

Relating the Acid-Base Compensatory Strategies of Brachyuran
Crabs to their Environments and Life Histories

by

Garett Joseph Patrick Allen

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

Department of Biological Sciences

University of Manitoba

Winnipeg

Copyright © 2022 by Garett Joseph Patrick Allen

Abstract

Brachyuran crabs are amongst the most habitat-diverse groups of animals on Earth. Different environments are associated with specific challenges that their inhabitants must overcome throughout their species' history to maintain physiological homeostasis, including the pH of the body fluids. The frequency and magnitude of acid-base challenges are environmentally dependent with some ecosystems experiencing regular seasonal fluctuations such as inland waters or coasts, some having diurnal fluctuations as occur in intertidal zones, whereas marine environments may face irregular but relatively large shifts from coastal upwellings or permanent extreme stress near hydrothermal vents. Given that brachyurans permanently inhabit these ecosystems, their acid-base regulatory processes and stress tolerances are presumably broad making them an interesting animal group to study connections between a species' life-history relates and physiology. Despite the diverse nature of brachyurans, their acid-base regulatory systems and stress tolerances are generally poorly understood. To date, only a few osmoregulatory-capable species are beginning to be characterized at the whole animal and mechanistic levels due to their historical use as osmoregulatory models.

This dissertation serves to broaden our understanding of brachyuran physiology and identify their various means to cope with acid-base challenges. Although the antennal glands of osmoregulatory-capable species are considered to be of negligible importance to their acid-base regulation, those of a typical stenohaline osmoconforming crab, *Metacarcinus magister*, appear to compliment the gills by regulating systemic bicarbonate ions (HCO_3^-) and eliminating acid-equivalents. The shallow hydrothermal vent crab, *Xenograpsus testudinatus* was found to be extremely hypercapnia-tolerant being capable of immense HCO_3^- accumulation and excreting CO_2 against inwardly-directed P_{CO_2} gradients – both of which seem impossible in other investigated animals. The semi-terrestrial crab, *Helice formosensis*, relies on the antennal glands and branchial action to prevent the accumulation of nitrogenous wastes that would otherwise disrupt their acid-base homeostasis, with some indications that they use the alanine-glucose cycle which was previously believed to be absent in aquatic species. While these results create many open questions, they provide clear examples of different adaptations and even organ usage within the investigated brachyuran species, several of which are fundamentally different from popular model species such as *Carcinus maenas* and *Callinectes sapidus*.

Acknowledgements

Completing this dissertation was an absolute adventure. Between conferences and fieldwork, I've travelled over 88,000 km, lost partial feeling in one of my feet, got seriously questioned several times in airports about my animal imports, and made so many wonderful friends both in and out of the academic community. It is impossible to individually thank everyone who has helped make this experience into what it became, including my funding sources (NSERC/UoM FWSP), but I hope that I have returned at least some equivalent to you.

They say to save the best for last, but it would feel wrong not to start with Dirk. As an advisor, you've given me an endless number of opportunities to explore my interests. You have listened when I needed to speak and spoke when I needed to listen – and for that, I am thankful. More importantly, you've been a great friend. Between the travels, the drinks, and the gigs I cannot even hope to explain how happy I am to have decided to work with you.

While every committee helps guide students through their program, mine has gone above and beyond in and out of our meetings. Gary, Jay, and Peter – you have always entertained my questions even when they are not remotely related to my thesis.

To my lab-mates, past and present, within Canada and Taiwan, there have been so many laughs, smiles, tears, and surprises that we have shared throughout the years. Alex... from the day I put the ammonia plates in backward you have always been there for me. You have a wonderful family and are as great a father as you are a friend. Thank you for all your help.

I guess that leaves my family – the people who will always love you even though you never shut up about crabs and lobsters. Perhaps I am lucky that most of you are Nova Scotian – we all do have at least some love for the weird, shelled critters. I feel that I have missed so much while being gone... but strangely it also feels like I have never left because of how much support you all have given me. Luckily, my family here has done an excellent job filling in for you all in person – you will have to thank them when you eventually meet. Although you have only met Mikyla, I'm sure you'll like the Nash family (and our many furry family members) just as much. Speaking of Mikyla, you've been the one that has kept me on track through the tail end of this adventure as you also finish yours. I can't wait to see where the next one takes us as you put up with my foolishness day after day. While we never know what life entails, I am sure it will at least bring us somewhere warmer than Winnipeg!

Dedication

Graduate school and downstream academic work can lead to some of our best life experiences. Travelling for conferences, pursuing genuinely interesting topics, piecing together little scientific puzzles the best we can... there is plenty of excitement given you have the passion. Unfortunately, it can also lead to some of our worst experiences. Constant pressure, pursuing perfection, a virtually unplannable future, and often moving far from friends and family... it's easy to miss the important parts of life that you can never get back. It is not difficult to find students and faculty experiencing a decline in their mental health as they go down the academic path. For some of the most educated members of society, it is surprising how it still seems to feel that seeking assistance is a sign of weakness.

I hope that those that read this document find what they need to get through their tough times. Maybe it is your friends or your family, maybe a pet, maybe a hobby, maybe it's a specialist, maybe you don't know what or who it is yet, but I do believe everyone has something that can brighten up their lives. I've certainly had personal obstacles throughout my journey that I am not sure I would have overcome without my support network. While this includes my entire family, it especially extends to my grandparents, James and Hilda. You have always told me to do what I love, regardless of what the world may think. You taught me that life is not about being perfect, it's about being whoever and whatever you become. Truly, you've been the real advisors to my life by just being yourselves. My only regret living how you taught me is that it brought me away from you both when Nan's time ran out – part of me surely left when she did.

Be proud of yourselves, acknowledge the work you've completed, and don't be afraid to take a step outside of your comfort zones. Life is not about being perfect, if anything it's more about learning how beautifully imperfect everyone and everything is.

This thesis is dedicated to my grandparents as well as whatever and whoever it is that helps you make it through your tough times.

Appreciate them for what they do for you, it will always be worth it.

Table of Contents

Abstract.....	i
Acknowledgements.....	ii
Dedication.....	iii
List of tables.....	vii
List of figures.....	vii
Chapter 1: General introduction.....	1
1.1. The interaction between species life history and physiological adaptations.....	1
1.2. General concepts of acid-base regulation.....	1
1.2.1. The importance of acid-base balance	1
1.2.2. The role of buffering agents	2
1.2.3. The role of respiratory gas exchange.....	3
1.2.4. The role of acid-base equivalent transport across epithelia.....	3
1.2.4.1. Acid-base regulation in the mammalian kidney	5
1.2.5. Coordination of regulatory action.....	6
1.3. Brachyuran crustaceans and environmental acid-base challenges.....	7
1.3.1. The broad habitat distribution of brachyurans.....	7
1.3.2. Changing environmental temperature	8
1.3.3. Hypercapnia.....	10
1.3.4. Non-hypercapnic environmental acidification	12
1.3.5. Oxygen bioavailability	15
1.3.6. Salinity.....	17
1.3.7. Emersion.....	20
1.4. Acid-base regulatory organs in brachyuran crabs	22
1.4.1. Branchial tissue.....	22
1.4.2. Antennal glands	30
1.4.3. Hypodermis and exoskeletal system.....	33
1.4.4. Gastrointestinal tract.....	34
1.5. Diversifying the field	34
1.6. Thesis goals and outline	35
Chapter 2: Acid-base compensation under acute acid-stress in an osmoconforming crab, <i>Metacarcinus magister</i>	37
2.1. Abstract	38

2.2. Introduction	39
2.3. Materials and methods	42
2.3.1. Animals.....	42
2.3.2. Whole animal experiments	42
2.3.3. Gill perfusion setup	43
2.3.4. Measuring transbranchial transport rates.....	44
2.3.5. Statistics.....	45
2.4. Results	46
2.4.1. Whole animal response to 200 $\mu\text{mol l}^{-1}$ NH_4Cl	46
2.4.2. Whole animal response to acid-stress and 200 $\mu\text{mol l}^{-1}$ NH_4Cl	46
2.4.3. Baseline transbranchial transport rates of perfused gills	50
2.4.4. Effects of pharmaceutical exposure on perfused gills	52
2.5. Discussion	57
2.5.1. Comparative consequences of acid-stress in aquatic crustaceans	57
2.5.2. Involvement of the gills and antennal glands in acid-base regulation of <i>M. magister</i>	59
2.5.3. Hypothetical mechanism of transbranchial CO_2 transport under control conditions ..	60
2.5.4. Hypothetical mechanism of transbranchial HCO_3^- transport under control conditions	61
2.5.5. Hypothetical mechanism of transbranchial ammonia transport under control conditions.....	63
2.5.6. Effects of acute acid-stress on transbranchial CO_2 and HCO_3^- transport	64
2.5.7. Effects of acute acid-stress on transbranchial ammonia transport	65
2.6. Conclusions	65
Transition to chapter 3	66
Chapter 3: Specialized adaptations allow vent-endemic crabs (<i>Xenograpsus testudinatus</i>) to thrive under extreme environmental hypercapnia.....	67
3.1. Abstract	68
3.2. Introduction	69
3.3. Materials and methods	72
3.3.1. Animals and acclimatory conditions	72
3.3.2. Hemolymph and water sampling.....	74
3.3.3. Whole animal ammonia excretion.....	76
3.3.4. Calculation of P_{NH_3}	76

3.3.5. Resting metabolic rate	77
3.3.6. Gill perfusions	78
3.3.7. Statistical analysis.....	79
3.4. Results	79
3.4.1. Hemolymph compensatory acid-base response.....	79
3.4.2. Isolated gill acid-base regulatory capacity under exposure to chronic (pH 6.50) and acute (pH 5.50) acidification	81
3.4.3. Whole animal response to acidification.....	83
3.5. Discussion	84
3.5.1. The compensatory accumulation of extracellular HCO_3^-	84
3.5.2. Excretion of CO_2 against an inwardly directed P_{CO_2} gradient.....	86
3.5.3. The role of ammonia in acid-base regulation	88
3.5.4. Vent-endemic adaptations	90
3.6. Conclusions	92
Transition to chapter 4	93
Chapter 4: Effects of emersion on acid-base regulation, osmoregulation, and nitrogen physiology in the semi-terrestrial mangrove crab, <i>Helice formosensis</i>	94
4.1. Abstract	95
4.2. Introduction	96
4.3. Materials and methods	99
4.3.1. Animal husbandry and experimental conditions	99
4.3.2. Hemolymph and urine carbonate equilibrium	100
4.3.3. Ammonia measurements	101
4.3.4. Major ion composition.....	101
4.3.5. Free amino acid composition.....	101
4.3.6. Whole animal ammonia excretion upon re-immersion	102
4.3.7. Statistical analysis.....	102
4.4. Results	102
4.5. Discussion	112
4.5.1. Immersed physiology	112
4.5.2. Emerged acid-base regulation.....	114
4.5.3. Emerged nitrogen physiology	116
4.5.4. Emerged water conservation	120

4.6. Conclusions	121
Chapter 5: General discussion and thesis summary.....	122
5.1. Comparing acid-base regulation of osmoregulatory-capable and incapable <i>Brachyura</i> .	122
5.2. Co-ordination between the gills and antennal glands.....	124
5.3. Brachyuran diversity extends into their acid-base regulatory strategies.....	126
5.4. Conclusions and future directions	127
Bibliography	129
Appendix and Supplemental Information.....	168

List of tables

Table 3.1. Water physiochemical parameters of acclimatory pH 6.50 (2.7 kPa P _{CO2}) and pH 5.50 (24.6 kPa P _{CO2}) tanks over the 14-day acclimatory periods as well as water samples obtained during animal collection at the vent site (N = 8). Acclimatory tank conditions were monitored every 1 – 2 days throughout experimentation. Data presented as means ± s.e.m; ‘N/a’ represents unmeasured parameters.....	74
--	----

Table 3.2. Major hemolymph cation and anion composition of freshly captured wild <i>X. testudinatus</i> (N = 6) as well as crabs exposed to either pH 6.50 (2.7 kPa P _{CO2} ; N = 6) or pH 5.50 (24.6 kPa P _{CO2} ; N = 6-7) for 14-days. Data presented as mean concentrations (mmol l ⁻¹) ± s.e.m; ‘N/a’ represents unmeasured parameters.	80
---	----

List of figures

Figure 1.1. Cladogram representation of the infraorder of decapod crustaceans based on the phylogenetic results of Wolfe et al. (2019). The infraorders are colour-coded to their more commonly used generalized names. Arrows indicate the gill morphology associated with various decapod infraorders.....	8
--	---

Figure 1.2. Images of an (A) intact phyllobranchiate gill (*ca.* 5 cm length) excised from the shallow hydrothermal vent crab, *X. testudinatus*, as well as a (B) stack of lamellae, (C) an individual lamella, and (D) a demonstration of a cannulated gill prepared for perfusion experiments. All images taken by Allen, G.J.P. under a dissection microscope. 24

Figure 2.1. Hemolymph and urine pH during 12-hours of exposure to either seawater adjusted to pH 8.10 (N = 7) or pH 7.10 (N = 6-7) containing 10 mmol l⁻¹ HEPES and 200 μmol l⁻¹ NH₄Cl. Asterisks indicate a significant difference between the hemolymph and urine pH at the same time point, whereas uppercase and lowercase letters denote significant differences between the pH of hemolymph and urine over time, respectively. 47

Figure 2.2. Hemolymph and urine P_{CO2} during 12-hours of exposure to either seawater adjusted to pH 8.10 (N = 7) or pH 7.10 (N = 6-7) containing 10 mmol l⁻¹ HEPES and 200 μmol l⁻¹ NH₄Cl. Asterisks or listed p-values indicate a significant difference between the hemolymph and urine P_{CO2} at the same time point. 48

Figure 2.3. Hemolymph and urine [HCO₃⁻] during 12-hours of exposure to either seawater adjusted to pH 8.10 (N = 7) or pH 7.10 (N = 6-7) containing 10 mmol l⁻¹ HEPES and 200 μmol l⁻¹ NH₄Cl. Asterisks indicate a significant difference between the hemolymph and urine [HCO₃⁻] at the same time point, whereas lowercase letters denote significant differences between the [HCO₃⁻] of the urine over time. 49

Figure 2.4. Hemolymph and urine [T_{Amm}] during 12-hours of exposure to either seawater adjusted to pH 8.10 (N = 7) or pH 7.10 (N = 3-7) containing 10 mmol l⁻¹ HEPES and 200 μmol l⁻¹ NH₄Cl. Asterisks indicate a significant difference between the hemolymph and urine [T_{Amm}] at the same time point, whereas uppercase letters denote significant differences between the [T_{Amm}] of the hemolymph over time. 50

Figure 2.5. Transport rates of H^+_{Eq} (A), CO_2 (B), HCO_3^- (C), and T_{Amm} (D) across the perfused gills of *M. magister* bathed in HEPES-enriched seawater of either pH 8.10 or 7.10 containing either 0 or 200 $\mu\text{mol l}^{-1}$ NH_4Cl . Asterisks denote significant differences between the transport processes. Data pertaining to crabs exposed to pH 8.10 conditions is based on 29-31 crabs whereas those pertaining to pH 7.10 are based on 36-39 crabs, with only one gill being used per crab per replicate. 52

Figure 2.6. Effects of basolaterally applied pharmaceuticals to the transport rates of H^+_{Eq} (A), CO_2 (B), HCO_3^- (C), and T_{Amm} (D) across the perfused gills of *M. magister* bathed in HEPES-enriched seawater of either pH 8.10 containing 200 $\mu\text{mol l}^{-1}$ NH_4Cl . Asterisks denote significant differences between the transport rate of pharmaceutically exposed gills and non-exposed gills. $N_{Ouabain} = 6$, $N_{KM91104} = 11$, $N_{Colchicine} = 6$, $N_{Acetazolamide} = 6$ 54

Figure 2.7. Effects of basolaterally applied pharmaceuticals to the transport rates of H^+_{Eq} (A), CO_2 (B), HCO_3^- (C), and T_{Amm} (D) across the perfused gills of *M. magister* bathed in HEPES-enriched seawater of either pH 7.10 containing 200 $\mu\text{mol l}^{-1}$ NH_4Cl . Asterisks denote significant differences between the transport rate of pharmaceutically exposed gills and non-exposed gills. $N_{Ouabain} = 8$, $N_{KM91104} = 8$, $N_{Colchicine} = 8$, $N_{Acetazolamide} = 9$ 55

