities did not affect growth or survival when reared in brackish water as opposed to what is seen when reared in full-strength seawater. While growth and survival are unimpaired, extracellular acid-base status varies between shrimp grown at low and high CO_2, suggesting a potential for chronic impairment of pH homeostasis. In chapter 4, I found that feeding leads to respiratory acidosis, likely driven by increased acid production from postprandial aerobic metabolism. This acid-base disturbance was not compensated through the accumulation of HCO_3^- as is seen with respiratory acidosis caused by increased environmental CO_2. Instead, acidosis was mainly recovered through large increases in ammonia and to a lesser degree titratable acid excretion. Overall, the data in this thesis provides valuable insights into understanding how global change and aquaculture influences the physiology of decapod crustaceans and provides fundamental information on feeding physiology to stimulate future research into the combined effects of feeding during aquaculture acid-base stressors.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dirk Weihrauch, for his support, encouragement, extreme patience, and unwavering belief in my abilities as a researcher. You gave me the opportunities to travel for research and gain life experiences I never thought I would have had. You created a research environment that fostered my curiosity and allowed me to try new things, fail, learn from my mistakes, and grow. I will be forever grateful for these opportunities. I will never forget the fun times in Germany, Sweden, and Italy. Although there have been tough times, there is no lab that I would have rather spent my Ph.D. years in. I would also like to thank my committee members, Drs. Jason Treberg, Gary Anderson, and Peter Eck for their constructive criticism and guidance along the way. Thank you for asking the tough questions that pushed me to be a better researcher and keep me on track when I got too ambitious. Thanks to my collaborators, Drs. Pung Pung Hwang, Yung-Che Tseng, and Frank Melzner, for everything they have done for me. My thesis would not be possible without your support.

To my fellow members of the crab lab, thank you for always being there to provide technical and moral support through both the highs and lows of my thesis work. Dr. Sandra Fehsenfeld, you were there from my very first day as a fresh undergrad in the lab; it has been a real privilege learning from you, and I wouldn't be the researcher I am today without you, thank you. A special thanks to Garett Allen (aka Carrot and every other nickname under the sun we called you), we started our Ph.D. journey together and I couldn't have asked for a better lab mate than you. You were always there to help me troubleshoot experiments no matter the time of day. You were always there to tag in and sampled for me so I could get some sleep when I was on non-stop sampling benders, and most importantly challenge me when I was being a know it all. I don't think I could have made it without your help. You truly are a brilliant researcher and friend, and I can't imagine I will ever have another lab mate quite like you. Haonan Zhuoyao, thank you for all the help over the years learning all about oocytes, the delicious cakes you baked, and hooking me up with your mom's delicious cooking. Stephanie Hans, thanks for always being there to lend a hand, all the hangouts, and the dumpling addiction that has given me the title of dumpling king! Ashley Tripp (Street Peach), you are hands down one of the sweetest and most kind-hearted people I have ever met. Huge thanks to both you and Chris for always being there to provide a helping hand in lab and in life, all the lemon squares, and good times. A big shout out to Maria and Mikyla (M&M),

you were such wonderful lab mates and even better friends. Thanks for being so wonderful with my little girls and putting up with me. It was a privilege getting to see you both become such brilliant researchers. Always remember DNase has nothing on you ☺. Aaron Klymasz-Swartz, you are one of the hardest working individuals I have met. Remember stay away from horses and tortoises dude!

I would also like to thank the various friends and colleagues who have provided technical advice, encouraged me to hang out to provide a distraction, or were just there to chat about the frustrations of research. Dr. Tony Signore, you are a brilliant researcher, a wonderful friend, and a role model of what a high caliber presenter should be. I am so glad I forced you to be my friend all those years ago. Dr. Rex Yoon, you are a gem! Selfless, genuine, and skilled, it has been a pleasure sharing an office with you and collaborating on so many projects. Luke Belding, we spent many years in our office together where you pulled me into a collaboration and taught me about the wonderful world of sturgeon physiology. I cherish all those moments we spent working together, brainstorming research ideas, struggling through our Ph.Ds., and talking about fishing. Your cheery attitude when challenged in life was a true inspiration. I miss you dearly. You may be gone, but I will never forget you.

Finally, I would like to thank my family. My parent Jose Antonio Quijada-Sanchez and Nora Rodriguez and my siblings Francisco Rodriguez, Mario Quijada-Rodriguez, Angela McEvoy, brother-in-law Caolan McEvoy and nephews Rory, Mateo, and Emilio who may not always understand what I do but have always supported me throughout my academic journey. Special thanks to my best friend and dear wife, Kelsey O'Brien. No words can describe how wonderful and supportive you have been throughout this journey, even when I didn't think I could pull through you always did. I am so lucky to have a wife willing to pull off single mom duty so I could spend all day and night in the lab doing experiments or writing my papers. Kelsey, you are the real MVP of my thesis. My two wonderful daughters Lily and Sierra, you two are my world and your cheery attitudes, hugs, kisses, and playfulness have been my motivation to push hard to the finish. Thanks to the many crabs that supported this research. I hope I kept your little bellies full of all you can eat scallops, fish, mussels, and octopus.

DEDICATION

This thesis is dedicated to my daughters, Lily Anne and Sierra Rey Quijada-O'Brien who inspired me throughout my thesis and to my late Grandmother Angela Quijada-Salguero who believed my intelligence and curiosity as a child could lead me to be a doctor one day. My little heart warrior, Sierra through your perilous journey with hypoplastic left heart syndrome you have taught me perseverance and are an inspiration of what can be accomplished in life no matter how difficult the road ahead seems.

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CHAPTER 1: GENERAL INTRODUCTION

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Parts of this chapter are part of a review in preparation titled “Acid-base regulation in decapod crustaceans: New mechanistic insights and unanswered questions”

Author contributions: ARQR and DW conceptualized the review. ARQR wrote the manuscript. DW revised the manuscript.

Crustaceans

Decapod crustaceans are estimated to have emerged nearly 455 million years ago during the Ordovician period of the Paleozoic era (Wolfe et al., 2019). The modern decapod groups underwent large family level divergence throughout the Cretaceous period of the Mesozoic era where atmospheric CO_2 levels above 1000 ppm (=~100 Pa, 1000 µatm) have been estimated (Orr et al., 2022; Wolfe et al., 2019). This suggests that in their evolutionary history, the modern lineages of decapod crustaceans would have adapted to chronically living at CO_2 tensions predicted for the end of the century (year 2100) due to anthropogenic activity. Presently, there are over 14,000 species of decapod crustaceans, which encompass the prawns, shrimps, crayfish, lobsters, anomuran crabs, and true crabs (brachyura) (De Grave et al., 2009). Decapod crustaceans are free-living species with diverse feeding strategies, including herbivores, carnivores, scavengers, deposit feeders, filter feeders, and opportunistic omnivores (Choy, 1986; Linton and Greenaway, 2007; Riisgård et al., 2015; Warner, 1977). Decapods also inhabit a variety of marine, freshwater, and terrestrial habitats, where they occupy various ecological niches including, variable and extreme environments such as, deserts, polar regions, hydrothermal vents, and intertidal zones (Daniels et al., 2006; Frederich et al., 2001; Greenaway and Taylor, 1976; Martin and Haney, 2005; Schubart et al., 1998; Yeo et al., 2008). Despite the wide variation in life histories among decapods, one generally common trait is the presence of active lifestyles with relatively high metabolic rates, which is believed to correlate with more efficient acid-base regulatory capabilities (Melzner et al., 2009). The acid-base regulatory robustness of decapods may partially explain how decapods have so successfully radiated into a variety of challenging

ecological niches and could prove vital to gaining insights into how decapods may respond to challenging aquaculture conditions and global change. This review will explore our current understanding of acid-base regulation in aquatic decapod crustacean. The focus will be on the mechanisms of acid-base regulation, responses to different acid-base disturbances, implications that acid-base regulation has on response to global change and intensive aquaculture, and finally, outstanding questions in the field.

General acid-base regulation

Like all animals, growth and survival in decapods depends on proteins and biochemical processes to maintain cellular energetic demands. As proteins are pH sensitive, decapods aim to maintain a relatively stable intra- and extracellular pH to prevent structural and functional changes in proteins that may alter cellular metabolism and basic physiological function (Somero, 1986). Routine cellular metabolism is constantly producing acid-base equivalents. Therefore, acid-base regulatory processes must work to maintain pH homeostasis. Decapods typically maintain a homeostatic pH of their extracellular fluids between 7.5-8.1, pCO_2 between 130-560 Pa, and HCO_3^- between 3-13 mmol L^{-1} (Table 1.1). However, extracellular pH is inversely proportional to ambient temperature and shifts to maintain constant relative alkalinity (i.e. constant $[OH^-]/[H^+]$) to keep the net charge of proteins constant (Truchot, 1978). This means that inter-species homeostatic pH in decapods is dynamic, and temperature must be considered when making cross-species and intra-species comparisons. Besides routine metabolism, other intrinsic factors such as exercise, feeding, and moulting may further challenge pH homeostasis requiring a compensatory acid-base regulatory response (Mangum et al., 1985; McDonald et al., 1979; Phlippen et al., 2000; Quijada-Rodriguez et al., 2022). As these challenges are a normal part of decapod life, the acid-base machinery can readily fully compensate for any changes in pH homeostasis. In addition, extrinsic factors like changes in ambient O_2, CO_2, ammonia, and salinity may also challenge pH homeostasis and lead to acute or sometimes chronic pH disequilibrium (Fehsenfeld and Weihrauch, 2017; Henry and Wheatly, 1992; Henry et al., 2012; Whiteley, 2011; Whiteley et al., 2018). While some decapods may readily compensate for deviations in pH due to extrinsic acid-base stressors, this capability will depend on the physiological plasticity of a given species and the degree and duration of environmental stress. The mechanisms that decapods use to regulate intra- and extracellular pH

include buffering, respiration, and ion exchange (Henry and Wheatly, 1992; Wheatly and Henry, 1992). The following sections will detail how decapods use these mechanisms to regulate pH homeostasis.

Table 1.1 Hemolymph pH, pCO_2, and HCO_3^- in decapod crustaceans. T represents water temperature in degrees Celsius, $pCO_{2\,W}$ is water partial pressure of CO_2 in pascals, pH_w is water pH, Sal. is water salinity in part per thousand (FW is generally deemed <0.5 ppt), pH_e is the hemolymph pH, pCO_{2e} is hemolymph partial pressure of CO_2 in pascals, $HCO_3^-{}_e$ is hemolymph bicarbonate, and Ref. refers to the literature reference where data was obtained from. ND indicates where measurements were not determined. ([1]Allen et al., 2020; [2]Allen et al., 2021; [3]Cameron, 1978; [4]Dejours and Beekenkamp, 1977; [5]Dissanayake and Ishimatsu, 2011; [6]Dissanayake et al., 2010; [7]Fehsenfeld and Weihrauch, 2013; [8]Fehsenfeld and Weihrauch, 2016; [9]Hammer and Pedersen, 2013; [10]Hans et al., 2014; [11]Harms et al., 2014; [12]Hu et al., 2016; [13]Klymasz-Swartz et al., 2019; [14]Pane and Barry, 2007; [15]Quijada-Rodriguez et al., 2021; [16]Quijada-Rodriguez et al., 2022; [17]Spicer et al., 2007; [18]Taylor and Wheatly, 1981; [19]Tripp et al., 2022; [20]Wheatly and Mcmahon, 1982; [21]Whiteley and Taylor, 1990; [22]Whiteley et al., 2001a)

Species	Grouping	T(ºC)	pCO_{2W} (Pa)	pH_W	Sal. (ppt)	pH_e	pCO_{2e} (Pa)	$HCO_3^-{}_e$ (mmol L^{-1})	Ref.
Callinectes sapidus	Brachyura	22	40	ND	FW	8	530	10.2	3
Eriocheir sinensis	Brachyura	23	131	7.41	FW	8.1	403	13.4	15
Eriocheir sinensis	Brachyura	12	ND	8.06	35	7.76	460	7	22
Carcinus maenas	Brachyura	14	53	7.7	10	7.9	263	6.6	7
Carcinus maenas	Brachyura	13.8	90	7.76	32	7.87	250	9	8
Carcinus maenas	Brachyura	14	49	8.1	32	7.82	230	5.5	16
Metacarcinus magister	Brachyura	14	49	8.1	32	7.93	132	4.9	10
Necora puber	Brachyura	15	20	7.96	34	7.9	190	6.6	17
Helice formosensis	Brachyrua	21	52	7.99	15.5	7.78	401	7.38	2
Hyas araneus	Brachyura	5	45	8.2	32	8.05	250	2.8	11
Chionoecetes tanneri	Brachyura	3	ND	7.85	34	7.8	240	5	14
Xenograpsus testudinatus	Brachyura	30	2765	6.49	35	7.42	2000	19	1
Xenograpsus testudinatus	Brachyura	28	ND	8	32	7.5	550	8.2	12
Homarus americanus	Lobster	16	48	8.13	34	7.87	200	5	13
Hommarus gamarus	Lobster	15	ND	ND	32-35	7.8	130	4.1	21
Procambarus clarkii	Shrimp	24	73	8.07	FW	7.54	578	4.47	19
Pacifastacus leniusculus	Shrimp	15	ND	7.39	FW	7.95	372	8.47	20
Palaemon elegans	Shrimp	15	90	7.96	32	7.7	220	3	6
Palaemon serratus	Shrimp	15	110	7.89	32	7.7	220	2.9	6
Pandalus borealis	Shrimp	7	52	8.1	35	7.76	250	5.1	9
Metapenaeus joyneri	Prawn	15	40	8.14	32	7.65	270	3.96	5
Austropotamobius pallipes	Crayfish	15	ND	ND	FW	7.9	400	6.9	18

Astacus leptodactylus	Crayfish	13	ND	ND	FW	7.8	300	4.73	4

Buffering

Buffering systems act as rapid mechanisms to minimize deviations in pH by reversibly binding H^+. While this mechanism is rather quick, it does not remove acid-base equivalents from the system and will only be effective until buffering capacity is exceeded. The buffering capacity of intra- and extracellular fluids in decapods is dictated by the combined capacities of bicarbonate and non-bicarbonate (e.g. proteins, inorganic phosphates, and ammonia) buffering systems. The bicarbonate buffering system and proteins mitigate changes in the extracellular fluids, where in decapods, the respiratory protein, hemocyanin accounts for as much as 95% of extracellular proteins and is the main protein buffer of the extracellular fluids (Uglow, 1969). Decapods normally regulate extracellular HCO_3^- to alter buffering in response to acid-base disturbances (Fehsenfeld and Weihrauch, 2016; Hans et al., 2014; Taylor and Wheatly, 1981; Whiteley and Taylor, 1990). Whether decapods also regulate hemocyanin concentrations to improve protein buffering is unclear. In Dungeness crab *Metacarinus magister*, hypercapnia has no effect on hemocyanin concentration (Hans et al., 2014), however, other factors that also influence acid-base status such as, feeding status, osmotic stress, and moulting affect hemocyanin concentration (Boone and Schoffeniels, 1979; Hagerman, 1983). Ammonia is another potential buffer in the extracellular fluids of decapods (Allen et al., 2020; Fehsenfeld and Weihrauch, 2013; Weihrauch and Allen, 2018). Extracellular ammonia in aquatic decapods is typically only between 50-400 μmol L^{-1} (Weihrauch et al., 2017) and therefore would have a much lower capacity to buffer pH change than HCO_3^-, which is typically 3-13 mmol L^{-1} (Table 1.1). Unlike in the extracellular fluids, proteins and inorganic phosphates are the predominant intracellular buffer (Roos and Boron, 1981; Wheatly and Henry, 1992). Direct measurements of pH in the hemolymph and various tissues and corresponding buffering capacities in decapods are limited to a few species (Gaillard and Malan, 1983; Milligan et al., 1989; Wheatly et al., 1991; Whiteley et al., 2001a). From these studies, decapods typically maintain their intracellular pH up to 0.6 pH units below extracellular pH or, in the case of the carapace fluids, 0.25 units above (Table 1.2). Comparisons of extracellular and intracellular buffering capacity in *Pacifastacus leniusculus* (See Table 1.2), show that the intracellular fluids, particularly in tissues that would more regularly become anaerobic, have a greater buffering capacity and should be able to better resist pH change, e.g. due

to lactic acid production. Buffering can only mitigate pH changes, so decapods rely on transport mechanisms to excrete acid-base equivalents to support buffering capabilities and re-establish pH homeostasis.

Table 1.2 Buffering capacity and acid-base parameters of intracellular and extracellular fluids in decapods under normal environmental conditions. T represents water temperature in degrees Celsius, Sal. is water salinity in part per thousand, pH is the pH of the corresponding tissue, HCO_3^- is the calculated bicarbonate concentration assuming pCO_2 in the tissue is equilibrated with the extracellular fluids, pCO_2 is the measured partial pressure of CO_2 in the extracellular fluids and assumed to be in equilibrium with the tissues and β refers to the buffering capacity of the tissue. ND indicates where measurements were not determined. Data taken from Gaillard and Malan, 1983; Milligan et al., 1989; Wheatly et al., 1991; Whiteley et al., 2001a.

Species	T(ºC)	Sal. (ppt)	Tissue	pH	HCO_3^- (mmol L^{-1})	pCO_2 (Pa)	β (mmol L^{-1} pH^{-1})
Callinectes sapidus	24	36	Hemolymph	7.6	4	333	ND
Callinectes sapidus	24	36	Heart	7.6	ND	ND	-45.82
Callinectes sapidus	24	36	Cheliped muscle	7.4	ND	ND	-58.27
Callinectes sapidus	24	36	Light levator muscle	7.3	ND	ND	-62.46
Callinectes sapidus	24	36	Dark levator muscle	7.4	ND	ND	-64.29
Pasifastacus leniusculus	12	FW	Hemolymph	7.85	4	333	-11.59
Pasifastacus leniusculus	12	FW	Heart	7.65	5	400	-18.69
Pasifastacus leniusculus	12	FW	Nerve	7.75	6.5	400	-57.68
Pasifastacus leniusculus	12	FW	Antennal gland	7.75	6.5	400	-17.61
Pasifastacus leniusculus	12	FW	Claw muscle	7.42	3	400	-20.84
Pasifastacus leniusculus	12	FW	Abdominal muscle	7.33	2.5	466	-34.57
Pasifastacus leniusculus	12	FW	Carapace	8.1	16	400	-17.21
Astacus leptodactylus	13	FW	Hemolymph	7.85	6	280	-11
Astacus leptodactylus	13	FW	Nerve	7.38	2	280	-25
Astacus leptodactylus	13	FW	Heart	7.41	2.5	280	ND
Astacus leptodactylus	13	FW	Claw muscle	7.32	2	280	-21
Astacus leptodactylus	13	FW	Abdominal muscle	7.23	1-2	280	ND
Eriocheir sinensis	12	35	Hemolymph	7.76	7	460	ND
Eriocheir sinensis	12	35	Leg muscle	7.3	3	ND	ND

Respiration

Owing to the low solubility of O_2 in water, aquatic decapods must maintain high ventilatory rates to extract sufficient O_2 and support cellular metabolism. Consequentially, this high

ventilation rate coupled with the high solubility of CO_2 in aquatic environments allows decapods to readily excrete CO_2 but restricts the ability to alter ventilation for acid-base regulation. Indeed, decapods do not regulate ventilation in response to hypercapnia (Batterton and Cameron, 1978; Jouve-Duhamel and Truchot, 1983). However, ventilation alters pH homeostasis in situations where the demand for O_2 changes, as respiratory CO_2 excretion rates are linked to O_2 uptake. For example, during environmental hypoxia, the increase in ventilation rate to improve O_2 uptake results in enhanced CO_2 excretion and extracellular alkalosis (Burnett and Johansen, 1981; Wilkes and Mcmahon, 1982). While not yet investigated in any crustaceans, ammonia elicits a hyperventilatory response in teleost fishes (Porteus et al., 2021; Zhang and Wood, 2009; Zhang et al., 2015). Recently, high environmental ammonia has been shown to stimulate an emersion response in *C. maenas,* like that used during hypoxia to aerate the gill chamber (Zimmer and Wood, 2017). This similarity in behaviour may suggest that ventilation could respond to ambient ammonia and play a greater role in acid-base regulation than previously thought. Given the limited capacity to acid-base regulate through ventilatory processes and dissociation of CO_2 into H^+ and HCO_3^- at physiological pH, decapods will have a higher capacity to regulate acid-base homeostasis through ion exchange mechanisms.

Branchial acid-base regulation

The gills of decapod crustaceans are the primary site of acid-base ion and gas exchange between the environment and extracellular fluids (Fehsenfeld and Weihrauch, 2017; Henry et al., 2012; Larsen et al., 2014). The gills are also involved in several other processes, including osmoregulation, Ca^{2+} transport and nutrient uptake (Allen and Weihrauch, 2021; Blewett and Goss, 2017; Henry et al., 2012). Within the decapods, three types of gills can be found: the phyllobranchiate gills of brachyuran and some anomuran crabs, the trichobranchiate gills of crayfish and lobster, and the dendrobranchiate gills of penaeoid and sergestoid shrimp (Freire et al., 2008). The phyllobranchiate gill contains an afferent and efferent vessel, which runs through the central shaft to which large numbers of flat lamellae are attached (Freire et al., 2008). These lamellae have a single epithelial cell layer covered by an ion-selective cuticle (Lignon, 1987). This review will focus on the phyllobranchiate gill of brachyuran crabs, as this is where most of the mechanistic work in decapods has been performed due to ease of perfusion for isolated gill studies.

The following sections will describe the different epithelial cell and gill types in brachyurans and the current understanding of transport mechanisms for acid-base equivalents (H^+, HCO_3^-, CO_2, ammonia).

Gill and epithelial cell types

The brachyuran gill has several epithelial cell types, including thin epithelial cells (chief cells), thick epithelial cells (striated cells), and pillar cells. The chief cells are believed to have a respiratory role due to thinness (1 - 5 µm thick), lack of extensive membrane infoldings, and lower mitochondrial abundance (Barra et al., 1983; Copeland and Fitzjarrell, 1968). The thick striated epithelial cells (10 - 20 µm thick) are often also referred to as ionocytes due to their extensive apical and basolateral folding and mitochondrial abundance (Barra et al., 1983; Compere et al., 1989; Copeland and Fitzjarrell, 1968; Luquet et al., 2002). Pillar cells are microtubule-rich cells that play a structural role, limiting changes/collapse of the hemolymph space (Compere et al., 1989; Goodman and Cavey, 1990). Pillar cells span into the hemolymph space where cells from opposite sides of the lamella meet to form a stalk that can be composed of multiple pillar cells spiralled together (Barra et al., 1983; Goodman and Cavey, 1990). The presence of these cell types in brachyurans will differ based on environmental salinity (Freire et al., 2008). In marine osmoconformers, all gills have thin chief cells and thin pillar cells. In osmoregulating crabs that maintain hyperosmotic hemolymph (relative to environment), anterior gills will look much like that of marine osmoconformers however, the posterior gills will have a large patch of thick striated cells and thick pillar cells, which are surrounded by thin chief cells and thin pillar cells (Barra et al., 1983; Compere et al., 1989; Copeland and Fitzjarrell, 1968). The presence of these cell types has been known for decades but, their acid-base regulatory roles (i.e. base-secreting and acid-secreting cells) are not entirely clear and may vary by species.

Preliminary hypotheses on the role of epithelial cell and gill types in acid-base regulation can be inferred from histological data coupled with recent gill perfusion and expression studies on *C. maenas*. Perfusion of *C. maenas* anterior (gills 4 - 6) and posterior (gills 7 - 9) gills suggest an equal capacity to alkalize extracellular fluids, and nearly identical ammonia transport capacity, except for gill 4, which has a slightly higher ammonia and proton transport capacity (Fehsenfeld and Weihrauch, 2013). The anterior gills have higher CO_2 excretion rates than the posterior gills

(Quijada-Rodriguez et al., unpublished). The posterior gills have a net HCO_3^- uptake, whereas the anterior gills have a net HCO_3^- excretion (Quijada-Rodriguez et al., unpublished). Comparisons between anterior and posterior gills suggest both gills are well equipped to regulate acid-base status, but the anterior gills rely on CO_2 and HCO_3^- excretion, whereas posterior gills rely on HCO_3^- retention and H^+ excretion. Gene expression profiles support the fact that the machinery is quite different between gill types and therefore likely also epithelial cell types. The posterior gills, which are abundant in thick epithelial cell types have higher expression of genes potentially involved in bicarbonate transport such as SLC4A1 (Cl^-/HCO_3^- exchanger), Na^+/HCO_3^- cotransporter, Na^+/K^+-ATPase, and carbonic anhydrases (Fehsenfeld and Weihrauch, 2013; Serrano and Henry, 2008). In contrast, anterior gills (thick cell absent) and posterior gills have essentially equal expression of H^+ (Na^+/H^+ exchangers and V-ATPase), CO_2 (Rh proteins), and ammonia transporters (Rh proteins) (Fehsenfeld and Weihrauch, 2013). Overall, these expression profiles likely suggest that there are differences in HCO_3^- mechanisms, whereas both gill types should have the same ability to transport CO_2 and H^+. While these results are informative for *C. maenas*, expression profiles of gill types may vary by species. For example, in freshwater *E. sinensis*, the vacuolar-type H^+-ATPase (V-ATPase) is only localized to the pillar cells whereas in brackish water acclimated *C. maenas* VHA is localized in all epithelial cell types (Freire et al., 2008; Weihrauch et al., 2001). Similarly, Rh protein expression in *E. sinensis* anterior gills is higher (Fig. 1.1) than in posterior gills instead of equally expressed as in *C. maenas* (Fehsenfeld and Weihrauch, 2013). To further elucidate the function of the different brachyuran cell and gill types, comparative studies investigating expression profiles and protein localization across different brachyurans are required.

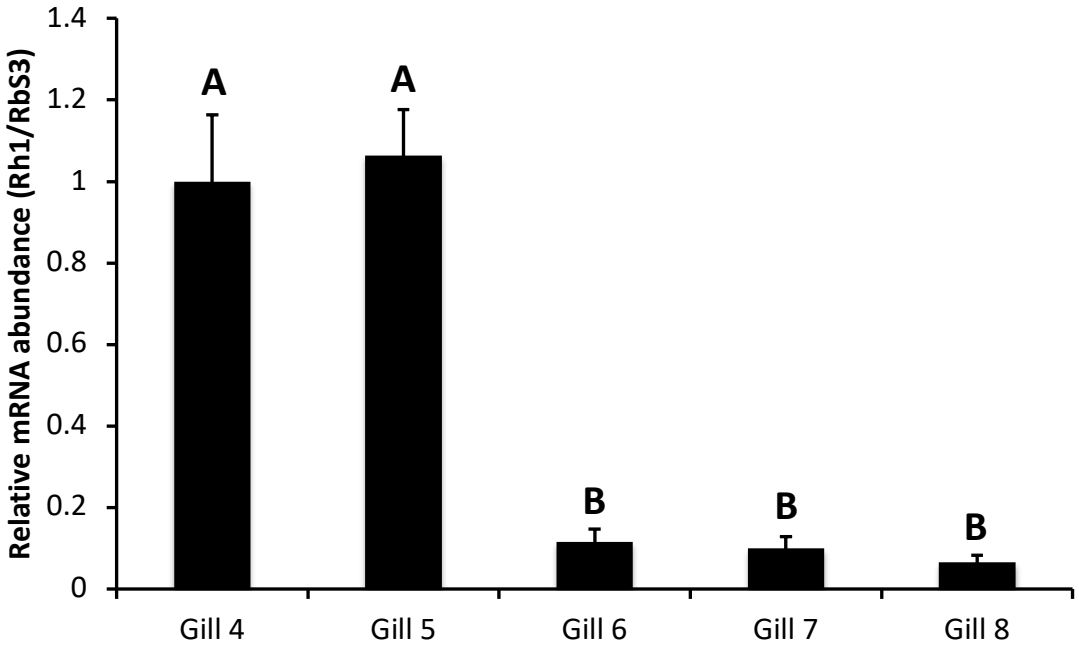

Figure 1.1 Relative mRNA transcript abundance of Freshwater acclimated Chinese mitten crab *Eriocheir sinensis* Rh protein 1(Rh1) normalized to the ribosomal protein S3 (RbS3) in the anterior (gills 4 and 5) and posterior (gills 6, 7, and 8) gills. EsRh1 primers 5'→3': Sense, CTTCATTCTCCGCCTCTTCG/ antisense, GTCCTCTGGTTATGCTGTCG; 124 bp amplicon. EsRbS3 primers 5'→3': Sense, AGTTTACTGACGGCCTCATG/ antisense, TTGATACCAAGCACACCCTG; 103 bp amplicon. 10 µL reaction with 400 nmol L^{-1} primer 1 µL cDNA and 2x SsoAdvanced Universal SYBR Green mix. Thermal cycling: 95ºC, 2 min followed by 40 cycles of 95ºC for 10s, and 60ºC for 10s. (Quijada-Rodriguez et al. unpublished)

Branchial transport mechanisms

Most of what is known regarding the cellular transport mechanisms in the crustacean gill is derived from studies investigating osmoregulation and ammonia excretion. Direct investigations on the cellular acid-base regulatory mechanisms have focused solely on the weak osmoregulating crab *Carcinus maenas* and strong osmoregulating crab *Neohelice granulata* (Fehsenfeld and Weihrauch, 2016a; Siebers et al., 1994; Tresguerres et al., 2008). As H^+ or NH_4^+ are commonly counter ions for Na^+ and similarly HCO_3^- for Cl^- then known osmoregulatory mechanisms are quite informative in understanding the cellular acid-base mechanisms in the crustacean gill (Mantel and Farmer, 1983; Truchot, 1983).

Gill perfusion techniques and tracer flux studies and electrophysiological measurements employing split gill lamella mounted in Ussing chambers have proven indispensable tools in understanding ion transport mechanisms in crustaceans. These approaches are commonly paired

with the application of pharmaceuticals that target specific transporters. Caution must be taken when interpreting the results of studies using pharmaceutical inhibitors as they are not always specific to a single target protein and apical application of certain inhibitors can cause false positives by interfering with the gill cuticle pores rather than the target protein (Onken and Riestenpatt, 2002). Fortunately, integrating molecular, biochemical assays, and microscopy approaches have supplemented isolated gill tissue approaches to minimize the pitfalls of pharmaceutical approaches. Based on the evidence derived from osmoregulation, ammonia excretion, and acid-base regulation studies, hypothetical working models for acid-base regulation in the gills of strong and weak osmoregulating euryhaline crustaceans will be discussed and are summarized in Fig. 1.2 and 1.3. For the purpose of this review, strong osmoregulating crustaceans are those capable of inhabiting freshwater (e.g. South American rainbow crab, *Neohelice granulata*) and weak osmoregulating crustaceans are those incapable of inhabiting oligohaline zone where salinity is below 5 ppt but can exist in brackish water environments (e.g. green crab, *C. maenas*). Due to a lack of studies on stenohaline osmoconformers, the mechanisms of acid-base regulation in these crustaceans are unknown.

Acid excretion mechanisms

The crustacean gills excrete acids as CO_2, H^+, or NH_4^+. While these pathways have a certain degree of commonality in transporters used, acid-base challenges may not stimulate all acid secretion pathways (see natural acid-base stressors). The following section will break down the currently hypothesized mechanisms CO_2 (Fig. 1.2), ammonia (Fig. 1.3), and H^+ (Fig. 1.4) transport across the gills of crustaceans. Where applicable, the variation in mechanisms used by weak and strong osmoregulators is highlighted.

CO_2 transport pathway

A portion of hemolymph acids are directly transported across the gills as CO_2. Using membrane-impermeable carbonic anhydrase inhibitors, it was shown that a hemolymph accessible membrane-bound carbonic anhydrase is likely in the gills of crustaceans allowing for dehydration of H^+ and HCO_3^- into CO_2 to promote rapid CO_2 excretion (Burnett and Mcmahon, 1985; Henry, 1987). A membrane-bound carbonic anhydrase gene (CAg) cloned from the gills of crustaceans

was believed to be the basolateral carbonic anhydrase responsible for mobilizing extracellular HCO_3^- for CO_2 excretion (Serrano and Henry, 2008; Serrano et al., 2007). Recent immunolocalization of CAg in *C. maenas* has revealed this isoform is not basolateral but primarily localized to the apical membrane of thick epithelial cell patch where its role in acid-base regulation is unknown (Quijada-Rodriguez, unpublished). Based on expressed sequence tag cDNA libraries, the gills of *C. maenas* express three additional carbonic anhydrase genes, including two vertebrate CA4-like (GB Accession #: DY657175.1 and DN202504.1) and one vertebrate CA7-like (GB Accession #: DY656586.1) gene. Interestingly, CA4 in chondrichthyan fishes is a membrane-bound plasma accessible isoform and is believed to contribute to CO_2 excretion by dehydration of extracellular HCO_3^- (Gilmour et al., 2007). It is possible that one of these identified CA4-like genes may be the putative basolateral membrane-bound carbonic anhydrase of the crustacean gill.

CO_2 diffusion through the basolateral and apical membrane of the gill epithelium may occur through simple diffusion or mediated by CO_2 channels. In aquatic organisms, Rh proteins and aquaporins have been shown to facilitate CO_2 transport (Perry et al., 2010; Talbot et al., 2015; Thies et al., 2022). Rh proteins have been identified in the gills of various decapods (Martin et al., 2011; Ren et al., 2015; Tripp et al., 2022; Weihrauch et al., 2004). To date, most of the work on Rh proteins in decapods has focused on a single isoform (Rh1). Through expression in *Xenopus* oocytes, *C. maenas* Rh1 (CmRh1) has been shown to facilitate CO_2 transport. Moreover, immunolocalization studies showed that CmRh1 is localized in the apical membrane of the gill epithelium, where it may mediate apical CO_2 excretion (Quijada-Rodriguez et al., unpublished). A basolateral Rh protein has yet to be identified, however, in the gills of another decapod, the crayfish *Procambarus clarkii*, a second Rh protein isoform is present and could act as a basolateral pathway for CO_2 transport (Tripp et al., 2022). Besides Rh proteins, three aquaporins have also been identified in the gills of *C. maenas*, including a classical aquaporin (CmAQP, GB Accession #: ON416879), an aquaglyceroporin (CmGLP, GB Accession #: ON416880), and big brain aquaporin (CmBIB, GB Accession #: ON416881) (Nash et al., 2022). The CO_2 transport capacity of these aquaporins has not been determined yet. However, CmAQP is localized on the apical membrane of crustacean gill epithelium (Nash et al., 2022). In the gazami crab *Portunus trituberculatus*, an aquaporin resembling CmAQP is upregulated when Rh1 is knocked down by RNAi, suggesting this aquaporin may compensate for the loss of Rh1 function (Si et al., 2018).