Figure 2.8. Proposed hypothetical model describing the transbranchial transport of H^+ , CO_2 , and HCO_3^- in *M. magister*. Transporters whose presence and/or cellular localization are unconfirmed are indicated by question marks. Active processes requiring the consumption of ATP are denoted by a yellow ‘spark.’ 56

Figure 2.9. Proposed hypothetical model describing the transbranchial transport of ammonia (NH_3/NH_4^+) in *M. magister*. Transporters whose presence and/or cellular localization are unconfirmed are indicated by question marks. Active processes requiring the consumption of ATP are denoted by a yellow ‘spark.’ 57

Figure 3.1. Hemolymph acid-base parameters of *X. testudinatus* acclimated over a 14-day period to seawater acidified to either pH 6.50 (2.7 kPa P_{CO2}; closed circles) or pH 5.50 (24.6 kPa P_{CO2}; open circles). Changes in extracellular pH (A; N = 6), HCO₃⁻ (B; N = 6), P_{CO2} (C; N = 6), and [NH₄⁺] (D; N = 5-7) were measured from pre-branchial hemolymph after 0, 1, 2, 7, and 14 days of acclimation. Environmental P_{CO2} levels are indicated by dashed lines to indicate the presence of inwardly directed P_{CO2} gradients (C). Asterisks denote significance based upon acclimation pH. Upper-case letters denote time-dependent differences within pH 6.50 exposed crabs whereas lower-case letters denote time-dependent differences within pH 5.50 exposed crabs. Data presented as means ± s.e.m, p < 0.05..... 81

Figure 3.2. Capacity of isolated perfused gill 5 of pH 6.50 acclimated *X. testudinatus* to alter pH (A), P_{CO2} (B), and HCO₃⁻ (C) of artificial hemolymph-like saline following a single gill passage. Gills were first exposed to pH 6.50 (2.7 kPa P_{CO2}) to determine their transport capacity under the acclimated condition. Gills were subsequently exposed acutely to pH 5.50 (24.6 kPa P_{CO2}) to observe changes in transport capacity. Degree of hemolymph alkalization by the gill is represented as ΔpH (A). Positive HCO₃⁻ and P_{CO2} excretion rates infer the molecule is lost to the environment as indicated by a lesser presence in the perfusate as compared to initial amounts within perfusion saline. Asterisks denote significance between initial and final pH or significant differences in transport rates depending on pH exposure. Data presented as means ± s.e.m, p < 0.05, N = 6 for all data points. 83

Figure 3.3. Whole animal metabolic rate (A) and ammonia excretion rates (B) were measured to indicate shifts in the crab's metabolism in response to acidification over a 14-day time course acclimation to either pH 6.50 (2.7 kPa P_{CO2}; open circles) or pH 5.50 (24.6 kPa P_{CO2}; closed circles). Metabolic rate (A) was determined as the rate of oxygen consumption per body mass per time (μg O₂ g⁻¹ h⁻¹) using closed-system respirometry (N = 6-9). Ammonia excretion rates (B) were determined as based on accumulation of ammonia within ambient water per body mass per time (μmol NH₄⁺ g⁻¹ h⁻¹; N = 6-9). Asterisks denote significance based upon acclimation pH. Upper-case letters denote time-dependent differences within pH 6.50 exposed crabs whereas

lower-case letters denote time-dependent differences within pH 5.50 exposed crabs. Data presented as means \pm s.e.m, $p < 0.05$ 84

Figure 4.1. pH of hemolymph and urine of immersed ($N_H = 22$, $N_U = 12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H = 18$, $N_U = 18$), 24 ($N_H = 12$, $N_U = 12$), 48 ($N_H = 6$, $N_U = 5$), and 160 ($N_H = 12$, $N_U = 12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M. 104

Figure 4.2. Partial pressure of CO_2 (P_{CO_2}) within the hemolymph and urine of immersed ($N_H = 22$, $N_U = 12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H = 18$, $N_U = 18$), 24 ($N_H = 12$, $N_U = 12$), 48 ($N_H = 6$, $N_U = 5$), and 160 ($N_H = 12$, $N_U = 12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M. 105

Figure 4.3. Concentrations of HCO_3^- within the hemolymph and urine of immersed ($N_H = 22$, $N_U = 12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H = 18$, $N_U = 18$), 24 ($N_H = 12$, $N_U = 12$), 48 ($N_H = 6$, $N_U = 5$), and 160 ($N_H = 12$, $N_U = 12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M..... 106

Figure 4.4. Concentrations of NH_4^+ within the hemolymph and urine of immersed ($N_H = 12$, $N_U = 12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H = 12$, $N_U = 12$), 24 ($N_H = 12$, $N_U = 6$), 48 ($N_H = 6$, $N_U = 5$), and 160 ($N_H = 12$, $N_U = 12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine

osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M..... 107

Figure 4.5. Whole animal ammonia excretion of crabs (N = 5) emersed for 160-hours immediately following re-immersion. Values are represented as the mean \pm S.E.M..... 108

Figure 4.6. Predominant free amino acids in the hemolymph (N = 6–12) and urine (N = 4–12) of immersed (0-h) or emersed crabs over a 160-hour time-course exposure. Where data is not available for urinary concentrations no detectable amino acid presence was found. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M. 109

Figure 4.7. Major ion composition of immersed (0-h) and emersed crabs' hemolymph (N= 6–12 for all ions) and urine (N = 5–12 for all ions) over a 160-hour time-course exposure. Tank parameters are included as comparison to the immersed crabs' environmental conditions at 15 ppt salinity. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M..... 110

Figure 4.8. Osmolality of the hemolymph and urine of immersed (N_H = 22, N_U = 12) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 (N_H = 18, N_U = 12), 24 (N_H = 12, N_U = 6), 48 (N_H = 6, N_U = 5), and 160 (N_H = 12, N_U = 11) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M..... 111

Figure 4.9. Rate of evaporative water loss as measured by changes in emersed crabs' total mass over a time course exposure. Emersed measurements were made after 6 (N = 12), 24 (N = 12),

48 (N = 12), and 160 (N = 24) hours of emersion. Values are represented as the mean \pm S.E.M.

..... 112

S.I. Figure 2.1. Transport rates of H^{+}_{Eq} (A), CO_2 (B), HCO_3^{-} (C), and T_{Amm} (D) across the perfused gills 4, 7, 8, and 9 of *M. magister* bathed in HEPES-enriched seawater of either pH 8.10 or 7.10 containing $0 \mu mol l^{-1} NH_4Cl$ 168

S.I. Figure 2.2. Transport rates of H^{+}_{Eq} (A), CO_2 (B), HCO_3^{-} (C), and T_{Amm} (D) across the perfused gills 4, 7, 8, and 9 of *M. magister* bathed in HEPES-enriched seawater of either pH 8.10 or 7.10 containing $200 \mu mol l^{-1} NH_4Cl$ 169

S.I. Figure 4.1. Hemolymph (N = 6–12) and urinary (N = 4-12) essential amino acid composition of immersed (0-h) and emersed crabs over a 160-hour time-course exposure. Where data in not available for urinary concentrations no detectable amino acid presence was found. Values are represented as the mean \pm S.E.M. 169

S.I. Figure 4.2. Hemolymph (N = 6–12) non-essential amino acid composition of immersed (0-h) and emersed crabs over a 160-hour time-course exposure. Where data in not available for urinary concentrations no detectable amino acid presence was found. Values are represented as the mean \pm S.E.M. 170

S.I. Figure 4.3. Urinary (N = 4–12) non-essential amino acid composition of immersed (0-h) and emersed crabs over a 160-hour time-course exposure. Where data in not available for urinary concentrations no detectable amino acid presence was found. Values are represented as the mean \pm S.E.M. 171

Chapter 1: General introduction

1.1. The interaction between species life history and physiological adaptations

Although organisms can be dramatically different, our collective lives depend on several common needs. One shared need is the maintenance of an internal environment that is sufficient to allow our cellular and enzymatic processes to occur at a sufficient rate to meet at least our basic needs. We generally refer to this process as the maintenance of physiological homeostasis, or more accurately as homeokinesis (Cannon, 1929; Macklem and Seely, 2010). As endogenous and exogenous stressors occur, our regulatory systems respond in an attempt to restore our internal fluids within an appropriate state that is either a specific value or an appropriately stable alternative (Torday, 2015). These regulatory processes have limits that differ between species, allowing certain species to exist in environments or through lifestyles that others cannot. For example, most aquatic animals can only survive within marine environments where ions are readily available whereas a small portion of their species diversity can permanently survive in ion-poor freshwater environments (Martin and Davis, 2001). The variation in the ability of organisms to tolerate stressors and maintain homeostasis stems, at least in part, through adaptations brought on by selective pressures caused by their environmental conditions and the life-histories of species or populations (Kozłowski, 1999; Ricklefs and Wikelski, 2002).

1.2. General concepts of acid-base regulation

1.2.1. The importance of acid-base balance

The pH of an organism's intracellular and extracellular fluids has a direct effect on nearly all biological processes due to the pH sensitivity of proteins (Riggs, 1988; Somero, 1986). As pH changes, the amphoteric components of amino acids may change their protonation state and thereby their net charge. Changes in the electrostatic interactions between a protein's amino acids affect its conformation and thereby alter its function (Baptista et al., 1997). Therefore, the efficiency of a protein's function will be optimal when the pH of its surrounding fluids promotes an optimal conformation. Organisms tightly regulate the pH of their intracellular and extracellular fluids to maintain fluid environments within a narrow range that allows their proteins to operate at rates that ensure proper cellular function. Although the specific regulated range of pH is species or even tissue-specific, the physiological range of extracellular fluids is generally pH 7.2 – 8.0 whereas the pH of the intracellular fluid is usually slightly more acidic,

resting between 7.0 – 7.4 with some variation between tissue types (Henry and Wheatly, 1992). Microenvironments such as acidified intracellular lysosomes or specialized fluids such as stomach acid may also be regulated to allow specialized enzymes such as acid hydrolases (de Duve, 1983) and pepsins (Fruton, 2002) to function under certain conditions. Should organisms fail to maintain the acid-base homeostasis of their vital fluids, enzymatic processes become compromised resulting in cellular dysfunction and eventual death. Regulatory actions serve to prevent such consequences by mitigating the magnitude of an acid-base disturbance and adjusting the net ratio of acidic and alkalotic equivalents within a fluid. While most cells are capable of a varying degree of intracellular pH regulation, the intracellular pH can only be restored once an appropriate extracellular acid-base homeostasis is established (Shartau et al., 2016). Therefore, most of this dissertation focuses on aspects of extracellular acid-base regulation and discusses intracellular aspects largely in a mechanistic context.

It is important to note that acid-base regulation refers to the net sum of all acidic and alkaline components within the fluid at any given time. Therefore, if a fluid's pH remains stable that does not necessarily mean that acidic or alkaline molecules have not been added to or removed from the solution – acid-base regulation is a continuous dynamic process even in a resting organism. Excretion of an acidic equivalent is equal to the retention of an alkaline equivalent and *vice-versa*. Similarly, molecules that bind or release free H^+ even momentarily equate to the net loss of an acid or base, respectively.

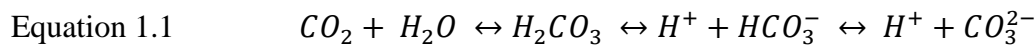
1.2.2. The role of buffering agents

The magnitude of an acid-base disturbance is mitigated by concentrating buffering agents within the bodily fluids. Buffering agents are amphiprotic molecules that exist in a pH-sensitive equilibrium – often simply referred to as a weak acid and its conjugate base, or *vice-versa*. Upon acidotic stress, the conjugate base reversibly binds free protons (H^+) and thereby reduces the magnitude of the acidotic disturbance. Conversely, alkalotic stress causes the weak acid to deprotonate thereby reducing the magnitude of the alkalotic disturbance. Without buffering agents, relatively minor acid-base disturbances would have a dramatic effect on the acid-base equilibrium of vital fluids. Although any amphiprotic molecule could technically act as a buffering agent, those that are physiologically valuable have an acid dissociation constant (pKa) near the fluid in question's regulated pH range. Extracellular fluids are generally buffered by

proteins/respiratory pigments and the carbonate equilibrium whereas intracellular fluids are more reliant on proteins and inorganic phosphates although both fluids may have other minor contributing agents.

1.2.3. The role of respiratory gas exchange

Respiration directly influences an organism's systemic acid-base equilibrium by manipulating CO₂ tensions within the extracellular fluid. While CO₂ itself may not appear to be an acid-base equivalent at first glance, its participation in the carbonate equilibrium (Eq. 1.1) means that its abundance within a fluid affects the ratio of the equilibrium's other components.



As respiration occurs, CO₂ flows down its partial pressure (P_{CO2}) gradient from the cytosol, into the extracellular fluid, and across the respiratory epithelium into the environment (Haldane and Priestley, 1905). Hyperventilation increases the rate that an organism excretes CO₂, causing the extracellular carbonate equilibrium to shift to the left following Le Chatelier's principle (Le Chatelier, 1884), increasing the favourability of HCO₃⁻ protonation and the formation of H₂CO₃. This reaction consumes free protons within the extracellular fluid, thereby promoting its alkalization. Conversely, hypoventilation reduces an organism's CO₂ excretion rate, causing the carbonate equilibrium to shift to the right resulting in an increased formation and subsequent dissociation of H₂CO₃ into a H⁺ and HCO₃⁻. This reaction increases the H⁺ concentration of the extracellular fluid, thereby promoting its acidification. Although its mechanistic role is discussed in greater detail throughout this chapter, it is important to note that the rate that an organism hydrates CO₂ and protonates HCO₃⁻ is incredibly fast due to its reversible catalysis by carbonic anhydrase activity within certain tissues and, where relevant, extracellular erythrocytes (Bundy, 1977; Henry and Cameron, 1982a).

1.2.4. The role of acid-base equivalent transport across epithelia

While respiration can manipulate CO₂ tensions to adjust the carbonate equilibrium, non-volatile acid-base equivalents such as H⁺, HCO₃⁻, and NH₄⁺ must be adjusted through transepithelial transport processes. Some acid-base transporters directly move acid-base

equivalents across phospholipid bilayers including the many isoforms of Na^+/H^+ -exchangers (NHEs), $\text{HCO}_3^-/\text{Cl}^-$ anion-exchangers (AEs), $\text{Na}^+/\text{HCO}_3^-$ cotransporters (NBCs), HCO_3^- accepting anion channels (Harms et al., 2014), and the V-type H^+ -ATPase (HAT) whereas other transporters such as the Na^+/K^+ -ATPase (NKA) help create an electrochemical gradient between the cell and the extracellular fluid that the many of the aforementioned transporters require to function (Boron et al., 1997; Claiborne et al., 2002; Fehsenfeld and Weihrauch, 2016a; Harms et al., 2014; Henry and Wheatly, 1992; Koeppen, 2009; Towle et al., 1997; Tresguerres et al., 2008). Given that many of these transporters rely on the exchange of Na^+ and Cl^- for H^+ and HCO_3^- , respectively, the activity of the carbonic anhydrase and osmoregulatory processes play a central role in transepithelial acid-base regulation (Burnett et al., 1985; Gilmour and Perry, 2009; Henry et al., 2003; Henry and Cameron, 1983, 1982b). Additionally, transporters that accept NH_4^+ either directly such as ammonium transporters (AMTs; Chasiotis et al., 2016; Durant and Donini, 2018) or in-place of K^+ , such as the NKA (Chew et al., 2014; Towle and Holleland, 1987; Weihrauch et al., 1998) and K^+ -channels (Carrisoza-gaytán et al., 2011; Fehsenfeld and Weihrauch, 2016b), play an important role due to the involvement of ammonia in renal (Koeppen, 2009; Weiner and Verlander, 2013) and branchial (Allen et al., 2020; Hans et al., 2018; Hu et al., 2017; Weihrauch and Allen, 2018) acid-base regulation. Some transporters such as Rhesus proteins even facilitate the transepithelial movement of gaseous CO_2 and NH_3 making their diffusive movement more efficient (Nawata et al., 2007; Soupene et al., 2002; Thies et al., 2022). Although the specific mechanistic interactions between these various transporters are complicated and dependent on often unknown factors such as their cellular localizations and transport characteristics, their combined function results in a net flux of acid-base equivalents that is important to intracellular and extracellular acid-base regulation (Boron, 2004). Although all cells may use any given combination of these transporters, regulation of the non-volatile acid-base equivalents within the extracellular fluid is performed by specific acid-base regulatory organs such as the mammalian kidney (Koeppen, 2009), the gills of water-breathing animals (Claiborne et al., 2002; Fehsenfeld and Weihrauch, 2017; Henry et al., 2012), and occasionally kidney-like ultrafiltration organs such as the antennal glands of crustaceans (Wheatly, 1985) that are capable of either producing a waste product that is physically removed from circulation (*e.g.* urine) or directly interacting and exchanging acid-base equivalents with an aqueous environment.

Although specific details of systemic acid-base regulation depend on the regulatory organ in question and the current state of an animal's extracellular acid-base status, a brief explanation of the regulatory action of the mammalian kidney may serve as a useful example due to its thorough characterization.

1.2.4.1. Acid-base regulation in the mammalian kidney

As extracellular fluid passes through the kidneys it undergoes ultrafiltration at the glomerulus allowing the filtrate to pass through the remaining sections of the nephrons where transepithelial transport processes reabsorb HCO_3^- , titrate urinary buffers, and excrete acid-equivalents to create acidic and ammonia-rich urine (Koeppen, 2009). The first sub-section of the nephron is the proximal tubule where most of the kidney's acid-secretion into the lumen and HCO_3^- reabsorption into the extracellular fluid occurs. Apical Na^+/H^+ exchanger and V-type H^+ -ATPase activity along the proximal tubule transport intracellular H^+ into the nephron's lumen (Koeppen, 2009). Intracellular carbonic anhydrase activity transforms intracellular CO_2 into H^+ that allows the acid-secretion to operate continuously while the coinciding HCO_3^- is moved across the basolateral membrane of the proximal tubule into the extracellular fluid by an electrogenic $1\text{Na}^+/3\text{HCO}_3^-$ cotransporter (Boron et al., 1997) and through anion exchangers. An apically-localized membrane-bound carbonic anhydrase isoform within the proximal tubule further enhances this reabsorptive process by protonating luminal HCO_3^- to form CO_2 that may then diffuse into the cytoplasm of the epithelial cells lining the proximal tubule to reach a similar outcome (Koeppen, 2009). In addition to this HCO_3^- reabsorptive mechanism, acid secreted into the nephron's lumen may react with urinary buffers and be temporarily neutralized (*e.g.* $\text{HPO}_4^{2-} + \text{H}^+ \rightarrow \text{H}_2\text{PO}_4^-$). Once this occurs, intracellular CO_2 is again hydrated and the consequent H^+ is transported into the lumen by acid-secreting transporters to re-acidify the urine while the coinciding HCO_3^- is transported across the basolateral membrane into the extracellular fluid (Koeppen, 2009).

Ammonia production and transepithelial movement also play an important role in the acid-base regulatory mechanism of the mammalian kidney. Cellular metabolism within the proximal tubule involves the catabolism of glutamine, resulting in the formation of NH_4^+ and HCO_3^- (Weiner and Verlander, 2013). Ammonium is then excreted into the nephron's lumen as an acid-equivalent while the HCO_3^- is reabsorbed across the basolateral membrane into the

extracellular fluid (Weiner and Verlander, 2013). Later sections of the nephron, namely the thick ascending limb of Henle's loop, reabsorb the luminary NH_4^+ using K^+ -transporters ($\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters and K^+ -channels Carrisoza-Gaytán et al., 2011; Weiner and Verlander, 2011) to be later re-secreted into the lumen within the collecting duct, where Rhesus-proteins promote the diffusive movement of intracellular NH_3 into the lumen that becomes acidified by acid-secreting cells (Koeppen, 2009; Weiner and Verlander, 2013, 2011).

Much like adjustments to respiratory rates can be used to regulate an animal's systemic acid-base status, transepithelial transport processes and associated metabolism within the regulatory organ are adjusted or can even alter their directionality in response to acid-base stressors (Claiborne et al., 2002; Fehsenfeld and Weihrauch, 2017; Koeppen, 2009). Generally speaking, these changes cannot occur within the same time-frame as respiratory adjustments as they require changes in the expression of relevant transporters or even hormonal influence although certain organisms such as aquatic crustaceans rely almost exclusively on adjustments to transepithelial transport mechanisms due to a limit capacity to alter respiration without compromising their oxygen bioavailability (Henry and Cameron, 1983; Wheatly and Henry, 1992).

1.2.5. Coordination of regulatory action

These acid-base regulatory mechanisms operate together to maintain systemic acid-base homeostasis; however, their relative importance to an acid-base disturbance depends on the type of disturbance. In straightforward cases, the source of an acid-base disturbance is compensated by the opposite regulatory response (Henry and Wheatly, 1992; Wheatly and Henry, 1992). For example, a respiratory acidosis is characterized by a significant reduction in extracellular pH that coincides with an increase in extracellular P_{CO_2} at constant $[\text{HCO}_3^-]$ is compensated by a metabolic alkalosis brought forth by an increase in extracellular HCO_3^- concentrations due to adjustments in renal or transepithelial transport mechanisms. Conversely, a metabolic acidosis is characterized by a decrease in extracellular pH that coincides with a decrease in extracellular $[\text{HCO}_3^-]$ at constant P_{CO_2} is compensated by a respiratory alkalosis brought forth by hyperventilation. Complex acid-base disturbances involving aspects of a respiratory and metabolic acidosis or alkalosis are counteracted through similar adjustments, depending on the severity of each component.

1.3. Brachyuran crustaceans and environmental acid-base challenges

1.3.1. The broad habitat distribution of brachyurans

Brachyuran crustaceans are incredibly habitat-diverse being found in aquatic environments ranging in salinities from freshwater systems to hypersaline lagoons and temperatures both polar and tropical (Martin and Davis, 2001). In addition, many brachyurans are described as terrestrial and semi-terrestrial (Greenaway, 1999; Hartnoll, 1988). Brachyurans are even found under extremophile conditions such as the deserts of Australia where drought conditions persist for years at a time (Greenaway and MacMillen, 1978; Linton and Greenaway, 1995) and hydrothermal vents where volcanic gas emissions create extremely acidic, hot, and often sulphurous environments (Ng et al., 2000; Rogers et al., 2012; Thurber et al., 2011). Many brachyurans inhabit aquatic environments of a breadth of salinities due to their ability to shift between an osmoconforming state (*i.e.*, maintaining their bodily fluid osmolality at approximately the same level as their immediate environment) and hyperosmoregulating state (*i.e.*, maintaining the osmolality of their bodily fluids above that of their immediate environment) as is well documented in the moderately euryhaline green shore crab, *Carcinus maenas* (Compere et al., 1989; Truchot, 1981), or the extremely euryhaline Chinese mitten crab, *Eriocheir sinensis* (Koch et al., 1954; Krogh, 1938). Some brachyurans exhibit a range of semi-terrestrialism such as those that undergo involuntary emersion in intertidal zones (Truchot, 1975a) or voluntary prolonged emersion due to an adopted behaviour as is common in mangrove-dwellers (Kathiresan and Bingham, 2001; Kristensen, 2008; Mchenga et al., 2007).

Due to their broad distribution and habitat diversity, brachyurans have developed their physiology and regulatory processes under the selective pressure of a breadth of environmental factors, many of which directly influence or challenge their ability to maintain acid-base homeostasis. The breadth of these adaptations make brachyurans a useful model to study how physiological processes such as acid-base regulation relate to the animal's predisposition to certain stressors. The following sub-sections generalize the effects and consequences of various environmental factors on the acid-base homeostasis of brachyuran species. Mechanistic details relating to the compensatory action of the crabs are discussed later in this dissertation (see section 1.4). Although this dissertation is heavily focused on brachyuran crabs, comparisons and examples are drawn from the broader order of decapod crustaceans (Fig. 1.1), especially when a particular subject is poorly or entirely uncharacterized in brachyurans. Although these

comparisons attempt to connect similarities between members of the Decapoda, the order is incredibly diverse including major differences at the anatomical level of their regulatory organs. For example, the heavily branched dendrobranchiate gills of prawns and the hair-like trichobranchiate gills of the lobsters, crayfish, and some anomurans have a greater surface area and are generally found in greater numbers than the phyllobranchiate gills of brachyurans and shrimps (Freire et al., 2008). Furthermore, the antennal glands of brachyurans lack the nephridial canal that is present in other decapod groups that is believed to be the ability of brachyuran species to manipulate the osmolality of their urine (Freire et al., 2008). Although there has not been an official consensus whether these differences infer a significant difference in the animals' acid-base regulatory system, it should be noted that it is a possibility that must be considered.

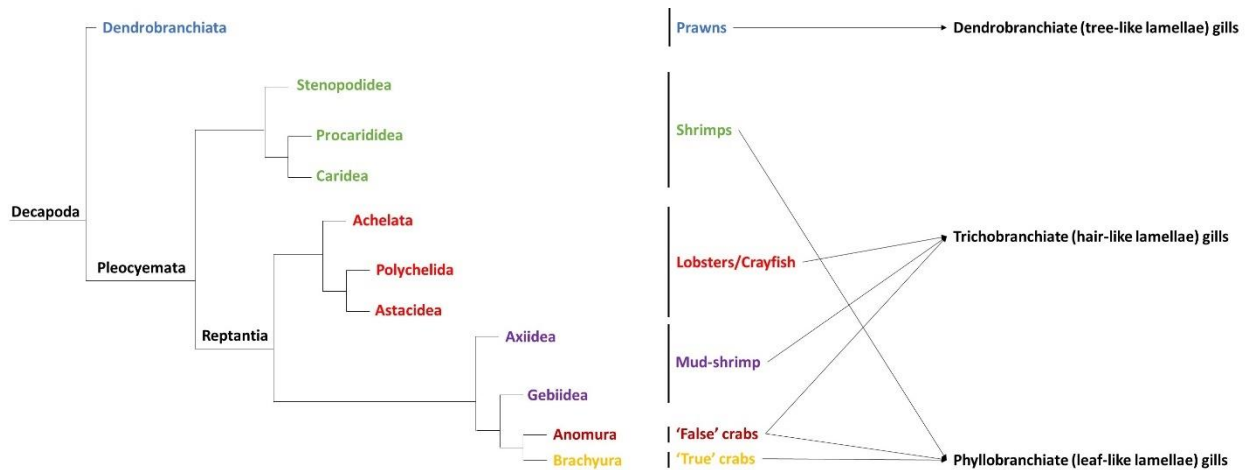


Figure 1.1. Cladogram representation of the infraorder of decapod crustaceans based on the phylogenetic results of Wolfe et al. (2019). The infraorders are colour-coded to their more commonly used generalized names. Arrows indicate the gill morphology associated with various decapod infraorders.

1.3.2. Changing environmental temperature

As temperature changes, it affects the autoionization constant (K_w) of water causing a shift in the ratio of H^+ to OH^- within the environment (Howell et al., 1973). The relationship between K_w and temperature is positively correlated meaning that an increase in temperature elevates K_w , resulting in an increased ratio of $H^+ : OH^-$ that acidifies the water and *vice-versa*. As this occurs, ectotherms - animals whose internal temperature mirrors that of their environment -

adjust their extracellular and intracellular fluids so that the net-difference in the pH of their bodily fluids and the environment remains constant – referred to as preservation of ‘constant relative alkalinity’ (Howell et al., 1973; Truchot, 1978, 1973). Therefore, temperature-driven changes in the pH of the water and the bodily fluids of ectotherms generally share a similar relationship where the change in pH per increase degree Celsius increase in environmental temperature reduces pH by about -0.017 units with some slight variation between species ($\Delta\text{pH}/\Delta t = -0.016 \text{ pH } ^\circ\text{C}^{-1}$). As such, changes in temperature result in the establishment of a new steady-state temperature-sensitive extracellular pH in ectotherms as has been documented in several species of decapod crustaceans (Howell et al., 1973; Qadri et al., 2007; Truchot, 1978, 1973; Wood and Cameron, 1985). While this would normally be averse to the animal, preservation of relative alkalinity conserves the net-charge of proteins and largely limits the consequences of a temperature-mediated acid-base challenge in comparison to other environmental stressors.

How brachyurans maintain constant relative alkalinity is not entirely understood and is somewhat controversial (Heisler, 1986), although the field currently suggests the involvement of passive protein buffering through the imidazole alaphastat hypotheses alongside slight changes in the extracellular carbonate equilibrium (Howell et al., 1973; Qadri et al., 2007; Truchot, 1978, 1973; Wood and Cameron, 1985). The imidazole alaphastat hypotheses of Reeves (1977, 1972) essentially suggest that changes in body fluid pH of ectotherms upon fluctuating temperatures are passively changed by fractional changes in the ionization of imidazole-rich solutions (for further discussion, see Burton, 2002). Since the sidechain of histidine residues is an imidazole group, it may be generalized that intracellular and extracellular proteins of ectotherms play a central role in maintaining acid-base homeostasis as temperatures change.

While the imidazole alaphastat hypotheses may account for a large proportion of temperature-mediated acid-base compensation, adjustments to the carbonate equilibrium also occur in brachyurans. Acute temperature fluctuations in ectotherms have an immediate inversed-relationship with pH and a positive-relationship with the P_{CO_2} of their extracellular fluid, presumably due to changes in the solubility and dissociation constants of the carbonate equilibrium (Truchot, 1978, 1976). This relationship is well-characterized in *C. maenas* where acute elevations in temperature immediately alter the solubility and dissociation constants of the

carbonate equilibrium (Truchot, 1976) causing extracellular pH to fall while P_{CO_2} rises (Truchot, 1978). Although the crab's extracellular pH remains constant, their extracellular P_{CO_2} and $[\text{HCO}_3^-]$ are gradually reduced over the next several hours of the exposure until a steady-state is reached (Truchot, 1978). This gradual adjustment to their extracellular carbonate equilibrium is believed to be regulated by changing the transepithelial flux rates of HCO_3^- , presumably across the gills (Cameron and Batterton, 1978a; Henry and Wheatly, 1992; Truchot, 1978; Wood and Cameron, 1985), rather than through respiratory adjustments that are regulated by extracellular oxygen tensions due to the physiochemical properties of aquatic environments (Dejours, 1975; Henry and Wheatly, 1992).

1.3.3. Hypercapnia

Hypercapnia refers to a significant elevation of P_{CO_2} within an environment. Although all aquatic environments may become hypercapnic, the frequency, magnitude, and duration of the event can be quite different. Marine environments face periodic bouts of hypercapnia caused by coastal upwellings as warm coastal water is moved away from the coastline and is replaced by deeper water from the opposing direction whose cold nutrient-rich nature carries a heightened load of P_{CO_2} . In the Benguela Current Large Marine Ecosystem, these upwellings can reduce oceanic pH from about pH 8.10 to pH 7.4 sometimes lasting up to 10-days (Knapp et al., 2016). Freshwater systems often carry varying levels of carbon sourced from detritus and surrounding terrestrial activity that can be seasonally dependent resulting in abrupt changes in their P_{CO_2} content by several hundred pascals (Pa) of CO_2 for several weeks or even months (Ran et al., 2017, 2015). Coastal brachyurans frequently inhabit tide-pools that become progressively hypercapnic as biotic activity releases CO_2 within their relatively small volume until the tidal cycle renews (Truchot, 1988, 1986). Furthermore, brachyurans are often one of the few endemic species found inhabiting hydrothermal vents where volcanic gas emissions can create extremely hypercapnic conditions where environmental P_{CO_2} can permanently exceed 30,000 Pa (Allen et al., 2020; Dahms et al., 2018; Martin and Haney, 2005; Ng et al., 2000; Thurber et al., 2011). While such extremes are outliers within aquatic environments, anthropogenic climate change is rapidly increasing the P_{CO_2} of aquatic environments causing their acidification and imposing a global acid-base disturbance on aquatic inhabitants (Hasler et al., 2016; IPCC, 2019; Whiteley, 2011; Woodward et al., 2010). Although the precise habitat and its associated hypercapnic-

potential apply differing magnitudes of acid-base stress on brachyuran species, their general response is relatively similar as described below.