Preliminary data on CmAQP also suggests this transporter does not mediate ammonia transport (Nash personal communication).

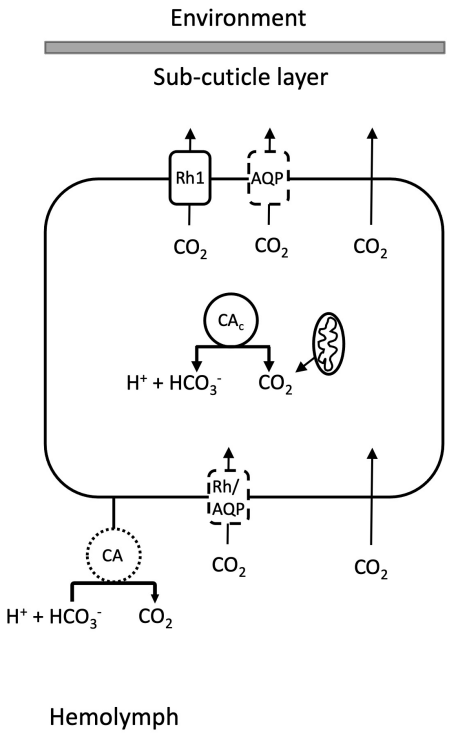

Figure 1.2 Hypothetical mechanism of CO_2 transport across the crustacean gill. Transporters with solid lines are known from localization studies or supported by molecular data. Transporters with dotted lines are supported by pharmacological data but molecular identity has not been confirmed. Transporters with dashed lines are hypothetical predictions from what is known in other aquatic taxa or based solely off molecular characterization of the isolated protein. For potential molecular identities refer to the text descriptions. Abbreviations are as follows: carbonic anhydrase (CA), cytoplasmic carbonic anhydrase (CAc), Rh protein (Rh), Rh protein 1 (Rh1), aquaporin (AQP).

Ammonia transport pathway

At physiological pH, ammonia mainly exists in the ionic NH_4^+ form (pKa ~9.3), which can act as an acid equivalent that is transported to regulate acid-base status. Ammonia transport in decapod crustaceans has been extensively reviewed (Weihrauch et al., 2004; Weihrauch et al., 2009; Weihrauch et al., 2017), so this section will only briefly review key transport pathways and integrate recently identified novel ammonia transporters in the gills of crustaceans and present new insights into the function of Rh proteins. Basolateral transport of ammonia occurs through K^+ channels and the Na^+/K^+ ATPase, which can accept NH_4^+ as a substrate in place of K^+ (Leone et

al., 2017; Towle and Holleland, 1987). The Na^+/K^+ ATPase is indeed involved in ammonia excretion, as inhibition through ouabain has been shown to reduce active ammonia excretion in the perfused gills of *Cancer pagurus* and *C. maenas* (Weihrauch et al., 1998; Weihrauch et al., 1999). Similarly, inhibition of K^+ channels by Cs^+, Ba^+ and ZD7288 demonstrated reduced ammonia excretion in *C. maenas* perfused gill preparations (Fehsenfeld and Weihrauch, 2016b; Weihrauch et al., 1998).

In aquatic organisms, Rh proteins function not only as CO_2 channels but also as ammonia channels (Perry et al., 2010; Thies et al., 2022). Investigations into the ammonia transport capabilities of the apically localized CmRh1 in *Xenopus* oocytes revealed that this Rh protein allows the transport of ammonia promoting membrane passage of NH_3 over NH_4^+ (Quijada-Rodriguez et al., unpublished). Based on the deduced amino acid sequence of the gene and the observed reliance on both NH_3 and NH_4^+ concentrations, it is likely that CmRh1 transports ammonia through NH_4^+ recruitment and NH_3 transport mechanism, where NH_3 is transported through the channel and the H^+ is shuttled back to its original location as suggested for other Rh proteins (Baday et al., 2015). However, as experiments on CmRh1 have focused solely on radiotracer fluxes and not electrophysiological measurements, it cannot be excluded that both NH_3 and H^+ are being transported across Rh proteins as proposed in more ancestral Rh proteins (Williamson et al., 2020). Even though the apical crustacean Rh protein may only move NH_3 across the membrane, a net NH_4^+ movement may occur as Na^+/H^+ exchangers have been proposed to work together with Rh proteins to create a *de-facto* Na^+/NH_4^+ exchange in what is known as the NHE-Rh metabolon (Wright and Wood, 2009).

Ammonia transport in weak osmoregulators, such as *C. maenas*, has been suggested to predominantly occur through vesicular transport coupled with the V-ATPase. This hypothesis was derived from the inhibition of active ammonia excretion in perfused gills preparations in the presence of the V-ATPase inhibitor bafilomycin and microtubule inhibitors such as colchicine, taxol, and thiabendazole (Weihrauch et al., 2002). As colchicine has been shown to not affect short circuit current (Weihrauch et al., 2002), this pathway would provide a NaCl independent pathway for ammonia excretion. Following the discovery of Rh proteins, in the gills of *C. maenas*, it was proposed that the V-ATPase creates acidified vesicles for acid trapping of ammonia via NH_3 transport by CmRh1 (Weihrauch et al., 2004). We now know CmRh1 is apically localized and,

therefore, is not involved in vesicular ammonia transport (Quijada-Rodriguez et al., unpublished). It remains to be identified whether other ammonia transporters, such as the predicted 2nd Rh protein, are co-localized with the V-ATPase to support this vesicular ammonia transport pathway.

In contrast to weak osmoregulating crustaceans, strong osmoregulators like *E. sinensis* are not dependent on vesicular ammonia transport (Quijada-Rodriguez unpublished). Instead, strong osmoregulators are believed to directly excrete ammonia across the apical membrane of the gill via an ammonia trapping mechanism (Weihrauch et al., 2017). This proposed mechanism relies on Na^+/H^+ exchangers or the apical V-ATPase found in strong osmoregulating crabs to generate an outwardly directed partial pressure gradient for NH_3 driving ammonia excretion through an apical Rh protein, which is subsequently protonated into NH_4^+ and trapped in the subcuticular space. An Rh protein resembling that of CmRh1 has been identified also in *E. sinensis*, however, whether an apical localization is present in *E. sinensis* has not yet been confirmed.

Recently, a novel ammonia transporter known as hippocampus abundant transcript 1 protein has been cloned in the gills of *C. maenas* (CmHIAT1) and functionally characterized in *Xenopus* oocytes (Fehsenfeld et al. unpublished). In *Xenopus* oocytes, CmHIAT1 was shown to exhibit a Na^+-dependent ammonia transport using radiolabelled methylamine as an ammonia analogue. Scanning ion-selective electrode technique (SIET) revealed this transporter is capable of active ammonia excretion. Analysis of branchial mRNA transcript abundance following high ammonia exposure revealed an upregulation of branchial CmHIAT1 but downregulation of CmRh1 after 6 hours and coincided with a reduction in hemolymph ammonia levels, suggesting HIAT1 may play a key role in regulating extracellular ammonia and therefore, also acid-base equivalents. The localization of HIAT1 in the branchial epithelium of crustaceans is currently unknown, but expression response and capacity to actively excrete ammonia against a high ammonia gradient would make it an excellent candidate for apical ammonia excretion, particularly under high (above extracellular ammonia levels) environmental ammonia conditions.

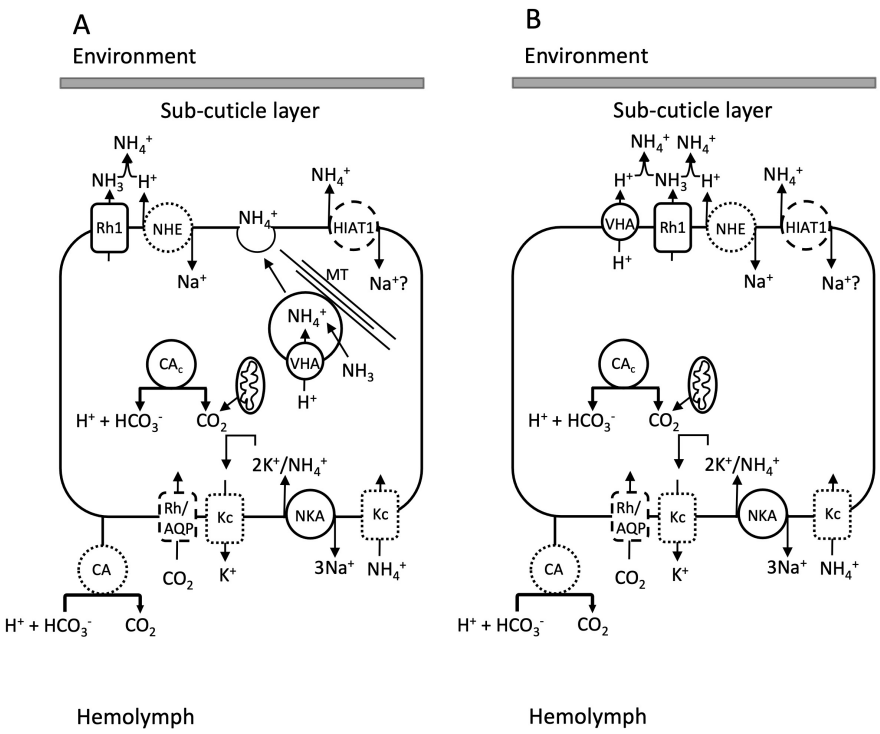

Figure 1.3 Hypothetical mechanisms of transporter mediated ammonia excretion in the gills of (A) weak and (B) strong osmoregulating crustaceans. While not displayed on these figures, membrane diffusion of CO_2 and NH_3 also occur. Transporters with solid lines are known from localization studies or supported by molecular data. Transporters with dotted lines are supported by pharmacological data but molecular identity has not been confirmed. Transporters with dashed lines are hypothetical predictions from what is known in other aquatic taxa or based solely off molecular characterization of the isolated protein. For potential molecular identities refer to the text descriptions. Abbreviations are as follows: carbonic anhydrase (CA), cytoplasmic carbonic anhydrase (CAc), Rh protein (Rh), Rh protein 1 (Rh1), aquaporin (AQP), Na^+/H^+ exchanger (NHE), Vacuolar type H^+-ATPase (VHA), Na^+/K^+ ATPase (NKA), K^+ channels (Kc), and hippocampus abundant transcript protein 1 (HIAT1).

H^+ transport pathway

The V-ATPase and Na^+/H^+ exchangers are the key H^+ transporters of the crustacean gill. As described for the CO_2 excretion pathway, hemolymph acids are transported into the gill epithelial cells as CO_2. Once in the cytosol, cytoplasmic carbonic anhydrase catalyzes the hydrolysis of CO_2 into H^+ and HCO_3^-, which are then transported across the apical or basolateral membrane depending on acid-base requirements. A cytosolic carbonic anhydrase gene (CAc) cloned from the gills of crustaceans is believed to mediate this cytoplasmic conversion of CO_2 to H^+ and HCO_3^- (Serrano and Henry, 2008; Serrano et al., 2007). Cytosolic H^+ are excreted across the apical membrane by either a Na^+/H^+ exchange or a Na^+ independent V-ATPase mechanism.

In strong osmoregulators like *N. granulata* and *Eriocheir sinensis*, the V-ATPase is localized in the apical membrane where it has been shown to mediate apical acid excretion under low pH stress (Freire et al., 2008; Tresguerres et al., 2008). In contrast, weak osmoregulators like *C. maenas*, have an intracellular localized V-ATPase, suggesting a vesicular acid secretion mechanism (Weihrauch et al., 2001). In perfused gill preparations of *C. maenas*, inhibition of microtubule networks by colchicine and inhibition of the V-ATPase by bafilomycin or KM91104 lead to reduced acid excretion (Fehsenfeld and Weihrauch, 2016a; Quijada-Rodriguez et al., unpublished). Further, colchicine was shown to not affect the short circuit current and therefore this microtubule-dependent acid secretion mechanism is independent of NaCl uptake mechanisms (Weihrauch et al., 2002). While the V-ATPase in weak osmoregulators is cytoplasmic under homeostatic conditions, the V-ATPase may translocate to other membranes when the animal is exposed to acid-base stressors. At least in the gills of chondrichthyan fishes, the V-ATPase is translocated to the basolateral membrane during alkalosis under the control of the enzyme soluble adenylyl cyclase (Tresguerres et al., 2010). Whether similar V-ATPase translocation occurs to that of chondrichthyans occurs in the crustacean gill remains to be investigated.

The electrochemical gradient created by the Na^+/K^+ ATPase energizes the excretion of H^+ by Na^+/H^+ exchange. Indeed, inhibition of the Na^+/K^+ ATPase in perfused gill preparations has demonstrated that this pump is involved in regulating acid excretion of both strong and weak osmoregulators (Fehsenfeld and Weihrauch, 2016a; Tresguerres et al., 2008). Further, mRNA abundance and enzyme activity of the Na^+/K^+ ATPase have been shown to increase during acute hypercapnia (~2 kPa pCO_2) compensation in the vent crab *Xenograpsus testudinatus*, supporting the importance of this pump in acid-base regulation (Hu et al., 2016). Na^+/H^+ exchange across the apical membrane of the crustacean gill is believed to occur by Na^+/H^+ exchangers (NHEs) of the cation proton antiporter 1 subfamily (CPA1). NHEs were initially proposed on the apical membrane of the gills of the weak osmoregulator, *C. maenas* and strong osmoregulator, *Callinectes sapidus* based on inhibition of Na^+ uptake by the rather unspecific inhibitor amiloride (Burnett and Towle, 1990; Siebers et al., 1987). Membrane vesicle preparation of *C. maenas* confirmed the presence of an electrogenic $2Na^+/H^+$ exchanger in the gills but could not differentiate between basolateral or apical localization (Shetlar and Towle, 1989). Subsequently, an NHE3 gene that mediates a Na^+ dependent H^+ excretion when expressed in *Xenopus* oocytes

was cloned from the gills of *C. maenas* (Towle et al., 1997). The stoichiometry of the NHE3 gene is unknown, however, like NHE3 in the gills of cephalopods and fish, it may also be apically localized for acid secretion but this has yet to be confirmed (Dymowska et al., 2012; Hu et al., 2014b).

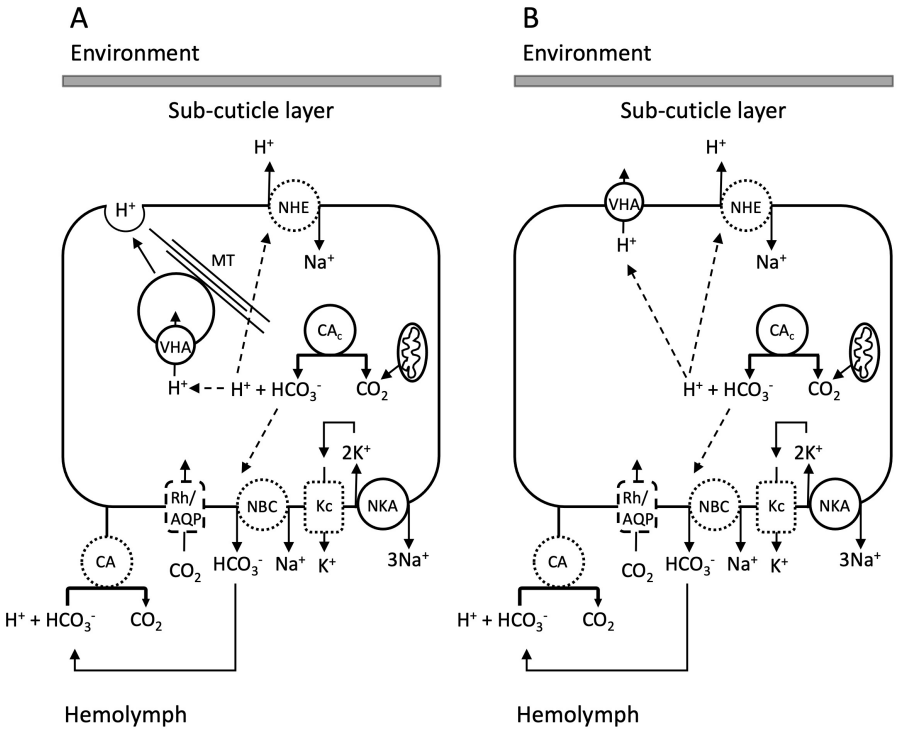

Figure 1.4 Hypothetical mechanisms of transporter mediated ammonia excretion in the gills of (A) weak and (B) strong osmoregulating crustaceans. While not displayed on these figures, membrane diffusion of CO_2 also occurs. Transporters with solid lines are known from localization studies or supported by molecular data. Transporters with dotted lines are supported by pharmacological data but molecular identity has not been confirmed. Transporters with dashed lines are hypothetical predictions from what is known in other aquatic taxa or based solely off molecular characterization of the isolated protein. For potential molecular identities refer to the text descriptions. Abbreviations are as follows: carbonic anhydrase (CA), cytoplasmic carbonic anhydrase (CAc), Rh protein (Rh), aquaporin (AQP), Na^+/H^+ exchanger (NHE), Vacuolar type H^+-ATPase (VHA), Na^+/HCO_3^- cotransporter (NBC), Na^+/K^+ ATPase (NKA), K^+ channels (Kc).

Base excretion mechanisms

Crustaceans excrete base through the gills as HCO_3^-, which is generally associated with Cl^- exchange (Allen et al., 2020; Cameron, 1985; Onken et al., 1991). The HCO_3^- transport pathways in crustacean gills are not well defined as they are mostly based on studies with inhibitors such as

DIDS and tenidap that are not specific to a single transporter but likely affect multiple HCO_3^- transporters (Ducoudret et al., 2001).

Mechanisms of base secretion have not been explicitly studied in weak osmoregulators and therefore remain relatively unknown. The mechanisms of base excretion in strong osmoregulators are mainly based on studies in *N. granulata* where branchial base excretion in perfused gills was stimulated through exposure to elevated environmental HCO_3^- and could be assessed by an increase in transepithelial potential difference (Tresguerres et al., 2008). Here, HCO_3^- excretion could be inhibited by exposing gills to Cl^- free conditions, suggesting a Cl^-/HCO_3^- exchanger. Pharmacological inhibition experiments revealed that base secretion depends on a carbonic anhydrase, the Na^+/K^+ ATPase, and Na^+/H^+ exchangers but not the V-ATPase (Tresguerres et al., 2008). These findings led to the generation of the currently hypothesized model for base secretion in strong osmoregulators presented in Fig. 1.5. In this model, membrane-bound carbonic anhydrase catalyzes the conversion of H^+ and HCO_3^- into CO_2, which diffuses into the cytosol. In the cytosol, cytoplasmic carbonic anhydrase converts CO_2 back to H^+ and HCO_3^-, where H^+ moves back into the hemolymph through Na^+/H^+ exchangers. Na^+ ions exchanged for H^+ by Na^+/H^+ exchangers are actively taken back into the hemolymph by the Na^+/K^+ ATPase. The accumulated HCO_3^- in the cytosol allows Cl^- uptake and HCO_3^- excretion by an apical Cl^-/HCO_3^- exchanger.

The molecular identity of most of the transporters in this HCO_3^- excretion pathway is currently unknown. The Na^+/K^+ ATPase has been shown in the *N. granulata* and basically, all crustaceans investigated to date to be on the basolateral membrane of all gill epithelial cells (Cieluch et al., 2004; Cieluch et al., 2007; Hu et al., 2016; Towle et al., 2001; Tresguerres et al., 2008). As described for acid excretion mechanisms, the cytosolic carbonic anhydrase is believed to be CAc, while the basolateral membrane-bound carbonic anhydrase is currently unknown. The molecular identity of the apical Cl^-/HCO_3^- exchanger in the crustacean gill remains to be identified but based on fish studies, it is likely a member of the SLC4 and/or SLC26 family (Evans, 2011). SLC4A1 (Cl^-/HCO_3^- exchanger) is expressed by the gills of *C. maenas* and *Procambarus clarkii* (Fehsenfeld and Weihrauch, 2013; Tripp et al., 2022). From microarray data, this gene is upregulated in posterior gills of *C. maenas* during acute dilute salinity stress, which coincides with active Cl^- uptake (Towle et al., 2011). At least in the hypodermis and gastrolith epithelia, SLC4A1 of *Cherax quadricarinatus* is found in the apical membrane where it is involved in HCO_3^- mobilization during

ecdysis and may have similar cellular localization in the gills (Abehsera et al., 2021). Based on transcriptome mining, a putative SLC26A4-6-like gene is present in *C. maenas* (GB Accession #: GFYW01194573). The mRNA transcript abundance of this gene in crustaceans gills has yet to be confirmed, but like in teleost fishes, this SLC26 gene may be involved in apical Cl^-/HCO_3^- exchange (Evans, 2011).

The identity of the basolateral Na^+/H^+ exchangers in the crustacean gills has also not yet been identified. In mammals, NHE1 is rather ubiquitously expressed on the basolateral membrane where it functions in the maintenance of intracellular pH (Donowitz et al., 2013). To date, only two NHE has been cloned in crustaceans, an NHE3-like and NHE2-like gene. NHE2 in *Litopenaeus vannamei* is unlikely the basolateral Na^+/H^+ exchanger as it has quite a low branchial mRNA abundance and is rather expressed in digestive tissues, the brain, and muscle. The NHE3 gene has not been localized in the crustacean gill but in fish and cephalopod gills, NHE3 is localized to the apical membrane (Dymowska et al., 2012; Hu et al., 2014b). Other NHE genes are likely present in crustaceans, as mammals and nematodes have 9 NHEs, and fish have at least 3 in the gills (Claiborne et al., 2002; Donowitz et al., 2013; Nehrke and Melvin, 2002).

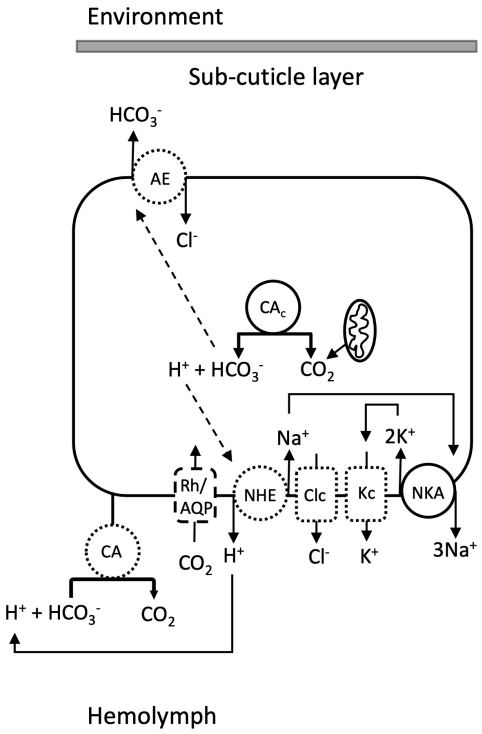

Figure 1.5 Hypothetical mechanism of HCO_3^- excretion in the gills of strong osmoregulators. Transporters with solid lines are known from localization studies in crustaceans or supported by molecular data. Transporters with dotted lines are supported by pharmacological data but molecular identity has not been confirmed. Transporters with dashed lines are hypothetical predictions from what is known in other aquatic taxa. Abbreviations are as follows: carbonic anhydrase (CA), cytoplasmic carbonic anhydrase (CAc), Rh protein (Rh), aquaporin (AQP), Na^+/H^+ exchanger (NHE), Vacuolar type H^+-ATPase (VHA), Na^+/K^+ ATPase (NKA), K^+ channels (Kc), Cl^- channels (Clc), and Cl^-/HCO_3^- anion exchanger (AE). Based on data from Tresguerres et al. (2008).

Extrabranchial acid-base regulation

The antennal gland (Green gland)

The antennal gland also referred to as the green gland, is at the anterior end of the animal near the base of the eyestalk or antenna. This tissue functions through the formation of the urine by ultrafiltration and subsequent modification as it passes through the proximal, distal, and end tubular region toward the bladder (Freire et al., 2008; Tsai and Lin, 2014). The known function of the antennal gland includes the regulation of divalent cations like Mg^{2+} and Ca^{2+} (Wheatly, 1985). The role of the antennal gland in acid-base regulation has only been investigated in four decapods so far with mixed results supporting its importance in acid-base regulation. In the blue crab *C. sapidus*, hypercapnia (2% CO_2) induced acidosis caused no changes in the urines' pH and had a

minimal contribution to total ammonia excretion (1 - 2%), showing a negligible role in acid-base regulation (Cameron and Batterton, 1978). In the amphibious thick crab *Helice formosensis*, the acid-base status of the urine was unchanged following emersion and closely matched the hemolymph acid-base status except for reduced ammonia concentrations following emersion (Allen et al., 2021). While the antennal glands of these two species had no acid-base regulatory response, in Dungeness crab *Metacarcinus magister* and signal crayfish *Pasifastacus leniusculus*, the antennal gland plays a minor role in acid-base regulation (Wheatly, 1985; Wheatly and Toop, 1989). In *M. magister*, exposure to low salinity results in an increase in HCO_3^- reabsorption and net acid excretion primarily as titratable acids with a small contribution by ammonia (Wheatly, 1985). Despite these changes in acid-base fluxes, the antennal gland was calculated to only contribute to about 10% of net base efflux and therefore has a minor contribution relative to branchial fluxes (Wheatly, 1985). Similar results were obtained for the signal crayfish exposed to hyperoxia (Wheatly and Toop, 1989), where a net HCO_3^- reabsorption was detected and an increase in renal acid excretion was measured to only account for 10% of the net excretion. However, in *P. leniusculus*, urinary acid excretion was primarily as ammonia rather than titratable acids as seen in *M. magister*, indicating species difference in antennal gland acid-base regulatory mechanisms. Recent work on *M. magister* exposed to low pH has corroborated the role of the antennal gland in HCO_3^- retention that was previously seen with low salinity exposure (Allen et al. unpublished). Further work is necessary to determine whether this function is species-specific or dependent on the environmental stressor applied and the cellular mechanisms involved.

The digestive tract

The digestive tract of decapods is a simple tube with diverticula that creates an interactive surface between the environment and internal body fluids. It can be divided into three sections: the cuticle-lined foregut (esophagus, cardiac stomach, and pyloric stomach), the midgut (hepatopancreas, midgut tube, and anterior/posterior midgut caeca), and the cuticle lined hindgut. The role of the digestive tract in crustacean acid-base regulation has not been explicitly investigated to date but based on histological and tangential studies on nutrient uptake and osmoregulation, some inferences can be made.

The foregut is where mechanical and chemical digestion occurs (Vogt, 2021). The stomach of crustaceans is typically slightly acidic, where a pH can be as low as 4.7 (Barker and Gibson, 1977;

Gibson and Barker, 1979). The acidity here is partially due to acidic mucopolysaccharides secreted by the oesophagus (Vogt, 2021). In *C. maenas*, feeding does not cause any obvious change in gastric pH, however, in some crustaceans, a slight alkalization could occur due to the buffering effect of food (Quijada-Rodriguez et al., 2022). Due to the relative stability of gastric fluid pH, pre- and postprandial, it is unlikely that the stomach leads to notable acid-base changes in the hemolymph as seen in some vertebrates where gastric acid secretion often causes a postprandial alkalosis (Niv and Fraser, 2002).

To our knowledge, there have been no direct measurements made of luminal fluid pH in either midgut or hindgut regions of the crustacean digestive tract. The lumen of the hepatopancreas is likely acidic as gastric fluids are normally acidic and low pH stimulates nutrient uptake in the hepatopancreas (Ahearn and Clay, 1988; Ahearn et al., 1986; Ahearn et al., 1992). Further, in the hepatopancreas, an apical electrogenic $2Na^+/1H^+$ exchange and basolateral electroneutral Na^+/H^+ exchange mechanism have been identified using brush border and basolateral membrane vesicle preparation (Ahearn et al., 2001; Duerr and Ahearn, 1996). Therefore, it is likely that proton extrusion, creating an acidic lumen for favourable nutrient uptake by the hepatopancreas, is driven by a Na^+/H^+ exchanger. This H^+ transport is coupled to a basolateral Cl^-/HCO_3^- exchange, which would allow intracellular pH maintenance by luminal directed proton secretion and hemolymph directed HCO_3^- transport (Ahearn et al., 1987). The presence of Na^+/H^+ exchange and Cl^-/HCO_3^- exchange processes in the hepatopancreas suggests this tissue is involved in acid-base regulation even if just during postprandial nutrient absorption.

Like the hepatopancreas, the midgut tube region is potentially also involved in acid-base regulation. The midgut of crustaceans is permeable to Na^+ and Cl^- (McNamara et al., 2005), so may have the capacity for Na^+/H^+ exchange and Cl^-/HCO_3^- exchange mechanisms. In the Whiteleg shrimp *L. vannamei*, an NHE2 gene has been cloned and is predominantly expressed in the intestine (midgut tube) (Li et al., 2019). This intestinal NHE is upregulated during acid stress and hemolymph pH is reduced when this gene is knocked down by RNAi, suggesting a role of this transporter in regulating the acid-base status of the hemolymph but also the midgut (Li et al., 2019). Compared to the midgut, the hindgut has a much lower ion permeability, likely to limit salt loss (McNamara et al., 2005). The role of the hindgut in ion regulation is much less clear when compared to the midgut. However, receptors for the crustacean hyperglycemic hormone (CHH)

have been identified in the hindgut, implying a possible ion regulatory role in this tissue (Chung and Webster, 2006). Overall, the digestive tract appears capable of moving acid-base equivalents to maintain a favourable pH along the digestive tract and to promote efficient nutrient absorption. Its overall contribution to maintaining extracellular acid-base status or compensating for changes due to intrinsic or extrinsic acid-base stressors, however, remains unknown.

Hypodermis

The hypodermis is a three layer tissue that lies directly below the crustacean carapace and regulates Ca^{2+} and HCO_3^- fluxes during the moult cycle (Henry and Kormanik, 1985; Roer, 1980; Roer and Dillaman, 1984). During intermoult, it is known that the carapace compartment is maintained 0.3-0.4 pH units more alkaline than the extracellular fluids to prevent carapace dissolution (Wheatly et al., 1991; Wood and Cameron, 1985). Presumably the hypodermis is working to maintain this alkaline compartment during intermoult and therefore might be involved in regulation of extracellular acid-base homeostasis through ion exchange between extracellular and carapace compartments. In fact, a recent study demonstrated that downregulation of SLC4A1 (Cl^-/HCO_3^- exchanger) and SLC4A7 (Na^+/HCO_3^- cotransporter) occurs during pre-moult, which coincides with when pre-moult extracellular alkalosis occurs in crustaceans (Abehsera et al., 2021; Mangum et al., 1985). During post-moult carapace calcification, the hypodermis is clearly involved in exchanging ions to increase Ca^{2+} and HCO_3^- uptake. At post-moult, HCO_3^- uptake is likely mediated by SLC4A1 (Cl^-/HCO_3^- exchanger), which is localized on the hypodermis just below the cuticle and is heavily upregulated at this point in time (Abehsera et al., 2021).

Natural acid-base challenges

Decapods may experience acid-base stressors during their daily life. These stressors can be intrinsic, such as during moulting, feeding, or exhaustive exercise. They may also experience extrinsic stressors due to changes in environmental parameters, such as O_2, CO_2, salinity, and ammonia. While extrinsic stressors are experienced in nature, crustaceans may also face extrinsic acid-base stressors in unnatural settings such as intensive aquaculture. Intrinsic and extrinsic acid-base disturbances are categorized as either respiratory or metabolic disturbances that are readily visualized through Davenport diagrams (Fig. 1.6). Respiratory acid-base disturbances occur when pH homeostasis is altered due to changes in pCO_2 levels. In contrast, metabolic acid-base

disturbances occur when pH homeostasis is altered through changes in HCO_3^- or organic acids (e.g., lactic acid). The following sections will briefly describe how different intrinsic and extrinsic stressors elicit respiratory and metabolic acid-base disturbances in aquatic decapods and the compensatory responses to acidosis and alkalosis. As mentioned previously, homeostatic pH is dynamic in decapods due to the inverse relationship with temperature (Truchot, 1978). Therefore, temperature will not be included as a stressor, but must be considered in cross-study comparisons.

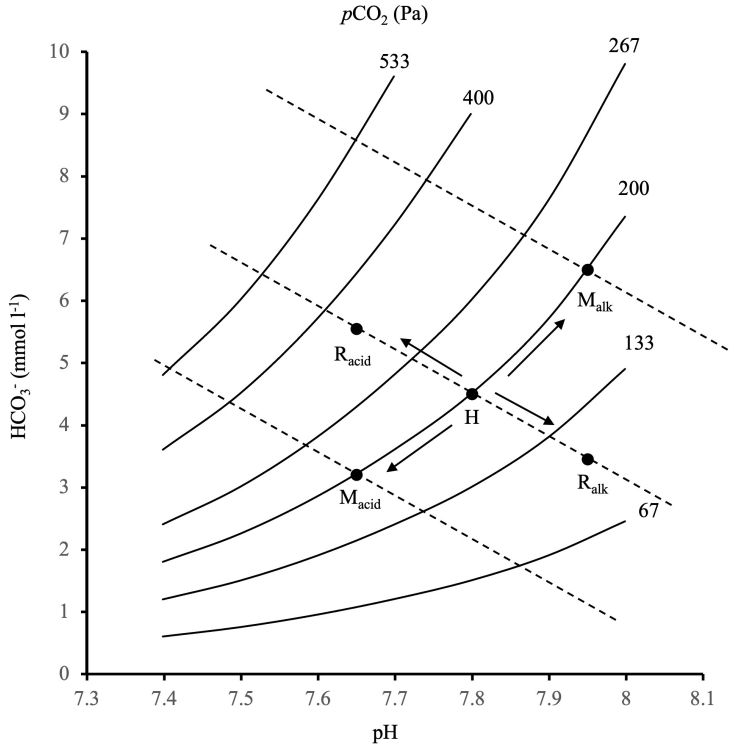

Figure 1.6 Davenport diagram showing possible respiratory and metabolic acid base disturbances in the hemolymph of green crab *Carcinus maenas* at 15 ºC and 35 ppt. Hemolymph HCO_3^- is plotted against hemolymph pH with CO_2 tensions overlain (solid lines). H represents the homeostatic acid-base status, M_{acid} represents metabolic acidosis, M_{alk} represents metabolic alkalosis, R_{acid} represents respiratory acidosis, and R_{alk} represents respiratory alkalosis. The dotted lines represent the non-bicarbonate buffering line of 7 slykes. This figure is redrawn using data from (Truchot, 1983).

Intrinsic acid-base stressors

The most extensively studied intrinsic acid-base stressor in decapods has been exhaustive exercise. Exercise leads to an increase in ventilation and heart rate to support aerobic demands (Booth et al., 1982; Hamilton and Houlihan, 1992; Mcmahon et al., 1979; Rose et al., 1998). This increase in aerobic metabolism leads to greater CO_2 production, which in most decapods results in elevated hemolymph pCO_2 levels and a respiratory acidosis (Booth et al., 1984; McDonald et al., 1979; Milligan et al., 1989; Rose et al., 1998; Waldron et al., 1986). Despite cardiorespiratory

changes to promote oxygen uptake and delivery, decapods also rely on anaerobic metabolism during exhaustive exercise, causing increases in hemolymph lactate up to 15 mmol L^{-1} (Booth et al., 1984; Hamilton and Houlihan, 1992; McDonald et al., 1979; Waldron et al., 1986). Therefore, in decapods, exercise results in an acidosis that is both metabolic (lactate accumulation) and respiratory (pCO_2 increase) in nature. One exception is *Homarus americanus*, which maintains lactate below 1 mmol L^{-1} partially due to its ability to maintain uncompromised hemolymph pO_2 and therefore, experiences only a respiratory acidosis (Rose et al., 1998). Recovery of exercise mediated acidosis occurs through elevated ventilation rate (increase CO_2 excretion), increased net acid efflux (increased titratable acid and ammonia efflux), and intracellular lactate metabolism (Booth et al., 1984; Hamilton and Houlihan, 1992; Mcmahon et al., 1979; Milligan et al., 1989; Rose et al., 1998).

Moulting is one of the most fundamental and energetically costly processes in crustaceans that is essential for growth. The moult cycle of crustaceans is divided into a pre-moult, moult, post-moult and intermoult stage. During moulting, the connective tissue of the old carapace is loosened, the organism escapes its old carapace, takes up water to expand the new flexible cuticle and hardens the new carapace. As the carapace of decapod crustaceans is composed of $CaCO_3$, it is inextricably linked to acid-base regulation to create an environment that promotes $CaCO_3$ precipitation and prevents dissolution. Currently, there are only a few studies on acid-base regulation regarding moulting and have, to my knowledge, only focused on the blue crab *Callinectes sapidus*. During pre-moult, *C. sapidus* experiences a metabolic alkalosis (increase in HCO_3^-) that is likely derived from environmental HCO_3^- as intermoult and shed carapace have similar mineral compositions indicating little mineral reabsorption (Cameron and Wood, 1985). This pre-moult alkalosis works to buffer subsequent acidosis that occurs at the onset of ecdysis, where impaired gas exchange leads to the accumulation of CO_2 (Mangum et al., 1985). During ecdysis, pO_2 levels drop, leading to increased lactate from anaerobic metabolism. This metabolic acidosis is countered by the pre-moult alkalosis decreasing pH to intermoult levels. Throughout post moult, extracellular acid-base status slowly returns to normal with a gradual reduction in HCO_3^- and lactate as well as the initiation of a large acid excretion and CO_2 uptake that promotes carapace calcification (Mangum et al., 1985).

Postprandial effects on decapod acid-base homeostasis have only just recently been investigated. In the red rock crab, *Cancer productus*, feeding was reported to have no effect on hemolymph pH, however, this study did not measure hemolymph pH until 12 hours postprandial at which point oxygen consumption returned to normal values and thus any specific dynamic action (SDA) related acidosis may have been compensated (McGaw et al., 2009). Recent work on *C. maenas* measuring nitrogen and acid-base homeostasis over the time course of digestion has shown a respiratory acidosis associated with feeding (Quijada-Rodriguez et al., 2022). Here, respiratory acidosis was not associated with any change in HCO_3^- but compensated by a large increase in net acid flux mainly achieved by ammonia excretion and, to a lesser extent, titratable acid flux and likely also ventilation.

Extrinsic acid-base stressors

Crustaceans may face environmental fluctuations in oxygen, leading to hyperoxia (above normal oxygenation) or hypoxia (below normal oxygenation). Hyperoxia naturally occurs in aquatic environments due to photosynthetic activity in tidal pools, estuaries, seagrass beds, lakes and streams (McArley et al., 2021). In contrast, hypoxia may occur in estuarine habitats when water stratification due to freshwater influx limits gas exchange between benthic and surface waters (Diaz, 2001). Anthropogenic nutrient pollution into freshwater and coastal habitats may also lead to hypoxia through eutrophication (Diaz, 2001). As mentioned previously, the ventilatory response in decapods depends on environmental oxygenation, with hyperoxia and hypoxia leading to reduced and increased ventilation, respectively (Batterton and Cameron, 1978; Jouve-Duhamel and Truchot, 1983; McMahon et al., 1974). These changes in ventilation cause respiratory acidosis during hyperoxia and respiratory alkalosis during hypoxia (Burnett and Johansen, 1981; McMahon et al., 1978; Taylor and Spicer, 1991; Truchot, 1983; Wheatly, 1987; Wheatly, 1989; Wheatly and Taylor, 1981; Wheatly et al., 1991; Wilkes and Mcmahon, 1982). Hyperoxia mediated acidosis of the extracellular fluids in decapods is typically only partially compensated (Truchot, 1983; Wheatly, 1987; Wheatly, 1989), while the intracellular pH of nerve, antennal gland and muscle has been shown to achieve full compensation, at least in the signal crayfish *P. leniusculus* (Wheatly et al., 1991). The compensatory response to hyperoxia-mediated acidosis includes the accumulation of HCO_3^- and increased acid efflux (titratable acid and ammonia) (Wheatly, 1987; Wheatly, 1989). For *P. leniusculus*, there is also evidence that HCO_3^- accumulation is linked to Cl^-

excretion and increased urinary HCO_3^- reabsorption (Wheatly, 1989; Wheatly and Toop, 1989). Like hyperoxia, hypoxia-mediated alkalosis is only partially compensated by decreasing extracellular HCO_3^- and with chronic or severe hypoxia, lactate accumulation may also partially offset the alkalosis (McMahon et al., 1978; Wheatly and Taylor, 1981; Wilkes and Mcmahon, 1982). With chronic hypoxia, ventilatory rates may be reduced slightly, allowing for recovery of pCO_2 but remain above control levels (McMahon et al., 1978). However, despite these ventilatory changes, alkalosis may persist through the accumulation of HCO_3^- (metabolic alkalosis) (McMahon et al., 1978). While incomplete recovery of hemolymph acid-base status appears the norm in decapods, it may be beneficial as pH can alter hemocyanin oxygen affinity, allowing decapods to modulate oxygen uptake during hypoxia and hyperoxia (Lallier and Truchot, 1989).

Elevated environmental CO_2 (hypercapnia) in aquatic environments occurs when respiratory activity exceeds photosynthetic activity in tidal pools, from coastal upwelling, and natural temporal fluctuation in biotic and abiotic factors in freshwater systems (Hasler et al., 2016; Saderne et al., 2013; Truchot, 1988). In aquaculture settings, high stocking densities have been found to increase CO_2 tension as high as 700 Pa (Zhang et al., 2019). Aquatic decapods typically experience respiratory acidosis upon exposure to elevated environmental CO_2 as hemolymph to environment CO_2 diffusion gradients are reduced and when ambient CO_2 exceeds normal extracellular levels, leading to CO_2 influx. Decapods can generally fully compensate for hypercapnia-induced acidosis, but the ability to compensate depends on species, CO_2 tension, and possibly osmoregulatory strategy. While not extensively tested, the osmoregulatory strategy of a species may limit the compensation of hypercapnic acidosis. Osmoconformers like *M. magister and Necora puber* achieve complete pH compensation when exposed to extreme hypercapnia (1 kPa pCO_2) while osmoregulators like *C. sapidus* and *C. maenas* only achieve partial compensation, suggesting osmoregulation may limit acid-base compensation (Cameron, 1978; Fehsenfeld and Weihrauch, 2016a; Pane and Barry, 2007; Spicer et al., 2007).

The compensatory response to hypercapnic acidosis is achieved through ion exchange and buffering as environmental CO_2 does not initiate a ventilatory response in decapods (Jouve-Duhamel and Truchot, 1983). Most aquatic decapods have been shown to compensate for hypercapnic acidosis through the accumulation of extracellular HCO_3^- (Allen et al., 2020; Cameron, 1978; Dissanayake et al., 2010; Hammer and Pedersen, 2013; Hans et al., 2014; Pane

and Barry, 2007; Quijada-Rodriguez et al., 2021; Spicer et al., 2007). An exception to this generally accepted compensatory mechanism is seen in the deep sea tanner crab *Chionoecetes tanneri*, which completely lacks the ability to accumulate HCO_3^- in response to environmental hypercapnia and does not elicit a compensatory response (Pane and Barry, 2007). In this species, the authors suggested that the stability of deep-sea environments and the low metabolic rate of this species has led to a lack of short-term acid-base regulatory capacity. In species that accumulate HCO_3^- in response to hypercapnia, it has been proposed that this occurs through Cl^-/HCO_3^- exchange with the environment as reductions in Cl^- flux coincide with HCO_3^- accumulation in hypercapnia exposed *C. sapidus* (Cameron, 1985). In the vent crab *Xenograpsus testudinatus*, hypercapnia induced an increase in extracellular HCO_3^- to above 90 mmol L^{-1}, which is coupled with an equimolar decrease in extracellular Cl^- (Allen et al., 2020). Earlier research had proposed that the carbonate of the carapace may also provide HCO_3^- for buffering during hypercapnia exposure, however, in *C. sapidus* it was determined that the carapace provides only a very small contribution (~7.5%)(Cameron, 1985). In *X. testudinatus*, no changes in extracellular Ca^{2+} or Mg^{2+} were observed following hypercapnia exposure, suggesting carapace dissolution for buffering was not occurring (Allen et al., 2020). The increase of acid secretion is another potential mechanism that may allow aquatic decapods to regulate acid-base status during hypercapnia exposure. In *C. sapidus*, hypercapnia induces an increase in apparent H^+ excretion (Cameron, 1985), while in *C. maenas* and *E. sinensis* hypercapnia induces an increase in ammonia excretion (acid equivalent) (Fehsenfeld and Weihrauch, 2013; Quijada-Rodriguez et al., 2021).

Decapods in estuarine environments experience fluctuations in salinity due to location in the estuary and changes in tide and freshwater run-off. Further, some species migrate between freshwater and marine habitats as part of their life cycle exposing them to osmotic challenges, e.g., the Chinese mitten crab *E. sinensis*, which spends most of its life in freshwater but migrates to marine habitats for breeding (Veilleux and Lafontaine, 2007). In all crustaceans studied so far, transfer from low to high salinity results in metabolic acidosis (decrease in HCO_3^-) coupled with respiratory alkalosis (decrease in pCO_2). In *C. maenas* and *P. leniusculus*, the decrease in HCO_3^- and pCO_2 results in a net acidosis, whereas in *E. sinensis*, these changes counteract each other, allowing for the maintenance of extracellular pH (Truchot, 1981; Truchot, 1992; Wheatly and Mcmahon, 1982). The opposite effect is seen in decapods moving from seawater into dilute

salinity, where now metabolic alkalosis occurs (Henry and Cameron, 1982; Truchot, 1981; Truchot, 1992; Whiteley et al., 2001a). While all decapods examined so far do experience a metabolic alkalosis when transferred to dilute salinity, the reasoning for the alkalosis is still debated and the time course of acclimation varies between species with some experiencing transient acidosis before alkalosis. In *C. sapidus*, no transient acidosis or change in pCO_2 was observed throughout the acclimation processes, however, an increase in strong ion difference ($[Na^+]$-$[Cl^-]$) was observed and implicated as a potential explanation for alkalosis (Henry and Cameron, 1982). In contrast, both *E. sinensis* and *C. maenas* have been shown to experience transient acidosis before the establishment of metabolic alkalosis (Truchot, 1981; Whiteley et al., 2001a). At least in *E. sinensis*, this transient acidosis has been shown to correlate with acidosis in muscle tissue and therefore is likely a result of acid-base exchange between the intra and extracellular fluid during cell volume and ionic regulation (Whiteley et al., 2001a). In *E. sinensis* and *C. maenas*, no indication of a change in strong ion difference had been noted however, the authors postulate that alkalosis may be linked to ion exchange promoting greater acid efflux (ammonia excretion) and exchange of osmolytes during cell volume regulation (Cl^-/HCO_3^- exchange) (Truchot, 1992; Whiteley et al., 2001a). Although some hypotheses have been put forward, the origin of acidosis and alkalosis during salinity transfers in decapods is still not fully understood.

In aquatic environments, ammonia, a weak base with a pKa of ca. 9.3, is usually abundant only in the low micromolar range. Decapods may naturally experience fluctuations in environmental ammonia, as sediments of benthic habitats can range from low micromolar to nearly 3 mmol L^{-1} (Weihrauch et al., 1999). In addition, crustaceans may face millimolar concentrations of ammonia from build-up in their burrows and tidal pools associated with high avian fecal material (Loder et al., 1996; Stoeckel et al., 2011). In aquaculture settings, ammonia is usually maintained lower than these more extreme environmental challenges but can still exceed 200 μmol L^{-1} (Romano and Zeng, 2013). The toxicity of ammonia and transport mechanisms in decapods has received considerable attention, however, the effect of elevated environmental ammonia on acid-base homeostasis is unclear. Recently, the effect of high environmental ammonia (HEA) on emersion response in *C. maenas* was investigated (Zimmer and Wood, 2017). Here, HEA increases hemolymph ammonia and lead to metabolic alkalosis. Due to the short nature of these experiments

(15 minutes in duration) the compensatory responses to regulate extracellular pH were not studied. Presumably, one might expect this compensatory response to involve elevated excretion of ammonia as decapods are capable of active ammonia excretion (Weihrauch et al., 2004). While no other studies have shown acid-base responses to HEA in decapods, alkalosis following exposure to HEA also occurs in teleost fishes (Cameron and Heisler, 1983; Wilson et al., 1994).

Physiological challenges of aquaculture and global change

Global change aquatic acidification

Increasing anthropogenic CO_2 emissions are predicted to lead to an increase of oceanic pCO_2 to 1000 μatm and a decrease in ocean surface pH of up to 0.3 pH units by the end of the century (IPCC, 2013). Since atmospheric changes of pCO_2 will translate to all bodies of water, freshwater systems will most likely also experience an increase in pCO_2 (Hasler et al., 2016). The high variation in biogeochemistry between freshwater bodies means rising atmospheric CO_2 may differentially affect freshwater systems (Hasler et al., 2016). While crustaceans naturally face fluctuations in environmental CO_2, these increases are rather acute compared to future global change-driven increases that will be a chronic issue. As previously discussed, acid-base regulation is an energetically costly process and the long-term energetic demands due to global change may affect long-term animal fitness. A recent meta-analysis synthesized the results of 55 studies on the effects of ocean acidification on decapods (Bednaršek et al., 2021). This study found negative effects on animal growth, survival, and aerobic metabolism, while pH regulation was usually well maintained. Interestingly, many freshwater systems have CO_2 tensions that exceed those predicted for marine systems and have been shown to cause negative effects on marine species (Raymond et al., 2013). Whether changes in freshwater pCO_2 from global change will also negatively impact decapods remains unclear.

Aquaculture challenges

Rising global populations and demand for food have led to the rapid rise in aquaculture over the last 40 years to account for as much as 46% of yearly global seafood production (FAO, 2020). Although crustaceans only account for 11.5% of global aquaculture production, they account for nearly ¼ of the global aquaculture economic value (FAO, 2020). The most cultured crustaceans are the Whiteleg shrimp *Litopenaeus vannamei* (52.9%), the Louisana crayfish *Procambarus*

clarkii (18.2%), Chinese mitten crab *Eriocheir sinensis* (8.1%), and Giant tiger prawn *Penaeus monodon* (8%), with other crustaceans accounting for the remaining 12.8% of crustacean aquaculture (FAO, 2020). The environmental impact of traditional pond aquaculture in coastal habitats has led to the development of more environmentally friendly recirculating aquaculture systems (Martins et al., 2010). To operate economically, aquaculture farms need to maintain high stocking densities, which can lead to the accumulation of metabolic wastes (CO_2 and ammonia) and depletion of oxygen (Skov, 2019). The toxic effects of elevated ammonia are well known in decapods (e.g. gill damage and immune suppression) and mitigation strategies are readily available to remove nitrogen from aquaculture systems (e.g. biofiltration and Bio-floc technology) (Crab et al., 2007; Romano and Zeng, 2013). Hypoxia in aquaculture systems may lead to changes in metabolism, suppress immunity, and reduce survival (Zheng et al., 2022). Like ammonia, hypoxia can be readily mitigated through various aeration strategies to increase pO_2 in the aquaculture system (Zheng et al., 2022). Direct studies on the effect of elevated CO_2 in aquaculture settings are nearly non-existent but based on the negative effects on growth and survival from ocean acidification research (Bednaršek et al., 2021), one might expect similar effects on decapods in aquaculture settings. Strategies to lower pCO_2 in aquaculture systems are much less effective than for dealing with high nitrogen or low O_2 (Brauner et al., 2019). The first issue for removing CO_2 from aquaculture systems stems from the high solubility of CO_2. Essentially, as the solubility of CO_2 in water is upwards of 30 times greater than O_2 then off-gassing CO_2 from an aquaculture system is far more difficult than replenishing O_2. Additionally, due to the dissociation of CO_2 into H^+ and HCO_3^-, most of the CO_2 in the system will be in the form of HCO_3^- and must be converted back to CO_2 before it can be off-gassed, which occurs at a rather slow rate in an aquaculture system (Brauner et al., 2019). It is apparent that mitigation of CO_2 in aquaculture systems is problematic and may negatively affect decapod species. Our current understanding of the effects of elevated CO_2 on aquaculture species is lacking and must be addressed to improve aquaculture productivity.

Thesis objective and chapter outline

This chapter summarizes the mechanisms and responses of decapod crustaceans to various intrinsic and extrinsic acid-base stressors with insights into the challenges of environmental CO_2 regarding global change and intensive aquaculture. The objectives of this thesis were to elucidate the physiological responses of euryhaline decapod crustaceans to intrinsic and extrinsic acid-base

stressors relevant to global change and aquaculture. The current review highlights a considerable gap in our understanding of the effects of elevated CO_2 on freshwater crustaceans and in aquaculture settings. The first two experimental chapters will address this knowledge gap using the euryhaline Chinese mitten crab *Eriocheir sinensis* (Chapter 2) and Whiteleg Shrimp *Litopenaeus vannamei* (Chapter 3). In addition, it is unknown how feeding affects acid-base regulation and nitrogen homeostasis in decapods. Yet, this is part of daily life and will interact with extrinsic stressors in aquaculture settings and likely influence responses to global change. Chapter 4 will provide an understanding of acid-base regulation in response to feeding to provide a foundation for future work on interactions between intrinsic and extrinsic acid-base stressors in aquaculture settings.

Chapter 2 investigates the effects of global change relevant freshwater acidification on various measures of animal performance on the euryhaline Chinese mitten crab *Eriocheir sinensis*. I tested the hypothesis that since freshwater systems regularly experience fluctuations in CO_2 that freshwater decapods would be well adapted to counter challenges with anthropogenic freshwater acidification and not experience detrimental impairment of animal performance. This hypothesis was tested by several measures of animal performance, including whole animal acid-base status, indicators of metabolic changes (ammonia excretion and oxygen consumption), calcification (carapace calcium content), locomotory behaviour, and survival.

Chapter 3 investigates the effects of recirculating aquaculture system CO_2 levels on growth, survival, and acid-base/nitrogen regulation of the Whiteleg shrimp *Litopenaeus vannamei*. I tested two hypotheses: (1) that chronic recirculating aquaculture system CO_2 levels do not affect acid-base homeostasis but come at increased energetic demand, and (2) that chronic metabolic cost of maintaining physiological processes under recirculating aquaculture system CO_2 conditions impairs growth and survival. These first hypotheses were tested by measuring whole animal acid-base status, hemolymph ammonia, and ammonia excretion rates of adult shrimp chronically exposed to recirculating aquaculture system conditions and normocapnic brackish water to gain an understanding of potential increases in protein catabolism and ability to maintain pH homeostasis. The second hypothesis was tested by measuring the growth and survival of juvenile shrimp for 63 days at low, moderate, and high recirculating aquaculture system CO_2 levels.

Chapter 4 investigates the effect of feeding (3% body weight) on acid-base regulation and nitrogen homeostasis in seawater-adapted green crab *Carcinus maenas*. I tested three hypotheses, (1) that feeding influences acid-base homeostasis through changes in gastric fluid pH, (2) that increased aerobic metabolism after feeding alters acid-base homeostasis through acid and ammonia production, and (3) postprandial acid-base and nitrogen homeostasis disturbances are compensated through buffering and acid-base equivalent/nitrogen exchange. These hypotheses were tested by measuring changes in gastric fluid pH, extracellular acid-base status, whole animal acid-base and nitrogen fluxes, and enzymatic activity of urea synthesis and catabolism pathways.

CHAPTER 2: ANTHROPOGENIC CO_2-MEDIATED FRESHWATER ACIDIFICATION LIMITS SURVIVAL, CALCIFICATION, METABOLISM, AND BEHAVIOUR IN STRESS-TOLERANT FRESHWATER CRUSTACEANS

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Published in: Biogeosciences. 2021. 18: 6297-6300

Author contributions: ARQR designed the study, performed experiments, analyzed data, and wrote the manuscript. PLK assisted with data collection. PHS assisted with data collection. MTH assisted with data collection. GJPA assisted with data analysis and drafting the manuscript. PPH provided financial support and analytical tools. YCT assisted in study design, manuscript editing, financial support, and analytical tools. DW assisted in study design, manuscript editing, financial support, and analytical tools

Abstract

Dissolution of anthropogenic CO_2 is chronically acidifying aquatic ecosystems. Studies indicate that ocean acidification will cause marine life, especially calcifying species, to suffer at the organism and ecosystem levels. In comparison, freshwater acidification has received less attention rendering its consequences unclear. Here, juvenile Chinese mitten crabs, *Eriocheir sinensis*, were used as a crustacean model to investigate the impact of CO_2-mediated freshwater acidification. Our integrative approach, investigating changes in the animal's acid-base homeostasis, metabolism, calcification, locomotory behaviour, and survival rate, indicates that this economically relevant crustacean will face energetic consequences from future freshwater acidification. These energetic trade-offs allow the animal to maintain its acid-base homeostasis at the cost of reduced metabolic activity, exoskeletal calcification, and locomotion, reducing the

animal's overall fitness and increasing its mortality. Results indicate that present-day Chinese mitten crab could be heavily affected by freshwater acidification like their marine counterparts and emphasize the importance of understanding the long-term implications of freshwater acidification on species' fitness.

Introduction

Rising levels of atmospheric CO_2 partially dissolve into marine systems, causing a decrease in oceanic pH referred to as ocean acidification. In marine species, ocean acidification has been demonstrated to impact development, metabolism, behaviour, and biomineralization, potentially leading to major ecosystem-level changes (Kroeker et al., 2013; Melzner et al., 2009; Tresguerres and Hamilton, 2017). It is generally believed that freshwater systems will also experience acidification (Hasler et al., 2016; Phillips et al., 2015; Weiss et al., 2018). However, the high variability in biogeochemistry between freshwater systems has been a limiting factor in modelling future freshwater scenarios (Hasler et al., 2016). Two recent case studies on different freshwater systems have suggested that the magnitude of CO_2 mediated acidification could be similar or even exceed predicted levels of ocean acidification (Phillips et al., 2015; Weiss et al., 2018). The potential that freshwater acidification may be of equal or greater severity than ocean acidification emphasizes the need to understand the biological responses and consequences to freshwater species.

Calcifying species are sensitive to acidification as dissolution of CO_2 reduces carbonate availability in parallel to pH, potentially increasing dissolution of their calcified exoskeleton (Feely et al., 2004; Roleda et al., 2012). To date, no comprehensive studies have investigated the various physiological and behavioural effects of realistic future levels of CO_2-mediated acidification on calcifying freshwater invertebrates. However, several studies have used high CO_2 levels beyond that relevant for potential future freshwater acidification to investigate acid-base regulation and calcification in freshwater calcifying invertebrates (Cameron, 1978; Cameron, 1985; David et al., 2020; Jeffrey et al., 2018a; Jeffrey et al., 2018b). Freshwater calcifying macro-organisms are largely limited to crustaceans and molluscs that comprise roughly 10% and 4% of freshwater species diversity, respectively (Balian et al., 2008). Crustaceans are arguably one of the most successful animal groups occupying almost all ecological niches across the globe, including freshwater, marine, and terrestrial habitats, making them a suitable model to study global change

consequences in a physiologically and ecologically robust group of species. Freshwater crustaceans occupy a key position in food webs where all crustacean life stages provide a vital food source for a wide range of juvenile and adult predators (Cumberlidge et al., 2009). Additionally, freshwater crustaceans provide vital ecological services as indicators of water quality, nutrient cycling of detritus and bioturbation of sediment (Cumberlidge et al., 2009). From an economic standpoint, freshwater crustaceans account for ~30 % (2.5 million tons) of aqua-cultured crustaceans worldwide, demonstrating that this group is an important human food source (Tacon, 2020). The ecological and economic importance of freshwater crustaceans, together with the apparent sensitivity of calcifying species to acidification based on marine studies, makes it imperative to determine whether freshwater crustaceans are sensitive to anthropogenic CO_2-mediated freshwater acidification.

The Chinese mitten crab (*Eriocheir sinensis*) is one of the most important freshwater crustaceans, accounting for the third largest crustacean aquaculture globally (FAO, 2018). This highly invasive catadromous species spends most of its life cycle in freshwater systems but has the physiological plasticity to migrate into marine environments where it reproduces (Veilleux and Lafontaine, 2007). The invasive status and aquacultural importance of *E. sinensis* have made it a well-studied freshwater crustacean model in biological research. Here, we used the juvenile life stage of *E. sinensis* as a freshwater crustacean model to investigate the effects of a potential future CO_2-mediated freshwater acidification scenario on acid-base regulation, metabolism, calcification, behaviour, and survival rate. Native to China's Yangtze river system, the third-largest river system in the world, juvenile *E. sinensis* in this habitat already experience regular fluctuations in freshwater pCO_2 from 681 to 3796 µatm (Ran et al., 2017), which may confer some pre-adaptation to elevated CO_2 because animals are accustomed to normal environmental CO_2 fluctuations. Crustaceans are believed to be more CO_2 tolerant than other calcifying organisms such as bivalves and coral because of their high metabolic activity and robust acid-base machinery, allowing for more efficient compensation of acid-base disturbances (Melzner et al., 2009). These combined predictors of CO_2 tolerance make *E. sinensis* an interesting model to study the effects of future CO_2 mediated freshwater acidification, as they may already possess the adaptations necessary to deal with future freshwater acidification conditions. Therefore, we hypothesized that *E. sinensis*

would be well-adapted to counteract challenges associated with fluctuating pCO_2 resulting from anthropogenic activity and not experience detrimental physiological or behavioural impairment.

Materials and methods

Animal maintenance

Wild-caught male and female juvenile Chinese mitten crab (*Eriocheir sinensis* 10-20 g) were purchased from the Chinese mitten crab Breeding Association of Taiwan. Crabs were maintained at the Academia Sinica Institute of Cellular and Organismal Biology aquatics facility (Taipei, Taiwan) in three 120-L aquariums with flow through dechlorinated Taipei tap water (in μmol L^{-1} Na^+ 237, K^+ 16, Ca^{2+} 216, Mg^{2+} 213, Cl^- 201; Y.C. Tseng pers. comm. See ringer measurement methods below) on a 14:10 h light-dark cycle with temperature ranging from 23 to 25 ºC. Water parameters for these holding tanks were the same as that of the control water used in the experimental acclimations. Juvenile crabs in non-experimental holding tanks were maintained at a density of roughly 100 individuals per tank with PVC pipes for shelter and a constant flow of freshwater to prevent the build-up of metabolic wastes. Crabs were fed *ad libitum* with oatmeal and mollusc meat three times per week and monitored for activity level and the presence of disease as general health indicators. Diet was selected to maintain an omnivorous diet as seen in the wild (Czerniejewski et al., 2010) and based on what is fed by our crab supplier (Y.C. Tseng pers. comm.). Crabs were fasted for 48 to 72 hours before sampling to minimize the effects of dietary intake on measured parameters.

Freshwater acidification

For experimental acclimation, crabs were sampled upon removal from the holding tanks (0-day time point) and transferred to flow through 10-L experimental tanks (6-7 crabs per tank, 4 tanks per treatment) containing either control or acidified freshwater (Table 2.1) with PVC pipes added for shelter. Acidified freshwater was achieved by injection of CO_2 directly into the experimental tanks by air stone to maintain environmental pH (pH controller, Aqua-MACRO). The pH controller system used in this study required that each tank had a pH probe, pH controller, CO_2 tank, gas regulating solenoid and air stone, thus meaning each tank in this study was independently pH/CO_2 regulated. CO_2 bubbling rate and freshwater flow rate were adjusted to minimize overshooting the target pCO_2 level. Following injection of CO_2 to regulate water pCO_2

we recorded a brief pCO_2 overshoot to a maximum level of 5625 µatm resulting from direct CO_2 injection into the experimental tanks by the pH controller. Water pH, total alkalinity, and temperature were regularly measured in the experimental tanks throughout the study. Water pH (NBS scale) and temperature were measured with a pH electrode (Accumet AP55 pH/ATC electrode, Ohio, USA) connected to a portable pH meter (Accumet AP71, Ohio, USA) calibrated with pH buffers (pH 4.00, 7.00, and 10.01) traceable to NIST standard reference material (Thermofisher Orion). Water alkalinity was measured by spectrophotometric assay on a Nanodrop 2000c (Thermo scientific, Wilminton, DE, USA) according to previously established protocols (Sarazin et al., 1999). Water pCO_2 was calculated with the CO2SYS excel add-in (Lewis and Wallace, 1998) using measured water temperature, pH and total alkalinity. Constants used for pCO_2 calculations include freshwater carbonate dissociation constants (K_1 and K_2) from Millero (1979), and $KHSO_4$ constants from Dickson (1990).

Table 2.1 Measured tank parameters for 7-day, 14-day, and 42-day experiments of control and CO_2 acidified freshwater (FW). Measured parameters include temperature, pH (NBS scale), total alkalinity (A_T), total CO_2 (C_T), and partial pressure of CO_2 (pCO_2).

	Temp (˚C)	pH	A_T (µmol L^{-1})	C_T (µmol L^{-1})	pCO_2 (µatm)
Control 7 day	23 ± 0.15	7.41 ± 0.02	501 ± 32	547 ± 36	1299 ± 121
Acidified 7 day	23 ± 0.15	6.73 ± 0.01	430 ± 13	614 ± 18	5109 ± 157
Control 14 day	24.6 ± 0.05	7.4 ± 0.01	517 ± 4	563 ± 5	1364 ± 46
Acidified 14 day	24.5 ± 0.13	6.8 ± 0.01	429 ± 7	589 ± 7	4633 ± 87
Control 42 day	24.5 ± 0.07	7.4 ± 0.01	529 ± 6	576 ± 7	1389 ± 31
Acidified 42 day	24.4 ± 0.1	6.8 ± 0.01	433 ± 4	592 ± 4	4634 ± 58

Hemolymph Acid-Base Status

Hemolymph acid-base experiments were conducted over seven days to determine if crabs could actively regulate acid-base status in the presence of future freshwater acidification conditions. Hemolymph samples (100 µL per crab) were taken at the base of a walking leg with a sterile syringe according to previous protocols for *E. sinensis* (Truchot, 1992). Samples from 2 to 3 crabs were pooled together (200-300 µL pooled hemolymph per n value) to get a sufficient volume for downstream analyses of ammonia, pH, and total carbon. Pooled hemolymph samples were gently mixed by slowly pipetting to avoid off gassing of CO_2 and disrupting hemolymph acid-base parameters. Measurements of pH and total carbon were performed immediately after

hemolymph collection and the remaining hemolymph was frozen at -20 °C for later analysis of ammonia. Hemolymph pH (200-300 µL samples) was measured in NBS scale using an InLab micro pH electrode calibrated with pH buffers traceable to NIST standard reference material (Thermofisher Orion). Hemolymph total carbon was measured in duplicate (50 µL per measurement) using the Corning 965 carbon dioxide analyzer (± 0.2 mmol L^{-1} precision) calibrated with $NaHCO_3$ standards ranging from 0 to 20 mmol L^{-1} to produce a standard curve with a minimum R^2 of 0.99. Hemolymph pCO_2 and HCO_3^- were calculated using a rearrangement of the Henderson-Hasselbalch equation with pK1 and αCO_2 values derived for *E. sinensis* hemolymph at 23 ºC (pK1= 6.079773, αCO_2= 0.00031263 mmol L^{-1} Pa^{-1} (Truchot, 1976; Truchot, 1992). Hemolymph ammonium was measured in triplicate (25 µL hemolymph per measurement) with a microplate reader (Molecular Devices, SpectraMax, M5) using an orthophthaldialdehyde fluorometric assay, which is insensitive to amino acids and proteins (Holmes et al., 1999). Ammonia standards were made from NH_4Cl in *E. sinensis* ringer (pH 8.1) containing (in mmol L^{-1}): 185 NaCl, 16 $CaCl_2$, 6 $MgCl_2$, 7 KCl, and 13 $NaHCO_3$. The ion concentrations for the ringer were based on ion composition measurement done on 4 juvenile Chinese mitten crabs in this study (in mmol L^{-1} Na^+ 191, K^+ 7.2, Ca^{2+} 16.3, Mg^{2+} 5.9, Cl^- 252). Concentrations of Na^+, K^+, Ca^{2+}, Mg^{2+} were measured by flame absorption spectrophotometry (Polarized Zeeman Atomic Absorption Spectrophotometer Z-5000, Hitachi High-Technologies, Tokyo, Japan), Cl^- was measured spectrophotometrically using the mercury (II) thiocyanate method (Florence and Farrar, 1971). HCO_3^- and pH values for the ringer were based on measurements taken from control crabs in this study and measured as described above.