Hypercapnic conditions acutely alter the acid-base homeostasis of aquatic organisms due to their dependence on the diffusive excretion of CO₂ down an environmentally directed P_{CO2} gradient. Under normal conditions, metabolic activity produces intracellular CO₂ that diffuses into the extracellular fluid until it reaches the respiratory organ that it diffuses across into the ambient environment. As environmental P_{CO2} levels rise, the favourability of this P_{CO2} gradient is reduced and the animal's ability to excrete CO₂ is impaired. If environmental P_{CO2} exceeds that of the extracellular fluid, the process becomes inverted. In such an instance, environmental CO₂ accumulates within the animal's extracellular fluid until an equilibrium is established. Metabolic activity then elevates extracellular P_{CO2} slightly restoring an environmentally-directed P_{CO2} gradient (Melzner et al., 2009). Although this allows the animal to continue to passively excrete CO₂, the influx of CO₂ causes the extracellular carbonate equilibrium to shift to the right. This relationship infers that hypercapnic stress invokes an acute respiratory acidosis lasting until hypercapnic conditions disperse or until sufficient acid-base compensatory action occurs (Truchot, 1975b). Brachyuran species compensate against hypercapnia-induced respiratory acidosis by invoking a metabolic alkalosis as indicated by the accumulation of HCO₃⁻ within their hemolymph while the elevated P_{CO2} levels of the extracellular fluid persist (Allen et al., 2020; Fehsenfeld and Weihrauch, 2017; Hans et al., 2014; Maus et al., 2018; Pane and Barry, 2007; Quijada-Rodriguez et al., 2021; Truchot, 1975b). Most of the accumulated HCO₃⁻ is collected from the environment through transbranchial transport processes (Fehsenfeld and Weihrauch, 2017, 2016a; Tresguerres et al., 2008), although a minority may originate from the dissolution of the carbonate-rich exoskeleton (Cameron, 1985a) and/or reabsorption of urinary HCO₃⁻ by the antennal glands (Wheatly, 1985). Although literature may suggest that brachyurans are only capable of partially counteracting the effects of moderate hypercapnia, their focus is heavily biased towards species capable of hyperosmoregulating (Burnett, 1997; Cameron, 1978; Fehsenfeld and Weihrauch, 2016a, 2013; Heisler, 1986; Quijada-Rodriguez et al., 2021; Wood and Cameron, 1985). Although few species have been studied, stenohaline osmoconformers appear to not only compensate faster than hyperosmoregulatory-capable species, they also tend to compensate completely even under relatively severe hypercapnic stress (*e.g.* > 1% CO₂/1013 Pa P_{CO2}; Allen et al., 2020; Hans et al., 2014; Pane and Barry, 2007; Small et al., 2010).

Although mechanistic details regarding acid-base regulatory transport processes are discussed in detail in section 1.4 of this dissertation, it is worth noting that this discrepancy may relate to the reduced osmoregulatory burden of stenohaline osmoconforming species given most of the transport of Na^+ and Cl^- across an epithelium usually relies on H^+ and HCO_3^- , respectively, as counter-ions (Henry et al., 2012; Henry and Wheatly, 1992). Actively hyperosmoregulating or even the potential to hyperosmoregulate may limit the ability of a species to perform transepithelial acid-base compensatory transport processes without compromising osmoregulatory needs, representing an interesting divide in adaptive strategies.

In addition to the induction of a respiratory acidosis, acute hypercapnia appears to have a suppressing effect on the metabolic rate of decapod crustaceans (Allen et al., 2020; Hans et al., 2014; Quijada-Rodriguez et al., 2021; Tripp et al., 2022). Whether or not metabolic suppression is beneficial or detrimental to the animals is controversial and may depend on its duration. Temporary or acute metabolic suppression may benefit the animal by reducing the production of endogenous wastes, many of which are acid-equivalents, while a subset of catabolic reactions that produce alkaline end-products such as HCO_3^- become more favourable, as suggested for the peanut worm, *Sipunculus nudus* (Langenbuch and Pörtner, 2002). Metabolic suppression is only beneficial if it is reversible once sufficient compensation is achieved, as has been documented in an extremely hypercapnia tolerant hydrothermal vent endemic species, *Xenograpsus testudinatus* (Allen et al., 2020). Conversely, chronic metabolic suppression would likely reduce the animal's growth, reproductive success, and overall fitness as suggested for many aquatic species (Langenbuch and Pörtner, 2002; Michaelidis et al., 2005; Portner et al., 2004). Although further clarity as to why the metabolic suppression occurs and what the potential benefits and consequences may be for brachyurans, relatively few studies have extended beyond measurements of oxygen consumption rates and ammonia excretion rates after relatively short time-periods (~ 1-2 weeks of exposure) making it difficult to make assumptions in the current state of the field.

1.3.4. Non-hypercapnic environmental acidification

Although less common than hypercapnic events, aquatic environments can become naturally acidified through the effects of acid rain or certain pollutants. The problem is a notable issue in poorly-buffered freshwater ecosystems where pH may reach acidities of ~4 - 5 pH

(Jeffries et al., 1979; Poléo et al., 1997; Schindler, 1988) whereas the immense volume and comparably concentrated carbonate equilibrium of marine and even brackish waters limit its effects. To my knowledge, direct acid-exposure has not been studied on brachyuran species before experiments performed in this dissertation (see chapter 2), presumably due to their greater association with marine and brackish-water environments in North America where acid rain was a predominant concern (Bridgman, 1989); however, several studies investigated its effects on freshwater crayfish (Ellis and Morris, 1995; Morgan and McMahon, 1982; Patterson and DeFur, 1988; Whiteley et al., 1995; Wood and Rogano, 1986).

Environmental P_{CO_2} does not undergo a substantial change when acidification is caused by non-carbonated acids; although, slight changes may occur due to changes in their carbonate equilibrium. Acidification of the extracellular fluid is therefore caused by an influx of environmental H^+ and/or indirect metabolic effects on the animal. Due to its positive charge, environmental H^+ cannot diffuse across phospholipid bilayers and must therefore penetrate the animal across their exposed epithelia through ion-exchange or across paracellular junctions. The bulk of a crustacean's exterior is covered by a calcified exoskeleton that is largely impermeable to ions and water (Lignon, 1986; Rasmussen and Andersen, 1996). While the cuticle of the gill is not freely permeable to all ions (Lignon, 1987), it does function as the main site where ions and small uncharged solutes permeate the organism as well as the site that dominates ion-exchange processes (Henry et al., 2012).

Most of the studies focusing on the non- CO_2 -driven acid-stress on the acid-base status of crustaceans have focused on crayfish. Although the gills of crayfish have a high capacity to actively absorb Na^+ and Cl^- from ion-poor freshwater environments (Freire et al., 2008; Mills and Geddes, 1980), many of these processes rely on an exchange for cytosolic H^+ and HCO_3^- that can directly alter the animal's acid-base status, potentially limiting their capacity to perform both regulatory tasks simultaneously. Furthermore, acidified conditions and the increased abundance of environmental H^+ may alter the efficiency of apical Na^+/H^+ exchangers, compromising some of the animal's osmoregulatory processes (Parks et al., 2008; Wood and Rogano, 1986). Additionally, the loss of HCO_3^- in exchange for Cl^- by presumed apical anion exchangers (AEs) is not beneficial for compensating an extracellular acidosis while the absorption of Cl^- is essential to hyperosmoregulation. Some indication has also demonstrated that the effectiveness

of $\text{HCO}_3^-/\text{Cl}^-$ exchange may be compromised in acid-stressed crayfish, potentially due to low intracellular HCO_3^- concentrations as a result of a severe metabolic acidosis (Wood and Rogano, 1986). While the branchial osmoregulatory and acid-base regulatory mechanisms are more complex than the activity of NHEs and AEs, these aspects present potential pitfalls in acid-exposed freshwater crustaceans.

Upon acute acid-exposure, crayfish species experience a severe complex acidosis characterized by an acute respiratory and metabolic acidosis that reduces extracellular pH by 0.4 – 0.5 units (Ellis and Morris, 1995; Morgan and McMahon, 1982; Patterson and DeFur, 1988; Whiteley et al., 1995; Wood and Rogano, 1986). The acute respiratory acidosis usually lasts between 24 – 48 hours during which time it accounts for approximately 40% of the-net acidification of the hemolymph (Wood and Rogano, 1986). It has been hypothesized that the respiratory acidosis relates to either respiratory changes or a thickened apical barrier across the gill epithelia (Patterson and DeFur, 1988; Wood and Rogano, 1986). These sources may be somewhat controversial given that Wood and Rogano (1986) found no change in extracellular lactate concentrations that may occur as a result of reduced aerobic metabolism and the fact that aquatic crustaceans are believed to regulate ventilation in response to circulating oxygen tensions and not acid-base disturbances (Dejours, 1975; Henry and Wheatly, 1992). Furthermore, the apparent ventilatory changes that occur in acid-exposed crayfish as described by Patterson and DeFur (1988), identified hyperventilation that would not explain the accumulation of extracellular P_{CO_2} but rather an attempt to eliminate it. It should be noted that these studies did not verify if the acidification caused a slight increase in P_{CO_2} due to shifts in the carbonate equilibrium nor did they consider the potential for the initial influx of H^+ into the extracellular fluid to cause a shift in the carbonate equilibrium that would also theoretically elevate extracellular P_{CO_2} . Regardless of the mechanism, the respiratory acidosis is corrected; however, the severe metabolic acidosis persists causing extracellular HCO_3^- concentrations to fall below 1 mmol l^{-1} after 10-14 days despite the animal's ability to limit further extracellular acidification resulting in mortalities (Ellis and Morris, 1995; Morgan and McMahon, 1982; Whiteley et al., 1995; Wood and Rogano, 1986). The attenuated acidosis may be the result of a sequestration of acid-equivalents within certain tissues (*e.g.*, muscle) or the fluid between the carapace and hypodermis as hypothesized by Wood and Rogano (1986), although this process has not been verified.

In summary, the limited studies concerning the consequences of acid-stress or non-hypercapnia mediated environmental acidification on decapod crustaceans are limited to freshwater crayfish. Despite their relatively ion-tight paracellular junctions (Freire et al., 2008) and the apparent ability to sequester acid-equivalents and stabilize an acidified extracellular pH, crayfish cannot prevent a lethal reduction in extracellular HCO_3^- and eventually succumb to their acidic environment. Whether a similar process occurs in marine osmoconforming crustaceans where osmoregulatory demands are lower and HCO_3^- bioavailability is greater, remains to be identified.

1.3.5. Oxygen bioavailability

While air-breathing animals are usually not limited by ambient oxygen bioavailability, the comparably low levels of oxygen contained within aquatic environments makes it difficult for water-breathing species to sufficiently oxygenate their tissues even under normoxic conditions (Kramer, 1987). Furthermore, the availability of oxygen in aquatic environments is sensitive to several factors including seasonal changes, the time of day, and anthropogenic activity. In small bodies of water with high biological activity (*e.g.* tide pools), photosynthetic activity can create hyperoxic environments reaching up to 600 Torr P_{O_2} whereas evenings become hypoxic reaching as low as 2 Torr P_{O_2} when photosynthesis halts and oxygen is consumed by inhabitants (Dejours, 1975; Truchot and Duhamel-Jouve, 1980). Coasts and freshwater environments experience fluctuating oxygen levels due to terrestrial run-off as seasons change or from anthropogenic activity and pollution (Davis, 1975; Rabalais et al., 2001). Depth is also a substantial factor with even intermediate depths containing a minimum oxygen layer that most organisms cannot tolerate (Childress and Seibel, 1998). Despite the potential adverse effects of fluctuating oxygen tensions, brachyurans are regularly found in these environments with burrow/burying species even experiencing virtual anoxia for several days due to their lifestyle (Bernatis et al., 2007; McGaw, 2005).

To sufficiently extract oxygen from their environment across their gills, crustaceans maintain a high ventilation rate under resting conditions in normoxic environments (Dejours, 1975; Henry and Wheatly, 1992). As a result, of their respiratory activity and the comparably high water solubility of CO_2 compared to O_2 (Dejours, 1975), crustaceans are limited in their ability to adjust their ventilatory activity to counteract an acid-base disturbance (Henry and

Wheatly, 1992; Mangum et al., 1985; Truchot, 1983). Ventilatory activity is instead regulated by changes in the extracellular oxygen levels of crustaceans causing hypoxic and hyperoxic environments to induce respiratory acid-base disturbances (Burnett, 1997; Dejours, 1975; Henry and Wheatly, 1992; McMahon, 1988). When environmental oxygens are low (*i.e.* hypoxia), aquatic species hyperventilate resulting in a respiratory alkalosis, whereas heightened environmental oxygen tensions (*i.e.* hyperoxia) lead to hypoventilation that invokes a respiratory acidosis (Truchot, 1988).

Upon a hyperoxia-mediated respiratory acidosis, brachyurans compensate through a metabolic alkalosis where it eliminates excess acid-equivalents across their branchial epithelia while the gills simultaneously transport HCO_3^- from their environment into the extracellular space (DeFur et al., 1980; Truchot, 1979; Wheatly, 1987). This response is similar to the previously described compensatory response used by brachyurans to counteract a hypercapnia-mediated respiratory acidosis, presumably due to both stressors being rooted by an increase in extracellular P_{CO_2} albeit for different reasons.

The response of brachyurans upon the development of a hypoxia-mediated respiratory alkalosis is more complicated. In the first few hours of hypoxic stress, hyperventilation increases the CO_2 excretion rate of crustaceans causing a reduction in extracellular P_{CO_2} as well as the remaining carbonate equilibrium causing hemolymph to become alkaline whose severity is exacerbated in dilute environments (Burnett and Johansen, 1981; Hagerman and Uglow, 1984; Johnson and Uglow, 1987; McMahon, 1988; Truchot, 1975). A net reduction in the metabolic production of CO_2 may also be involved in this process as indicated in hypoxia-stressed *C. maenas* (Burnett and Johansen, 1981). If hypoxic conditions pass a critical threshold, generally below 20 Torr (Fogue et al., 1992; Johnson and Uglow, 1987; McMahon, 1988; Morris and Butler, 1996; Truchot, 1975), lactic acid accumulation increases due to a greater dependence on anaerobic metabolism that acts as a metabolic acidosis that may even cause a slight acidification of the extracellular fluid. This acidosis is largely hypothesized and not directly documented as crustaceans appear to simultaneously accumulate extracellular HCO_3^- to a degree that either restores their normoxic extracellular pH or even causes a slight alkalization (Johnson and Uglow, 1987; Morris and Butler, 1996; Truchot, 1975). The direct source of this HCO_3^- is controversial with some researchers implying exoskeletal carbonate reserves are mobilized due to the

occasional observation of hypoxia-mediated elevations of extracellular Ca^{2+} levels (Hagerman and Uglow, 1984; Johnson and Uglow, 1987) or through changes in transbranchial transport rates of acid-base equivalents.

1.3.6. Salinity

The majority of brachyuran species are stenohaline marine species (Martin and Davis, 2001) that osmoconform to their environment, meaning that the osmolality of their bodily fluids is approximately equivalent to their environment. While these species may regularly experience notable fluctuations in environmental salinity, many intertidal crabs face daily changes in salinity due to tidal activity (Simonik and Henry, 2014; Truchot, 1988) whereas some species even migrate from freshwater to seawater as part of their reproductive cycle (Veilleux and De Lafontaine, 2007) or lifestyle (Henry and Cameron, 1982b). These euryhaline, osmoregulatory-capable crabs possess specialized posterior gills that undergo a salinity-sensitive transformation that increases their surface area, their abundance of ion-transport machinery, and mitochondrial abundance allowing them to actively absorb Na^+/Cl^- from their dilute environment across their gill epithelium (Barra et al., 1983; Compere et al., 1989; Freire et al., 2008; Henry et al., 2012). As a result, their bodily fluid osmolality is continuously held above that of their environment with some species such as *C. maenas* being moderate hyperosmoregulators and others being strong hyperosmoregulators such as *E. sinensis* (Freire et al., 2008; Koch et al., 1954; Krogh, 1938; Siebers et al., 1982). In addition to the active absorption of environmental Na^+/Cl^- across their gills, the tissues within hyperosmoregulating crabs must adjust their internal osmolality to match that of their hemolymph to avoid cellular deformation or even rupturing. In brachyurans, these anisotonic cell volume regulatory mechanisms involve metabolic adjustments where the production of non-essential amino acids is increased so that they may be transported out of the cell into the extracellular fluid as well as an efflux of ions, presumed to be mostly K^+ , Cl^- , and HCO_3^- , through basolateral channels (Gilles and Péqueux, 1981; Lang and Paulmichl, 1995). Given that both the osmoregulatory changes in branchial transport processes and the anisotonic response involve the movement of acid-base equilibrium between the intracellular, extracellular, and environment, it is unsurprising that changes in salinity challenge the acid-base equilibrium of brachyurans (Henry and Wheatly, 1992; Whiteley et al., 2001).

Although the consequences of an osmotically induced acid-base disturbance have only been studied in a few brachyuran species, their findings indicate that hyperosmoregulatory capable species all experience an immediate transient hemolymph acidosis lasting several hours followed by a persistent metabolic alkalosis (Henry and Wheatly, 1992; Whiteley et al., 2001). The source of these acid-base disturbances is poorly understood; however, several signs point towards their cause being indirect consequences of anisosmotic cell volume regulation. During the first 6-hours of hypoosmotic stress, the extracellular fluid of *E. sinensis* becomes acidotic (*ca.* $\Delta\text{pH}_e = -0.3$) while hemolymph HCO_3^- levels fall by approximately 50% (Truchot, 1992; Whiteley et al., 2001). While this occurs, the intracellular pH of the leg muscle is alkalized (*ca.* $\Delta\text{pH}_i = +0.2$) due to a 50% increase in HCO_3^- concentrations (Whiteley et al., 2001). The belief is that hypoosmotic stress induced an immediate metabolic acidosis within the muscle's intracellular fluid – the veracity and potential cause of which remains unknown – that led to an increase in the efflux of acid-equivalents from the tissue into the extracellular fluid causing it to become acidotic (Whiteley et al., 2001). After 6 hours, this process is reversed as intracellular HCO_3^- , presumably through HCO_3^- -accepting anion channels (Harms et al., 2014; Lang and Paulmichl, 1995), and non-essential amino acids are transported into the extracellular space (Whiteley et al., 2001) as would be expected during a cell volume regulatory decrease (Gilles and Péqueux, 1981). As a result, the extracellular fluid undergoes a metabolic alkalosis that is transient in some species (Truchot, 1981) and persistent in others (Henry and Cameron, 1982b; Truchot, 1992). Elevated extracellular ammonia concentrations have also been detected in crabs experiencing acute hypoosmotic stress and may contribute to the development of the metabolic alkalosis, although its contribution appears to be relatively small (Mangum et al., 1976; Whiteley et al., 2001).

An additional source of an osmotically induced acid-base disturbance is through the related changes in transbranchial transport processes. Active absorption of Na^+ and Cl^- across the gills of hyperosmoregulating brachyurans involves several transporters that are indirectly fueled by the electrochemical gradients maintained across the branchial epithelia by the Na^+/K^+ -ATPase and V-type H^+ -ATPase (Burnett and Towle, 1990; Chung and Lin, 2006; Leone et al., 2017; Lucu and Flik, 1999; Luquet et al., 2002b; Onken and Putzenlechner, 1995; Postel et al., 1998; Siebers et al., 1985, 1982; Tsai and Lin, 2007; Weihrauch et al., 2004a). These ATPases actively ensure intracellular Na^+ concentrations are low, and that the epithelium is hyperpolarized – that

is, that it holds a greater electronegative potential than its surrounding environment and extracellular fluids. These cellular properties attract environmental Na^+ towards the apical gill epithelium where they are transported into the cell through Na^+ -channels, Na^+/H^+ exchangers, and potentially $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters (Burnett and Towle, 1990; Lucu and Siebers, 1986; Onken et al., 2000; Onken and Riestenpatt, 2002; Towle et al., 1997; Towle and Weihrauch, 2001; Tresguerres et al., 2008). While the aforementioned Na^+/K^+ -ATPase is likely responsible for most of the movement of intracellular Na^+ across the basolateral membrane, $\text{Na}^+/\text{HCO}_3^-$ cotransporters may also participate (Boron et al., 1997; Tresguerres et al., 2008). Environmental Cl^- is transported across the apical membrane in exchange for HCO_3^- by anion exchangers while the hyperpolarized nature of the branchial epithelia promotes the movement across the basolateral epithelium through Cl^- -channels (Genovese et al., 2005; Onken et al., 2000; Onken and Graszynski, 1989; Onken and Putzenlechner, 1995; Onken and Riestenpatt, 2002; Riestenpatt et al., 1995). Carbonic anhydrase activity, which in itself is positively-correlated with the movement of crabs into dilute environments (Henry et al., 2003; Serrano et al., 2007), is connected to the absorption of both Na^+ and Cl^- as it provides the intracellular $\text{H}^+/\text{HCO}_3^-$ counter-ions used by the apical exchangers (Böttcher et al., 1991; Burnett et al., 1985; Henry and Cameron, 1983). The fact that the gill is the predominant site of osmoregulatory and acid-base regulatory action and the shared involvement of many of the same transporters links the two processes (Allen and Weihrauch, 2021; Fehsenfeld and Weihrauch, 2016a; Henry et al., 2012; Henry and Wheatly, 1992). While this relationship is well known, it is not clear how they exactly affect the animal's acid-base equilibrium. The greater influx of transbranchial Na^+ should correlate to an increase in acid-excretion rates whereas the influx of Cl^- should equate to an increase base-efflux across the gill. Depending on a particular species, these processes may negate one another (*i.e.* net-zero acid-base flux), leaving anisosmotic changes to drive acid-base disturbances, whereas other species may have a net-acid or net-base flux across their gills. This may be a secondary result of adjustments to the strong ion difference (SID) as the crabs adjust their intracellular environments to maintain electroneutrality (Cameron, 1989; Stewart, 1983) or it could be caused by the direct need to maintain acid-base homeostasis, although the field has yet to distinguish between the two given the difficulties of accurately quantifying SID *in vivo*.

The effect of forced hypoosmotic stress on the acid-base equilibrium of stenohaline osmoconforming species has only been studied in two species. The Dungeness crab,

Metacarcinus magister, experiences a similar acid-base disturbance as hyperosmoregulatory crabs upon changing from 35 to 20 ppt. although at a faster pace with smaller net-changes in their extracellular pH. The initial respiratory acidosis is relatively mild ($\Delta\text{pH}_e = -0.2$) lasting less than 12-hours at which point *M. magister* begins rapidly accumulating extracellular HCO_3^- with a slight alkalization of its extracellular fluid ($\Delta\text{pH}_e = -0.1$) after 72-hours (Wheatly, 1985). The velvet crab, *Necora puber*, does not experience any acid-base disturbances at all when moved from 35 to 20 ppt. environments, maintaining constant hemolymph pH for at least 50 hours despite its extracellular Cl^- concentrations rapidly falling to equilibrate with their ambient environment (Whiteley et al., 2001).

1.3.7. Emersion

Emersion is a complex physiological stressor experienced by many semi-terrestrial intertidal and mangrove-dwelling brachyuran species due to environmental factors (e.g. tidal motion) or behaviour (Kathiresan and Bingham, 2001; McMahon, 1988; Pfister, 2007). As brachyurans emerge from aquatic environments, they retain water within their branchial chamber allowing their gills to continue to regulate their physiological state (Henry et al., 2012). Branchial chamber water reserves become increasingly hazardous as time passes due to ionic imbalances and the accumulation of wastes, requiring the animal to eventually replenish its water reserve or face physiological consequences (Durand and Regnault, 1998; Simonik and Henry, 2014; Wolcott, 1992). Furthermore, evaporative water loss from the body or the loss of water from urine and feces may concentrate wastes within the branchial chamber. Respiratory processes are also affected by emersion. Oxygen is more readily available in aerial environments making it possible, but not necessarily beneficial, for brachyurans to hypoventilate and consequentially experience hypercapnic stress (Truchot, 1975a). These factors are also all subject to fluctuations in temperature that are more pronounced in aerial environments than aquatic environments. Given that these factors are all individual acid-base stressors and the fact that brachyurans have a broad spectrum of aerial tolerance/terrestrialization (Greenaway, 1999; Hartnoll, 1988; Watson-Zink, 2021) the relationship between emersion and their acid-base status is complicated.

Although species differ in their ability to tolerate emersion, they all appear to experience an acute respiratory acidosis caused by hypoventilation and the accumulation of CO_2 within the

branchial chamber's water reserves (Allen et al., 2021; Burnett and McMahon, 1987; DeFur et al., 1983; DeFur and McMahon, 1984; Luquet and Ansaldo, 1997; Truchot, 1975a; Truchot, 1979). This response is slightly different in osmoconforming intertidal species such as the red rock crab, *Cancer productus*, and the xanthid mud-crab, *Eurytium albidigitum*, which also experience an accumulation of lactic acid in their hemolymph causing a mixed respiratory and metabolic acidosis (Burnett and McMahon, 1987; DeFur et al., 1983; DeFur and McMahon, 1984). These species are capable of partially counteracting their mixed acidosis for several hours through the accumulation of extracellular HCO_3^- ; however, after 4 hours their extracellular pH peaks in acidity indicating they cannot sustain their compensatory response (Burnett and McMahon, 1987). Intertidal species that are more active during emersion are capable of fully compensating their extracellular pH through branchial ion-exchange processes between the branchial chamber water reserve and their hemolymph. Studies performed on the rainbow crab, *Neohelice (Chasmagnathus) granulata*, suggest that compensation occurs through Na^+/H^+ exchange whereas hemolymph Cl^- levels are unchanged in both media (Luquet and Ansaldo, 1997), suggesting their accumulation of extracellular HCO_3^- is of an independent, presumably metabolic, source. Once branchial water reserves become unfavorable for compensatory transport processes (4 hours in *N. granulata*; Luquet and Ansaldo, 1997), their hemolymph becomes somewhat more acidotic and evidence of Na^+/H^+ exchange mechanisms disappears limiting their ability to remain emersed. Brachyurans that undergo prolonged emersion from a behavioral standpoint either in the absence or in conjunction with intertidal action appear capable of fully counteracting signs of a respiratory acidosis (Burnett and McMahon, 1987) and may even experience an over-compensatory metabolic alkalosis (Allen et al., 2021). As with other species, HCO_3^- accumulates within the hemolymph to counteract the respiratory acidosis; however, they generally have a greater reliance on urine reprocessing that allows them to potentially volatilize gaseous wastes (CO_2/NH_3 ; Allen et al., 2021; De Vries et al., 1994; Wolcott, 1991) and use their ultrafiltrating antennal glands to adjust the ionic composition of the brachial chamber waters by diluting the reserves with urine that is recollected by the crab. Accumulation of extracellular ammonia has been hypothesized to be a consequence of chronic emersion in fully-terrestrial and semi-terrestrial brachyurans (Durand and Regnault, 1998; Regnault, 1994; Wood et al., 1986; Wood and Boutilier, 1985). In theory, such an occurrence would offer an additional means to reduce emersion-induced acid-stress by combining NH_3 and

H⁺ to form NH₄⁺; however, these studies have largely found that ammonia does not accumulate in most species leaving a perplexing question as to how brachyurans prevent the toxic and acid-base disruptive effects of nitrogenous waste accumulation (Larsen et al., 2014).

1.4. Acid-base regulatory organs in brachyuran crabs

Crustaceans possess several organs capable of contributing to their systemic acid-base status including the gills, antennal glands, the hypodermis and exoskeletal system, and the gastrointestinal tract. Studies have largely focused on the gills due to their overwhelming contribution to the animal's acid-base status among other regulatory processes (Allen and Weihrauch, 2021; Henry et al., 2012). The antennal glands have received some attention; however, most literature has investigated their role through the eyes of relatedness to osmoregulation rather than acid-base regulation directly (Cameron and Batterton, 1978b; Truchot, 1979; Wheatly, 1985). The importance of the hypodermis and exoskeletal system is contested and often based purely on changes in extracellular Ca²⁺ and Mg²⁺ concentrations as HCO₃⁻ reserves are adjusted leaving virtually no understanding as to how the process works mechanistically (Cameron, 1985a, 1981; DeFur et al., 1980; Small et al., 2010). Even less studied is the gastrointestinal tract which, to my knowledge, is purely hypothetical given the lack of literature regarding acid-base equivalent fluxes across the gut in decapod crustaceans. The following sections summarize the relevant anatomical and vascular features of the regulatory organs, how they differ amongst certain types or species of brachyurans, and information regarding their acid-base regulatory mechanisms.

1.4.1. Branchial tissue

Brachyuran gills regulate many of the fundamental systemic processes through the transbranchial transport of ions/acid-base equivalents and gases. Their anatomical structure and direct interaction with the environment and hemolymph allow them to transport acid-base equivalents as required to maintain systemic homeostasis. Most of the regulatory transport appears to relate to the direct transport of H⁺ as well as the transport of carbonate species (CO₂ and HCO₃⁻) and ammonia (NH₃/NH₄⁺), although there may be other acid-base equivalents that participate at a minor level. The following sections detail the physical properties of the gills and summarized descriptions of the hypothetical transport mechanisms for the main acid-base equivalents. Please note, direct transport of H⁺ is discussed indirectly due to its connection to the

processes and the fact that most studies investigated transbranchial transport processes through raw changes in pH rather than titrating the amount of H⁺ specifically transported across the epithelia. These mechanisms encompass some information where evidence is provided at both the molecular and physiological levels; however, many aspects are based on molecular information that remains to be verified in future studies. Although these mechanisms occur simultaneously and may interconnect with one another, their transport mechanisms are described separately for the sake of clarity.

1.4.1.1. Branchial structure and vasculature

Crustacean gills are often referred to as multifunctional super-organs due to their predominant regulation of the animal's systemic osmoregulatory, acid-base regulatory, respiratory, and nitrogenous waste excretory needs. Brachyuran crabs possess paired phyllobranchiate gills consisting of symmetrical stacks of leaf-like lamellae that are in direct contact with their ambient environment allowing for a high surface area that is capable of respiratory gas and ion exchange processes (Fig. 1.2; Freire et al., 2008). Hemolymph that has passed through the closed portion of the crab's circulatory system is pooled within open sinuses that directly perfuse each gill (McGaw and Reiber, 2015, 2002). Hemolymph that enters each gill's singular afferent vessel moves across the lamellae towards the efferent vessel whereas branchial water passes in the opposing direction creating a counter-current flow that maximizes processes such as O₂ uptake and CO₂ excretion (Freire et al., 2008). Once hemolymph enters the afferent vessel, unidirectional valves prevent backflow (Taylor and Taylor, 1986) forcing hemolymph into the heart for recirculation (McGaw and Reiber, 2015, 2002). This hemolymph flow pattern exposes the gills to the net-metabolic output of the extra-branchial tissues while creating a favorable environment for respiratory and ion exchange processes, resulting in the production of post-branchial hemolymph that is slightly alkalized and low in metabolic wastes (*e.g.* CO₂ and ammonia; Allen and Weihrauch, 2021).

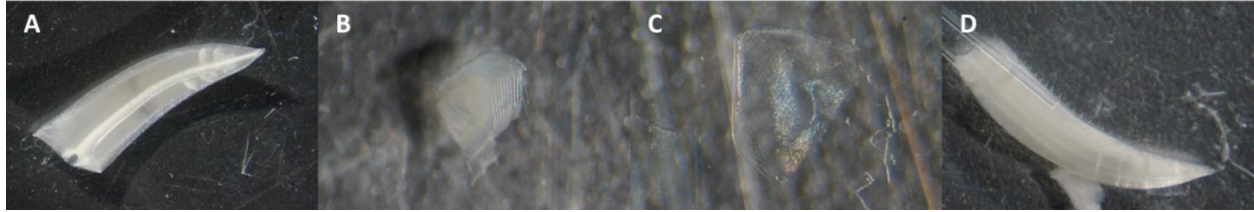


Figure 1.2. Images of an (A) intact phyllobranchiate gill (*ca.* 5 cm length) excised from the shallow hydrothermal vent crab, *X. testudinatus*, as well as a (B) stack of lamellae, (C) an individual lamella, and (D) a demonstration of a cannulated gill prepared for perfusion experiments. All images taken by Allen, G.J.P. under a dissection microscope.

Brachyuran gills possess two functionally distinct epithelial types commonly termed ‘thin’ and ‘thick’ cell patches that are relevant to their regulatory function. Thin epithelia are approximately 1-2 μm thick and contain few mitochondria as would be expected for epithelia that are primarily involved in respiratory gas exchange (Barra et al., 1983). Thick epithelia are 10-20 μm thick with heavy degrees of cellular infolding with an abundance of mitochondria, as would be expected of an ion-transport epithelia in an osmoregulatory tissue (Barra et al., 1983). The abundance of these epithelial types correlates with the osmoregulatory-state of brachyurans. The gills of osmoconforming species appear to be virtually homogeneously limited to thin epithelia, with small thick epithelial regions surrounding the afferent vessel (Copeland and Fitzjarrell, 1968), presumably due to the animal’s negligible need to perform transbranchial Na^+/Cl^- transport (Freire et al., 2008). Hyperosmoregulatory capable crabs transform their gills upon acclimation to dilute environments creating distinct anterior and posterior gills. Anterior gills, generally pairs 1-6, retain the high degree of thin epithelial cells present in their osmoconforming state, suggesting they remain responsible for respiratory gas exchange (Compere et al., 1989). Posterior gills, generally pairs 7-9, increase the abundance of their thick epithelia and their overall expression of transport machinery to support the mass transbranchial transport of environmental Na^+/Cl^- into their hemolymph (Compere et al., 1989; Goodman and Cavey, 1990; Siebers et al., 1982). In addition to their specialized gills, the paracellular junctions of osmoregulatory-capable crab’s branchial epithelia are ‘tighter’ to ions in comparison to osmoregulatory-incapable crabs whose epithelia are considered ‘ion-leaky’ (Luquet et al., 2002a; Onken and McNamara, 2002; Weihrauch et al., 1999). Due to their higher ionoregulatory capabilities, the posterior gills of osmoregulating crabs are generally responsible for a greater

deal of acid-base regulatory transport whereas the anterior gills are predominately used to exude CO₂ (Fehsenfeld and Weihrauch, 2016a, 2013; Genovese et al., 2005).