Ammonia excretion and oxygen consumption

Ammonia excretion and oxygen consumption were measured over a seven-day acclimation to control and acidified freshwater. These two parameters were measured on individual crabs haphazardly selected from the four control and four acidified freshwater aquaria. Ammonia excretion and oxygen consumption measurements were performed in parallel to hemolymph sampling however, crabs were first randomly selected and placed into respirometry chambers before selecting crabs for hemolymph sampling to avoid using crabs recently sampled for hemolymph. Ammonia excretion experiments were performed in plastic Tupperware filled with

200 mL of filtered control or acidified freshwater. Crabs were given 30 minutes to acclimate to the experimental chambers before initiation of water sampling, as ammonia excretion is elevated for a short time directly after handling (Hans et al., 2014). Water samples (1 mL) for ammonia analysis were collected directly after 30 and 90 minutes of being placed in the experimental chambers. Ammonia concentrations of the water at the 30 and 90-minute time points were determined using the aforementioned orthophthaldialdehyde fluorometric assay (Holmes et al., 1999). Ammonia excretion rates were calculated according to the Eq. (1):

$$Ammonia\ excretion\ rate = \frac{(Amm_{90} - Amm_{30}) \times V}{t \times m}$$

where Amm_{90} is the water ammonia concentration at 90 minutes, Amm_{30} is the water ammonia concentration at 30 minutes, V is the chamber volume during the flux period in litres, t is the flux time in hours, and m is the fresh weight of the crab in grams.

The oxygen consumption rate was measured by closed-system respirometry in custom-made 3 L glass respiration chambers containing filtered (0.2 μm) freshwater. To achieve the correct experimental CO_2 tension, respirometry chambers were submerged in large 18 L buckets of filtered freshwater and a pH controller (Aqua-MACRO) was used to regulate the injection of CO_2 as described above for experimental tanks. Crabs were transferred to the submerged respirometry chambers and given 15 minutes to adjust to fully oxygenated respiration chambers before being sealed. Chambers were placed horizontally, allowing for lateral crab movement in the chamber, and oxygen saturation was measured continuously every 15 seconds for 30 minutes at 23 °C. The oxygen sensor (PreSens oxygen micro optode, type PSt1, PreSens Precision Sensing GmbH, Regensburg, Germany) was attached to the top of the chamber and connected to an OXY-4 mini multichannel fibre optic oxygen transmitter (PreSens Precision Sensing GmbH, Regensburg, Germany). Oxygen saturation was always maintained above 80%. Respiration chambers without a crab were used to determine any potential background bacterial respiration for each trial. Preliminary trials demonstrated that crab movement and ventilation rate in the chamber was sufficient to mix the water within the chamber and prevent oxygen stratification, as indicated by a linear decline in oxygen availability. While this approach allows for the measurement of oxygen consumption, some limitations must be considered. Logistical constraints prevented the use of an intermittent flow respirometry approach where the animal could have been given a long amount of time to acclimate to the respirometry chamber. This technical limitation means that the

reported measurements cannot be considered a resting metabolic rate as the handling stress, brief air exposure and transfer to a novel environment may have influenced the animal's metabolic rate. However, we would like to point out that in previous trials from our lab using an intermittent flow respirometry setup on green crabs *Carcinus maenas*, crayfish *Procambarus clarkii*, and lobsters *Homarus americanus*, that crustaceans placed in respirometry chambers will stabilize oxygen consumption to a resting rate in under 30 minutes (Gwangseok R. Yoon pers. comm).

Carapace calcification

To assess carapace calcification, changes in the calcium content relative to carapace mass was measured at one, two, three and six weeks of high CO_2 exposure according to previously established protocols (Spicer and Eriksson, 2003). In brief, a piece of carapace (ca. 2.5 cm^2, 15.2 ± 0.4 mg) was removed from the dorsal carapace. The weighed piece of carapace was digested in HNO_3 (13.1 N) at 60°C for 16 hours. Digested samples were then diluted to a final HNO_3 concentration of 2 % v/v. The carapace Ca^{2+} content was measured by atomic absorption spectrophotometer (Z-8000; Hitachi). Standard solutions from Merck (Darmstadt, Germany) were used to make the Ca^{2+} standard curve.

Locomotory behaviour Assay

A 24 x 24 cm square, novel, opaque tank was used in the open field test to assess changes in movement of juvenile crabs after a seven-day exposure to control and freshwater acidified conditions. Acclimated crabs were transferred to the novel tank containing control or acidified freshwater and given 5 minutes to acclimate, as done in previous crustacean behavioural studies (Robertson et al., 2018). After acclimation, crab activity was recorded with a digital camera (UI-3240CP Rev.2, Ids, Germany) for 5 minutes (300 seconds) and videos of the movement were processed with the image analysis Ethovision XT motion tracking software (v. 7.0, Noldus, Netherlands). In this study, four factors were measured; distance covered (cm), velocity (cm s^{-1}), movement (time in movement, seconds) and mobility (time in mobile state, seconds). We defined movement as the duration for which the central body point (whole body) was changing location. Mobile state was defined as the duration in which crabs exhibited any movement, even if the center point of the animals remained in the same location, for example, appendage movement.

Statistical analysis

Statistical analyses were conducted using JMP Pro 16 (Cary, NC, USA) and GraphPad Prism 8.4.2 (San Diego, CA, USA). Data were analyzed for outliers by the ROUT test with a Q value of 1 %. For all data, heterogeneity of variance was tested by Levene's test and normal distribution of residuals by Shapiro-Wilk test. Two transformations were done so that data could meet the assumptions of normal distribution and homogeneity of variance. A Johnson SB transformation was applied to hemolymph pCO_2 data and a square root transformation was applied to ammonia excretion rate data. In this study, hemolymph parameters, ammonia excretion, and oxygen consumption data were analyzed by a two-way ANOVA post hoc Dunnett's test. For Dunnett's test, comparisons were made to the zero-day control with time and pCO_2 values as fixed factors. Carapace calcification data were analyzed by two-way ANOVA post hoc Tukey HSD with time and pCO_2 values as the fixed factors. Behavioural data displayed a high degree of co-linearity between dependent variables, violating the assumptions of the MANOVA test. Therefore, we analyzed behavioural data using a student's t-test except for appendage movement time. Appendage movement time data violated assumptions of student's t-test, so were analyzed by the Wilcoxon test. Survival curves were analyzed for significant differences by the Mantel-Cox test. The survival curve hazard ratio was determined by the Mantel-Haenszel test. For all data sets, p values ≤ 0.05 were considered significant. Data are presented as mean ± standard error (SEM). Statistical output results are written in text or summarized in Table 2.2.

Table 2.2 Statistical results of two-way ANOVAs from hemolymph acid-base parameters, oxygen consumption, ammonia excretion and carapace calcification experiments. For hemolymph acid-base measurements, response variables were hemolymph pH, HCO_3^-, pCO_2, or ammonia with time and CO_2 as fixed independent variables. For whole animal experiments, response variables were oxygen consumption rate and ammonia excretion rate with time and CO_2 as fixed independent variables. For carapace calcification experiments, the response variable was carapace calcium content with time and CO_2 as fixed independent variables. P-values below 0.05 are considered statistically significant and are bolded.

Two-way ANOVA					
Dependent Variable	**Independent Variable**	**df**	**df_{error}**	**F ratio**	**p-value**
Hemolymph pH	Time	4	84	1.36	0.25
	CO_2	1	84	15.63	**0.0002**
	Time x CO_2	4	84	3.8	**0.0068**
Hemolymph HCO_3^-	Time	4	84	2.85	**0.028**
	CO_2	1	84	0.94	0.33
	Time x CO_2	4	84	0.83	0.51
Hemolymph pCO_2	Time	4	84	3.22	**0.016**
	CO_2	1	84	16.24	**0.0001**
	Time x CO_2	4	84	1.74	0.15
Hemolymph Ammonia	Time	4	74	2.52	**0.048**
	CO_2	1	74	1.41	0.24
	Time x CO_2	4	74	3.33	**0.015**
O_2 consumption	Time	4	47	2.04	0.1
	CO_2	1	47	40.95	**<0.0001**
	Time x CO_2	4	47	3.39	**0.016**
Ammonia excretion	Time	4	76	3.01	**0.023**
	CO_2	1	76	0.7	0.4
	Time x CO_2	4	76	2.91	**0.027**
Carapace Ca^{2+}	Time	3	68	3.98	**0.011**
	CO_2	1	68	8.59	**0.0046**
	Time x CO_2	3	68	4.33	**0.0074**

Results

Probability of Survival

The effect of freshwater acidification on survival was determined by generating survival curves for crabs in control and acidified freshwater (Fig. 2.1). There was a significant difference in the probability of survival between the control and acidified freshwater environments (Mantel-Cox log rank test, X^2_1=9.41, p=0.0022, Fig. 2.1). Crabs in the acidified freshwater tanks had a 50% mortality compared to 15 % mortality in control freshwater tanks. Calculation of the Mantel-Haenszel hazard ratio indicates that crabs in acidified freshwater have a 3.68 times greater probability of mortality than the crabs held under control conditions.

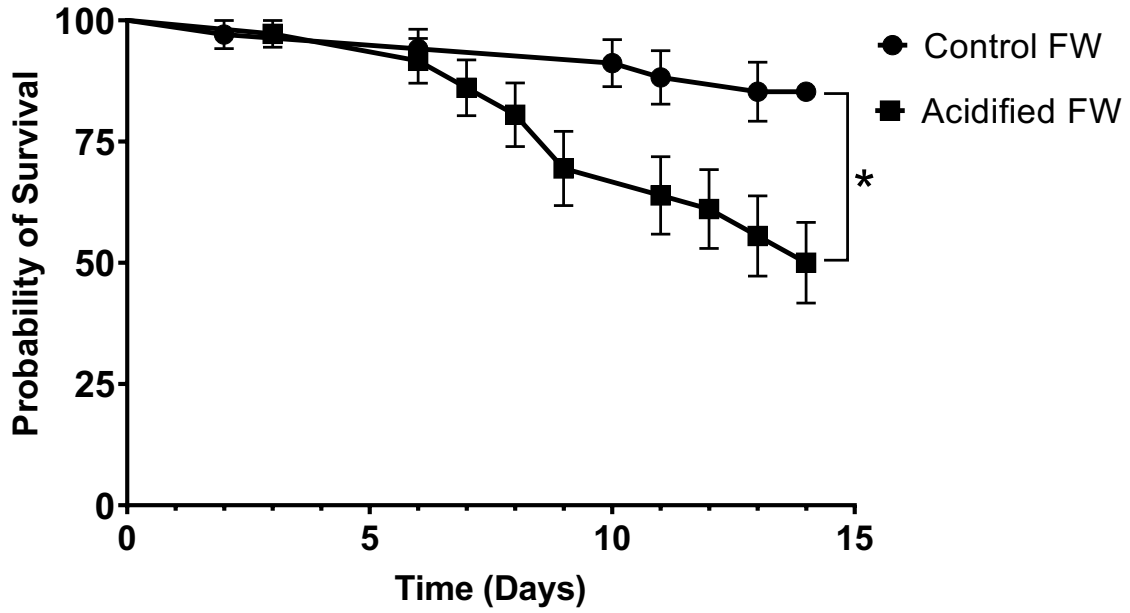

Figure 2.1 Survivorship curves of juvenile Chinese mitten crab, *Eriocheir sinensis*, over 14 days of exposure to control (pH 7.4, 1364 µatm pCO_2) or CO_2-acidified (pH 6.8, 4633 µatm pCO_2) freshwater. Data are presented as probability of survival +/- SEM. (N=34 for control freshwater and N=36 for acidified freshwater). Statistical significance was assessed by Mantel-Cox test * indicating significant difference between probability of survival between control and freshwater acidified crab populations.

Acid-base status

Chinese mitten crab maintained in control freshwater showed no changes in hemolymph pH, bicarbonate, pCO_2, or ammonia throughout the experimental time course (Fig. 2.2; Table 2.2). In contrast, acidified freshwater had a significant effect on hemolymph pH, bicarbonate, pCO_2, and ammonia (Fig. 2.2; Table 2.2). Exposure to acidified freshwater induced a respiratory acidosis indicated by a decline in hemolymph pH (pH 8.11 ± 0.015 to 8.03 ± 0.0019) and an increase in hemolymph pCO_2 (404 ± 23 Pa to 486 ± 26 Pa; 1 µatm = 0.101325 Pa) within the first six hours of exposure (Fig. 2.2a, c). Hemolymph acidosis was maintained for two days. Recovery of hemolymph pH occurred by day seven, although hemolymph pCO_2 remained elevated (499 ± 20 Pa). Recovery of hemolymph pH coincided with increases in hemolymph HCO_3^- (16.7 ± 0.78 mmol L^{-1}) and ammonia (136 ± 2.9 µmol L^{-1}; Fig. 2.2b, d). No significant changes in hemolymph HCO_3^- and ammonia were observed until seven and two days of exposure, respectively, suggesting a delayed extracellular pH regulatory response.

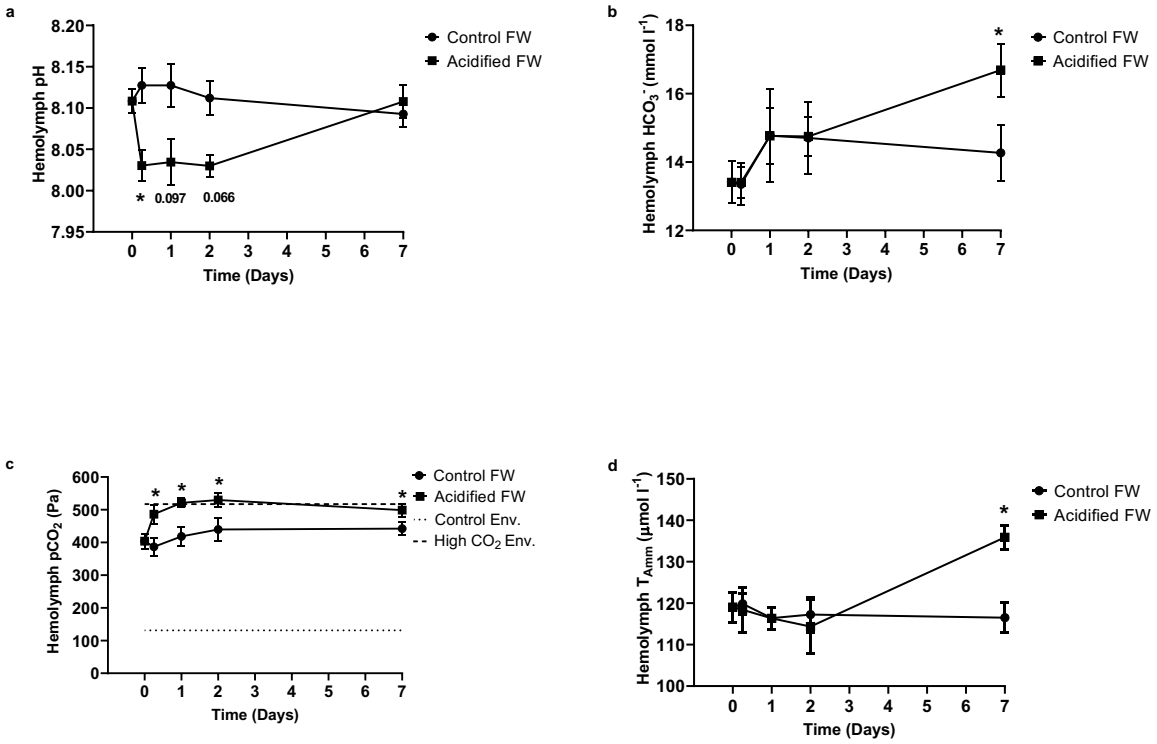

Figure 2.2 Changes in extracellular (a) pH, (b) HCO_3^-, (c) pCO_2, and (d) ammonia of juvenile Chinese mitten crab, *Eriocheir sinensis*, during a 7-day time course of exposure to control (pH 7.41, 1299 µatm pCO_2) or CO_2-acidified (pH 6.73, 5109 µatm pCO_2) freshwater. Data are presented as mean +/- SEM. (N=6-14, 2-3 crabs pooled per N value). Statistical significance was assessed by two-way ANOVA followed by a post-hoc Dunnett's test with * indicating significant difference from day zero measurements. P-values near but not <0.05 are written above corresponding data point.

Metabolism

Metabolic changes were quantified through ammonia excretion rate and oxygen consumption rate. Ammonia excretion rate was used as an indicator of potential shifts in protein catabolism. Oxygen consumption rate was used as an indicator of changes in aerobic metabolism. Control crabs exhibited steady oxygen consumption rates and ammonia excretion rates throughout the measured time course (Fig. 2.3; Table 2.2). Crabs exposed to freshwater acidification experienced a significant reduction in oxygen consumption rate within six hours that was maintained throughout the rest of the time course (Fig. 2.3a; Table 2.2). Ammonia excretion rates were also significantly affected by freshwater acidification (Fig. 2.3b; Table 2.2). Initially, ammonia excretion rates were unchanged until the second day of exposure (Fig. 2.3b). On the

second day of exposure, ammonia excretion rates doubled and remained elevated for the duration of the seven-day time course (Fig. 2.3b).

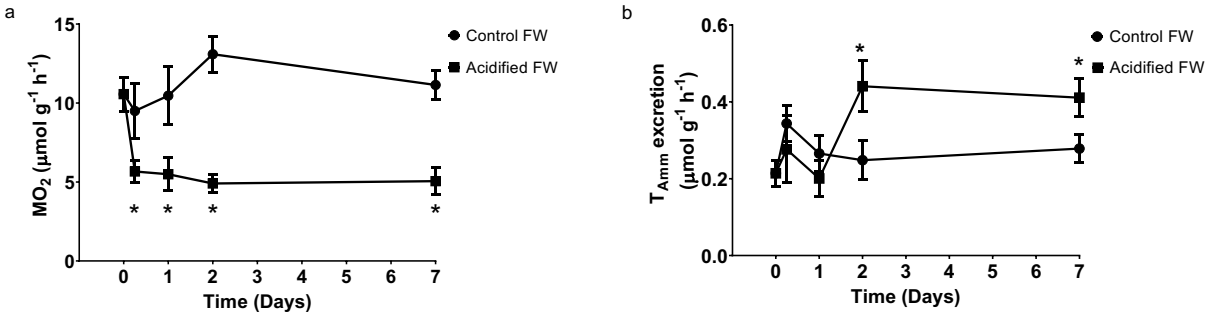

Figure 2.3 Changes in whole animal (a) oxygen consumption rate (MO_2) and (b) ammonia excretion rate of juvenile Chinese mitten crab, *Eriocheir sinensis*, during a 7-day time course of exposure to control (pH 7.41, 1299 µatm pCO_2) or CO_2-acidified (pH 6.73, 5109 µatm pCO_2) freshwater. Data are presented as mean +/- SEM. (N=5-6 for oxygen consumption and N=7-12 for ammonia excretion). Statistical significance was assessed by two-way ANOVA followed by a post-hoc Dunnett's test. Significant differences from day zero measurements are indicated by *. P-values near but not <0.05 are written above corresponding data point.

Carapace calcification

Changes in calcification were quantified as the change in the crab's exoskeletal calcium content following exposure to freshwater acidification conditions. Calcification was measured several times over a six-week acclimation, as several studies on marine crustaceans report changes in calcification after 20+ days of acclimation (Long et al., 2013; Ries et al., 2009; Taylor et al., 2015). Overall, there was a significant time, pCO_2 and interactive time and pCO_2 effect on calcification (Table 2.2). Post hoc analysis suggests there were no significant changes in carapace calcification in the first two weeks of exposure to freshwater acidification (Fig. 2.4). However, after three and six weeks of exposure, a significant decline in carapace calcium content to 84.1 ± 2.9 % and 85.2 ± 3.3 % of control crab levels was observed (Fig. 2.4).

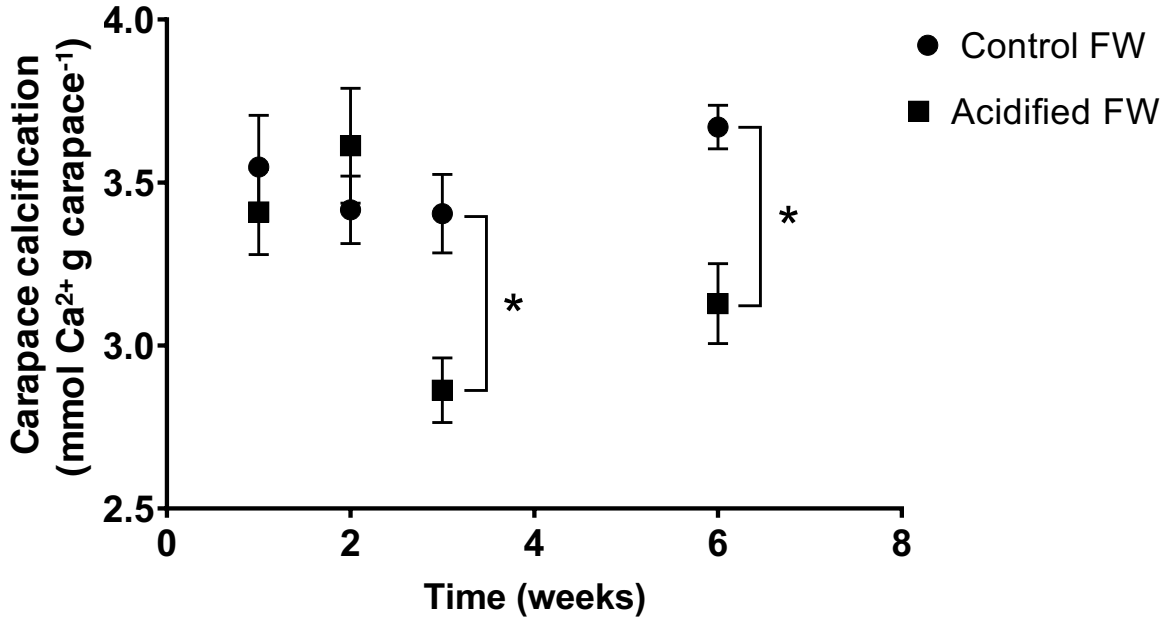

Figure 2.4 Changes in carapace calcium content of juvenile Chinese mitten crab, *Eriocheir sinensis*, over a 6-week exposure to control (pH 7.4, 1389 µatm pCO_2) or CO_2-acidified (pH 6.8, 4634 µatm pCO_2) freshwater. Data are presented as mean +/- SEM. (N=6-12). Statistical significance was assessed by two-way ANOVA followed by a post-hoc Tukey HSD test with * indicating significant difference between control and acidified FW crabs for each respective week.

Locomotory Behaviour Assay

An open field test was used to quantify locomotory behavioural changes over a five-minute recording period in a novel arena (Table 2.3). Crabs exposed to acidified freshwater covered less distance than crabs in control freshwater (student's t-test, t_{35}=-2.5, p=0.017, Table 2.3). Crabs in acidified freshwater also had a lower velocity than crabs in control freshwater after the seven-day exposure (student's t-test, t_{35}=-2.37, p=0.024, Table 2.3). Movement and mobility were also quantified. Movement was defined as the crab changing its relative location in the arena. Mobility was defined as the movement of body appendages even if the crab's location did not change. There was a significant decrease in movement (student's t-test, t_{35}=-2.55, p=0.015, Table 2.3) and mobility (Wilcoxon test, Z=2.08, p=0.037, Table 2.3) following the seven-day exposure to acidified freshwater.

Table 2.3 Changes in locomotory behaviour of juvenile Chinese mitten crab, *Eriocheir sinensis*, after a seven-day exposure to control (pH 7.41, 1299 µatm pCO_2) or CO_2-acidified (pH 6.73, 5109 µatm pCO_2) freshwater. Data are presented as mean +/- SEM. (N=18-19). Statistical significance was assessed by student's t-test or Wilcoxon test for mobility time with * indicating significant difference between control and acidified FW treatments.

	Distance moved (cm)	Velocity (cm s^{-1})	Movement time (s)	Mobility time (s)
Control FW	761 ± 46	2.53 ± 0.15	148 ± 7	215 ± 5
Acidified FW	601 ± 45*	2.04 ± 0.15*	119 ± 9*	179 ± 14*

Discussion

Anthropogenically driven aquatic acidification has the potential to negatively impact both freshwater and marine life. Meta-analyses of biological responses to ocean acidification suggest that marine crustaceans generally experience minimal consequences to pCO_2 tensions (~1000 µatm) predicted to occur by the year 2100 (Kroeker et al., 2013; Melzner et al., 2009; Wittmann and Pörtner, 2013). Acidification to levels expected for the year 2300 (~2000 µatm) negatively impacts about half of the studied marine crustaceans (Wittmann and Pörtner, 2013). In contrast, the biological responses of any freshwater invertebrate to realistic future CO_2 mediated freshwater acidification remain unknown. In the present study, we aimed to demonstrate the physiological and behavioural consequences of a future CO_2 mediated freshwater acidification scenario on a juvenile freshwater crustacean, the Chinese mitten crab, *Eriocheir sinensis*. Our results suggest that freshwater juvenile Chinese mitten crab experience significant impairment of metabolism, calcification, locomotory behaviour and survival when exposed to freshwater acidification (4633-5109 µatm pCO_2). The high energetic demands to sustain essential physiological processes such as acid-base regulation may cause energetic reallocation that impairs several physiological processes and alters animal fitness.

Plausibility of Freshwater Acidification Conditions

Modelling of future CO_2 mediated freshwater acidification for the year 2100 is nearly non-existent, making the plausibility of the pCO_2 levels used in this study difficult to assess. The control pCO_2 levels used in this study reflect the average pCO_2 measured in 13 stations along the mainstem of the Yangtze River system (excluding Nanjing station, which is at the mouth of the river and

influenced by coastal upwelling) (Ran et al., 2017). The future freshwater acidification conditions used in this study represents a 3500+ µatm increase in pCO_2 from control levels. This increase is roughly 1000+ µatm higher than the highest average level recorded by the 13 stations along the mainstem of the Yangtze river (Ran et al., 2017). While future CO_2 mediated acidification models are not available for the Yangtze River, the relationship between changes in freshwater pCO_2 in other freshwater systems as a response to changes in atmospheric pCO_2 may provide indications of plausible future increases in pCO_2. Weiss et al. (2018) tracked changes in pCO_2 of four freshwater bodies in Germany between 1981-2015 and reported that freshwater pCO_2 increased by an average of 561 µatm over this time period while atmospheric pCO_2 increased by ~60 µatm from 340 to 399 µatm (National Oceanic and Atmospheric Administration; www.esrl.noaa.gov/gmd/dv/iadv). This relationship suggests that for every 1 µatm increase in atmospheric pCO_2, these freshwater bodies increased by 9.35 µatm. Since atmospheric pCO_2 is projected to rise to approximately 985 µatm by the year 2100 (IPCC, 2013) this would mean that freshwater pCO_2 in these systems could rise by as much as 5469 µatm. Assuming this relationship is accurate, the pCO_2 levels used in this study would be within a range that could feasibly occur in the Chinese mitten crab's native environment by the year 2100. Further, it should be noted that while freshwater systems average pCO_2 levels of 3100 µatm (streams and rivers) and 1410 µatm (lakes), the pCO_2 levels used for acidified freshwater in this study are within ranges that can already be seen in freshwater systems globally (Raymond et al., 2013). For example, the Mackenzie, Mississippi, Ohio and Elbe rivers, suggesting that acidification scenario used in this study is conceivable for freshwater (Cole and Caraco, 2001; Raymond et al., 2013).

Probability of Survival

Sensitivity to aquatic acidification is quite variable in marine crustaceans. In mid to high intertidal and burrowing species including porcelain crabs (*Petrolisthes cinctipes*, *Petrolisthes manimaculus*, and *Porcellana platycheles*), burrowing shrimp (*Upogebia deltaura*), and barnacles (*Semibalanus balanoides* and *Elminius modestus*), minimal changes in survival probability are reported at pCO_2 tensions ranging from 1395 to 2707 µatm (Donohue et al., 2012; Findlay et al., 2010; Page et al., 2017). Presumably, the variability in CO_2 levels experienced in burrows and intertidal zones has driven the evolution of adaptation for greater CO_2 tolerance in these groups of crustaceans. We predicted that juvenile Chinese mitten crab would also have an elevated CO_2

tolerance and face minimal changes in survival probability because the Yangtze river normally fluctuates by as much as 3000 µatm (Ran et al., 2017). Despite being a freshwater organism with strong ionoregulatory capabilities, our results show a sharp decrease in survival rate of Chinese mitten crabs over 14 days of exposure to 4633 µatm pCO_2 (Fig. 2.1). Such rapid decreases in survival have also been observed in non-burrowing crustaceans or crustaceans that do not inhabit high intertidal regions including brine shrimp (*Artemia sinica*), red king crab (*Paralithodes camtschaticus*), and low intertidal long-clawed porcelain crab, (*Pisidia longicornis*), exposed to 1500, 1637, and 5821 µatm pCO_2, respectively (Long et al., 2013; Page et al., 2017; Zheng et al., 2015). It might be tempting to conclude that low survival in Chinese mitten crabs compared to tolerant mid to high intertidal and burrowing marine crustaceans is simply due to the greater pCO_2 tensions used in the present study (4633 µatm). However, in the intertidal broad-clawed porcelain crab (*Porcellana platycheles*) pCO_2 levels of 5821 µatm have been shown to not affect the probability of survival after 24 days of exposure (Page et al., 2017). It should also be mentioned that for all mortalities in this experiment there were no obvious signs of disease and intact bodies of deceased crabs were collected, suggesting that the elevated CO_2 treatment and not disease or cannibalism was the reason for increased mortality. Therefore, the low survival rates in the present study suggest a high susceptibility to acidification and contradict our hypothesis that inhabiting a highly fluctuating CO_2 environment would confer tolerance to future freshwater acidification.

Physiological Responses

Juvenile Chinese mitten crab effectively recovered extracellular pH following respiratory acidosis resulting from freshwater acidification by the accumulation of extracellular HCO_3^- as a buffer (Fig. 2.2). Compensation of acid-base homeostasis under freshwater acidification was not surprising given that strong acid-base regulatory capabilities are typically seen in highly active organisms such as fish, cephalopods and crustaceans (Melzner et al., 2009). Similar recovery of extracellular pH to elevated environmental CO_2 has also been observed in Dungeness crab (*Metacarcinus magiste*r) and velvet crab (*Necora pub*er) exposed to even higher pCO_2 tensions (10000+ µatm; Pane and Barry, 2007; Spicer et al., 2007). In contrast, green crab (*Carcinus maenas*) and blue crab (*Callinectes sapidus*) have been shown to not fully compensate extracellular pH at 10000+ µatm CO_2 levels (Cameron, 1978; Fehsenfeld and Weihrauch, 2016a). However, measurements in these species were only done over 48 hours and more time may have been

required for the animals to recover, as seen in our study, where recovery was only observed after seven days. The compensatory responses to acidosis in crustaceans generally includes respiratory CO_2 excretion, H^+ excretion typically through Na^+/H^+ or NH_4^+ exchange and accumulation of extracellular HCO_3^- as a buffer, where HCO_3^- is derived through either branchial Cl^-/HCO_3^- exchange and, to a lesser degree, from calcified structures (e.g. exoskeleton) (Wheatly and Henry, 1992). In freshwater crustaceans, acid-base regulation occurs mainly within the gills (Henry et al., 2012), where the Na^+/K^+-ATPase and H^+-ATPase generate the electrochemical gradients that drive ion exchange (Leone et al., 2017). The Na^+/K^+-ATPase alone may already account for over 20 % of an animal's energetic budget (Milligan and McBride, 1985). The increase in ion transport that must occur to re-establish and maintain acid-base homeostasis in the face of freshwater acidification could pose an increased energetic demand. In fact, in sea urchin larvae pCO_2 tensions of 800 µatm have been shown to double ion transport ATP demands (Pan et al., 2015). It is therefore conceivable that the energetic cost for long-term maintenance of acid-base homeostasis under freshwater acidification may come at substantial energetic cost, which could have negative implications on other physiological parameters and thereby animal fitness.

Heightened energetic demands to maintain crucial physiological processes during exposure to environmental CO_2 acidification can occur through reallocation of energy budgets or through modification of metabolism to increase energy supplies. In fact, in the marine brittle star, *Amphiura filiformis*, exposure to CO_2 tensions ranging from 1000 to 8000 µatm for 40 days caused an increase in metabolic rate (increased energy budget)(Wood et al., 2008). This metabolic change was postulated to fuel increased calcification observed in this species (Wood et al., 2008). In contrast, the metabolic rate of juvenile European lobster (*Homarus gammarus*) remained unchanged when exposed to 1100 and 8000 µatm CO_2 (Small et al., 2020). However, in *H. gammarus,* branchial Na^+/K^+ ATPase activity was increased, demonstrating a reallocation of energy supplies despite maintaining an unchanged energy budget (Small et al., 2020). Unlike juvenile European lobster and brittle star, juvenile Chinese mitten crabs experienced a decrease in oxygen consumption (potentially decreased energy budget). Despite reductions in oxygen consumption, crabs could still re-establish extracellular pH through HCO_3^- accumulation, suggesting a potential reallocation of energy supplies to essential ionoregulatory processes.

Typically, a reduction in oxygen consumption, as seen in the present study, is observed when an organism cannot compensate for a reduction in extracellular pH (Pörtner et al., 2004). While in juvenile Chinese mitten crabs, this could be the case at the initial two days of the time course, by day seven extracellular pH was fully compensated yet, oxygen consumption rates were reduced. It is known that high environmental pCO_2 levels can trigger an accumulation of compounds such as adenosine that can lead to reduced oxygen consumption as observed in the peanut worm, *Sipunculus nudus* (Reipschläger et al., 1997). A similar mechanism could conceivably be in place that led to reduced oxygen consumption in the Chinese mitten crab as a strategy to conserve energy supplies to promote survival upon exposure to short-term stressors like high environmental pCO_2 levels. Such an adaptation may be present in Chinese mitten crab as these crabs would regularly experience short-term fluctuations in environmental CO_2 of their natural habitat. In fact, in the Mediterranean mussel (*Mytilus galloprovincialis*) chronically reduced oxygen consumption rates lasting up to 90 days have been observed to allow survival following exposure to ocean acidification (5026 µatm pCO_2, Michaelidis et al., 2005). Reducing oxygen consumption is a viable strategy used by many organisms to survive short-term periods of environmental stress (Guppy and Withers, 1999). However, it is a less viable long-term strategy as reduction in metabolic rate reduces energy availability for costly physiological processes such as calcification and protein synthesis, which would ultimately affect growth and reproductive success as reported in freshwater pink salmon (*Oncorhynchus gorbuscha*) and marine amphipod (*Gammarus locusta*) (Borges et al., 2018; Ou et al., 2015).

Besides reduced oxygen consumption, freshwater acidification led to an increase in extracellular concentrations and excretion of ammonia, a metabolic product of protein catabolism. Elevated excretion of ammonia may function as an excretable acid equivalent to assist the maintenance of pH homeostasis, a mechanism suggested for the brackish water green crab (*Carcinus maenas*) and hydrothermal vent crab (*Xenograpsus testudinatus*) (Allen et al., 2020; Fehsenfeld and Weihrauch, 2013). Furthermore, the previously mentioned reduction in oxygen consumption and increased ammonia excretion (decrease in O:N ratio) indicates that juvenile Chinese mitten crabs have a greater reliance on protein catabolism as an energy source under elevated environmental CO_2. Similar decreases in oxygen consumption and increases in ammonia excretion have been observed in the Mediterranean mussel (*M. galloprovincialis*, 5026 µatm pCO_2,

15-90 days) and brittle star (*A. filiformis*, 6643 µatm pCO_2, 28 days), where catabolism of amino acid may provide metabolic bicarbonate to further help sustain pH homeostasis (Hu et al., 2014a; Michaelidis et al., 2005). While potentially beneficial for sustaining acid-base status, elevated protein catabolism requires a consistent source of protein through either a high protein diet or increased food consumption, which if not met could cause muscle wastage an effect seen in brittle star during heightened energetic demands of ocean acidification (Wood et al., 2008). Interestingly, feeding rate has been shown in juvenile European lobster (*H. gammarus*) and green crab (*C. maenas*) to decline as a result of elevated environmental CO_2 making a greater reliance on protein catabolism during energetically constricted times a potentially precarious situation for juvenile Chinese mitten crab (Appelhans et al., 2012; Small et al., 2020).

Carapace calcification is an energetically costly process related to growth and predation defence in crustaceans that freshwater acidification and the associated metabolic changes could impair. Decapod crustaceans are believed to be the least susceptible of calcifying organisms to aquatic acidification as their exoskeletal $CaCO_3$ exists in the more stable calcite form providing greater resilience to dissolution in contrast to bivalves and corals (Ries et al., 2009). Indeed, the marine crustacean carapace is well protected from aquatic acidification mediated dissolution with reports of either no change or an increase in calcification being typically observed (Kroeker et al., 2013; Ries et al., 2009; Whiteley, 2011). However, in the present study, juvenile Chinese mitten crab had reduced levels of carapace calcification as reflected by a lower carapace calcium content after three and six weeks of exposure (Fig. 2.4). While not as common, examples of reductions in carapace calcification have been observed in marine crustaceans, including several porcelain crabs and the tanner crab, *Chionoecetes bairdi* (Long et al., 2013; Page et al., 2017). In crustaceans, carapace dissolution may occur to support extracellular pH buffering that normally occurs through branchial HCO_3^- uptake by providing an alternative source of HCO_3^-(Cameron, 1985; Defur et al., 1980). In the present study, extracellular pH was recovered long before carapace dissolution was apparent, therefore it is less likely that the carapace is mobilized as a source of HCO_3^-. Instead, reductions in carapace calcium content most likely reflect an alteration in the rate of calcification or acid mediated dissolution of the carapace. As carapace formation and maintenance is an energetically expensive process requiring careful ion regulation by numerous organs, the aforementioned changes in whole animal energetics due to freshwater acidification could have

negative implications on animal fitness either by weakening the exoskeleton or impairing post-moult calcification, which can hamper growth and leave animals vulnerable to predation.

Behavioural Responses

Elevated freshwater pCO_2 altered locomotory behaviour in juvenile Chinese mitten crabs. Crabs in acidified freshwater covered less total distance during movement and did so at a lower velocity. No studies have previously examined changes in crustacean distance covered in the presence of elevated environmental CO_2. However, reduced speed of movement has also been reported in Shiba shrimp (*Metapenaeus joyneri*) exposed to CO_2 levels of 9079 µatm; however, unlike in Chinese mitten crab, this shrimp did not experience a reduction in oxygen consumption rate correlated with locomotory impairment (Dissanayake and Ishimatsu, 2011). While not measured in our study, in Shiba shrimp there was a reduction in aerobic scope, which would likely lead to reduced aerobic performance and reduced movement (Dissanayake and Ishimatsu, 2011). Similar alterations in aerobic scope could partially be behind the reductions in velocity seen in juvenile Chinese mitten crab, however, this is entirely speculative and there are many cases where elevated CO_2 does not alter aerobic scope (Lefevre, 2016). In addition to moving slower, Chinese mitten crab spent less time moving their entire body throughout the novel arena and less time moving only their appendages while staying at a fixed location. Reduced movement time and appendage movement were also seen in the hermit crab (*Pagurus bernhardus*) exposed to 12000 µatm CO_2 (de la Haye et al., 2011). In contrast, the isopod (*Paradella dianae*) experienced no change in swim time or crawling time when exposed to 2085 µatm pCO_2 despite a measured metabolic depression (Alenius and Munguia, 2012). Differences in the effect of CO_2 on movement time may result from the CO_2 levels employed, but further studies on a greater variety of species are required to determine potential patterns for crustaceans. It is plausible that overall locomotory behaviour is reduced in this study due to alterations in neurological function resulting from ionic imbalances or other CO_2-mediated effects that may occur from elevated environmental CO_2 (For review of neural effects of aquatic acidification see Tresguerres and Hamilton, 2017). With a potential reduction in overall energy availability, crabs may reduce energy expenditure through locomotion to conserve energy stores for physiological processes more crucial to surviving the physiological distress caused by freshwater acidification. The overall reductions in locomotion observed in juvenile Chinese mitten crab could have negative consequences for their survival, as

reduced movement would make these crabs more vulnerable to predation, reduce migratory capabilities and reduce foraging ability.

Conclusion

In conclusion, we found impairment of survival, metabolism, calcification, and locomotion with exposure to a potential future CO_2 mediated freshwater acidification scenario. Energy availability was reduced despite heightened ionoregulatory energetic demands. Changes in the animals' energy budgets likely result in a greater dependency on protein catabolism as an energy source to allow for extracellular pH recovery at the cost of reducing their exoskeletal calcification and locomotion. We found that despite successful acid-base compensation, survival rates declined with 3.8 times greater probability of mortality under acidified freshwater conditions. While our study suggests negative impacts of freshwater acidification, these results should be assessed with caution as the assumed acidification levels are based on a relationship between changes in atmospheric CO_2 and freshwater CO_2, which remains to be more effectively modelled. Nevertheless, this study shows that despite inhabiting an environment that experiences regular fluctuations in pCO_2 the Chinese mitten crab may be at risk to future freshwater acidification. This emphasizes the importance of modelling acidification in freshwater systems to accurately assess biological consequences of global change. Based on our findings that a physiologically robust species displays sensitivity to future freshwater acidification, further research investigating the effect of freshwater acidification on a wide range of freshwater species from all phyla are required to better identify the effects of anthropogenic CO_2 accumulation on freshwater ecosystems.

TRANSITION TO CHAPTER 3

In the previous chapter, I found that acute CO_2 mediated freshwater acidification impacted survival, calcification, oxygen consumption, protein catabolism, and locomotory behaviour. It was apparent that elevated CO_2 creates an energetic trade-off, which could impact animal fitness. However, chapter focused mostly on acute responses to elevated CO_2 and does not provide insight into the long-term consequences of elevated CO_2. The aquaculture industry has for a long-time farmed crustaceans at high stocking densities with CO_2 levels that exceed that of global change relevant CO_2 mediated aquatic acidification. Therefore, aquaculture systems may provide fantastic models to understand long-term effects of elevated CO_2 on crustaceans. In chapter 3, we partnered with Förde Garnelen GmbH shrimp aquaculture facility in Northern Germany to determine levels of CO_2 in this facility and gain access to animals that have chronically been raised in high CO_2. By mimicking this high CO_2 system in the lab, I investigate the effects varying CO_2 tensions on growth, survival, acid-base regulation, and nitrogen balance in the most widely aquacultured crustacean, the Whiteleg shrimp *Litopenaeus vannamei*.

CHAPTER 3: EFFECTS OF ELEVATED CO_2 IN RECIRCULATING AQUACULTURE SYSTEMS ON GROWTH, SURVIVAL, ACID-BASE, AND NITROGEN PHYSIOLOGY OF THE WHITELEG SHRIMP, *LITOPENAEUS VANNAMEI*

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In preparation for submission to the Journal Aquaculture research

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ARQR contributed to study design, performed experiments, analyzed data, and wrote the manuscript. ES performed experiments. CH contributed to study design, performed experiments, and assisted with manuscript writing. KT performed experiments. FM contributed to study design, revised the manuscript, acquired funding, and analytical tools. DW contributed to study design, revised the manuscript, acquired funding, and analytical tools.

Abstract

Accumulation of CO_2 in recirculating shrimp aquaculture systems may negatively impact animal physiology and impair growth and survival. In the present study, we measured the carbonate chemistry of a recirculating shrimp aquaculture system and subsequently exposed juvenile and adult Whiteleg shrimp, *Litopenaeus vannamei*, to either CO_2 levels found in this aquaculture facility or CO_2 levels normally found in the Baltic Sea, where this facility is located, to investigate the impact of CO_2 on growth, survival, acid-base and nitrogen physiology of farmed shrimp. In contrast to previous studies showing reduced growth and survival in *L. vannamei* reared at 34 ppt salinity, our results show that when reared at 17ppt *L. vannamei* experienced no effect of CO_2 on growth and survival. Furthermore, we described the acid-base regulatory capabilities of adult *L. vannamei* for the first time. We showed that after chronic high CO_2 exposure adult shrimp were

unable to maintain acid-base homeostasis at normal levels reported for this species. We also found that nitrogen homeostasis was not impacted by elevated CO_2 indicating unchanged protein catabolism. Results suggest that CO_2 tolerance in this shrimp species may depend on environmental salinity and that the ability to maintain acid-base homeostasis may decline with chronic exposure to elevated CO_2. This indicates that elevated CO_2 in aquaculture conditions may compromise aquaculture productivity depending on interaction with other environmental factors within the aquaculture system and potentially the time of CO_2 exposure.

Introduction

Traditional shrimp pond aquaculture has a large environmental impact on coastal ecosystems. The establishment and operation of shrimp ponds destroy important coastal habitats (e.g. salt marshes and mangroves) and release waste effluents that may contain large pathogen loads, pharmaceuticals, and particulate organic matter that alter the water quality of coastal regions (Barraza-Guardado et al., 2013; Páez-Osuna, 2001). Recirculating aquaculture systems , on the other hand, can provide a more environmentally friendly alternative to pond aquaculture systems (Martins et al., 2010). However, the high stocking densities needed to profitably use recirculating aquaculture systems may lead to the build-up of metabolic wastes such as CO_2 in the culture water, which can impact the physiology of the cultured animals and thereby the overall productivity of the aquaculture system.

Biological responses to chronically elevated aquatic CO_2 have been studied extensively in the context of ocean acidification. Calcifying species such as corals, bivalves, echinoderms and crustaceans are believed to be particularly susceptible to aquatic acidification as dissolution of CO_2 reduces carbonate availability and pH, potentially increasing the dissolution of $CaCO_3$ exoskeletons (Feely et al., 2004; Roleda et al., 2012). Numerous consequences of CO_2 mediated acidification have been reported for marine organisms including reduced growth and survival, disruption of acid-base homeostasis, increased pathogen susceptibility, behavioural impairment, and altered metabolism (Hernroth et al., 2012; Kroeker et al., 2013; Melzner et al., 2009; Meseck et al., 2016; Tresguerres and Hamilton, 2017; Whiteley, 2011). Decapod crustaceans are generally more tolerant to acidification due to the evolution of strong acid-base regulatory capabilities to deal with elevated CO_2 loads resulting from their high activity level compared to sessile or immobile invertebrate species (Melzner et al., 2009). However, decapod crustaceans exposed to

ocean acidification (1000+ µatm) have been shown to experience detrimental effects (Bednaršek et al., 2021). Shrimp aquaculture facilities may operate at CO_2 levels above 4000µatm (Zhang et al., 2019), which is far greater acidification stress than ever expected during ocean acidification. At these aquaculture-relevant CO_2 levels, meta-analyses indicate that more than 80% of marine crustaceans face negative effects (Wittmann and Pörtner, 2013), suggesting a potential for impairment of animal performance and productivity in aquaculture settings.

The Whiteleg shrimp (*Litopenaeus vannamei*) is the most important cultured crustacean, accounting for as much as 53% (4.1 million tonnes) of crustaceans farmed worldwide (FAO, 2018). Native to the Eastern Pacific Ocean from Mexico to Peru, adult *L. vannamei* breed in the open ocean and post larvae migrate to coastal estuaries where they stay until adulthood (FAO, 2006). The migration to coastal estuaries exposes juvenile *L. vannamei* to a wide range of environmental salinities as well as fluctuating O_2 and CO_2 levels that naturally occur in these coastal habitats (Burnett, 1997). Organisms inhabiting highly variable environments such as coastal estuaries are typically well adapted to tolerate variation in environmental parameters (Magozzi and Calosi, 2015). However, even organisms adapted to variable environments may be susceptible to chronic environmental stress, as would be present in an aquaculture setting due to the high metabolic demands causing energetic trade-offs, which in turn result in reduced animal fitness (Magozzi and Calosi, 2015). The physiological plasticity of organisms inhabiting variable environments and the potential for energetic trade-offs from chronic stress led to our hypotheses that (1) *L. vannamei* would maintain acid-base homeostasis but increase protein catabolism under recirculating aquaculture systems CO_2 levels and (2) the chronic metabolic cost of maintaining key physiological processes would lead to an energetic trade-off impairing growth and survival under elevated environmental CO_2. In the present study, we obtained *L. vannamei* from the Förde Garnelen GmbH (Strande, Germany) recirculating aquaculture shrimp farm. We used measurements of the carbonate chemistry in the farm to test our hypotheses by (1) measuring changes in acid-base and nitrogen homeostasis of adult shrimp held under normal Baltic seawater and Förde Garnelen GmbH recirculating aquaculture shrimp farm CO_2 tensions and (2) measuring growth and survival of juvenile shrimp reared over 63 days under low (normal Baltic seawater), medium and high (Förde Garanelen GmbH farm levels) CO_2.

Materials and methods

Shrimp farm water parameters

Before our experiments, we collected culture water parameters of the 'Förde Garnelen' recirculating aquaculture systems to access the system's carbonate chemistry. We measured temperature and salinity as well as pCO_2 (using a CONTROS HydroC® sensor, 4H-Jena engineering, Kiel, Germany) at the shrimp culture tank outflow and took water samples to measure total alkalinity (A_T). Water samples for A_T were fixed with HgCl (Dickson et al., 2003). A_T was measured through potentiometric open-cell titration with hydrochloric acid using a VINDTA autoanalyzer (Dickson et al., 2007; Mintrop et al., 2000). Water carbonate chemistry parameters were calculated in CO2SYS (Lewis and Wallace, 1998), with K1 and K2 dissociation constants (Roy et al., 1993) and measured temperature, salinity, and A_T and pCO_2 (Table 3.1).

Table 3.1 Carbonate chemistry parameters in the 'Förde Garnelen' recirculating aquaculture systems measured before our experiments (mean ± SEM).

Parameter	Mean ± SEM
Salinity (ppt)	16.5±0.1
Temperature (ºC)	26±0.2
C_T (µmol kg^{-1})	2794±1
pH (NBS Scale)	7.2±0.01
A_T (µmol kg^{-1})	2623±3
pCO_2 (µatm)	6366±4
HCO_3^- (µmol kg^{-1})	2576±2.5
CO_3^{2-} (µmol kg^{-1})	21.26±0.3
Ω Calcite	0.6±0.01
Ω Aragonite	0.4±0.01

Experiment 1 - Juvenile shrimp growth and survival

Juvenile *L. vannamei* (450 shrimp, 280±4 mg), approximately two weeks post-larval stage, were obtained from the Förde Garnelen GmbH aquaculture facility (Strande, Germany) and transported to the GEOMAR Helmholtz Centre for Ocean Research aquatics facility (Kiel, Germany). Post-larval shrimp at the Förde Garnelen GmbH aquaculture facility were originally sourced from the Shrimp Improvement System LLC (Florida, USA). The animals were cultured in a flow-through system containing 30 aquaria (12 L) that were distributed equally across three

insulated 130 L water baths to maintain a constant temperature in the aquaria. Each aquarium was spray painted black on the outside to prevent the visualization of shrimp in adjacent tanks. The water baths were heated to 26 ± 0.5 ºC using three 300W heaters (AquaMedic, Germany), with the water being circulated by EHEIM pumps. Baltic Sea water flowing into the culture aquaria was pre-heated in a basin below the water baths, aerated with pressurized air, and passed through a UV water sterilizer (HW UV water sterilizer, model 500, HW Wiegandt, Krefeld, Germany) before being pumped into the culture aquaria. Water in-flow rates (85 mL min^{-1}) were checked weekly to determine that flow rates remained constant. Shrimp were maintained at 24 hours of light to replicate conditions of the aquaculture facility. Shrimp were fed *ad libitum* with a mixed diet of pellet feed and *Mytilus chilensis* meat obtained from the aquaculture facility. Pellet food (40% protein, 9% fat, 9% ash, 2.5% fibre, and 1.4% phosphorous) was fed in the mornings and minced mussel meat was fed in the evenings. An initial feed amount of 5% of the body weight was used but increased as the shrimp grew. Excess food and feces were removed every second day.

For the growth and survival experiments, all culture aquaria were initially aerated with air containing 5000 µatm pCO_2 to maintain shrimp arriving from the aquaculture facility at high CO_2 levels, similar to those animals have experienced at the aquaculture facility. Three days after arrival from the aquaculture facility, shrimp from each tank were individually netted, dried carefully with a paper towel, and weighed in a container with 100 mL of culture water that had been zeroed on a balance (Sartorius LC series 200 balance). One day after weighing, the 30 culture tanks (with 15 shrimp per 630 cm^2 tank) in the three water baths were randomly assigned to either low, medium or high CO_2 and directly aerated with air containing either 400, 2400, or 6000 (initially 5000) µatm pCO_2. Due to facility limitations, the high CO_2 treatment group could only be run at 5000 µatm pCO_2 in the beginning. However, to more closely mimick the aquaculture facility, the high CO_2 level was increased to 6000 µatm after 2 weeks. Shrimp were grown for a total of 63 days under the three treatment conditions at which point shrimp were weighed again (Sartorius U4600p balance) and the number of shrimp alive per tank was counted. During the experiment, temperature (Cond 315i, WTW), salinity (Cond315i, WTW), and pH (Cond 3110, WTW, Probe: SenTix81, WTW) were measured 2-3 times weekly. Ammonia levels were measured weekly with an ammonium quick test kit (JBL GmbH, Neuhofen, Germany) to determine that buildup was not occurring in the aquaria. Water samples for total inorganic carbon were fixed with HgCl (Dickson

et al., 2003) and measured with an AIRICA analyzer (Marianda GmbH) calibrated to certified reference materials (Dickson et al., 2003). Water carbonate chemistry parameters were calculated in CO2SYS (Lewis and Wallace, 1998) using $KHSO_4$ constants (Dickson, 1990), K1 and K2 dissociation constants (Roy et al., 1993) and measured temperature, pH (NBS scale), salinity, and total inorganic carbon. Water parameters for growth and survival experiments are displayed in Table 3.2.

Table 3.2 Environmental tank carbonate chemistry during the 63-day juvenile shrimp growth and survival experiments. Values are mean ± SEM.

Parameter	Low CO_2	Medium CO_2	High CO_2
Salinity (ppt)	16.5±0.04	16.5±0.04	16.5±0.05
Temperature (ºC)	26.5±0.05	26.6±0.05	26.6±0.05
C_T (µmol kg^{-1})	1995±5	2085±15	2162±6
pH (NBS Scale)	7.9±0.01	7.6±0.02	7.3±0.02
A_T (µmol kg^{-1})	2063±7	2025±15	2046±6
pCO_2 (µatm)	1070±42	2235±92	4788±179
HCO_3^- (µmol kg^{-1})	1884±6	1975±15	1998±5
CO_3^{2-} (µmol kg^{-1})	79±2.7	43±1.8	21±1.1
Ω Calcite	2.2±0.08	1.2±0.05	0.6±0.03
Ω Aragonite	1.4±0.04	0.7±0.03	0.4±0.02

Experiment 2 - Adult shrimp acid-base and nitrogen regulation

Adult Whiteleg shrimp (*L. vannamei*) weighing 23.57-33.40 g (150+ days in aquaculture) were also obtained from the Förde Garnelen GmbH aquaculture facility (Strande, Germany). Shrimp were maintained at the GEOMAR Helmholtz Centre for Ocean Research aquatics facility (Kiel, Germany) in 12 darkened 10 L tanks with flow-through (100 mL min^{-1}) Baltic Sea water. Baltic Sea water was pumped into a water basin, and heated to 27ºC with 300W heaters (AquaMedic, Germany) before flowing into the individual aquaria. Shrimp were maintained at a density of 2-3 shrimp per tank for the duration of the experiment and fed daily (approximately 5% of their body mass) with mussel meat. Feeding was withheld 48 hours before performing hemolymph sampling and ammonia excretion experiments to avoid any postprandial effects on acid-base and nitrogen physiology. Upon the initial arrival of shrimp from the aquaculture facility, all aquaria were aerated with air containing 6000 µatm CO_2 (mimicking aquaculture facility water pCO_2). Shrimp were maintained under these aquaculture conditions for two weeks at the GEOMAR before the start of

experiments. After this acclimation phase, 6 of the 12 tanks were switched from aeration with air containing 6000 µatm CO_2 to normal pressurized air (ca. 400 µatm pCO_2, low CO_2 group). Shrimp were maintained under either high or low CO_2 for two weeks before measurement of hemolymph parameters and ammonia excretion. Tank parameters were measured every other day during the acclimation process as described above for growth and survival experiments (Table 3.3).

Table 3.3 Environmental tank carbonate chemistry during the two-week adult shrimp acclimations. Values are mean ± SEM.

Parameter	Low CO_2	High CO_2
Salinity (ppt)	17±0.1	17±0.1
Temperature (ºC)	27±0.1	27±0.05
C_T (µmol kg^{-1})	2000±7	2187±5
pH (NBS Scale)	7.9±0.02	7.2±0.01
A_T (µmol kg^{-1})	2063±7	2046±6
pCO_2 (µatm)	1140±96	5496±153
HCO_3^- (µmol kg^{-1})	1890±7	2009±1
CO_3^{2-} (µmol kg^{-1})	75.9±3.1	16.5±0.4
Ω Calcite	2.1±0.1	0.5±0.01
Ω Aragonite	1.3±0.1	0.3±0.01

For hemolymph sampling, shrimp were taken directly from their tank and hemolymph (0.5 mL) was drawn from the pericardial cavity by puncturing the intersegment membrane between the cephalothorax and first abdominal segment with a sterile syringe. This entire sampling process was done within a minute to minimize the effects of air exposure on hemolymph acid-base status. Hemolymph was immediately centrifuged (10,000g, 3 minutes) to remove hemocytes and pH was subsequently measured at the experimental temperature with a micro pH electrode (InLab Micro Combination pH electrode, Mettler Toledo, Greisensee, Switzerland). Hemolymph total carbon was measured in duplicate (50 µl each) using the Corning 965 carbon dioxide analyzer (±0.2 mmol L^{-1} precision) calibrated with a standard curve generated using a $NaHCO_3$ dilution series of 40, 20, 10, 5, and 0 mmol L^{-1} in double distilled water with a minimum R^2 of 0.99. The remaining hemolymph was immediately frozen at -20 ºC until later analysis of ammonia. Hemolymph pCO_2 and HCO_3^- were calculated through rearrangement of the Henderson hasselbalch equation with constants pK1 and αCO_2 derived from Truchot (1976).

For determination of hemolymph ammonia, samples were deproteinized in 4 volumes of 6% perchloric acid on ice for 5 minutes and centrifuged to remove protein precipitate. Sample supernatant was subsequently neutralized with 2 mol L^{-1} KOH, centrifuged to remove $KClO_4$ precipitate and diluted 2 fold with MilliQ water before analysis by colorimetric salicylate-hypochlorite assay (Verdouw et al., 1977). Hemolymph deproteinization was necessary to avoid the inhibitory effects of proteins on this assay.

For ammonia excretion experiments, shrimp were placed in small tanks containing 2 L of Baltic Sea water aerated with either air containing 6000µatm CO_2 or normal pressurized air (ca. 400 µatm pCO_2). Shrimp were allowed to acclimate to these tanks for 30 minutes to minimize elevations in ammonia excretion observed in crustaceans directly after handling (Hans et al., 2014). After this acclimation time, a 10 mL water sample was taken for determination of initial ammonia concentration (zero hour time point) and shrimp were then allowed to excrete for one hour after which a second 10 mL water sample was taken (one-hour time point). Ammonia concentrations of the water sample at zero and one hour were determined by colorimetric salicylate-hypochlorite assay (Verdouw et al., 1977) measured on a microplate spectrophotometer (PowerWave HT, BioTek Model XS2). Ammonia excretion rates (E_{Amm}) were calculated according to the following equation:

$$E_{Amm} = \frac{(Amm_f - Amm_i) \times V}{t \times m}$$

Where Amm_f is the final ammonia concentration measured at the one-hour time point, Amm_i is the initial ammonia concentration measured at the zero hour time point, V is the volume in the tank during the one hour flux period in litres, t is the flux time in hours, and m is the mass of the fresh weight of the shrimp in grams.

Statistics

Statistical analyses were conducted using JMP Pro 16 (Cary, NC, USA). For all data, homogeneity of variance was tested by Levene's test and normal distribution of residuals by the Shapiro-Wilk test. If data failed the assumptions for parametric analysis a log transformation was initially used to determine if data could be analyzed through parametric methods. Log transformation was applied to hemolymph HCO_3^- and weight gain with runt excluded data. For growth and survival experiments, data meeting assumptions for parametric analysis were analyzed

by one-way ANOVA and data that failed assumptions for parametric analysis were analyzed by a Kruskal-Wallis test. Hemolymph parameters and ammonia excretion rates were analyzed by a student's t-test. For all data, N=1 is equivalent to one aquarium. For all data sets, p values ≤ 0.05 were considered significant. Data are presented as mean ± standard error (SEM).

Results

Experiment 1- Juvenile shrimp growth and survival

Environmental parameters during the juvenile shrimp growth and survival experiments are summarized in Table 3.2. The percentage of shrimp exhibiting stunted growth (< 3 grams, ~ > 2 standard deviations from treatment average) was not significantly affected by environmental pCO_2 levels (Kruskal-Wallis test; X^2_2=2.26, p=0.32, Table 3.3). As the inclusion of runt shrimp in the growth analysis may mask or amplify the effects of CO_2, we performed two statistical analyses on growth data, either including or excluding runt shrimp. Initial mean weights of juvenile shrimp in growth and survival trials were kept the same between the three treatment groups (Kruskal-Wallis test; X^2_2=5.25, p=0.07, Table 3.3). After 63-days of growth, average weight gain per shrimp (Kruskal-Wallis test; X^2_2=0.54, p=0.62) and specific growth rate (one-way ANOVA; $F_{2,27}$=0.72, p=0.5) showed no difference between the three CO_2 treatment groups when runt shrimp were included in the analysis (Table 3.4). The exclusion of runt shrimp from the analysis of average weight gain (one-way ANOVA; $F_{2,27}$=0.51, p=0.61) and specific growth rate (Kruskal-Wallis test; X^2_2=2.01, p=0.37) revealed that the lack of response in growth rate due to CO_2 was not due to the presence of runts in the analysis (Table 3.4). Total biomass production by individual aquaria after 63-days of growth was not significantly affected by environmental CO_2 levels (one-way ANOVA; $F_{2,27}$=1.26, p=0.3, Table 3.3).

Also, survival was not significantly different in juvenile shrimp exposed to the three CO_2 treatment levels after 63 days (one-way ANOVA; $F_{2,27}$=1.63, p=0.21, Table 3.4).

Table 3.4 Survival and growth measures of juvenile *L. vannamei* reared in low, medium and high CO_2 for 63 days. Statistical analysis was performed either using a one-way ANOVA or Kruskal-Wallis test and no statistical significance was identified.

Parameter	Runts	Low CO_2	Medium CO_2	High CO_2
Initial body weight (g)	Included	0.28±0.014	0.30±0.016	0.26±0.013
Final body weight (g)	Excluded	9.34±0.47	9.15±0.30	8.79±0.38
	Included	8.67±0.59	8.41±0.28	8.42±0.45
Weight gain (g)	Excluded	9.06±0.47	8.84±0.30	8.53±0.37
	Included	8.39±0.58	8.10±0.28	8.16±0.45
Specific growth rate (%/day)	Excluded	5.6±0.09	5.4±0.12	5.6±0.10
	Include	5.4±0.12	5.3±0.12	5.5±0.11
Biomass production (g)	Included	58±4.8	67±3.8	67±3.5
Survival (%)	Included	70±5.6	79±4.7	81±5.3
Shrimp Runts (%)	Included	13±3.6	10±3.2	5.9±2.7

Experiment 2 - Adult shrimp acid-base and nitrogen regulation

Environmental parameters for adult shrimp acclimations are summarized in Table 3.3. Shrimp transferred from high pCO_2 (5496 µatm) to low pCO_2 levels (1140 µatm, normal Baltic Sea water pCO_2 levels) for two weeks had a hemolymph pH of 7.71 ± 0.01, hemolymph [HCO_3^-] of 5.50 ± 0.13 mmol L^{-1}, and hemolymph pCO_2 of 376 ± 13 Pa (Fig. 3.1). In contrast, shrimp continuously maintained at high pCO_2 (5496 µatm) had acidic hemolymph compared to low CO_2 exposed shrimp as indicated by a reduced hemolymph pH (T_{12}=-3.68, p=0.0031) and increased hemolymph pCO_2 (T_{12}=13.8, p<0.0001). The observed acidosis was at least partially compensated for as suggested by a significant increase in hemolymph bicarbonate (T_{12}=23.14, p<0.0001) that was nearly double that recorded in shrimp maintained in normal seawater pCO_2 tensions.

Ammonia excretion rates and hemolymph ammonia levels can provide indications of changes in ammoniagenesis resulting from shifts in protein catabolism. Shrimp maintained under high pCO_2 levels showed no significant difference in ammonia excretion rates to shrimp under normal Baltic Sea water pCO_2 levels (T_{16}=0.53, p=0.6, Fig. 3.2). Although not significant, hemolymph ammonia in shrimp maintained at high pCO_2 levels was reduced by 14% (or 96 µmol L^{-1}) when compared to shrimp kept at low pCO_2 levels (T_{10}=-2.16, p=0.056, Fig. 3.2).

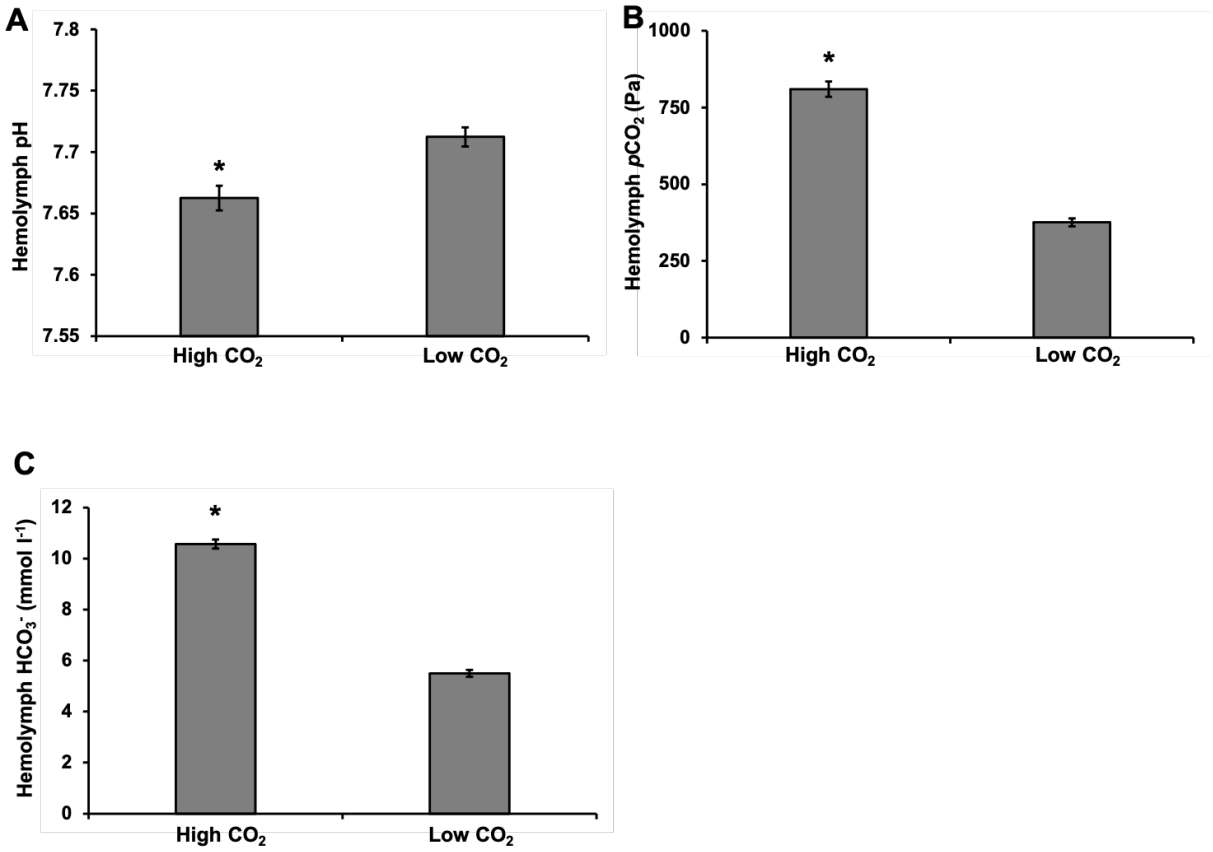

Figure 3.1 Changes in hemolymph (a) pH, (b) pCO_2, and (c) HCO_3^- of adult Whiteleg shrimp, *Litopenaeus vannamei*, after a two-week acclimation to low CO_2 (17 ppt, 27 ºC, pH 7.9, 1140 µatm pCO_2) or high CO_2 (17 ppt, 27º C, pH 7.2, 5496 µatm pCO_2). Data are presented as mean +/- SEM. (N=5-6). Statistical significance was assessed by a student's t-test and indicated by *.