The apical epithelia of the gills are covered by a thin (2 µm) cuticle, largely composed of chitin-protein matrices that provide structural support to the organs while also acting as an ion diffusion barrier (Compère et al., 1998; Roer and Dillaman, 1984). Although this porous barrier is not biologically active, it has a selective permeability to certain ions being substantially more permeable to cations than anions in *C. maenas* (Lignon, 1987; Onken and Riestenpatt, 2002; Weihrauch et al., 2002). These pores are capable of being blocked by at least some large compounds, such as amiloride, which reduces the ionic permeability of the cuticle through steric inhibition (Onken and Riestenpatt, 2002). While different species possess differing levels of cuticular permeabilities and therefore cannot be freely assumed equal across crustacea (Avenet and Lignon, 1985; Lignon, 1987, 1986), the general concept of the cuticle as a molecular sieve is likely shared.

1.4.1.2. Transbranchial CO₂ transport

As hemolymph passes through the gills, CO₂ rapidly diffuses across the branchial epithelia and into the surrounding environment due to the presence of an environmentally directed P_{CO₂} gradient (Melzner et al., 2009). Modelling has suggested that most of the CO₂ held within the hemolymph is excreted across the gills immediately upon their perfusion (Aldridge and Cameron, 1979) indicating they are highly CO₂ permeable. While epithelia are generally permeable to CO₂ due to the gas being non-polar, brachyuran gills contain Rhesus proteins (Fehsenfeld and Weihrauch, 2013; Martin et al., 2011) that are recognized as CO₂/NH₃ gas-accepting channels in several organisms (Endeward et al., 2008; Nakada et al., 2007; Nawata et al., 2010; Soupene et al., 2002; Thies et al., 2022) and likely serve to enhance the movement of these gases across the gill.

Assuming that the model is correct and extracellular CO₂ is excreted almost immediately upon hemolymph entering the gill, further CO₂ excretion during the hemolymph's transit through the gill would be heavily reduced by reduction in extracellular P_{CO₂} levels unless extracellular HCO₃⁻ is either transported across the basolateral membrane and converted to CO₂ or it is converted to CO₂ within the hemolymph directly. Several transporters are hypothesized to be capable of transporting HCO₃⁻ across the basolateral membrane, as described in section 1.4.1.3.,

although their characterization and veracity remain to be investigated. Assuming at least one such transporter can import extracellular HCO_3^- into the cytosol of brachial cells, intracellular carbonic anhydrase activity may protonate this HCO_3^- forming CO_2 that may then be excreted (Böttcher et al., 1991; Genovese et al., 2005; Henry and Cameron, 1983, 1982b). Several studies have proposed that a basolaterally-localized membrane-bound carbonic anhydrase isoform may act directly on the carbonate equilibrium of the hemolymph (Burnett, 1984; Burnett et al., 1985; Burnett and McMahon, 1985; Genovese et al., 2005), functioning similarly to the membrane-bound carbonic anhydrase found in vertebrate lungs (Klocke, 1978). Such an isoform of carbonic anhydrase would continually replenish the P_{CO_2} levels within the hemolymph as it passes through the gill, maximizing CO_2 excretion. While a putative membrane-bound carbonic anhydrase is believed to exist in brachyurans (Böttcher et al., 1991; Burnett, 1984; Serrano et al., 2007) its localization has not been confirmed. One or both of these carbonic anhydrase isoforms appears to be relevant to CO_2 excretion based on pharmaceutical studies with a *ca.* 50% inhibition occurring in perfused gills of seawater-acclimated *C. maenas* (Fehsenfeld and Weihrauch, 2016a). The relevance of carbonic anhydrase in transbranchial CO_2 excretion may increase in dilute media, given their mRNA expression levels increase 8-fold upon acclimation to dilute environments (Henry et al., 2003; Serrano et al., 2007).

1.4.1.3. Transbranchial HCO_3^- transport

Studies have demonstrated that brachyuran gills are capable of excreting HCO_3^- into their environment as well as secreting HCO_3^- into its extracellular space depending on the osmoregulatory and acid-base regulatory needs of the animal (Allen et al., 2020; Cameron, 1985a; Cameron and Batterton, 1978a; Fehsenfeld and Weihrauch, 2016a; Genovese et al., 2005; Hans et al., 2014; Henry and Cameron, 1982b; Maus et al., 2018; Pane and Barry, 2007; Quijada-Rodriguez et al., 2021; Siebers et al., 1994; Tresguerres et al., 2008; Truchot, 1975b; Truchot, 1981, 1983). In the case of *C. maenas* and *N. granulata*, HCO_3^- has even been implicated as the sensory component that signals for the gill to adjust systemic acid-base status even when isolated from the animal during perfusion experiments (Siebers et al., 1994; Tresguerres et al., 2008). Despite its central role in systemic acid-base regulation, HCO_3^- transport across the gills is poorly understood and largely built around a combination of hypothetical $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers (AEs) and $\text{Na}^+/\text{HCO}_3^-$ cotransporters (NBCs) whose identities, localizations, and transport characteristics are not defined beyond pharmaceutical

sensitivities that cannot distinguish between the two types of transporters (Fehsenfeld and Weihrauch, 2016a; Tresguerres et al., 2008). Some evidence for the importance of at least the AE lies in the general observation that the accumulation of HCO_3^- is usually paralleled by a decrease in extracellular Cl^- (Allen et al., 2020); however, this may be related to the preservation of the fluid's electroneutrality (Cameron, 1989; Stewart, 1983).

Studies at the molecular level have similarly struggled to identify the importance of HCO_3^- transporters in acid-base regulation with only minor changes in mRNA expression levels being detected upon exposing the whole animals to acid-base stressors. For example, a microarray study performed on the gills of brackish-water acclimated *C. maenas* following 3 or 7 days of acclimation to hypercapnia found a very slight but significant 1.1-fold reduction in branchial mRNA levels of an AE (GenBank: CX994129.1) with no significant change to NBC mRNA expression levels (Fehsenfeld et al., 2011). In a follow-up study, Fehsenfeld and Weihrauch (2013) identified that the same AE and NBC were expressed higher within the posterior gills than their anterior equivalents; however, the only significant change in their expression under 7-days of hypercapnic stress was a slight downregulation in AE mRNA expression in gill 4. In the osmoconforming great spider crab, *Hyas araneus*, hypercapnia did not affect mRNA expression of any AEs, in fact, the only HCO_3^- transporter that was affected was an anion channel known as bestrophin whose expression significantly increased (Harms et al., 2014). Bestrophin may play a currently unknown role in the acid-base regulation of aquatic animals given that it shows early signs of being a crucial component to acid-base regulation in the mammalian eye where it facilitates the transport of Cl^- and HCO_3^- across the retinal pigment epithelium (Qu and Hartzell, 2008).

While the literature has struggled to provide direct evidence for the exact roles of the various branchial HCO_3^- transporters, some authors have attempted to produce hypothetical models for the freshwater-acclimated rainbow crab, *N. granulata* (Tresguerres et al., 2008) and seawater-acclimated *C. maenas* (Fehsenfeld and Weihrauch, 2016a). Assuming the gills are in a state requiring a net-excretion of HCO_3^- from the animal's hemolymph, extracellular CO_2 diffuses into the branchial epithelia directly or following conversion of extracellular HCO_3^- by an unconfirmed basolaterally-localized membrane-bound carbonic anhydrase (Burnett, 1984; Fehsenfeld and Weihrauch, 2016a; Tresguerres et al., 2008). In *C. maenas*, but not *N. granulata*,

a basolateral electroneutral $\text{Na}^+/\text{HCO}_3^-$ cotransporter was hypothesized to directly import extracellular HCO_3^- into the cytosol, similar to those observed within the thick ascending loop of Henle within the mammalian kidney's nephrons (Krapf, 1988). Intracellular carbonic anhydrase is then hypothesized to hydrate cytosolic CO_2 forming HCO_3^- that is then excreted into the environment by apical $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers. Protons that are produced by the hydration of CO_2 may be excreted into the environment or intracellular vesicles by Na^+/H^+ exchanger or a V-type H^+ -ATPase or transported into the hemolymph *via* electrogenic $2\text{Na}^+/\text{H}^+$ exchangers believed to be expressed along the basolateral membrane. Basolateral Na^+/K^+ -ATPase activity provides the driving force for these processes by creating a hyperpolarized epithelium and low intracellular Na^+ environment (Fehsenfeld and Weihrauch, 2016a; Siebers et al., 1994; Tresguerres et al., 2008).

Explanation of the mechanism used by the gills to transport HCO_3^- into its hemolymph under acidotic conditions is less understood and involves many assumptions. Intracellular HCO_3^- likely crosses the basolateral epithelium through an electrogenic $\text{Na}^+/\text{HCO}_3^-$ cotransporter that must overcome the unfavorable transport of Na^+ and HCO_3^- against their chemical gradients (Tresguerres et al., 2008). Such a feat is a major function of an electrogenic $\text{Na}^+/\text{HCO}_3^-$ cotransporter found within the proximal tubule of the mammalian kidney (Boron et al., 1997) whose stoichiometry is shifted from $1\text{Na}^+:3\text{HCO}_3^-$ to $1\text{Na}^+:2\text{HCO}_3^-$ upon phosphorylation, resulting in a change in its directionality (Gross et al., 2001). Such an NBC is not confirmed in brachyuran gills; however, its presence would explain the bi-directional movement across the basolateral epithelium where a $1\text{Na}^+:3\text{HCO}_3^-$ stoichiometry may be electrogenic enough, assuming the tissue is hyperpolarized by Na^+/K^+ -ATPase activity and/or V-type H^+ -ATPase activity, to counteract the unfavorable movement of Na^+ and HCO_3^- against their concentration gradient. A basolateral anion exchanger was hypothesized to be involved in the extracellular accumulation of HCO_3^- in *H. araneus*; however, the study was based purely on mRNA level changes and did not provide evidence as to how such a transporter would function. Basolateral anion channels such as bestrophin-1 (Harms et al., 2014) may also aid in the accumulation of extracellular HCO_3^- similar to how the hyperpolarized branchial epithelia move Cl^- across basolateral Cl^- -channels (Onken and Graszynski, 1989). As HCO_3^- accumulates within the hemolymph, apical $\text{Cl}^-/\text{HCO}_3^-$ may alter its directionality due to reduced intracellular HCO_3^- concentrations thereby allowing for uptake of environmental HCO_3^- (Harms et al., 2014;

Tresguerres et al., 2008). Intracellular carbonic anhydrase is likely involved in the process due to its ability to alter intracellular H^+ and HCO_3^- concentrations (Burnett et al., 1985; Tresguerres et al., 2008), whereas a basolateral membrane-bound carbonic anhydrase would seem counter-productive to HCO_3^- accumulation. The feasibility of these hemolymph-directed HCO_3^- transport mechanisms remain to be elucidated, especially across different species and salinities where electrochemical gradients and the burden of osmoregulation differ.

1.4.1.4. Transbranchial ammonia transport

Excretion across the gills acts as a potential means to regulate systemic acid-base homeostasis while simultaneously mitigating the toxic effects associated with the accumulation of extracellular ammonia (Ip and Chew, 2010; Larsen et al., 2014; Weihrauch and Allen, 2018). Brachyurans are capable of passively excreting gaseous ammonia (NH_3) and actively excreting ionic ammonium (NH_4^+) through separate mechanisms. Passive excretion of NH_3 is achieved by an environmentally directed P_{NH_3} gradient from the metabolically active intracellular fluids, through the extracellular fluid, and into the environment (Kormanick and Cameron, 1981). Much like CO_2 , NH_3 is accepted by Rhesus proteins that are highly expressed in the gills (Fehsenfeld and Weihrauch, 2013; Martin et al., 2011; Nawata et al., 2010; Si et al., 2018; Weihrauch et al., 2004b; Wright and Wood, 2009) allowing for diffusive movement to occur more rapidly across the tissue.

Ammonium accounts for the majority of total ammonia within an animal's body fluids due to the molecule's relatively alkaline pKa (*ca.* 9.3, Cameron and Heisler, 1983). Due to its ionic charge, NH_4^+ cannot diffuse across the membrane; however, it shares a similar hydration radius and charge to K^+ allowing it to be accepted by most K^+ transporters including the Na^+/K^+ -ATPase (Chew et al., 2014; Leone et al., 2017; Towle and Holleland, 1987) and K^+ -channels (Carrisoza-gaytán et al., 2011; Fehsenfeld and Weihrauch, 2016b; Weihrauch et al., 1998). The gills of brachyuran crabs actively transport extracellular NH_4^+ into the cytosol using the basolateral Na^+/K^+ -ATPase (Fehsenfeld and Weihrauch, 2016c; Weihrauch et al., 2002, 1999) and K^+ channels (Fehsenfeld and Weihrauch, 2016b; Weihrauch et al., 1998). Rhesus-protein expressing vesicles acidified through either V-type H^+ -ATPase activity or Na^+/H^+ exchange (Fehsenfeld and Weihrauch, 2016a; Weihrauch et al., 2001) are then believed to trap cytosolic NH_3 , effectively mitigating its ability to cause cytotoxic effects. Acidified ammonia-rich vesicles

are then moved along the microtubule network towards the apical membrane where ammonia is excreted *via* exocytosis (Ren et al., 2015; Weihrauch et al., 2002). This active mechanism can occur independent of environmental conditions and is believed to be the dominant pathway which brachyurans use to excrete ammonia against unfavorable conditions (Weihrauch and Allen, 2018). Such an example is observed in the gills of *M. magister* where ammonia excretion continues to occur against a 16-fold hemolymph-directed gradient (Martin et al., 2011). These conditions were applied acutely, suggesting the gills do not need to adjust their branchial transport profile and may use this mechanism even in the absence of unfavorable conditions.

1.4.2. Antennal glands

The antennal glands of crustaceans are considered structural analogs to the nephrons within the mammalian kidney and are involved in aspects of osmoregulation, acid-base regulation, and nitrogen excretion in brachyurans (Allen et al., 2021; Chung and Lin, 2006; De Vries et al., 1994; Freire et al., 2008; Holliday, 1980; Tsai and Lin, 2014; Wheatly, 1985; Wolcott, 1991). In comparison to the gills, the role of the antennal glands in these regulatory processes has often been considered to be minor (Henry et al., 2012; Henry and Wheatly, 1992; Wheatly and Henry, 1992); however, this broad claim appears to be based on only three studies, two of which measured acid-base parameters on aquatic species, and only one of which exclusively used an acid-base stressor (Cameron and Batterton, 1978b; Lockwood and Riegel, 1969; Wheatly, 1985).

1.4.2.1. Structure of the brachyuran antennal glands

Antennal glands are paired organs found in the crab's anterior region near the base of the eyestalks responsible for the production of urine that exudes from the animal's nephropores (Freire et al., 2008). Although the structure of the organs differ in some species, brachyuran antennal glands generally consist of a coelomosac where ultrafiltration occurs, a complex set of coiled tubules called the labyrinth, and a bladder – lacking the nephridial canal present in most other decapods (Freire et al., 2008; Peterson and Loizzi, 1974; Tsai and Lin, 2014). Hemolymph is directed to the antennal gland through defined arteries (McGaw and Reiber, 2015, 2002) where it contacts the coelomosac and undergoes an ultrafiltration process. The filtrate is then passed through the labyrinth whose convoluted maze of tubules reabsorbs and secretes ions to form urine that is eventually deposited into the bladder which may also perform some degree of

transepithelial transport processes on its own (Freire et al., 2008; Holliday, 1980). Some attempts to characterize different cell types within the antennal glands have indicated distinct coelomic cells, labyrinthine cells, and end-labyrinthine cells in a semi-terrestrial crab, *Ocypode stimpsoni* (Tsai and Lin, 2014). In this species, coelomic cells are generally Na⁺/K⁺-ATPase-poor and express a Na⁺/H⁺ exchanger, Na⁺/K⁺/2Cl⁻ cotransporter, and the V-type H⁺-ATPase within a large vacuole whereas labyrinthine cells and end-labyrinthine cells possess much higher Na⁺/K⁺-ATPase immunoreactivity along their basolateral membrane with an apical expression of the Na⁺/H⁺ exchanger and V-type H⁺-ATPase (Tsai and Lin, 2014). Labyrinthine and end-labyrinthine cells are distinguished from each other by a basolateral and apical expression of the Na⁺/K⁺/2Cl⁻, respectively (Tsai and Lin, 2014). The presence of similar cell types in other brachyuran species remains to be verified, especially given that terrestrial and semi-terrestrial crabs such as *O. stimpsoni* generally make more extensive use of their antennal glands for regulatory function than most fully aquatic species (Allen et al., 2021; De Vries et al., 1994; Greenaway, 1999; Tseng et al., 2020; Wolcott, 1991).