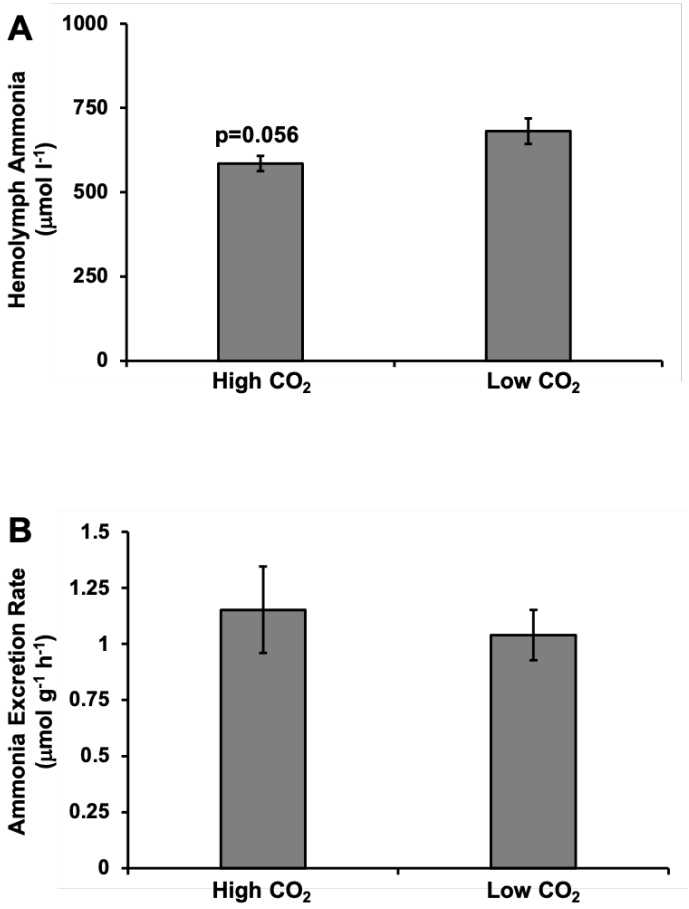

Figure 3.2 Changes in (a) hemolymph ammonia and (b) ammonia excretion rates of adult Whiteleg shrimp, *Litopenaeus vannamei*, after a two-week acclimation to low CO_2 (17ppt, 27ºC, pH 7.9, 1140µatm pCO_2) or high CO_2 (17 ppt, 27 ºC, pH 7.2, 5496 µatm pCO_2). Data are presented as mean +/- SEM. (N=5-6). Statistical significance was assessed by a student's t-test. P-values near but not <0.05 are written above the corresponding data point.

Discussion

Litopenaeus vannamei is the most widely farmed crustacean in the world, yet, it remains unclear how the accumulation of CO_2 in high-density recirculating aquaculture systems affects the aquaculture productivity of this important species. In the present study, we measured the carbonate chemistry of a recirculating aquaculture systems facility in Northern Germany from where we acquired juvenile and adult *L. vannamei* to test the effects of recirculating aquaculture systems CO_2 levels on growth, survival, acid-base and nitrogen regulation. We showed that CO_2 levels similar to those found in the recirculating aquaculture systems facility did not affect the growth or survival of *L. vannamei* in early post-larval stages indicating minimal impacts of CO_2 on aquaculture productivity during this period. We also, for the first time, investigated the acid-base

regulatory capabilities of this species and found that *L. vannamei* under chronically elevated recirculating aquaculture systems CO_2 levels were unable to fully compensate for the respiratory acidosis, which may lead to disruption of the animal's physiology the longer they remain under aquaculture conditions, which could have negative implications on productivity. We also found that protein metabolism remained unchanged in response to changes in environmental CO_2, which suggests that elevated CO_2 does not lead to increased ammonia waste production, nor does it require depletion of protein stores, which will not further impair aquaculture productivity.

Growth and survival in juvenile shrimp

Investigations into the effects of elevated environmental CO_2 on long-term growth rate in post-larval crustaceans have focused on decapod crustaceans where a decrease in growth rate has generally been reported (Kurihara et al., 2008; Long et al., 2013; Muralisankar et al., 2021; Wickins, 1984). In our study, we found growth rates (weight gain and specific growth rate) remained unchanged regardless of the applied CO_2 tension in *L. vannamei* (Table 4). However, another recent study also on *L. vannamei* found contradictory results to the present study, with a significant reduction in growth rate (weight gain and specific growth rate) between shrimp reared at 1104 μatm and shrimp reared at 2946 and 4742 μatm (Muralisankar et al., 2021). While the CO_2 tensions between these two studies on *L. vannamei* were very similar, the shrimp density in our study was about double, while the salinity was half. In *L. vannamei*, increased density has been previously shown to reduce specific growth rates while a salinity around 17-20 ppt has been suggested as the optimal salinity for maximum growth rate (Li et al., 2007; Wasielesky et al., 2013). This is confirmed in our study where we observed a two to three-fold higher specific growth rate than reported for full-strength seawater acclimated *L. vannamei* by Muralisankar et al. (2021). As salinity and not density is likely driving this increased growth rate in our shrimp, the improvement of growth rate due to the lower salinity in the current study may be negating the potential reductions in growth rate due to aquaculture CO_2 tensions that have been reported for shrimp reared in full-strength seawater. All crustacean studies showing reduced growth rates with increased environmental CO_2 (2000+ μatm) have been performed on crustaceans reared in full-strength seawater (Kurihara et al., 2008; Long et al., 2013; Muralisankar et al., 2021; Wickins, 1984). The few studies where CO_2 was not shown to affect growth rate in full-strength seawater crustaceans, were likely due to the low CO_2 tensions (~1000 μatm) used in those studies (Kurihara

et al., 2008; Lowder et al., 2017). For example, the Pacific grass shrimp (*Palaemon pacificus*) showed no changes in growth rate after 30 weeks when reared in 1000 vs. 400 μatm (control) CO_2 but experienced a significant decrease in growth rate after 15 weeks when exposed to 1900 μatm pCO_2 (Kurihara et al., 2008). Our results - when compared to previous studies in marine crustaceans - suggest that the effect of environmental CO_2 on growth rate may depend on not just the CO_2 tension but also on environmental salinity, a concept that to our knowledge has not been empirically tested and must be examined.

Stunted growth in shrimp is a commonly observed problem associated with strains of farmed shrimp, that can have a large impact on aquaculture profitability (Flegel, 2012). Several pathogens have been correlated with the stunted growth pathology including hepatopancreatic parvovirus, IHHNS virus, Monodon baculovirus, and Laem-Singh virus (Flegel, 2012). Studies on the effects of ocean acidification have shown the potential for elevated environmental CO_2 levels to suppress the immune system in several invertebrates including crustaceans increasing their susceptibility to pathogens (Hernroth et al., 2012; Meseck et al., 2016). Therefore, the accumulation of CO_2 in high-density aquaculture systems could lead to an increased prevalence of stunted shrimp. We determined that the prevalence of stunted shrimp was not significantly increased with increasing CO_2 levels (Table 3.3). In fact, we found an overall greater number of shrimp with stunted growth in the low CO_2 treatment compared to the high CO_2 treatments although this was not statistically different. The immune system of crustaceans is not universally suppressed in response to acidification. For example, in the brine shrimp (*Artemia sinica*), elevated environmental CO_2 has been shown to increase mRNA transcript abundance of key genes related to immune response (Zheng et al., 2015), suggesting CO_2 could have either a species-specific suppressive or stimulatory effect. While the effects of CO_2 on the immune system of *L. vannamei* have not been thoroughly studied, one can conclude that the stunted growth pathology that is economically challenging in shrimp aquaculture is unlikely to result from elevated environmental CO_2 levels.

Besides growth rates, the survival rate is one of the key parameters in assessing the productivity and overall economic success of an aquaculture system. Elevated environmental CO_2 has been shown to have variable effects on survival in decapod crustaceans. Here, we found a 35-40% mortality rate after 9 weeks of growth that was independent of environmental CO_2 levels (Table 3.4). The mortality rates in the current study appeared to be largely a result of cannibalism (pers.

obs.), which is not surprising given the stocking density, lack of shelter in the experimental tanks, and frequent moulting, which could conceivably make shrimp particularly vulnerable to predation. It is also possible that mortalities in the current study stem from pathogens commonly seen in aquaculture shrimp, which have been correlated to increased cannibalistic behaviours (Romano and Zeng, 2017). Nevertheless, juvenile *L. vannamei* in the current study appeared tolerant to elevated environmental CO_2 levels. Decapod crustaceans that inhabit estuaries such as porcelain crabs (*Petrolisthes cinctipes*, *Petrolisthes manimaculus*, and *Porcellana platycheles*) and barnacles (*Semibalanus balanoides* and *Elminius modestus*) have also been reported to experience minimal mortalities due to elevated environmental CO_2 levels (Findlay et al., 2010; Page et al., 2017). It has been suggested that the variability in CO_2 experienced in estuaries and particularly high intertidal regions has driven the evolution of greater tolerance to CO_2-mediated acidification in species inhabiting these variable environments (Page et al., 2017). One might assume that as the native habitat of *L. vannamei* includes coastal estuaries, this species too would have evolved similar CO_2 tolerance. However, elevated environmental pCO_2 levels decreased survival rates of full-strength seawater acclimated *L. vannamei* (Muralisankar et al., 2021). In conjunction with the contradicting results from the current study, these results suggest that in *L. vannamei* survival, like growth rates under elevated environmental pCO_2 levels could depend on environmental salinity.

Acid-base and nitrogen physiology in adult shrimp

Maintaining acid-base homeostasis is fundamental to an animal's physiology as slight changes in intra- or extracellular pH can impair proper protein function and regulation disrupting essential physiological processes. Decapod crustaceans are generally known as relatively strong acid-base regulators as their high metabolic capacity has pre-dispositioned this group of species to have robust acid-base machinery to deal with metabolic acid production (Melzner et al., 2009). Indeed, in crustaceans including green crab (*Carcinus maenas*), and prawns (*Palaemon elegans* and *Palaemon serratus*) hemolymph pH is readily maintained after chronic exposure (10 weeks *C. maenas* and 30 days prawns) to 3500-3700 μatm CO_2 (Appelhans et al., 2012; Dissanayake et al., 2010). While we predicted that *L. vannamei* would be capable of readily maintaining acid-base homeostasis due to its life history, our study showed that, when chronically exposed to CO_2 tensions of ca. 5500 μatm, hemolymph pH is compromised, suggesting an inability to fully compensate for the respiratory acidosis that is experienced at recirculating aquaculture system like

CO_2 levels. The hemolymph pH levels found in this study for shrimp transferred to 1100 µatm CO_2 for two weeks is similar to pH levels recorded in other studies for *L. vannamei* and similar to the acid-base status observed in other shrimp species under normal CO_2 tensions indicating that the pH values seen under high CO_2 levels as applied in the current study are indeed a result of an uncompensated acidosis (Fehsenfeld and Weihrauch, 2017; Zhou et al., 2009). While hemolymph pH was not fully compensated, our results demonstrate at least a partial compensation as HCO_3^- was significantly accumulated in the hemolymph to partially buffer the changes in pH associated with increased hemolymph pCO_2 (Fig. 3.1), a mechanism commonly seen in decapod crustaceans in response to acidosis (Wheatly and Henry, 1992). It should be noted that, while we maintained adult shrimp for two weeks under high CO_2 before being assigned to the high CO_2 or low CO_2 treatments, these shrimp came directly from a RAS where they were grown from the post-larval stage in high CO_2 levels (ca. 6000 µatm) for as much as 150 days before arriving at our facilities. Therefore, these shrimp were at CO_2 levels above 5500 µatm for as much as 30 weeks and the high metabolic cost to maintain pH homeostasis during this chronic exposure may have led to a loss of pH regulatory capability. Unfortunately, to our knowledge, only one study has investigated the maintenance of acid-base homeostasis under chronic exposure to elevated environmental CO_2 levels on a scale of several months in crustaceans. This one study on the green crab (*Carcinus maenas*) and edible crab (*Cancer pagurus*) showed that hemolymph pH was maintained stable after as long as 9 months of exposure to CO_2 tensions of 1000 µatm (Whiteley et al., 2018). However, the CO_2 tensions used on *C. maenas* and *C. pagurus* were low compared to our high CO_2 treatment and very similar to our low CO_2 treatment but also similar to levels that can normally be experienced in coastal habitats such as the Baltic Sea (Thomsen et al., 2017) making cross-study comparisons difficult. As we were unable to monitor hemolymph acid-base status from early juvenile to late adult stages, it remains to be determined if *L. vannamei* is simply incapable of compensating for the acid-base disturbance caused by higher CO_2 tensions such as those in RAS or whether a loss of acid-base regulatory capability occurs from months-long chronic exposure to elevated CO_2.

Ammonia is a metabolic end product of protein catabolism that can serve as an indicator of metabolic changes but may also act as an acid-base equivalent (Weihrauch and Allen, 2018). The lack of change in ammonia excretion rates in shrimp maintained at high CO_2 tensions suggests that

unlike results reported for the green crab (*C. maenas*) and the shallow hydrothermal vent crab (*Xenograpsus testudinatus*), *L. vannamei* is not reliant on ammonia to excrete acidic equivalents in the form of NH_4^+ for acid-base regulation (Allen et al., 2020; Fehsenfeld and Weihrauch, 2013). These results are in line with the fact that acid-base homeostasis was not maintained in *L. vannamei* in RAS-like CO_2 levels. In response to elevated environmental pCO_2 levels, it has been suggested that some invertebrates shift to a greater dependence on amino acids catabolism to support the high metabolic demand of acid-base regulation while also potentially supplying metabolically-sourced HCO_3^- for buffering (Hu et al., 2014a; Michaelidis et al., 2005). The fact that ammonia excretion rates are unchanged and hemolymph ammonia is not significantly elevated but instead slightly decreased indicates that amino acid catabolism is not increasing with elevated CO_2. However, as metabolic rates (oxygen consumption) were not measured in this study we cannot conclude whether greater reliance on protein catabolism for the total energy budget is occurring under high CO_2 conditions as would be the case if the total energy budget were to decrease (reduced oxygen consumption) but protein catabolism maintained consistent.

Conclusion

In conclusion, RAS-like CO_2 levels do not appear to affect the growth and survival of 17 ppt acclimated *L. vannamei* during the first 11 weeks post-larval, suggesting that aquaculture productivity during this time is likely unaffected by CO_2. However, we found evidence that adult shrimp (~30 weeks post-larval) were unable to maintain acid-base homeostasis following chronic exposure to RAS-like CO_2 levels. Since we found physiological impairment in adult shrimp, it is possible that negative effects on the growth and survival may not manifest until after exposure for longer than 11 weeks. Comparison to other studies on seawater acclimated *L. vannamei* revealed that environmental salinity may have an important interactive effect on the growth and survival of shrimp during exposure to elevated environmental CO_2 levels. This implies that growing *L. vannamei* at lower environmental salinities could be beneficial for avoiding unwanted CO_2 effects on growth and survival in an aquaculture setting. Future studies should directly address the effect of salinity on CO_2 tolerance of *L. vannamei* and whether the effects of elevated pCO_2 levels on growth and survival in 17 ppt salinity acclimated shrimp changes between early and late post-larval development. Overall, this study provides a framework to begin understanding the long-term effects that the accumulation of CO_2 in RAS can have on shrimp aquaculture productivity.

TRANSITION TO CHAPTER 4

In the first two experimental chapters, I explored the acute (Chapter 2) and chronic (Chapter 3) effects of elevated aquatic CO_2 (extrinsic stressor) on the physiological responses of euryhaline decapod crustaceans. The results of chapter 2 suggest that acute elevated CO_2 may lead to energetic trade-offs that may impact animal fitness. In Chapter 3, I found that despite impacts on acid-base physiology of *L. vannamei* growth and survival in this species is not affected when reared in brackish water as opposed to previous studies showing an effect when reared in full strength seawater. Most studies investigating acid-base disturbances in the presence of extrinsic acid-base stressors work on fasted animals to avoid any potential intrinsic acid-base changes due to feeding. However, in nature and aquaculture settings crustaceans need to feed and may experience an extrinsic stressor during digestion. It is currently unknown how feeding alters acid-base homeostasis in decapod crustaceans; therefore, Chapter 4 will address this gap in knowledge to provide a basis for studies to investigate how feeding may alter responses to extrinsic acid-base stressors.

CHAPTER 4: POSTPRANDIAL NITROGEN AND ACID-BASE REGULATION IN THE SEAWATER ACCLIMATED GREEN CRAB, *CARCINUS MAENAS*

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Published in: Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 2022. 267: 111171

Author contributions:

ARQR designed the study, performed experiments, analyzed data, and wrote the manuscript. GJPA assisted with data collection and revised the manuscript. MTN assisted with data collection and revised the manuscript. DW contributed to study design, manuscript editing, financial support, and analytical tools.

Abstract

The effects of feeding (meal of 3% of body mass) on acid-base and nitrogen homeostasis were investigated in the seawater acclimated green shore crab, *Carcinus maenas*. Feeding did not change gastric fluid pH (~pH 6); however, feeding was associated with a respiratory acidosis. Hemolymph HCO_3^- did not increase during this acidosis, although titratable and net acid efflux changed from an uptake to an excretion. Feeding affected the crabs' nitrogen homeostasis causing a substantial increase in hemolymph ammonia and urea concentrations after six hours. At this point, hemolymph urea accounted for ~1/3 of nitrogenous waste accumulated within the hemolymph, suggesting a potential role in ammonia detoxification. The postprandial increase in hemolymph ammonia coincided with an 18-fold increase in ammonia excretion rates that accounted for the majority of net acid excreted by the crabs. Urea excretion rates did not increase after feeding; however, branchial urease activity increased, implying that the gills may possess a mechanism to form excretable ammonia through the catabolism of urea. Our results demonstrate that despite an acidic gastric compartment, *C. maenas* does not experience a postprandial alkaline tide and that any feeding related acid-base challenges are primarily derived from metabolic acid production. Our findings also indicate that unlike the bicarbonate buffering acid-base

compensatory response induced by hypercapnia and emersion, acid-base challenges upon feeding are compensated through changes in the excretion of acid equivalents, mainly in the form of ammonia.

Introduction

Feeding and digestion are essential for nutrient acquisition in crustaceans. The last several decades of research concerning crustacean digestive physiology have focused on gastric processing, nutrient acquisition, neuroendocrine control, digestive enzymes and metabolism (McGaw and Curtis, 2013; Vogt, 2021; Whiteley et al., 2001b). Feeding is also known to affect acid-base regulation in mammals, amphibians, reptiles, and most gastric fishes through the generation of a postprandial alkaline tide (Niv and Fraser, 2002; Wang and Rindom, 2021; Wang et al., 2001; Wood, 2019). When gastric cells secrete HCl into the stomach lumen to form an extremely acidified compartment for digestion, gastric cells simultaneously transport HCO_3^- into the extracellular fluids to maintain intracellular pH, resulting in an extracellular metabolic alkalosis referred to as an alkaline tide. Crustaceans also possess an acidic stomach, however, their gastric pH is less acidic (pH 4.7-6.7) than that of the aforementioned vertebrates (stomach typically pH 1 to 3) (Koelz, 1992; Linton and Greenaway, 2004; Vogt, 2021). To date, postprandial changes in gastric fluid pH have not been investigated in crustaceans. It remains unclear whether feeding leads to further acidification of the stomach, which may lead to an extracellular acid-base challenge in these animals similar to that reported in several vertebrates.

Crustaceans experience an increase in metabolic rate after feeding, known as specific dynamic action (Secor, 2009; Whiteley et al., 2001b). Specific dynamic action results from the energetic demands of feeding, gastric processing, nutrient acquisition and protein synthesis (Houlihan et al., 1990; McGaw and Van Leeuwen, 2017; Secor, 2009). Increased rates of aerobic metabolism cause the production of volatile (e.g. CO_2) and non-volatile (e.g. H^+, NH_4^+) acids and nitrogenous wastes (e.g. NH_4^+) to increase (Pörtner, 1989). As feeding stimulates aerobic metabolism, the accumulation of acids and nitrogenous wastes may lead to an intrinsic challenge of acid-base and nitrogen homeostasis in crustaceans.

Although feeding associated changes in acid-base balance have not been investigated in crustaceans, its effect on nitrogen metabolism has in the context of growth optimization in aquaculture. In several crustaceans, feeding increases ammonia excretion rates and, while not

directly shown, likely increases hemolymph ammonia concentrations (Crear and Forteath, 2002; Koshio et al., 1993; Ponce-Palafox et al., 2017; Radford et al., 2004; Rosas et al., 1996; Wang et al., 2019). Since ammonia is an amphoteric molecule, its postprandial accumulation suggests it may affect the animal's acid-base status. In addition to its potential to disrupt acid-base homeostasis, ammonia is toxic to most animals, including crustaceans (Larsen et al., 2014). Its postprandial accumulation may lead to an increase in the synthesis of less toxic nitrogenous wastes such as urea as shown in the Dungeness crab, *Metacarcinus magister*, and the swimming crab, *Portunus trituberculatus*, following exposure to elevated environmental ammonia (Martin et al., 2011; Ren et al., 2015).

The present study addresses the considerable gap in our understanding of the consequences of feeding on acid-base regulation and nitrogen homeostasis in crustaceans. To date, most research concerning challenges to acid-base and nitrogen homeostasis in crustaceans focuses on the consequences of environmental factors such as hypercapnia, elevated environmental ammonia concentrations, emersion, salinity, and oxygen bioavailability using chronically starved animals (summarized in Fehsenfeld and Weihrauch, 2017). Since feeding is an essential and regular process, it is important to understand its effects on acid-base and nitrogen homeostasis as these effects will occur regardless of environmental stressors. The green shore crab, *Carcinus maenas*, was used as a model to investigate postprandial changes in acid-base and nitrogen homeostasis in crustaceans. *Carcinus maenas* was selected as it is one of the most extensively studied crustacean models regarding acid-base regulation and nitrogen excretion (Fehsenfeld and Weihrauch, 2017; Weihrauch et al., 2017). We monitored postprandial changes in acid-base and nitrogen homeostasis over a 36-hour period coinciding with the approximate time of complete gastric clearance in *C. maenas* at our experimental temperature according to previous studies (McGaw and Penney, 2014). Through gastric fluid pH measurements, we determined whether crustaceans employ an extreme acidification mode of digestion similar to vertebrates exhibiting an alkaline tide. We also measured hemolymph acid-base and nitrogen status to determine whether crabs experience a postprandial acid-base and ammonia challenge and whether crabs synthesize urea for postprandial ammonia detoxification. Through whole animal acid-base and nitrogen fluxes and enzyme activity measurements, we investigated the mechanisms by which *C. maenas* compensate for postprandial acid-base and nitrogen challenges. The hypotheses of this study were that (1)

feeding would alter acid-base status in *C. maenas* through changes in gastric acidification, (2) feeding would create a change in acid-base and nitrogen homeostasis through acid and ammonia production due to the specific dynamic action of food, and (3) *C. maenas* would compensate for postprandial acid-base and ammonia challenges through changes in acid-base and ammonia flux and detoxification of ammonia through the synthesis and excretion of urea. Further, unexpected results regarding the accumulation and excretion of urea prompted an investigation into the enzymatic synthesis and catabolism of urea by arginase and urease.

Materials and methods

Animal care

Male green crabs (*Carcinus maenas*) were collected from Northern Placentia Bay, NL, CA and transported to the Duff Roblin Animal Holding Facility (University of Manitoba, Winnipeg, MB, CA). The general population of crabs were maintained in a recirculating seawater system composed of three 1200 L tanks and a sump with artificial seawater (Fritz Reef Pro Mix, Fritz Aquatics, Mesquite, TX, USA) at a salinity of 32 ppt, 14 ºC, pH 8.1, 12h:12h light:dark cycle. Tanks were supplemented with PVC pipes for shelter. A total of 59 individuals weighing between 40 and 67 grams were used for this study. Experimental crabs were transferred and held in two aerated ~220 L seawater aquaria (max. 12 crabs per aquaria) with similar conditions to general population tanks. Each aquarium was connected to a biological filter (Eheim professional 4+, Eheim GmbH, Deizisau, Germany) and maintained in a temperature controlled environmental room (Conviron, Winnipeg, MB, CA). Crabs were given one week to acclimate to the new housing conditions before experiments were performed, during which time crabs were fed *ad libitum* with bay scallops three times per week. Crabs were fasted for a minimum of 72 hours prior to experiments as it has been previously reported that gastric clearance in this species at our experimental temperature may take as long as 36 hours (McGaw and Penney, 2014).

Feeding protocol

For all experiments, crabs were individually transferred to aerated plastic containers (21 cm L x 13.5 cm W x 8 H cm) containing 2 L of artificial seawater. Crabs were then air exposed for less than one minute to weigh the animals. After weighing, crabs were given 30 to 60 minutes to recover from handling stress. Before feeding, a control sampling period was completed to

determine the animals' baseline hemolymph parameters, gastric pH, and flux rates. Following the baseline sampling period, water was refreshed in all holding containers and half of the crabs were fed bay scallops equivalent to 3 % of their body mass and given 20 minutes to feed. All animals in the fed group consumed their entire meal within this 20-minute period. After feeding, water was refreshed in all holding containers and the 36-hour sampling time course began, with the water being completely refreshed every 12 hours.

Gastric fluid pH

Twelve individuals were used for this experiment and sampled independent of whole animal flux, hemolymph, and enzyme activity experiments. Gastric fluid pH was determined by taking fluid samples from the cardiac stomach of fed and unfed crabs, as previously described (Linton et al., 2014). In brief, crabs were held upside down and maxillipeds were lifted with a pipette tip. A small piece of PE160 tubing connected to a 200 µL pipette tip was gently slid through the mouth into the cardiac stomach. Gastric fluid was slowly pipetted out of the cardiac stomach (~100 µL), transferred into a 1.5 mL microcentrifuge tube, and floated in a water bath held at 14 ºC. Gastric fluid pH was immediately measured while in the water bath using a micro pH electrode (InLab Micro, Mettler Toledo, Columbus, OH, USA) connected to a pH meter (model 225, Denver Instruments, Gottingen, Germany). The effects of repeated sampling on gastric pH were accounted for by performing measurements on an unfed group of crabs in parallel over the same time course. Sampling was performed at 0, 3, and 6 hours postprandial. These sampling times were based on previous studies on this species showing roughly 50 and 25 % of food matter remains present in the foregut at 3 and 6 hours postprandial, respectively (McGaw and Penney, 2014). Samples between 0 and 3 hours could not be collected as the food pieces in the cardiac stomach were too large and clogged the tubing, preventing the collection of gastric fluids without overly stressing the crabs.

Hemolymph acid-base and nitrogen parameters

Twelve individuals were used for this experiment and sampled independent of whole animal flux, gastric fluid pH, and enzyme activity experiments. Hemolymph samples were taken at 0, 6, 12, 24, and 36 hours after feeding to monitor postprandial changes in acid-base and nitrogen status. Hemolymph (~200 µL) was taken by puncturing the arthrodial membrane at the base of a walking

leg with a sterile syringe. Hemolymph was then transferred to a 1.5 mL tube and floated in a water bath held at 14 ºC. Hemolymph pH was immediately measured by a micro pH electrode (InLab micro, Mettler Toledo, Columbus, OH, USA) and a 15 µL sample was taken with a gas tight Hamilton syringe for immediate analysis of total CO_2. The remaining hemolymph was centrifuged at 10,000 x *g* for 3 minutes and subsequently frozen at -80 ºC for later analysis of urea and ammonia. The potential effects of repeated short-term air exposure on the hemolymph parameters were accounted for by measuring a group of unfed crabs in parallel over the experimental time course.

Hemolymph total CO_2 was measured in triplicate by a custom-built gas sparging chamber coupled with a Li-850 (LI-COR, Lincoln, NE, USA) infrared CO_2 gas analyzer (Allen et al., 2021; Lee et al., 2018). In brief, a 5 µL hemolymph sample was injected through a septum injection port into a glass sparging chamber containing 0.1 mmol L^{-1} HCl with antifoam SE-15 (1 µL mL^{-1}) to convert total inorganic carbon into CO_2. By continuously passing pure N_2 gas (80 mL N_2 gas min^{-1}, GFC17, Aalborg, Orangeburg, NY, USA) through the bottom of the sparging column, CO_2 was released from the sample and passed through the top of the chamber directly into the Li-850 CO_2 analyzer. The CO_2 pulse measured by the Li-850 analyzer was used to determine the amount of CO_2 injected into the chamber as described in Lee et al. (2018) and compared to pulses produced by a $NaHCO_3$ standard curve to determine total CO_2 in the hemolymph sample. Using the measured hemolymph total CO_2 and pH, hemolymph pCO_2 and HCO_3^- were calculated using a rearrangement of the Henderson-Hasselbalch equation using the pK1 and αCO_2 determined for *C. maenas* hemolymph at 32 salinity and 14 ºC extrapolated from nomograms provided by Truchot (1976).

Hemolymph samples for ammonia and urea determination were deproteinized to avoid the interference of proteins on the spectrophotometric assays used. Deproteinization was achieved by mixing 100 µL of hemolymph with 100 µL of 8% perchloric acid that was then incubated on ice for 5 minutes. Samples were subsequently centrifuged (4 ºC, 10,000 x *g*, 5 minutes) and the supernatant was neutralized with 0.4 volumes of 3 mol L^{-1} KOH. Neutralized samples were centrifuged (4 ºC, 10,000 x *g*, 5 minutes) to remove $KClO_4$ precipitate. The neutralized supernatants were used for spectrophotometric ammonia and urea analysis. Ammonia (expressed as µmol L^{-1}) was analyzed spectrophotometrically using a microplate version of the salicylate-

hypochlorite assay described by Verdouw et al. (1977). Urea (expressed as μmol L^{-1}) was analyzed spectrophotometrically using a microplate version of the diacetyl monoxime assay described by Rahmatullah and Boyde (1980). To confirm that the diacetyl monoxime assay was not detecting any non-urea compounds in the hemolymph a pooled sample from control crabs and a pooled sample from 6-hour postprandial crabs was measured in the presence and absence of urease (10U mL^{-1}, 30 min at room temperature). The urease treated 0-hour control sample had no detectable urea whereas the urease treated 6-hour postprandial sample had a small ~4 μmol L^{-1} detection, which was about 3% of the urea concentration measured in the untreated sample (6 hours postprandial sample no urease added) and therefore deemed negligible.

Whole animal fluxes

Eleven individuals (5 control and 6 fed) were used for this experiment and sampled independent of hemolymph, gastric fluid pH, and enzyme activity experiments. Whole animal ammonia, urea, and titratable alkalinity flux rates were measured over a 42-hour period in fed and unfed crabs. The general crab feeding protocol described above was employed. Fluxes were determined by measuring ammonia, urea, and titratable alkalinity (equivalent to inverse titratable acidity) of water samples at the beginning and end of each flux period. In this study, five flux periods were measured -6 to 0, 0 to 6, 6 to 12, 12 to 24 and 24 to 36 hours. Full water changes were performed at 0, 12, and 24 hours to reduce the accumulation of waste products. For ammonia and urea determination, 1 mL water samples were taken and frozen (-20 ºC) for later analysis as previously described for hemolymph. Titratable alkalinity was determined using a double endpoint titration. For titrations, water samples (20 mL) were aerated for 30 minutes with CO_2-free air (air bubbled through 2 mol L^{-1} NaOH to remove CO_2) and titrated to pH 4.5 with standardized HCl (0.1 N). Samples were continuously aerated with CO_2-free air for an additional 15 minutes until pH was stabilized to remove excess CO_2 in the sample and then further titrated to pH 3.8 (first endpoint). Once pH stabilized at pH 3.8, samples were back titrated with standardized NaOH (0.05 N) to the initial sample pH (second endpoint). Titrants were dispensed by pipettes calibrated to 2, 10, and 100 μL. As a burette was not used for titration, the calibration of the pipettes was periodically checked throughout the analysis to assure continuous accurate titrant volume pipetting. All water samples for acid-base titrations were stored at 4 ºC and analyzed within 24 hours of collection.

$$(1)\ Titratable\ alkalinity = \frac{((A * A_V) - (B * B_V)}{S_V}$$

$$(2) Titratable\ acid\ flux = -\frac{(TA_f - TA_i) * V}{t * m}$$

Titratable alkalinity of water samples was calculated according to equation (1) where A is the concentration of the standardized HCl (Eq L^{-1}), A_V is the volume of HCl added to titrate the sample to pH 3.8 (L), B is the concentration of standardized NaOH (Eq L^{-1}), B_V is the volume of NaOH added to titrate back to initial pH (L), and S_V is the volume of the sample titrated (L). Titratable acid flux over a flux period was calculated according to equation (2), where TA_f is the final titratable alkalinity (Eq L^{-1}), TA_i is initial titratable alkalinity (Eq L^{-1}), V is the volume of water in the flux chamber (L), t is flux time (hours), and m is the crab weight (grams). Net acid fluxes were calculated through the addition of ammonia flux and titratable acid flux where positive values indicate a net acid uptake or base excretion, and negative values indicate a net acid excretion or base uptake.