1.4.2.2. Apparent role of the antennal glands in acid-base regulation

The antennal glands have the potential to regulate systemic acid-base status by adjusting the acid-base status of the urine before its excretion. This topic is relatively unexplored in aquatic brachyurans with most studies focusing on the antennal glands' ability to regulate the systemic concentration of divalent cations (*e.g.* Ca²⁺ and Mg²⁺; Holliday, 1980; Wheatly, 1985; Wheatly et al., 2002) and sulfate ions (SO₄²⁻; Riegel and Cook, 1975). Under acute hypoosmotic stress (20 ppt.), the antennal glands of *M. magister* are capable of reabsorbing HCO₃⁻ from the glandular lumen indicating it can selectively manipulate urinary acid-base equivalents (Wheatly, 1985). In hypercapnia-exposed freshwater acclimated blue swimming crabs, *Callinectes sapidus*, the antennal glands did not appear to perform any significant manipulation of the acid-base equivalents within the urine, although urinary pH was considerably more alkaline than the blood under hypercapnic stress (+0.32 units) and remained stable throughout the exposure (Cameron and Batterton, 1978b). Other studies exposing seawater-acclimated *C. maenas* to hypercapnic and hypoxic stress deemed the antennal glands as negligible acid-base regulatory organs as occlusion of the nephropores did not alter the net-base efflux rate of the intact animal (Truchot, 1979). Occlusion of the nephropores is likely a poor method to determine such a factor as it may cause internal stress as the urine cannot escape the bladder, potentially causing extra-glandular

compensatory action to occur. While it may be unwise to deem the antennal glands as negligible manipulators of the systemic carbonate system, these results do provide some basis to deem them of significantly less importance than the gills.

Antennal glands may also manipulate the acid-base status of brachyurans by adjusting the concentration of urinary ammonia. Studies have investigated this possibility to some degree with results largely identifying that the urine contains approximately the same concentration of ammonia as the hemolymph (Cameron and Batterton, 1978b; Wheatly, 1985). While excreting hemolymph-level concentrations of ammonia through the urine is of notable importance to the animal's systemic state, its importance depends on the flow-rate of the urine. Marine brachyurans excrete urine at an inconsistent rate with a more consistent and rapid rate occurring as environmental salinities decrease, presumably to excrete excess water from the body fluids (Cameron and Batterton, 1978b; Freire et al., 2008; Holliday, 1980; Riegel and Cook, 1975; Wheatly, 1985). Unfortunately, the only freshwater study has suggested urine in *C. sapidus* is relatively ammonia-poor and likely does not excrete enough ammonia to be relevant to systemic acid-base regulation (Cameron and Batterton, 1978b).

The importance of the antennal gland in acid-base regulation and nitrogenous waste excretion is much more pronounced in semi-terrestrial crabs that remain emersed for prolonged durations. For example, the ghost crab, *Ocypode quadrata*, concentrates ammonia within its acidic isotonic urine (pH 5.50) reaching concentrations upwards of 100 mmol l^{-1} that is recycled into its branchial chamber allowing some NH_3 and presumably CO_2 to volatilize during the process (De Vries et al., 1994; Wolcott, 1991).

While further work on the antennal glands is required to determine if their contribution in brachyuran acid-base regulation in both aquatic and semi-terrestrial species is truly negligible in comparison to the gill, it cannot be dismissed that these organs work together within an intact animal. Most studies explore only one organ or deem the antennal glands to be unimportant by either occluding the nephropore or stating urinary flow is too intermittent. Likely, the gills act as the predominant site for HCO_3^- absorption from the environment while the antennal glands may act as a site that regulates how much HCO_3^- (or possibly H^+) is lost over time, especially in aquatic species where urine cannot be recycled (Wheatly, 1985).

1.4.3. Hypodermis and exoskeletal system

Crustaceans are characterized for their calcified structures – particularly their thick exoskeleton that is often discussed as a structural and defensive non-biologically active organ. While the exoskeleton certainly does serve those functions, its composition includes large amounts of CaCO_3 and MgCO_3 salts making it an enormous potential source of base-equivalents carried by the animal (Roer and Dillaman, 1984). The hypodermis – an epithelial layer that could be considered the outer ‘skin’ of crustaceans – is capable of reversibly calcifying and solubilizing the exoskeleton during the moult cycle (Roer, 1980). While the precise mechanism of calcification and solubilization is outside of the interest of this dissertation, calcification appears to be related to the alkalization of the fluid between the hypodermis and exoskeleton whereas solubilization presumably relates to its acidification (Cameron, 1985b; Cameron and Wood, 1985; Wood and Cameron, 1985). Extracellular CO_2 and HCO_3^- are transported across the hypodermis where the alkalized fluid compartment causes their speciation to shift towards CO_3^{2-} to permit calcification. Under solubilizing conditions, acidification of the fluid compartment causes this process to reverse, allowing CO_2 or HCO_3^- to be directed back into the extracellular fluid. While the transporters and mechanisms of this process are not fully understood, its reversible nature has led to many authors investigating whether it is exploited to counteract acid-base disturbances (Cameron, 1985a, 1981; DeFur et al., 1980; Small et al., 2010).

Mobilization of exoskeletal CO_3^{2-} to buffer the extracellular fluid is generally monitored by an increase in Ca^{2+} and/or Mg^{2+} within the hemolymph in parallel to the accumulation of HCO_3^- , rather than direct measurements on the exoskeletal system itself. Cameron (1981) provided evidence that this process is likely highly important in fully-terrestrial brachyurans whereas its importance to aquatic species is relatively low. Defur et al. (1980) found that although seawater-dwelling red rock crabs, *Cancer productus*, do not mobilize exoskeletal CO_3^{2-} during immersed acid-base stress, they appear to be capable of mobilization during emersion based on the appearance of a 2-fold increase in hemolymph Ca^{2+} concentrations after 1 hour of emersion. More recent studies have suggested that ocean acidification and anthropogenic climate change may affect the calcification of calcifying invertebrates (Whiteley, 2011); however, even relatively high hypercapnic exposures do not appear to invoke a clear mobilization of exoskeletal carbonate stores (Small et al., 2010).

1.4.4. Gastrointestinal tract

The gastrointestinal tract is likely the least studied putative acid-base regulatory organ in brachyuran crustaceans – in fact, I am not aware of any published studies that have directly measured changes in acid-base fluxes across the gastrointestinal epithelia under an environmental acid-base challenge. The gut is largely involved in the reabsorption of ions and water in terrestrial (Greenaway et al., 1990) and hypo- and hyperosmoregulating brachyurans (Mantel and Farmer, 1983). Given that most osmoregulatory transport is linked to an exchange of acid-base equivalents, these processes may be involved in systemic acid-base regulation (Wheatly and Henry, 1992); however, the role of the gut in an osmoconforming crab is even less understood.

1.5. Diversifying the field

Within the last few decades, a substantial shift in our understanding of brachyuran acid-base regulation has occurred allowing us to begin forming a mechanistic understanding of at least the branchial components. Most of this detailed understanding has related to the wealth of knowledge brought forth by early osmoregulatory studies on the gills of *C. maenas*, *C. sapidus*, *E. sinensis*, and *N. granulata*. Virtually all the mechanistic information concerning brachyuran acid-base physiology stems from the use of these osmoregulatory capable species, particularly *C. maenas*, under all sorts of stressors. As outlined throughout this dissertation, brachyurans are extremely diverse – as such, it is difficult to say how far the field’s current knowledge extends. Stenohaline osmoconformers that represent the majority of brachyuran species (Martin and Davis, 2001) have more or less yet to be studied mechanistically although whole animal studies suggest they are more tolerant and more fully compensate for most acid-base stressors than their osmoregulatory relatives (Hans et al., 2014; Pane and Barry, 2007; Small et al., 2010). The most effort into their mechanistic details is a purely molecular-based study on *H. araneus* whose deep-sea dwelling lifestyle has been found to correlate with poor acid-base compensation (Harms et al., 2014). Beyond the gills, only one study investigated the potential importance of the antennal glands, albeit under hypo-osmotic stress, finding they may be capable of regulating systemic HCO_3^- levels (Wheatly, 1985). Even under the stress of emersion, our knowledge is largely limited to intertidal species that may not reflect the processes used by species that emerge for more than *ca.* 6-hour intervals. Certainly, it is not a problem to understand these few species at a detailed level and it would be unrealistic to suggest all species be investigated; however, the

diversity of brachyurans and their manipulatable gills (Allen and Weihrauch, 2021) represents a rare opportunity to observe how essential physiological processes occur in a broad spectrum of environments and life histories.

1.6. Thesis goals and outline

The contents of this dissertation intend to broaden our understanding of brachyuran acid-base regulatory processes, including related aspects of nitrogen regulation and osmoregulation where possible, in species of different environments. Beyond the goal of the dissertation, the hope is that the presented work can provide a basic understanding of how such processes occur in stenohaline marine species and perhaps identify interesting reasons to study a broader species diversity.

This initial chapter served to not only provide a generalized consensus on the types of environmental stressors experienced by aquatic brachyurans but also their general means of maintaining acid-base homeostasis. Additionally, the information was presented in a way that should bring some of the field's shortcomings to the reader's attention, including a bias towards osmoregulatory capable species and the dismissal of extra-branchial organs in their acid-base regulatory processes. In chapter 2, the acid-base compensatory response of *M. magister*, a stenohaline osmoconforming crab, was investigated at the whole animal and branchial levels under acid-mediated environmental acidification. While the crab's gills certainly were involved in the compensatory process, albeit not in the direct fashions identified in osmoregulatory capable species, the antennal glands played a critical role in the regulation of systemic HCO_3^- reserves while likely acting as a major site for acid-excretion. Chapter 3 focuses on specialized adaptations to extreme hypercapnia in a hydrothermal vent-endemic species, *X. testudinatus*, as a representative of the extremophile brachyurans commonly found in such harsh environments. While it was expected that the crab would be highly acid-tolerant, results suggest that the species, and their isolated gills, are capable of excreting CO_2 against hemolymph-directed P_{CO_2} gradients – a process that is fundamentally different from our CO_2 excretory mechanisms of animals in general. Chapter 4 investigated how a relatively uncharacterised semi-terrestrial mangrove crab, *Helice formosensis*, attempts to regulate its acid-base status under prolonged emersion – a stressor that the field largely understands from a purely intertidal viewpoint. The study identified that the crab reprocesses its urine within the branchial chamber allowing it to

undergo prolonged emersion by preventing acidotic stress (although they may be limited by a progressive alkalosis). Curiously, nitrogenous wastes do not accumulate during emersion, as documented in other terrestrial species; however, the crab presents metabolic signs of a functional Cahill cycle which has previously been unidentifiable in aquatic species. The general discussion held in chapter 5 summarizes these findings and discusses open questions that became apparent throughout the completion of this dissertation.

**Chapter 2: Acid-base compensation under acute acid-stress in an osmoconforming crab,
*Metacarcinus magister***

Garett Joseph Patrick Allen* and Dirk Weihrauch

Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada, R3T
2N2

Prepared for submission to: Comparative Biochemistry and Physiology Part A: Molecular and
Integrative Physiology.

Author contributions:

G.J.P.A. designed the study, performed the experiments, analyzed and interpreted results, and
wrote the manuscript. D.W. aided in the study's design, manuscript editing, and provided
financial assistance.

2.1. Abstract

Although the acid-base regulatory capacity of several brachyuran species has been studied, mechanistic details are biased towards euryhaline, osmoregulating species such as *C. maenas*. Osmoregulating species possess specialized gills with relatively ion-tight paracellular pathways capable of dramatically transforming their ion-transport machinery to hyperosmoregulate in dilute media while osmoconforming in seawater. Conversely, the gills of marine osmoconformers are incapable of actively absorbing Na^+/Cl^- in dilute media and possess ion-leaky paracellular junctions that presumably facilitate the equilibration of some environmental and extracellular factors. Due to the interconnectedness of transporters used for transbranchial osmoregulation and acid-base balance, marine osmoconformers may not be accurately described by the hypothetical transport models of euryhaline crabs. In the presented study, the consequences of acid-mediated environmental acidification (pH 7.1) on the systemic and urinary acid-base status of the osmoconforming Dungeness crab, *Metacarcinus magister*, were investigated. Crabs fully corrected the brief respiratory acidosis induced by environmental acidification within 12-hours of exposure through a concerted process involving the gills and antennal glands. Perfused gills indicate that acid-exposure induces a directional shift in their transbranchial HCO_3^- transport resulting in the net-accumulation of extracellular HCO_3^- while the antennal glands increase their HCO_3^- reabsorption rates while increasing their acid-equivalent secretion rates. When combined, the crab prevents the loss of HCO_3^- to their environment while excreting an acidified and low- HCO_3^- containing urine. Perfused gills indicate that transbranchial acid-base regulation is highly dependent on the Na^+/K^+ -ATPase and carbonic anhydrase that likely indirectly activate a hypothetical basolateral $\text{Na}^+/\text{HCO}_3^-$ cotransporter and apical $\text{HCO}_3^-/\text{Cl}^-$ anion exchanger.

2.2. Introduction

Due to the pH sensitivity of proteins, living systems all share the need to regulate their acid-base status (Riggs, 1988; Somero, 1986). The capacity of a species to counteract acid-base disturbances and the mechanisms they rely on are presumably related to their life history and predisposition to acid-base challenges. Brachyuran crustaceans are amongst the most environmentally diverse animal families inhabiting terrestrial and aquatic ecosystems of various salinities, temperatures, depths, and acidities (Martin and Davis, 2001). Many species bury themselves for extended periods where poor water circulation and waste accumulation ($\text{NH}_3/\text{NH}_4^+$, H^+ , CO_2) produce physiologically challenging environments rarely encountered by their pelagic relatives (McGaw, 2005). Due to their varying degree of predisposition to acidic stressors, brachyurans are interesting comparative animal models for studying acid-base regulatory mechanisms.

Brachyuran gills are responsible for most of the animal's acid-base regulatory needs through the transbranchial transport of acid-base equivalents between the hemolymph and the environment (Henry et al., 2012). Although several acid-base equivalents are transported across the gill, those pertaining to the carbonate equilibrium are the most influential. Excretion of CO_2 is believed to rely on the maintenance of an environmentally directed CO_2 partial pressure (P_{CO_2}) gradient (ΔP_{CO_2}) that allows the gas to passively diffuse from the hemolymph, across the branchial epithelia, and into the environment (Melzner et al., 2009). Rhesus proteins expressed within the gills are believed to facilitate this process as they have been shown to function as CO_2 channels and thereby alter the gill's CO_2 permeability (Geyer et al., 2013; Perry et al., 2010; Thies et al., 2022). Transbranchial transport of H^+ is generally achieved either directly *via* the V-type H^+ -ATPase as is common in freshwater species (Freire et al., 2008) or exchange for Na^+ *via* Na^+/H^+ exchangers (Fehsenfeld and Weihrauch, 2016c; Towle et al., 1997; Tresguerres et al., 2008). Relatively little mechanistic information has described transbranchial HCO_3^- transport mechanisms; however, the bi-directional transport is believed to depend on a combination of various $\text{HCO}_3^-/\text{Cl}^-$ anion exchangers and $\text{Na}^+/\text{HCO}_3^-$ cotransporters (Braun et al., 2009; Fehsenfeld and Weihrauch, 2016a; Parks et al., 2007; Tresguerres et al., 2008). These transport processes are all linked by branchial carbonic anhydrase activity that replenishes the intracellular concentrations of H^+ , CO_2 , and HCO_3^- that serve as substrates for the various transporters (Burnett et al., 1985; Henry et al., 2003; Henry and Cameron, 1983).