Enzymatic assays

Twenty-four individuals were used for this experiment and sampled independent of acid-base flux, gastric fluid pH, and hemolymph experiments. In this experiment, the feeding protocol previously described was used, except that at the start of the experiment, the unfed crabs (0-hour control) were sacrificed and sampled for gill and hepatopancreas tissue. Further, after feeding, fed animals were returned to the 220 L aquaria instead of being maintained in the 2 L experimental containers to maintain negligible environmental waste levels. For tissue sampling, crabs were sacrificed by inserting a nail through the ventral body at the anterior tip of the tail to destroy the ventral ganglia. The hepatopancreas, anterior gill 5 and posterior gill 9 were dissected, flash frozen in liquid nitrogen, and stored at -80 ºC until enzymatic analyses were performed. Gills 5 and 9 were pooled together for enzyme activity assays to have one representative anterior gill and one representative posterior gill to account for any potential difference in activity due to variations between these gill types.

Tissues for urease and arginase assays were homogenized in 5 volumes of 25 mmol L^{-1} potassium phosphate buffer (pH 7.5) with 1 mmol L^{-1} dithiothreitol (DTT) by a Qiagen Tissuelyser II (Valencia, CA, USA) for 2 minutes at 30 Hz with the sample adapter pre-chilled in a -20 ºC

freezer. A subset of each homogenate was diluted 1:10 with 25 mmol L^{-1} potassium phosphate buffer (pH 7.5) for protein quantification using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). The remaining samples were frozen at -80 ºC and assays were performed on the subsequent day.

Urease activity was measured by detecting the production of ammonia in the presence of urea using a microplate assay based on the protocol previously described by Linton and Greenaway (1998). In brief, assays were conducted in triplicate in a final volume of 200 µL containing 20 µL of tissue homogenate, 160 µL of 25 mmol L^{-1} potassium phosphate buffer (pH 7.5) and 20 µL of 10 mmol L^{-1} urea. For each homogenate, a triplicate set of blank assays was run in parallel, where urea was replaced with 20 µL of 25 mmol L^{-1} potassium phosphate buffer (pH 7.5). Assays were incubated for 1 hour (hepatopancreas and 0 h control gill homogenates) or 20 minutes (6 and 12 h postprandial gills) at 25 ºC. Preliminary test assays demonstrated that urease activity was linear over the assay period and that doubling/halving protein concentration doubled/halved assay activity. Assays were terminated by the addition of 40 µL of 3 mol L^{-1} perchloric acid and subsequently incubated for 10 minutes at 4 ºC for complete deproteinization and centrifuged (4,500 x *g*, 4 ºC, 5 minutes, Multifuge X3R with microplate adapter, Thermo Scientific). Supernatants were then neutralized with 36.5 µL of 3 mol L^{-1} KOH and centrifuged (4,500 x *g*, 4 ºC, 5 minutes, Multifuge X3R with microplate adapter, Thermo Scientific) to remove $KClO_4$ precipitate. The ammonia concentration of neutralized supernatants was then analyzed by salicylate hypochlorite ammonia assay (Verdouw et al., 1977).

Arginase activity was measured by detecting the production of urea using a microplate assay modified based on previously described protocols (Felskie et al., 1998; Jung et al., 2021). In brief, assays were conducted in triplicate in a final volume of 200 µL containing 1 mmol L^{-1} $MnCl_2$, 60 mmol L^{-1} glycine buffer (pH 9.5), 250 mmol L^{-1} L-arginine, and 20 µL of tissue homogenate. Prior to beginning assays, 20 µL of tissue homogenate was mixed with 20 µL of 2.5 mmol L^{-1} $MnCl_2$ to activate the enzyme. For each homogenate, a triplicate set of blank assays was run in parallel, where L-arginine was omitted. Assays were incubated for 60 minutes (gill homogenates) or 20 minutes (hepatopancreas) at 25 ºC. Preliminary test assays demonstrated that arginase activity was linear over the assay period and that doubling/halving protein concentration doubled/halved assay activity. Assays were terminated by the addition of 40 µL of 3 mol L^{-1} perchloric acid, incubated

for 10 minutes at 4 ºC for complete deproteinization and centrifuged (4,500 x *g*, 4 ºC, 5 minutes, Multifuge X3R with microplate adapter, Thermo Scientific). The urea concentration of deproteinized supernatants was then analyzed by diacetyl monoxime urea assay (Rahmatullah and Boyde, 1980).

Enzyme activity for both assays were calculated according to equation (1) where R_A is the ammonia or urea concentration (μmol L^{-1}) of the assay containing urea or L-arginine substrate, R_B is the ammonia or urea concentration (μmol L^{-1}) of the blank assay, V is assay volume (L), T is time in minutes and P is the amount of protein in the tissue homogenate added to the assay in milligrams.

$$(1)\ Enzyme\ activity = \frac{(R_A - R_B) * V}{T\ x\ P}$$

Scallop ammonia and urea content

To determine the dietary ammonia and urea content of food used in this study, 0.25 g bay scallop muscle was homogenized in 3 volumes of 8 % perchloric acid by Qiagen Tissuelyser II (Valencia, CA, USA) for 2 minutes at 30 Hz with the sample adapter pre-chilled in a -20 ºC freezer. Homogenates were placed at 4 ºC for 10 minutes to deproteinize and then centrifuged (10,000 x *g*, 4 ºC, 5 minutes). Supernatants were neutralized with 0.2 volumes of 3 mol L^{-1} KOH, centrifuged, and then diluted 1:5 with MilliQ water. Ammonia and urea concentrations were then analyzed by the previously described salicylate-hypochlorite ammonia assay and diacetyl monoxime urea assay (Rahmatullah and Boyde, 1980; Verdouw et al., 1977).

Statistics

Statistical analyses were conducted using JMP Pro 16 (Cary, NC, USA). For all data, normal distribution of residuals was tested by Shapiro-Wilk test and assessment of Q-Q plots. Sphericity was tested by Mauchly's sphericity test for hemolymph, gastric pH, and whole animal flux data. Homogeneity of variance was tested using the Levene's test for arginase and urease assay data. Two transformations were used so that parametric statistical tests could still be used to assess data violating the assumption of normal distribution. Cube root transformations were used for hemolymph urea, ammonia flux, titratable acid flux and net acid flux data. A reciprocal transformation was used for gill urease data. If the assumption of sphericity was passed for repeated measures data, then a two-way ANOVA repeated measures test was used with time and

treatment (fed *vs.* unfed) as the independent variables. Hemolymph HCO_3^-, gastric pH, ammonia flux and net acid flux were the dependent variables assessed by a two-way ANOVA repeated measures test. If the assumption of sphericity was violated for repeated measures data, then a repeated measures MANOVA with Greenhouse-Geisser or Huynh-Feldt correction was used with time and treatment (fed *vs.* unfed) as independent variables. Hemolymph pH, hemolymph pCO_2, hemolymph ammonia, hemolymph urea, urea flux and titratable acid flux were the dependent variables assessed by repeated measures MANOVA. For arginase and urease activity data, a one-way ANOVA (urease activity and gill arginase activity) or Kruskal-Wallis (hepatopancreas arginase activity) test was used with time as the independent variable. For all statistical tests where statistical significance was detected, a multiple pairwise comparison by post-hoc Tukey's HSD test was used. For all data sets, p values ≤ 0.05 were considered significant. Data are presented as means ± standard error (SEM).

Results

Gastric fluid pH

Gastric fluid pH was measured to determine whether crabs alter gastric pH during digestion and whether this influences postprandial acid-base homeostasis. Under fasting conditions, unfed and fed crab groups had a gastric fluid pH of 5.99 ± 0.09 (n = 6) and 6.08 ± 0.06 (n = 6), respectively. There was no significant main or interactive effect of feeding and time on gastric fluid pH (Fig. 4.1, Table 4.1). Interestingly, after feeding, gastric fluid pH in the fed group appeared less variable than in the unfed group (Fig. 4.1).

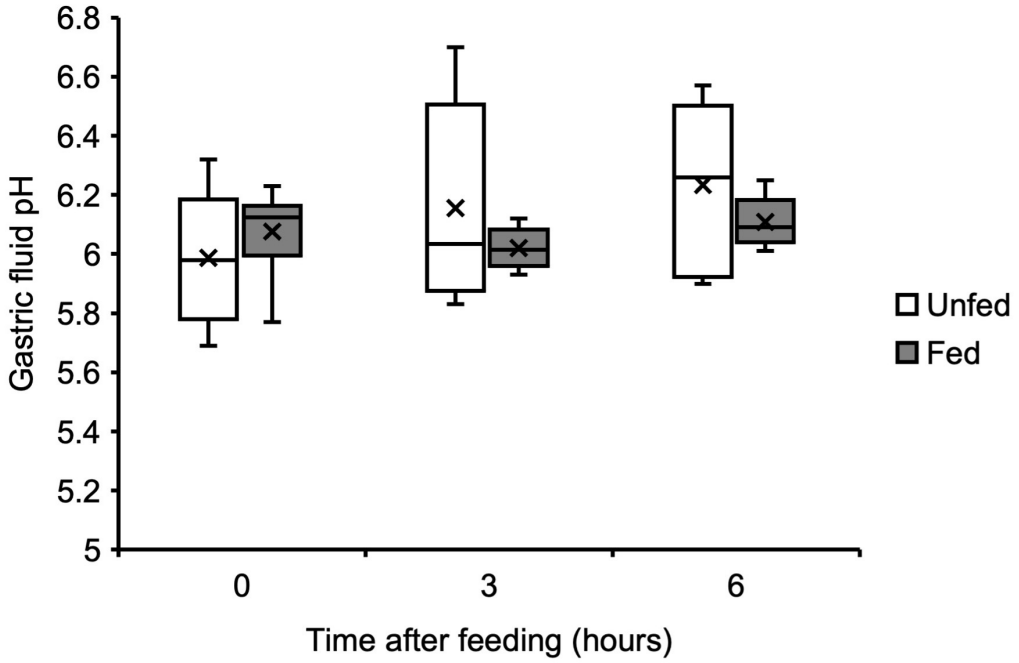

Figure 4.1. Gastric fluid pH collected from the cardiac stomach of unfed (unfilled) and fed (grey filled) crabs over a 12-hour experimental period. Feeding occurred immediately after the 0-hour sampling and crabs were repeatedly sampled. The fed and unfed crab groups were sampled in parallel. The "x" indicates the mean, the inner line of the box is the median, the upper and lower edges of the box are the 1st and 3rd quantile, and the outer whiskers indicate the maximum and minimum values recorded. n = 6.

Hemolymph acid-base and nitrogen status

Hemolymph acid-base and nitrogen status was measured to determine if feeding alters hemolymph acid-base status and increases hemolymph ammonia. The hemolymph acid-base status of fed crabs differed from that of unfed crabs during the first 12 hours of digestion (Fig. 4.2). Unfed green crabs did not change their hemolymph pH, HCO_3^-, pCO_2, ammonia or urea over the 36-hour time course (Fig. 4.2, Fig. 4.3). Feeding led to a significant decrease in hemolymph pH (~0.1 pH units) at six hours postprandial (Fig. 4.2a). This hemolymph pH reduction coincided with an increase in hemolymph pCO_2 from 224 ± 24 to 338 ± 40 Pa (n = 6) but no change in hemolymph HCO_3^- (Fig. 2). Crabs recovered from the acidosis by 24 hours postprandial, at which point hemolymph pH and pCO_2 returned to control levels (Fig. 4.2a,c). Fed crabs never significantly accumulated hemolymph HCO_3^- throughout the 36-hour time course (Fig. 4.2b). Feeding also altered hemolymph nitrogen status (Fig. 4.3). Hemolymph ammonia levels increased from 306 ± 36 to 641 ± 72 μmol L^{-1} at six hours postprandial (Fig. 4.3a, n = 6). Similarly, hemolymph urea

increased after feeding from 24 ± 9 to 168 ± 26 μmol L^{-1} six hours postprandial (Fig. 4.3b, n = 6). By 24 hours postprandial, both hemolymph ammonia and urea were no longer significantly different from baseline levels (Fig. 4.3). The detailed statistical summary of main and interactive effects of feeding and time on hemolymph parameters is presented in Table 4.1 and 4.2.

Bay scallop muscle ammonia and urea concentrations were measured to determine whether increases in hemolymph ammonia and urea levels could be attributed to dietary ammonia and urea uptake. Scallop muscle ammonia concentrations were measured to 314 ± 55 μmol kg^{-1} (n = 8) and urea concentrations were 64 ± 11 μmol kg^{-1} (n = 7). Based on the measured average scallop ammonia and urea concentrations, the potential change in hemolymph ammonia and urea due to dietary intake was calculated to be 31.4 and 6.35 μmol L^{-1}, respectively. This calculation is based on the assumptions that hemolymph volume is 30% of the crab's weight (Robertson, 1960), a food consumption of 3% of crab weight (see methods), and that 100% of the dietary ammonia and urea is transported into the hemolymph.

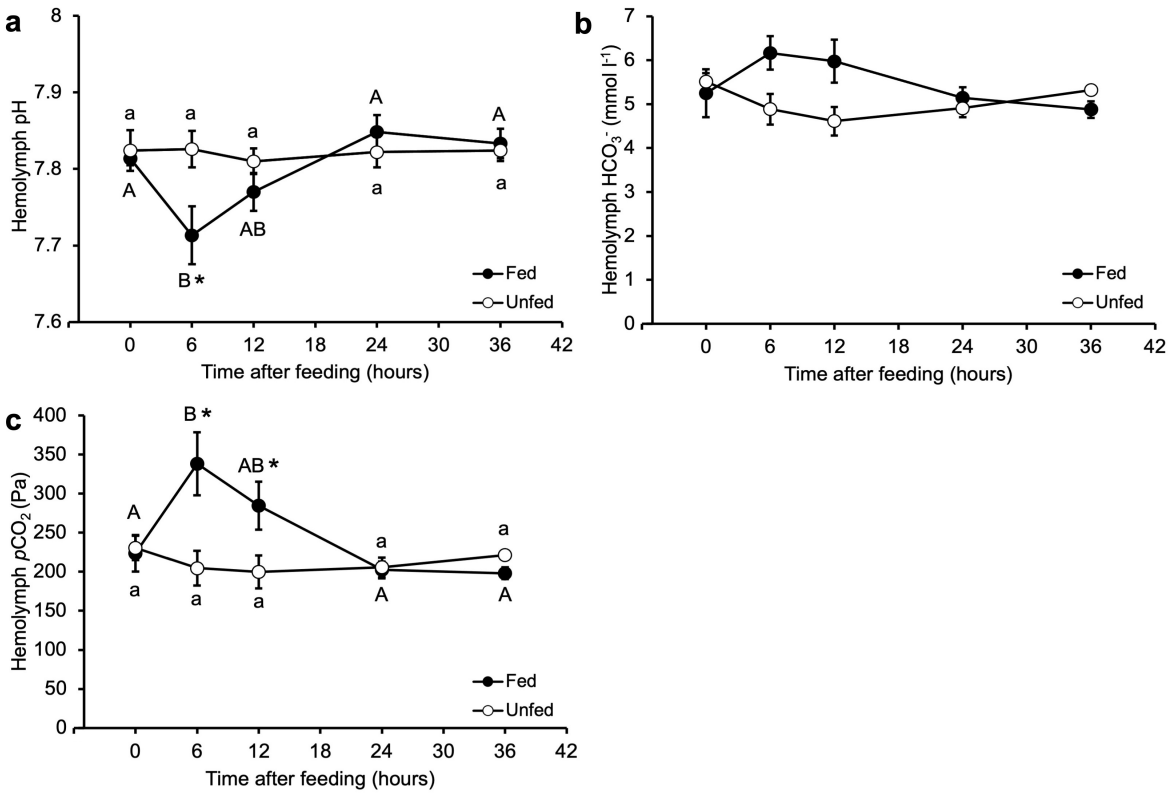

Figure 4.2 Hemolymph (a) pH, (b) HCO_3^-, and (c) pCO_2 of unfed (unfilled circle) and fed (filled circles) crabs over a 36-hour sampling period. Feeding occurred immediately after the 0-hour sampling and crabs were repeatedly sampled. The fed and unfed crab groups were sampled in parallel. Values are mean ± SEM. n = 6 for fed group and n = 5 for unfed group. Time points with the same letter are not significantly different. Upper case letters are used to compare significance within the fed group. Lower case letters are used to compare significance within the unfed group. Asterix indicate significant differences between fed and unfed group at a particular time point.

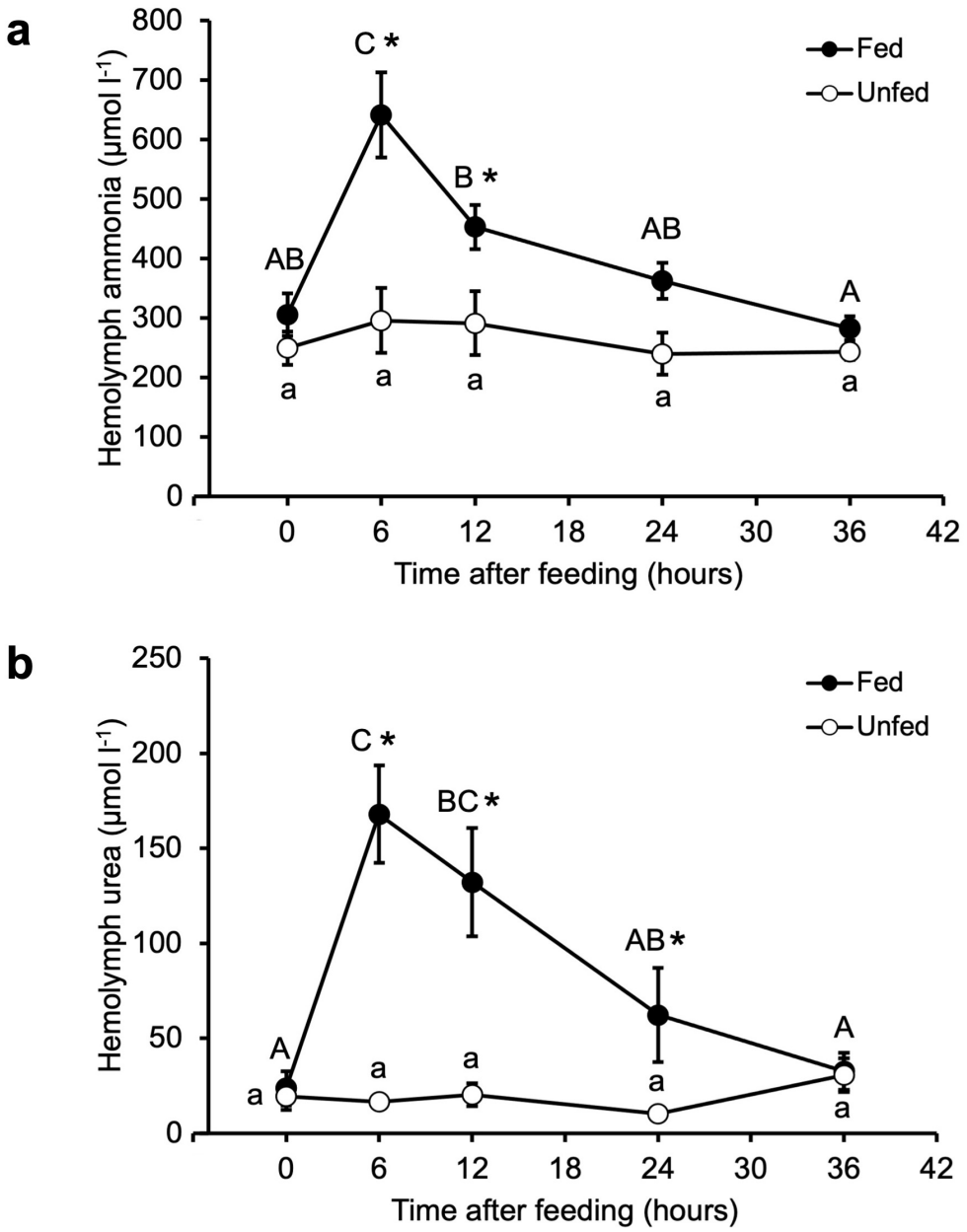

Figure 4.3 Hemolymph (a) ammonia and, (b) urea of unfed (unfilled circle) and fed (filled circles) crabs over a 36-hour sampling period. Feeding occurred immediately after the 0h sampling and crabs were repeatedly sampled. The fed and unfed crab groups were sampled in parallel. Values are mean ± SEM. n = 6 for fed group and n = 5 for unfed group. Time points with the same letter are not significantly different. Upper case letters are used to compare significance within the fed group. Lower case letters are used to compare significance within the unfed group. Asterix indicate significant differences between fed and unfed group at a particular time point.

Acid-base equivalent and nitrogen fluxes

Acid, ammonia, and urea fluxes were measured to determine how crabs were compensating for the accumulation of nitrogen and acids after feeding. Unfed crabs exhibited a significant variation in ammonia, titratable and net acid fluxes over the 36-hour time course (Fig. 4.4). All negative flux rates indicate an excretion from animal into the water whereas positive flux rates indicate an uptake from water into the animal. The average ammonia flux rates in unfed crabs over the 36-hour time course ranged from -96 ± 33 to -21 ± 3 nmol g^{-1} h^{-1} (n = 6, Fig. 4.4a). The average titratable and net acid flux rates in the unfed crabs ranged from 171 ± 37 to -32 ± 34 nmol g^{-1} h^{-1} (n = 5) and 107 ± 26 to -54 ± 32 nmol g^{-1} h^{-1} (n = 6), respectively (Fig. 4b,c). In contrast, urea fluxes in the unfed crabs did not significantly vary over time (Fig. 4.5). Despite variations in the flux rates of unfed crabs, feeding significantly altered acid-base and nitrogenous waste fluxes (Fig. 4.4, Fig. 4.5). In the first six hours postprandial, ammonia flux rates increased from -72 ± 16 to -1358 ± 176 nmol g^{-1} h^{-1} (Fig. 4.4a, n = 6). The ammonia flux rates remained significantly elevated throughout the time course, with a -197 ± 45 nmol g^{-1} h^{-1} rate measured at 24 to 36-hour postprandial time period (Fig. 4.4a, n = 6). The total ammonia excreted over the 36-hour time course in unfed and fed crabs was 1.03 ± 0.24 µmol g^{-1} and 17.21 ± 2.47 µmol g^{-1}, respectively (n = 6, $t(10) = 9.95$, $p < 0.0001$, student's t-test). Titratable acid fluxes into the water also increased in the first six hours postprandial from 132 ± 54 to -382 ± 171 nmol g^{-1} h^{-1} (Fig. 4.4b, n = 6). Titratable acid flux rates returned to control levels by the 24 to 36-hour flux period (Fig. 4.4b). The total titratable acid efflux (or base influx) into water over the 36-hour time course was calculated using the -6 to 0 h flux rate as a baseline. Fed crabs had a significantly higher total titratable acid efflux (or base influx) into the water than unfed crabs over the 36-hour time course with an efflux of 9.00 ± 1.89 and 3.71 ± 1.17 µmol g^{-1} in fed and unfed crabs respectively (n = 6, $t(10) = 2.38$, $p = 0.039$, student's t-test). From titratable acid and ammonia flux rates, a net acid flux rate was calculated. Net acid flux rates into the water were significantly elevated in the first six hours postprandial from 59 ± 61 to -1740 ± 319 nmol g^{-1} h^{-1} (Fig. 4.4c, n = 6). Similar to ammonia flux rates, net acid flux rates remained elevated throughout the time course, with a -135 ± 75 nmol g^{-1} h^{-1} rate measured at the 24 to 36-hour postprandial time period (Fig. 4.4c, n = 6). Total net acid efflux (or base influx) into the water over the 36-hour time course was also calculated using the -6 to 0-hour flux rate as a baseline. Total net acid efflux was 1.29 ± 1.25 µmol

g^{-1} in unfed crabs and increased to 23.61 ± 3.56 μmol g^{-1} in fed crabs (n = 6, t(10) = 5.92, p = 0.0001, student's t-test). In contrast to unfed crabs, urea flux rates in fed crabs varied over the time course (Fig. 4.5); however, urea flux rates were never significantly different from -6 to 0-hour baseline rate. Compared to the unfed crabs, the 0 to 6-hour urea flux rate was significantly different in fed crabs. When considering total urea excreted over the 36-hour time course, there was no significant difference in total urea excretion between unfed and fed crabs, with excretions of 195 ± 49 nmol g^{-1} and 493 ± 128 nmol g^{-1}, respectively (n = 6, t(10) = -2.17, p = 0.07, unequal variance t-test). The detailed statistical summary of main and interactive effects of feeding and time on flux rates is presented in Table 4.1 and 4.2.

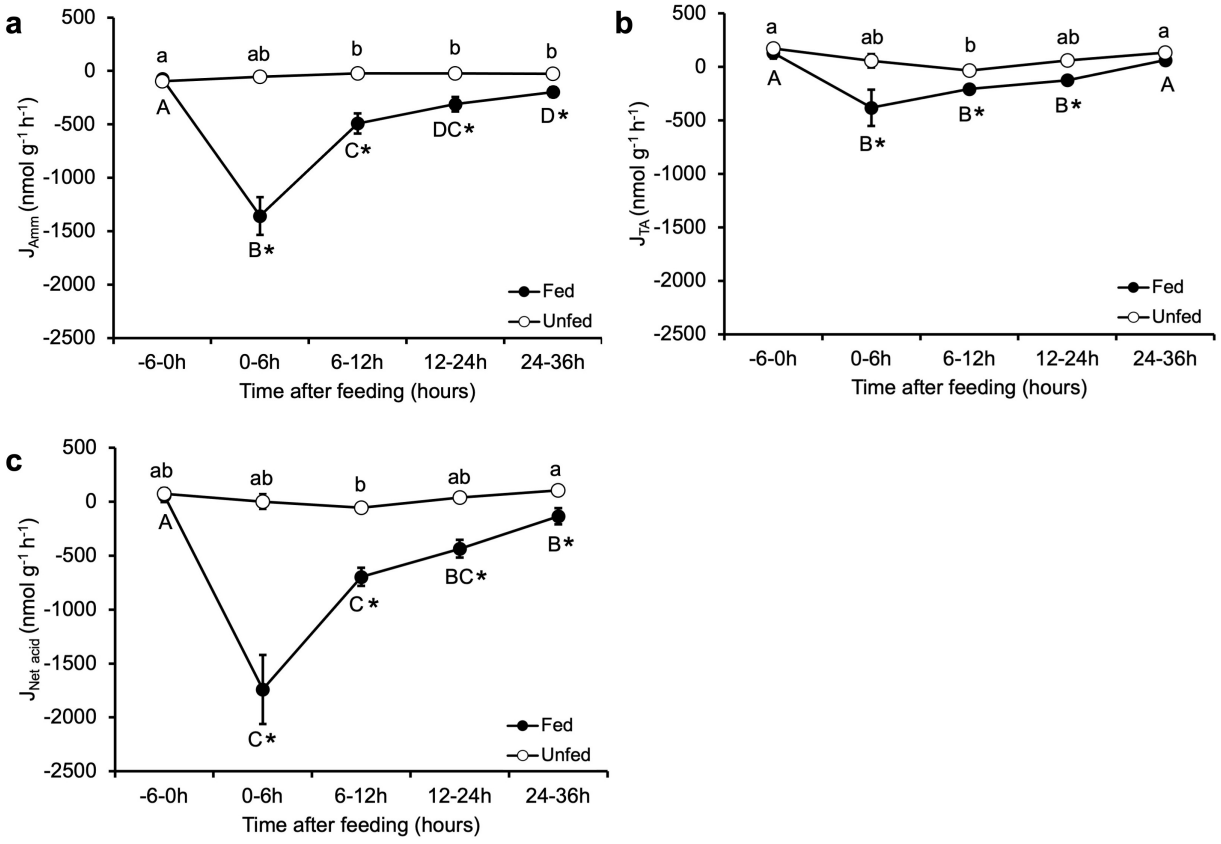

Figure 4.4 Whole animal flux rates of (a) ammonia, (b) titratable acid, and (c) net acid in unfed (unfilled circle) and fed (filled circles) crabs over a 36-hour sampling period. Negative flux rates indicate excretion from the animal into the environment. Positive flux rates indicate uptake from the environment into the animal. For acid-base fluxes, an acid excretion could also indicate a base uptake and vice versa. Feeding occurred immediately after the 0-hour sampling and crabs were repeatedly sampled. The fed and unfed crab groups were sampled in parallel. Values are mean ± SEM. n = 6. Time points with the same letter are not significantly different. Upper case letters are used to compare significance within the fed group. Lower case letters are used to compare significance within the unfed group. Asterix indicate significant differences between fed and unfed group at a particular time point.

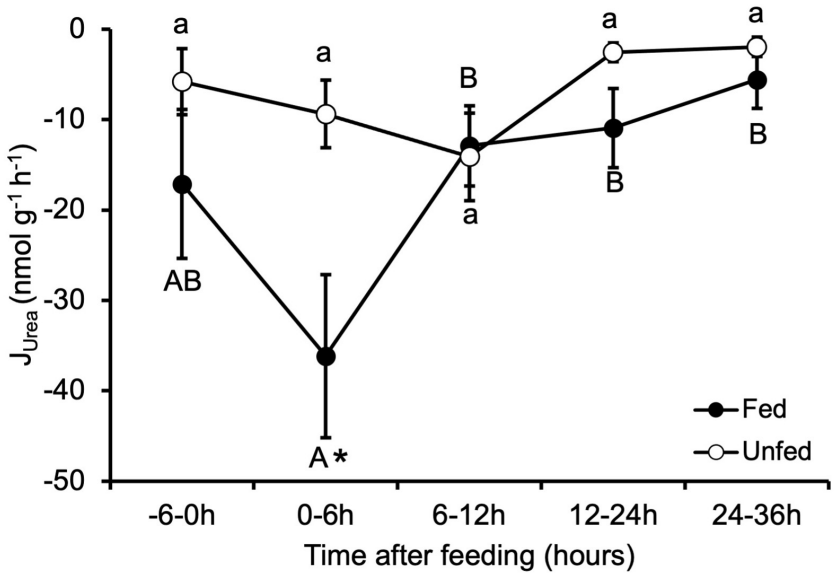

Figure 4.5 Whole animal urea flux rates in unfed (unfilled circle) and fed (filled circles) crabs over a 36-hour sampling period. Negative flux rates indicate excretion from the animal into the environment. Positive flux rates indicate uptake from the environment into the animal. Feeding occurred immediately after the 0-hour sampling and crabs were repeatedly sampled. The fed and unfed crab groups were sampled in parallel. Values are mean ± SEM. n = 6. Time points with the same letter are not significantly different. Upper case letters are used to compare significance within the fed group. Lower case letters are used to compare significance within the unfed group. Asterix indicate significant differences between fed and unfed group at a particular time point.

Branchial and hepatopancreas arginase and urease activity

As hemolymph urea increased but excretion rates remained unchanged after feeding, we measured arginase and urease activity to better understand how urea was being synthesized and catabolized after feeding. Under control conditions, hepatopancreas arginase activity was higher than in the gills (n = 8, Z = 3.31, p = 0.0009, Wilcoxon test). Hepatopancreas arginase activity decreased after feeding from 14.5 ± 1.35 to 6.58 ± 0.26 nmol mg^{-1} Pr min^{-1} at six hours postprandial (n = 8, Fig. 4.6a). Arginase activity remained below control levels at 12 hours postprandial in the

hepatopancreas (Fig. 4.6a). In contrast, branchial arginase activity increased at six hours postprandial from 2.16 ± 0.25 to 3.08 ± 0.31 nmol mg^{-1} Pr min^{-1} (Fig. 4.6b, n = 8). By 12 hours postprandial, branchial arginase activity decreased below control levels (Fig. 4.6b).

Urease activity in the gills was higher than the hepatopancreas, where urease activity was nearly undetectable (n = 8, Z = 3.31, p = 0.0009, Wilcoxon test). Hepatopancreas urease activity (0.028 ± 0.008 nmol mg^{-1} Pr min^{-1}) remained unchanged after feeding (Fig. 4.7a, n = 8). Branchial urease activity increased at six hours postprandial from 1.27 ± 0.11 to 3.64 ± 0.11 nmol mg^{-1} Pr min^{-1} (n = 8, Fig. 4.7b). Branchial urease activity remained elevated relative to control levels by 12 hours postprandial (Fig. 4.7b). The detailed statistical summary of main and interactive effects of feeding and time on enzyme activity is presented in Table 4.3.

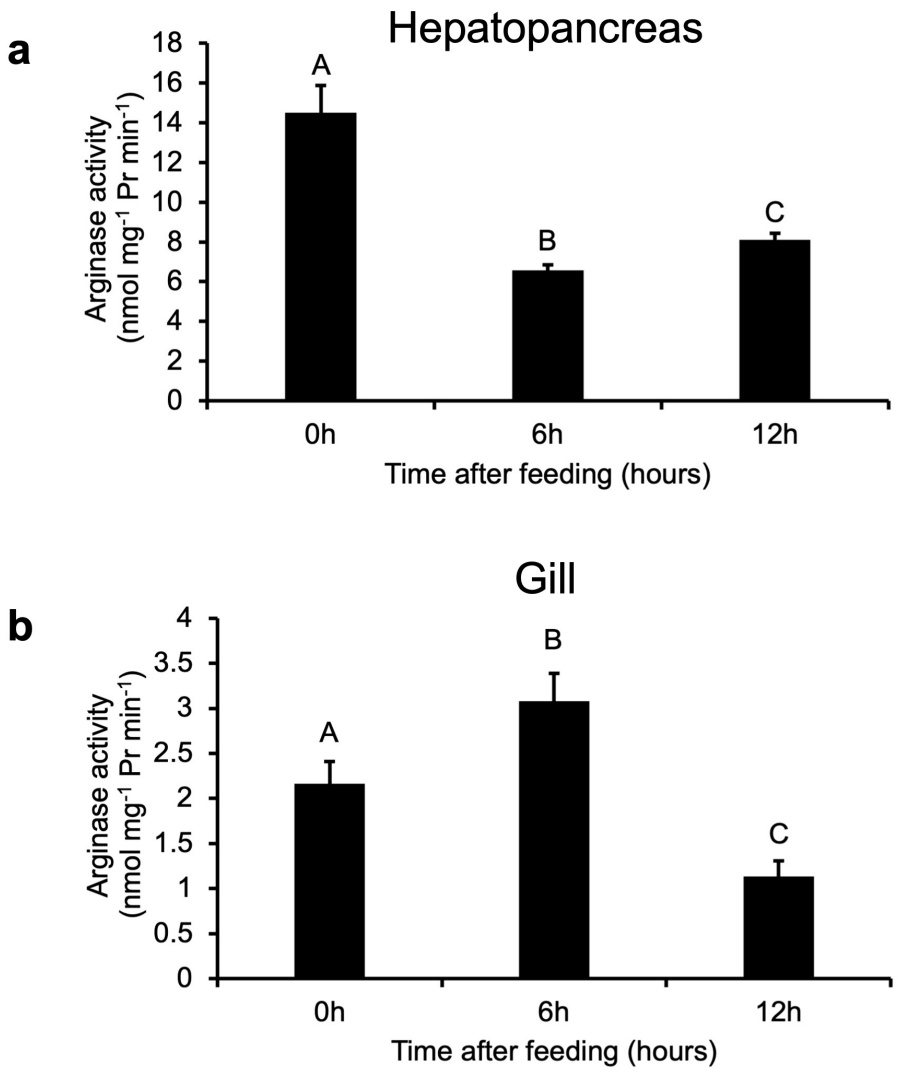

Figure 4.6 Arginase activity in (a) hepatopancreas, and (b) gills at 0, 6, and 12 hours after feeding. Activity is measured based on the production of urea over the assay time. Feeding occurred after sampling 0-hour time point crabs. Values are mean ± SEM. n = 8. Time points with the same letter are not significantly different.

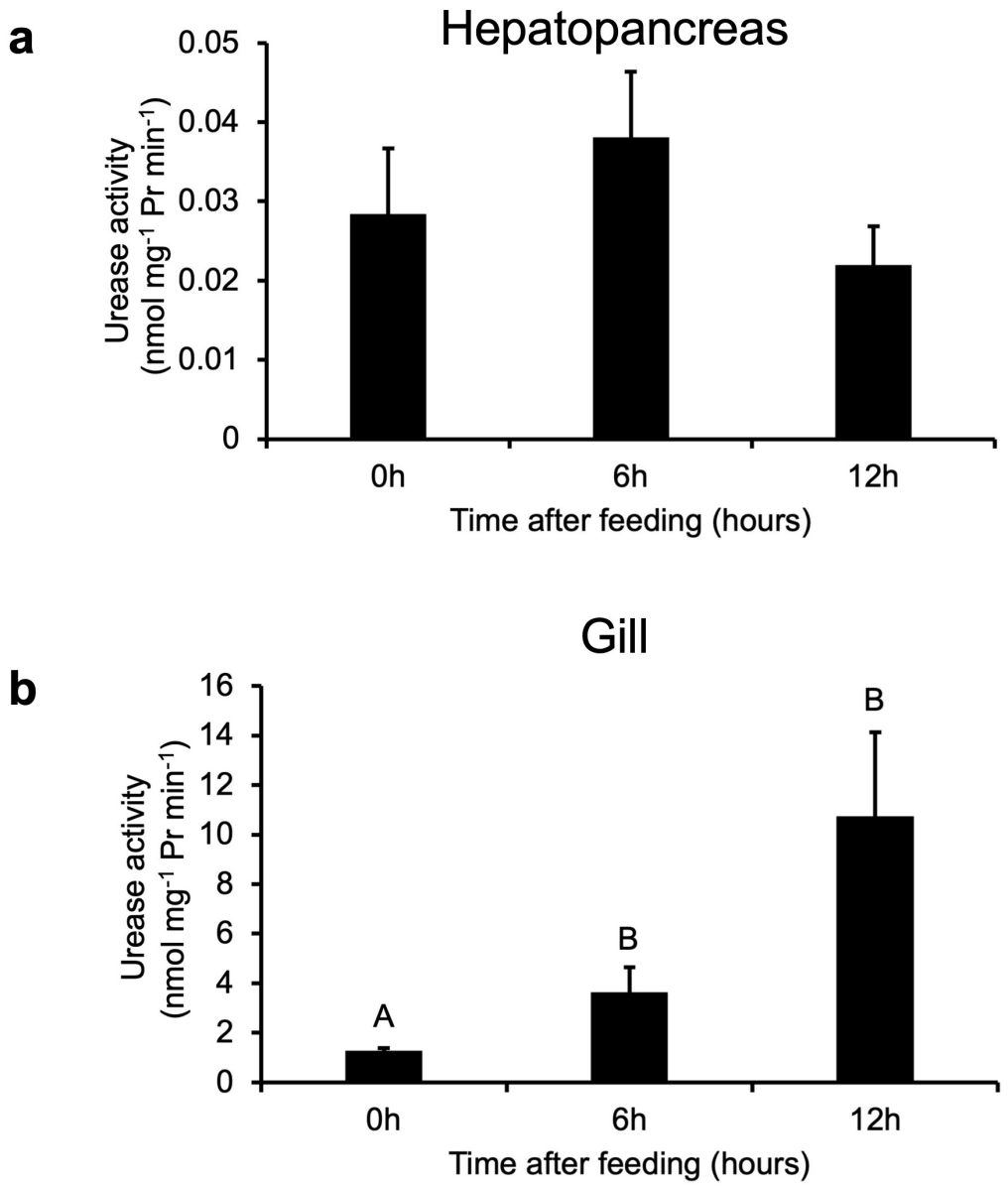

Figure 4.7 Urease activity in (a) hepatopancreas, and (b) gills at 0, 6, and 12 hours after feeding. Activity is measured based on the production of ammonia over the assay time. Feeding occurred after sampling 0-hour time point crabs. Values are mean ± SEM. n = 8. Time points with the same letter are not significantly different.

Table 4.1 Statistical results of two-way repeated measures ANOVAs. Dependent variables assessed include hemolymph HCO_3^-, gastric fluid pH, ammonia flux and net acid flux. The fixed independent variables were time and feeding status. P-values below 0.05 are considered statistically significant and are bolded.

Dependent variable	Independent variable	df	df_{error}	F ratio	p-values
Hemolymph HCO_3^-	Time	4	36	0.99	0.43
	Feeding status	1	9	1.78	0.21
	Interaction	4	36	4.31	**0.0059**
Gastric pH	Time	2	20	2.64	0.1
	Feeding status	1	10	0.28	0.6
	Interaction	2	20	2.17	0.14
Ammonia flux	Time	4	40	30.57	**<0.0001**
	Feeding status	1	10	56.12	**<0.0001**
	Interaction	4	40	39.29	**<0.0001**
Net acid flux	Time	4	40	14.64	**<0.0001**
	Feeding status	1	10	32.08	**0.0002**
	Interaction	4	40	4.22	**0.0061**

Table 4.2 Statistical results of repeated measures MANOVAs. For hemolymph parameters, dependent variables assessed include hemolymph pH, pCO_2, ammonia and urea. For whole animal flux rates, dependent variables assessed include urea and titratable acid flux. The fixed independent variables were time and feeding status. Abbreviations are as follow: Hemo. is hemolymph, Amm. is ammonia, Tit. Is titratable. P-values below 0.05 are considered statistically significant and are bolded.

Dependent variable	Independent variable	df	df_{error}	F ratio	p-values	G-G corr ε	H-F corr. ε
Hemo. pH	Time	2.6	23.38	3.46	**0.038**	0.65	-
	Feeding status	1	9	4.09	0.21		
	Interaction	2.6	23.38	3.36	**0.042**		
Hemo. pCO_2	Time	2.68	24.13	3.43	**0.038**	0.67	-
	Feeding status	1	9	4.09	0.074		
	Interaction	2.68	24.13	5.44	**0.00067**		
Hemo. Amm.	Time	2.07	18.65	12.5	**0.0003**	0.52	-
	Feeding status	1	9	10.71	**0.0096**		
	Interaction	2.07	18.65	6.5	**0.0068**		
Hemo. Urea	Time	4	36	10.05	**<0.0001**	-	1
	Feeding status	1	9	11.4	**0.0082**		
	Interaction	4	36	11.68	**<0.0001**		
Urea flux	Time	2.2	22	3.43	**0.047**	0.55	-
	Feeding status	1	10	3.99	0.07		
	Interaction	2.2	22	3.43	**0.047**		
Tit. acid flux	Time	1.76	17.57	12.73	**0.0006**	0.44	-
	Feeding status	1	10	23.93	**0.0006**		
	Interaction	1.76	17.57	1.8	0.1958		

Table 4.3 Statistical results of one-way ANOVAs and Kruskal-Wallis test. Dependent variables assessed by one-way ANOVA include branchial urease and arginase activity and hepatopancreas urease activity. Dependent variables assessed by Kruskal-Wallis test include hepatopancreas arginase activity. The fixed independent variable was time after feeding. P-values below 0.05 are considered statistically significant and are bolded.

One-way ANOVA						
Dependent variable	Independent variable	Tissue	df	df_{error}	F ratio	p-values
Urease activity	Time	Gill	2	21	15.78	**<0.0001**
Arginase activity	Time	Gill	2	21	15.19	**<0.0001**
Urease activity	Time	HP	2	21	1.24	0.31
Kruskal-Wallis test						
Dependent variable	Independent variable	Tissue	df	X^2	p-values	
Arginase activity	Time	HP	2	18.62	**<0.0001**	

Discussion

The present study examined the effect of feeding on acid-base and nitrogen regulation in a marine decapod crustacean, *Carcinus maenas*. We present the first study examining acid-base and nitrogen regulatory changes in a crustacean over the complete course of digestion. We did not find evidence supporting our hypothesis that postprandial gastric acidification would lead to changes in hemolymph acid-base status of *C. maenas*. In contrast, our results support the hypothesis that feeding causes changes in acid-base and nitrogen homeostasis through increased aerobic metabolism due to the specific dynamic action of food. Finally, we also found evidence that compensation of postprandial acid-base and ammonia challenges occurs through increased acid and ammonia efflux from the animal and the synthesis of urea for ammonia detoxification.

Postprandial gastric acidification

Digestive gastric acid secretion may lead to changes in extracellular acid-base status (Niv and Fraser, 2002; Wang et al., 2001; Wood, 2019). In contrast to vertebrates that experience an alkaline tide, our results suggest that *C. maenas* does not alter its extracellular acid-base status due to gastric acid secretion after feeding. The gastric fluid pH of *C. maena*s (pH ~6) measured in the present study are within the range reported for other crustaceans (pH 4.7-6.7) (Linton and Greenaway, 2004; Vogt, 2021). Postprandial gastric fluid sampling demonstrated that *C. maenas* maintains

gastric fluid pH stable after feeding; however, the abundance of proteins in the food likely led to a slight buffering of the gastric fluids. In fact, the buffering effect of food has been documented in teleost fishes, where the gastric chyme pH typically alkalizes after feeding (Wood, 2019). This suggests that after feeding *C. maenas* may secrete acid into the cardiac stomach to counter protein buffering and maintain gastric fluid pH stable. It would be expected that any postprandial gastric acid secretion would be accompanied by an equimolar transport of base (HCO_3^-) into the extracellular fluids to maintain intracellular pH as seen in vertebrate gastric cells (Niv and Fraser, 2002). Results from the current study show that *C. maenas* did not accumulate hemolymph HCO_3^- and did not experience an alkalosis after feeding despite maintaining gastric fluid pH (Fig. 4.2), indicating that postprandial gastric pH regulation does not alter postprandial extracellular acid-base status. It is possible that different regions of the digestive tract are secreting base to compensate for gastric acid secretion as suggested in gulf toadfish, *Opsanus beta* (Taylor and Grosell, 2009), however, this is speculative and requires further studies on acid-base equivalent transport by different regions of the crustacean digestive tract.

Postprandial acidosis and ammonia accumulation

Aerobic metabolism leads to nitrogenous waste and acid production (Pörtner, 1989). Feeding induces a 3-fold increase in metabolic rate of *C. maenas* under similar experimental conditions (temperature and feeding protocol) to the present study (McGaw and Penney, 2014). Our measurements of hemolymph pH and ammonia after feeding are consistent with the prediction of increased acid and nitrogenous waste production due to aerobic metabolism. The measured accumulation of ammonia observed in the current study also corroborates with the previous hypotheses of several crustacean aquaculture studies, suggesting an increased postprandial ammoniagenesis due to protein catabolism (Crear and Forteath, 2002; Koshio et al., 1993; Ponce-Palafox et al., 2017; Radford et al., 2004; Rosas et al., 1996; Wang et al., 2019). Regarding acid-base status, the increased hemolymph pCO_2 alongside the observed decrease in extracellular pH and stable HCO_3^- indicates the occurrence of postprandial respiratory acidosis. To our knowledge, a respiratory acidosis after feeding has not been previously observed in any animal. A postprandial acidosis has been previously observed in the agastric killifish *Fundulus heteroclitus* (Wood et al., 2010); however, the acidosis in this fish was metabolic in nature (i.e., reduction in extracellular HCO_3^-) and not associated with an increase in extracellular pCO_2 levels. Interestingly, the

respiratory acidosis generated by feeding in the current study is comparable to the degree of respiratory acidosis (pH change) reported during a 6 hour air exposure and 6 hour environmental hypercapnia exposure (1% CO_2) in *C. maenas* (Fehsenfeld and Weihrauch, 2016a; Simonik and Henry, 2014). Respiratory acidosis in crustaceans has typically been associated with impaired gas exchange such as during emersion or from uptake of environmental CO_2 during exposure to environmental hypercapnia (Fehsenfeld and Weihrauch, 2016a; Fehsenfeld and Weihrauch, 2017; Simonik and Henry, 2014). Postprandial respiratory acidosis in this study was not associated with elevated environmental CO_2 and unlikely associated with impairment of gas exchange. In fact, feeding in crustaceans generally leads to an increased heart rate, cardiac output, and ventilation rate (McGaw, 2005; McGaw, 2006a; McGaw and Reiber, 2000), which would actually improve CO_2 excretion. When the cardiorespiratory changes are considered together with our measured acid flux rates, it is evident that the acidosis experienced after feeding is due to increased metabolically derived acid production and not impaired gas exchange or CO_2 influx as seen during emersion and hypercapnia.

Postprandial acid-base compensatory mechanisms

Crustaceans typically counter extracellular acidosis by several strategies including respiratory CO_2 excretion, H^+ excretion by Na^+/H^+ exchange, ammonia excretion, and accumulation of extracellular HCO_3^- as a buffer by Cl^-/HCO_3^- exchange (Fehsenfeld and Weihrauch, 2017; Wheatly and Henry, 1992). In the present study, an increase in ammonia and titratable acid excretion but not accumulation of extracellular HCO_3^- accompanied feeding (Fig. 4.2b, Fig. 4.3c). Although ammonia can be excreted as a weak acid (NH_4^+) or base (NH_3), Weihrauch et al. (1998) demonstrated that branchial ammonia excretion in *C. maenas* occurs predominantly by transporter mediated excretion of ionic NH_4^+ at physiologically relevant hemolymph ammonia levels. In the present study, ammonia accounted for 73% of the net acid excretion with the remainder coming from titratable acidity. To our knowledge, titratable acid and ammonia flux rates have not been simultaneously measured in crustaceans during an acid-base stress. Similar measurements have been performed on fish and, unlike in *C. maenas*, such a heavy reliance on ammonia excretion for net acid-base flux has not been reported (Bucking and Wood, 2008; Cooper and Wilson, 2008; Taylor and Grosell, 2009; Wood and Bucking, 2011; Wood et al., 2007; Wood et al., 2010). Based on the previously reported increases in O_2 consumption found in *C. maenas* after feeding,

postprandial respiratory CO_2 excretion was also likely increased as branchial O_2 uptake would be paralleled by CO_2 excretion (McGaw and Penney, 2014). Therefore, the postprandial compensatory acid-base response appears to be achieved through a combination of changes in CO_2, ammonia, and a slight amount of titratable acid excretion.

The compensatory response to the postprandial respiratory acidosis identified in the present study differs from the response to emersion and hypercapnia mediated respiratory acidosis. In contrast to postprandial responses, compensation of respiratory acidosis during emersion or environmental hypercapnia in *C. maenas* appears primarily dependent on extracellular HCO_3^- accumulation to buffer the increase in extracellular pCO_2 (Fehsenfeld and Weihrauch, 2013; Fehsenfeld and Weihrauch, 2016a; Simonik and Henry, 2014; Truchot, 1975). In addition, a potential role for ammonia excretion in compensation of acidosis during environmental hypercapnia has been demonstrated (Allen et al., 2020; Fehsenfeld and Weihrauch, 2013; Weihrauch and Allen, 2018). During emersion ammonia excretion is not important in acidosis compensation as the gills do not have an abundance of water to facilitate ammonia excretion (Simonik and Henry, 2014). Finally, titratable acid flux into the branchial chamber has not been measured during emersion, but during hypercapnia, titratable acid flux does not change when measured using single endpoint titrations (Truchot, 1979). As titratable acid and ammonia fluxes have not been simultaneously measured in studies on the effects of hypercapnia and emersion on acid-base regulation, it is difficult to determine if, in general, ammonia is the predominant contributor to net acid flux in *C. maenas* or whether this mechanism is specific to postprandial recovery. Overall, our results show that postprandial acidosis recovery is more heavily dependent on acid excretion compared to extrinsically derived respiratory acidosis, which is more dependent on HCO_3^- buffering. The molecular mechanism responsible for postprandial acid secretion remains to be investigated; however, based on previous mechanistic studies on unfed *C. maenas* it likely involves a combination of Na^+/K^+ ATPase, Na^+/H^+ exchangers, Rh proteins, V-type H^+-ATPase, and vesicular ammonia trafficking (Fehsenfeld and Weihrauch, 2016a; Weihrauch et al., 2002).

Ammonia detoxification

Accumulation of ammonia leads to toxic effects in most animals, including crustaceans, and must be effectively excreted or detoxified (reviewed in Larsen et al., 2014; Weihrauch et al., 2017). As mentioned previously, the increase in postprandial aerobic metabolism led to a two-fold

increase in hemolymph ammonia levels. To compensate for extracellular ammonia loads, *C. maenas* increased ammonia excretion 18 times over control values in the first six hours after feeding. These first six hours of excretion accounted for nearly 50% of ammonia excreted over the entire 36-hour time course, suggesting a high capacity for rapid ammonia excretion. By the completion of the absorption and digestion portion (~first 20 hours for *C. maenas* at 15 ºC; McGaw and Penney, 2014) of the crustacean digestive cycle, 86% of ammonia excreted over the complete digestive cycle had been excreted. As the gills of crustaceans are the major site of ammonia excretion (Cameron and Batterton, 1978), one might assume the majority of postprandial ammonia excretion is due to branchial fluxes. However, previous studies using isolated gill perfusions of brackish water *C. maenas*, demonstrated that an increase of hemolymph ammonia from 300 to 640 μmol L^{-1}, as measured in this study after feeding, only results in a roughly two-fold increase in branchial ammonia excretion (Weihrauch et al., 1998). Although this gill perfusion study was done on brackish water *C. maenas* and the present study on seawater *C. maenas*, it has been shown that ammonia excretion between isolated gill preparations does not vary with salinity (Fehsenfeld and Weihrauch, 2016a). The discrepancy between previous gill perfusion studies and the whole animal ammonia fluxes in the present study may have several explanations. First, there may be some sort of stimulatory effect that is not present during gill perfusion that increases postprandial branchial ammonia excretion such as hormones. Second, there may be a considerable contribution to postprandial ammonia excretion by a non-branchial tissue, such as the antennal glands or intestine. Finally, catabolism of nitrogenous compounds within the gill after feeding may increase the branchial ammonia excretion rates without changing hemolymph ammonia levels such as urease-mediated production of ammonia (see urease mechanism below). Although it is not abundantly clear how *C. maenas* achieves such a rapid ammonia clearance after feeding, our results suggest that detoxification of ammonia is being primarily achieved through the direct excretion of ammonia.

Several studies have demonstrated that urea may be produced as a mode of detoxification when hemolymph ammonia increases in crustaceans (Cheng et al., 2004; Hong et al., 2007; Martin et al., 2011; Ren et al., 2015). In *C. maenas*, hemolymph urea increased approximately seven-fold at six hours postprandial. At this time point, urea accounted for roughly 1/3 of measured hemolymph nitrogen. In contrast to hemolymph urea, urea excretion rates did not significantly change after

feeding. Despite stable urea excretion rates, hemolymph urea eventually returned to control levels by 36 hours postprandial. Based on calculations of dietary urea concentrations, the maximum increase in hemolymph urea that could be achieved directly from dietary urea uptake was a mere ~6 μmol L^{-1}. Taken together, these results suggest an increase in postprandial urea synthesis and, due to the stable urea excretion rates, a subsequent conversion into another nitrogenous product, rather than direct excretion.

Urea can be synthesized through the ornithine-urea cycle, uricolysis, or arginolysis (Wright, 1995). Crustaceans are believed to lack a functional urea cycle (Claybrook, 1983). Additionally, arginine is an essential amino acid in crustaceans that cannot be synthesized and must be attained through feeding (Claybrook, 1983). Therefore, the increase in hemolymph urea in the present study must either be derived through uricolysis or arginolysis. To determine whether urea was produced due to the increase in dietary arginine uptake, branchial and hepatopancreatic arginase activity levels were measured. At six hours postprandial, arginase activity decreased in the hepatopancreas. Due to the importance of the hepatopancreas in nutrient uptake (i.e. amino acids) (Vogt, 2021), this reduction in arginase activity may minimize catabolism during arginine uptake so that this essential amino acid can be distributed to other tissues where arginine stores are important. At the gills, a 50% increase in arginase activity was detected at six hours postprandial and decreased below baseline levels at 12 hours postprandial. This result suggests a potential importance of arginine in fuelling early postprandial branchial metabolism. However, due to the high branchial urease activity, the gills were unlikely to significantly contribute to the measured increase in hemolymph urea. The increases in postprandial hemolymph urea are likely a result of arginolysis of consumed arginine or uricolysis. In aquatic crustaceans, the hepatopancreas activity of xanthine dehydrogenase and xanthine oxidase are comparable to terrestrial crustaceans, suggesting an ability to produce uric acid (Lallier and Walsh, 1991). Additionally, uricase activity is present in the hepatopancreas of aquatic crustaceans, allowing for uricolysis (Lallier and Walsh, 1991). In *C. maenas*, hypoxia has been shown to stimulate the synthesis and accumulation of extracellular uric acid, demonstrating a capacity to synthesize this nitrogenous waste (Lallier and Truchot, 1989). A role for uric acid in ammonia detoxification has been suggested during ammonia stress in the Kumura shrimp, *Marsupenaeus japonicus*, where elevated environmental ammonia leads to catabolism of uric acid and increases in urea levels in the hepatopancreas and hemolymph (Cheng

et al., 2004). Taken together, it is conceivable that urea synthesis and ammonia detoxification may partially occur through uricolytic routes.

Beside amino acid catabolism, ammonia can be produced through the breakdown of urea by urease. As no considerable increase in urea excretion was detected after feeding, urease activity was measured to determine whether urea was eventually converted back to ammonia for excretion. Hepatopancreas urease activity in *C. maenas* was nearly undetectable and unchanged after feeding as has been previously reported in other crustaceans (Hanlon, 1975). Interestingly, a substantial urease activity was detected in the gills relative to hepatopancreas. Branchial urease activity significantly increased after feeding and corresponds with decreasing hemolymph urea, suggesting the conversion of urea to ammonia at the gills. Once urea is converted to ammonia at the gills, ammonia can be readily excreted into the environment down the ammonia gradient. A similar mechanism has been proposed in the terrestrial crab, *Gecarcoidea natalis*, where uric acid is catabolized in the branchiostegite into urea and subsequently transported into the gills through the hemolymph where urease converts urea into ammonia for direct excretion (Linton and Greenaway, 1998; Linton et al., 2017). While energetically costly, this mechanism would work to maintain circulating ammonia levels reduced and avoid ammonia toxicity.

Conclusions

In summary, we have provided a fundamental understanding of the effects of feeding on acid-base regulation and nitrogen homeostasis in a decapod crustacean, *C. maenas*. We found that *C. maenas* does not experience a postprandial alkaline tide but experiences a respiratory acidosis and accumulation of ammonia presumably due to increased acid production related to aerobic metabolism. Acidosis compensation occurred through acid excretion predominantly as ammonia. We found that postprandial increases in extracellular ammonia may be detoxified through the synthesis of urea, which is subsequently catabolized into ammonia by the gills for direct excretion. Future research will address the molecular mechanisms in the gills that are activated after feeding to enhance acid and ammonia excretion. Based on our results, it would be interesting for future studies to address the interactive effects of feeding and other extrinsic acid-base stressors (e.g., elevated CO_2) as this could have direct implications of our understanding of crustacean acid-base regulation in intensive recirculating aquaculture systems, tidal regions, or future ocean acidification.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

Despite crustaceans being the second most valuable contributor to global fisheries and aquaculture (FAO 2020), our understanding of the effects of global change on decapods and acid-base stressors in crustacean aquaculture is still rather limited. This thesis set out to yield new insights into how intrinsic and extrinsic acid-base stressors that may be experienced in intensive aquaculture and due to global change impact acid-base regulation and physiological responses in decapod crustaceans. The experimental chapters of the thesis focused on the effects of global change relevant freshwater acidification on the Chinese mitten crab, *Eriocheir sinensis* (Chapter 2), the effects of elevated CO_2 in recirculating aquaculture systems on acid-base regulation and aquaculture productivity in the Whiteleg shrimp, *Litopenaeus vannamei* (Chapter 3), and the effects of feeding on acid-base regulation in the green shore crab, *Carcinus maenas* (Chapter 4). Chapter 5 will highlight the major findings of each thesis chapter, how these different studies are interlinked in our understanding of crustacean acid-base regulation and future directions.

Effects of elevated CO_2 on decapods

Since the early 2000s, there have been hundreds of studies on the effects of ocean acidification on marine life (Riebesell and Gattuso, 2014). Various studies on crustaceans have reported negative effects at CO_2 levels expected for the end of the century in marine systems (~1000 µatm) (Bednaršek et al., 2021). In aquaculture systems, CO_2 tensions often exceed levels expected for ocean acidification and which are associated with physiological impairment (Skov, 2019). Interestingly, the crustacean aquaculture industry has operated under these conditions for decades without reports in the scientific literature of severe negative effects due to elevated CO_2. Similarly, the CO_2 tension in freshwater systems, particularly streams and rivers on average, also exceeds 3000 µatm and may be further acidified by global change possibly threatening freshwater crustaceans (Hasler et al., 2016; Raymond et al., 2013). The first two experimental chapters of this thesis investigated how acid-base regulation and physiological responses are influenced by elevated CO_2 in the context of global change mediated freshwater acidification and intensive aquaculture.

In chapter 2, I hypothesized that as freshwater systems experience normal fluctuations in CO_2, then freshwater inhabitants must be well adept at tolerating increases in CO_2 and face minimal

physiological impairment. Chapter 2 demonstrated that *E. sinensis* could fully compensate for deviations in extracellular pH resulting from exposure to 5103 µatm pCO_2 through the accumulation of extracellular HCO_3^-, which is the general response seen in decapods exposed to elevated CO_2 (Fehsenfeld and Weihrauch, 2017). Further results demonstrated that while acid-base status was compensated, there was a cost associated with acid-base regulation that may have altered energetic allocation, resulting in increased mortality and decreases in calcification, metabolic rate, and locomotory behaviour. This concept of energetic reallocation leading to declines in certain physiological processes following exposure to hypercapnia has also been suggested in various marine invertebrates, including lobster, sea urchins, and sea stars (Pan et al., 2015; Small et al., 2020; Stumpp et al., 2012; Wood et al., 2008). As highlighted throughout the discussion of chapter 2, when compared to the vast crustacean literature regarding ocean acidification, some marine decapods have similar responses to *E. sinensis* while others were more tolerant, making generalizations of decapod responses to global change quite difficult. Being the first study on global change relevant freshwater acidification in a decapod or any other species other than pink salmon (*Oncorhynchus gorbuscha*; Ou et al., 2015) made comparisons with freshwater species difficult. However, the common response between the pink salmon and the present study on *E. sinensis* is that global change-driven freshwater acidification may cause physiological impairment that could have negative implications on animal fitness. Despite negative physiological effects observed in chapter 2, this study was rather acute and leaves uncertainty on the long-term implications of elevated CO_2 on *E. sinensis*. This was one of the first studies exploring global change relevant freshwater acidification on aquatic animals and clearly, there is a lot of work to be done to further understand what implications global change-driven CO_2 will have on freshwater crustaceans and other taxa.

Chapter 3 investigated whether elevated CO_2 in recirculating aquaculture systems affects acid-base regulation and if it truly impairs growth and survival, as suggested by ocean acidification research. Our results showed that, when *L. vannamei* is taken from high CO_2 aquaculture waters and placed under "normal" CO_2 tensions, extracellular pH increased to normal acid-base status levels reported for this species, suggesting an uncompensated chronic acidosis under aquaculture conditions. As few studies on decapods have performed long-term exposures to CO_2, it remains unclear whether loss of pH regulation is a common occurrence. At least in the stenohaline crab

Cancer pagurus exposure to 1000 µatm pCO_2 also lead to loss of pH balance after 3 months, while animals survived at least another 6 months (Whiteley et al., 2018). In contrast, this same study showed *C. maenas* could maintain pH balance over 12 months. It is possible that chronic CO_2 exposure may lead to an energetic compromise where complete pH recovery is lost so that other physiological processes also essential for survival can be fueled. Chapter 3 also demonstrated that no effect on growth or survival was experienced by juvenile shrimp reared in half-strength seawater with low, medium, or high CO_2 tensions (1000, 2234, 4788 µatm pCO_2). This result was the opposite of what has been reported for *L. vannamei* reared at these similar CO_2 tensions but in full-strength seawater (Muralisankar et al., 2021). These discrepancies between responses of *L. vannamei* reared at full-strength and half-strength (this thesis) seawater may suggest that intra-species responses to elevated CO_2 might depend on environmental salinity providing a very intriguing future avenue of research for aquaculture growth optimization in response to elevated CO_2.

Feeding physiology and implications on aquaculture

Acid-base regulation in decapods has been studied for decades and, although it is known in other animal taxa that feeding can cause deviations in pH homeostasis, the consequences of feeding on crustacean acid-base status were unknown. Chapter 4 addressed this shortcoming by investigating how acid-base status, nitrogen levels, gastric pH, and acid-base fluxes changed after feeding in a seawater crustacean. Results demonstrated an acute postprandial respiratory acidosis that was compensated through increased ammonia and titratable acid fluxes but not the accumulation of HCO_3^-. Measurements of gastric pH gave no indication that the extracellular acidosis was being derived due to changes in gastric fluid acid-base status. Instead, the timing of acid-base changes followed a similar pattern to postprandial oxygen consumption shown by McGaw and Penney (McGaw and Penney, 2014), suggesting that acidosis is linked to acid production from aerobic metabolism. Overall, this chapter provides a fundamental understanding of acid-base changes due to feeding and demonstrates that feeding like elevated CO_2 causes respiratory acidosis but is compensated differently due to the abundance of ammonia, which can be excreted as an acid-base equivalent. This chapter shows that, while intrinsic and extrinsic acid-base stressors may produce a common acid-base challenge (e.g. respiratory acidosis), the compensatory responses may vary based on the source of the challenge. Therefore, one might

expect that changes in branchial mRNA and protein expression after feeding might differ from that experienced during hypercapnia exposure despite both eliciting a respiratory acidosis. While I had hoped to further understand whether the digestive tract influences extracellular acid-base status, there was no apparent change at least in the stomach and attempts to measure pH in other regions of the digestive tract were unsuccessful. Future work measuring mRNA and protein expression changes along the digestive tract and the capacity to acidify or alkalize luminal fluid in tissues isolated from fed and fasted animals may provide better insights into the role of the digestive tract in whole animal acid-base regulation.

Impact and future directions

Chapter 2 has shown that the levels of CO_2 expected from global change-driven freshwater acidification may pose challenges to aquatic species like ocean acidification. While I caution against making bold claims that species are threatened with extinction based on rather acute studies, it suggests that long-term studies and investigations into a variety of freshwater animal taxa are necessary to gain better insights into the susceptibility of freshwater species to global change. Along the lines of long-term studies, transgenerational adaptation to elevated CO_2 has been shown in some marine species and could aid in providing further insights into the potential susceptibility of different species to freshwater acidification.

Chapters 2 and 3 have shown that responses to elevated CO_2 are quite diverse within decapods with environmental salinity and osmoregulatory strategy, potentially influencing CO_2 tolerance and physiological responses. Chapter 3 demonstrated that when reared in a low salinity environment, impairment of growth and survival was not present, suggesting that activation of osmoregulatory machinery may influence CO_2 tolerance. Chapter 2 suggested that energetic demands of acid-base regulation during high CO_2 exposure may lead to impairment of other physiological processes. As osmoregulation may have substantial energetic costs, it may limit the energetic availability for acid-base regulation and influence CO_2 tolerance and physiological responses to elevated CO_2. Clearly, there is an interaction between osmoregulation and responses to elevated CO_2 that must be further explored and could shed light on species susceptibility to global change and improvement of aquaculture productivity.

Chapter 4 developed the fundamental understanding of acid-base responses to feeding, as typically the effect of acid-base stressors on extracellular acid-base status is measured on fasted

animals. Previous decapod studies have shown that the physiological demand of feeding and acid-base stressors like emersion or salinity change leads to increased mortality (McGaw, 2006b; McGaw et al., 2009). McGaw et al.(2009) also showed that the degree of respiratory acidosis experienced during emersion is greater if crabs had been fed up to 12 hours before emersion. With some basic understanding of postprandial effects on acid-base status now established in crustaceans, this work can now be directly applied to aquaculture to understand whether feeding and acid-base stressors that are commonly found in aquaculture settings like CO_2 interact to create more extreme acid-base challenges and alter mortality. These follow-up studies could then help determine acceptable levels of CO_2 in aquaculture settings to minimize any interactive effects with feeding stress and any associated effects on aquaculture productivity.

In conclusion, while this thesis focused on decapod crustaceans, the challenges investigated are faced by other animal taxa like fishes and invertebrates. Therefore, while responses may differ between animal taxa many of the fundamental principles addressed in this thesis and questions raised could provide comparisons and a better understanding of the diverse physiology of other animal groups.

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