In addition to adjusting the carbonate equilibrium, ammonia (*i.e.* total NH_3 and NH_4^+ , T_{Amm}) and its transbranchial transport can be used to counteract acid-base disturbances as occurs within the mammalian kidney (Weiner and Verlander, 2011). Due to its relatively alkaline acid-dissociation constant (pKa 9.3; Cameron and Heisler, 1983), physiological pH causes >99.5% of ammonia to exist in its acid-equivalent form, NH_4^+ . Under acid-base disturbances, crustaceans often alter their ammonia excretion rate to change their net-acid efflux rates. For example, *C. maenas* increases its ammonia excretion rate 18-fold to eliminate acid-equivalents and nitrogenous wastes that accumulated within its hemolymph after feeding (Quijada-Rodriguez et al., 2022). Several invertebrates including the American horseshoe crab, *Limulus polyphemus* (Hans et al., 2018), the common octopus, *Octopus vulgaris* (Hu et al., 2017), and the hydrothermal vent crab, *X. testudinatus* (Allen et al., 2020), are believed to regulate systemic ammonia concentrations to maintain a pool of excretable acid-equivalents that can be similarly mobilized across the gills under acid-base disturbances (Weihrauch and Allen, 2018). Branchial excretion of gaseous NH_3 occurs passively down its P_{NH_3} gradient where its transbranchial passage is facilitated by Rhesus proteins (Martin et al., 2011; Nakada et al., 2007; Wright and Wood, 2009). Some species, especially those inhabiting poorly-buffered freshwater environments (Weihrauch and Allen, 2018), rely on HAT and NHE activity to create an acidic boundary layer along the gill's apical epithelium, forming an acid-trap that converts excreted NH_3 to NH_4^+ (Wilson et al., 1994). Brachyurans are also capable of actively transporting NH_4^+ across the basolateral gill epithelium and excreting it against unfavorable environmental conditions. Due to the similarity in their hydration radii and molecular charge, NH_4^+ can be accepted by many K^+ transporters including the Na^+/K^+ -ATPase (Leone et al., 2017; Towle and Holleland, 1987; Weihrauch et al., 1998). Once within the cytosol, NH_3 is believed to enter Rhesus-protein expressing vesicles where it is converted to NH_4^+ and subsequently trapped by vesicular V-type H^+ -ATPase or Na^+/H^+ exchangers activity (Fehsenfeld and Weihrauch, 2016a; Ren et al., 2015; Weihrauch et al., 2002). Although these branchial mechanisms may allow crustaceans to use ammonia as an acid-base compensatory molecule, ammonia is also acutely toxic (Larsen et al., 2014; Wilkie, 1997). As such, compensatory mechanisms that rely upon or relate to the accumulation of ammonia within the hemolymph may pose a threat to the animal if critical systemic levels are reached whereas mechanisms that increase ammonia's excretion may prove beneficial to the animal beyond its acid-base regulatory needs.

Hypothetical mechanisms of transbranchial H^+ , CO_2 , HCO_3^- , and NH_3/NH_4^+ transport in crustaceans are largely based on euryhaline species such as *C. maenas* (Fehsenfeld and Weihrauch, 2016a), *N. granulata* (Tresguerres et al., 2008), and *C. sapidus* (Burnett et al., 1985; Cameron and Batterton, 1978a; Henry and Cameron, 1982b). Euryhaline osmoregulating crabs possess gills capable of undergoing anatomical transformations that dramatically alter branchial transport properties allowing them to hyperosmoregulate in dilute media and osmoconform in seawater (Fehsenfeld and Weihrauch, 2016a, 2013; Freire et al., 2008). Furthermore, the paracellular junctions between branchial cells of osmoregulatory active crabs are fairly ion ‘tight,’ limiting the passive flux of ions down their concentration gradients (Weihrauch et al., 1999). Marine osmoconforming crabs, the majority of brachyuran species (Martin and Davis, 2001), cannot transform their branchial transporter machinery in response to osmotic stress (Compere et al., 1989) and possess ion ‘leaky’ paracellular junctions that may increase the likelihood that environmental factors penetrate their extracellular space (Weihrauch et al., 1999). These differences suggest that current hypothetical working models do not accurately describe those of marine osmoconformers.

Despite their ion ‘leaky’ paracellular pathways and limited osmoregulatory capacity, gills of *M. magister* are capable of active ammonia excretion (Martin et al., 2011) and counteracting hypercapnia-driven acid-base disturbances (Hans et al., 2014; Pane and Barry, 2007). While hypercapnic conditions cause CO_2 to rapidly diffuse across phospholipid bilayers, the degree of acidosis an animal experiences upon an acid-mediated environmental acidification may relate to its paracellular tightness and counteractive transport mechanisms. Due to their limited occurrence in a natural setting, literature describing the acid-base regulatory response to purely acid-mediated, as opposed to CO_2 -mediated, environmental acidification in invertebrates is sparse having only been studied in freshwater crayfish. Despite their ion ‘tight’ epithelia, *Procambarus clarkii* exposed to H_2SO_4 -acidified waters of pH 3.8 experience a large extracellular metabolic acidosis where hemolymph pH was reduced by up to 0.5-units (Morgan and McMahon, 1982; Patterson and DeFur, 1988; Wood and Rogano, 1986; Zanotto and Wheatly, 1993). Crayfish did not accumulate extracellular HCO_3^- to counteract these disturbances as has been shown for most crustaceans exposed to hypercapnic conditions but rather suffered from a chronic loss of HCO_3^- (Morgan and McMahon, 1982; Patterson and DeFur, 1988; Wood and Rogano, 1986).

In the presented study, acid-base regulatory characteristics of intact Dungeness crabs and their isolated gills were investigated under control (pH 8.1) and HCl-acidified environmental conditions (pH 7.1). The gills and antennal glands allowed the Dungeness crab to maintain constant extracellular HCO_3^- concentrations while excreting acid-equivalents to prevent the severe acidosis experienced by freshwater crayfish during acid-exposure. Pharmaceutical manipulations of perfused gills were also used to develop hypothetical working models for acid-base regulation in the marine osmoconformer.

2.3. Materials and methods

2.3.1. Animals

Male Dungeness crabs were purchased from commercial supermarkets (The Real Canadian Superstore, Winnipeg, Manitoba, Canada) and housed in recirculating seawater systems in the Duff Roblin Animal Holding Facility of University of Manitoba for a minimum of 7 days before experimentation. Artificial seawater (Fritz Reef Pro Mix™, U.S.A.) was maintained at *ca.* 32 ppt, pH 8.10, and 14 °C on a 14:10 hour light:dark cycle. Crabs were provided plastic shelters and fed frozen scallops *ad libitum* 3-times per week. Crabs were fasted for 48-hours before experimentation to mitigate the effects of feeding on the animal's physiological state.

2.3.2. Whole animal experiments

Dungeness crabs were exposed to either control (10 mmol l⁻¹ HEPES, 200 μmol l⁻¹ NH₄Cl, 32 ppt. salinity Fritz RPM, pH 8.10) or acidified seawater (10 mmol l⁻¹ HEPES, 200 μmol l⁻¹ NH₄Cl, 32 ppt. salinity Fritz RPM, pH 7.10) over a 12-hour time course. HEPES was added to the seawater to mitigate aeration and metabolic wastes from changing the pH during experiments. Equimolar ammonia to the crab's hemolymph concentration (Hans et al., 2014; Martin et al., 2011) was added to the seawater to limit the animal's ability to passively excrete ammonia down its P_{NH₃} gradient to better target its transport mechanism. The pH of seawater was adjusted using 1N HCl and NaOH. Crabs were individually housed in 20-gallon aquaria within a controlled environmental room to maintain water at 14°C throughout the experiment.

Hemolymph and urine were sampled prior to the beginning of the experiment as well as 3-, 6-, and 12-hours post-exposure. Hemolymph (*ca.* 1 ml) was collected by puncturing the arthroal membrane of the rear walking leg with a gas-tight Hamilton syringe. Urine was aspirated from deflected nephropores using a pipet as described by Wolcott (1991). The anterior

of the crab was patted dry and the first 10 μl of urine excluded from the sample to mitigate environmental contamination. The pH of hemolymph and urine was measured immediately using an InLab Micro pH electrode (Mettler-Toledo, Ohio, USA) attached to a pH-ISE meter model 225 (Denver Instruments, Gottingen, Germany). Total CO_2 was then measured using methods described by Lee et al. (2018). In brief, 5 μl of sample was injected through a septum using a gas-tight Hamilton syringe and into a glass spargine column filled with 0.1 mmol l^{-1} HCl to transform the dissolved inorganic carbon into CO_2 . Nitrogen gas (100 $\text{ml N}_2 \text{ gas min}^{-1}$, GFC17, Aalborg, Orangeburg, NY, USA) was passed through the chamber forcing the CO_2 into an infrared CO_2 detector (LI-850 infrared $\text{CO}_2/\text{H}_2\text{O}$ gas analyzer, LI-COR, Lincoln, NE, USA). Total CO_2 was then assessed by determining the integral of raw data collected by LI-8 x 0 software (v1.0.2, LI-COR) of samples compared to those of NaHCO_3 standards. Rearrangement of the Henderson-Hasselbalch equation (Eq. 2.1) was then used to determine the sample's P_{CO_2} using the CO_2 solubility constant ($\alpha = 0.043 \text{ mmol l}^{-1} \text{ torr}^{-1}$) and dissociation constant of carbonic acid ($\text{pK}_{a1} = 6.00$) experimentally determined by Truchot (1976) for the hemolymph of *C. maenas* at 14°C and 32 ppt. salinity.

$$\text{Equation 2.1.} \quad P_{\text{CO}_2} = \frac{TCO_2}{[(1 + \text{antilog}(\text{pH} - \text{pK}_{a1})) * \alpha \text{CO}_2]}$$

The concentration of HCO_3^- within the samples were then determined using equation 2.2

$$\text{Equation 2.2.} \quad [\text{HCO}_3^-] = TCO_2 - (P_{\text{CO}_2} * \alpha \text{CO}_2)$$

Hemolymph and urine samples were then frozen at -20°C until they were deproteinized using 6% perchloric acid and neutralized using KOH before measuring their ammonia concentrations using the methodology described by Quijada-Rodriguez et al. (2022) using an Orion 9512 ammonia gas sensing ISE electrode (Fisher Scientific, Ottawa, ON, Canada) attached to a pH/mV/temperature/ISE meter (Accumet Excel XL25, Fisher Scientific) following protocols described by Weihrauch et al. (1998).

2.3.3. Gill perfusion setup

Crabs taken from their general holding tanks were anaesthetized on ice and terminated by severing the central ganglia before exposing the branchial chamber. Posterior (#7-9) gills were then excised from the animal and immediately submerged in an artificial hemolymph-like saline (in mmol l^{-1} : 380 NaCl, 7.5 KCl, 10 CaCl_2 , 20 MgCl_2 , 30 Na_2SO_4 , 5 NaHCO_3 , 0.5 glucose, 0.5

glutamine, 0.1 glutathione, 0.2 NH₄Cl; pH 8.0; Hans et al., 2014; Martin et al., 2011). Following the protocol described by Siebers et al. (1985), heat-stretched polyethylene-50 tubing connected to a peristaltic pump (Sci 323 Watson-Marlow Bredel Pump, Falmouth, Cornwall, England) was then used to cannulate and perfuse artificial hemolymph-like saline through the afferent vessel of the gill at a rate of $178.8 \pm 4.8 \mu\text{l min}^{-1}$. An additional piece of heat-stretched polyethylene-50 tubing was used to cannulate the efferent vessel to collect perfusate exiting the gill. A custom-made neoprene clamp was then situated around the base of the gill to gently compress the tissue and secure the cannulas. Clamped and cannulated gills were then submerged in a continuously aerated bathing solution composed of 32 ppt. artificial seawater (Fritz RPM, U.S.A.) supplemented by 10 mmol l⁻¹ HEPES and adjusted to pH 8.10 or 7.10 using 1N HCl and NaOH. HEPES was necessary as the aeration of the bathing media was found to quickly alkalize the solution in preliminary experiments. When required, ammonia concentrations in the bathing solution were adjusted to 200 $\mu\text{mol l}^{-1}$ using NH₄Cl.

2.3.4. Measuring transbranchial transport rates

Following setup, gills were perfused for 15-minutes to allow them to equilibrate to the ammonia-free HEPES enriched bathing media and perfusion solution. Bathing media was then replenished, and the gill's baseline transport rates were measured over 30 minutes. Ammonia-free HEPES enriched bathing media was then replaced with a similar solution containing 200 $\mu\text{mol l}^{-1}$ NH₄Cl and perfusate collected for 30-minutes to determine how the gill's transport rates were affected by equimolar NH₄Cl conditions that promote active ammonia excretion mechanisms due to the limited ability of ammonia to diffuse down its P_{NH₃} gradient. Equimolar ammonia HEPES-enriched bathing media was then replenished and pharmaceutical enriched perfusion saline was perfused through the gills for a 30-minute incubation period before a 30-minute collection period. Pharmaceutical-free artificial hemolymph-like saline was then passed through the gill for a 30-minute pre-collection washout period followed by a final 30-minute recovery collection period. This perfusion protocol lasted approximately 3.25 hours which is within the 6-hour viability period of perfused gills isolated from *M. magister* in previous studies (Hans et al., 2014; Martin et al., 2011). Ouabain was used to target the Na⁺/K⁺-ATPase (5 mmol l⁻¹; Weihrauch et al., 1998a), KM91104 was used to target the V-type H⁺-ATPase (20 $\mu\text{mol l}^{-1}$; Kartner et al., 2010), colchicine was used to target the microtubule network (200 $\mu\text{mol l}^{-1}$; Weihrauch et al., 2002), and acetazolamide was used to target carbonic anhydrase (1 mmol l⁻¹;

(Weihrauch et al., 1998). The perfusion saline for all experiments where gills were planned to be exposed to either KM91104 or acetazolamide included DMSO (1% final volume) to match the composition of the pharmaceutically enriched saline. All pharmaceuticals were applied to the basolateral epithelium of the gills due to the potential interference of the cuticle upon apical application (Onken and Riestenpatt, 2002). Only gills that continued to be successfully perfused throughout the entire duration of the experiment were accepted as data included in the pharmaceutical experiments. As such, control rates used in the pharmaceutical analyses are based on a slightly lower sample number than the rates reported in the non-pharmaceutical analyses.

Perfusate pH, total carbon, and ammonia were measured using the methods previously described for hemolymph and urine. Total carbon was measured using either a Corning 965 Total CO₂ analyzer (Olympic Analytical Services, England) or the methods of Lee et al. (2018). Ammonia concentrations within perfusate samples were measured using an Orion 9512 ammonia gas sensing ISE electrode (Fisher Scientific, Ottawa, ON, Canada) attached to a pH/mV/temperature/ISE meter (Accumet Excel XL25, Fisher Scientific) following protocols described by Weihrauch et al. (1998). Perfusate P_{CO2} and [HCO₃⁻] were determined mathematically using equations 2 and 3, respectively. Transport rates of acid-base equivalents across the gill were described as the per mass and time net-change in an acid-base equivalent abundance following passage through the gill. Post-hoc experiments where perfusion saline was perfused through the cannulas in absence of a gill were used to correct transport rates for any adsorption of acid-base equivalents by the tubing.

2.3.5. Statistics

Statistical analyses were performed using GraphPad Prism (v.9.3.1; GraphPad Software, La Jolla California, U.S.A., www.graphpad.com). Data was tested for Gaussian distribution and homogeneity of variance using the Shapiro-Wilk and Browne-Forsyth tests, respectively. Outliers within data sets were then identified and removed following a Grubb's outlier test ($\alpha = 0.05$). Statistical differences in time-course exposure experiments were assessed using a mixed-effects three-way ANOVA and Tukey's post-hoc test where environmental pH, fluid type, and time were the independent variables. Statistical differences between perfused gills were assessed using a paired or unpaired Student's t-test. Statistical differences were deemed significant when

$p < 0.05$. All biological replicates are defined as individual crabs and data is presented as the mean \pm S.E.M.

2.4. Results

2.4.1. Whole animal response to 200 $\mu\text{mol l}^{-1}$ NH_4Cl

Before the animal's exposure to 200 $\mu\text{mol l}^{-1}$ NH_4Cl (*i.e.* control $t = 0\text{h}$), their hemolymph is maintained slightly alkaline compared to their urine (+0.11 pH; Fig. 2.1). Hemolymph also contained significantly more CO_2 (1.9-fold; hemolymph $P_{\text{CO}_2} = 191 \pm 12$ Pa P_{CO_2} ; urine $P_{\text{CO}_2} = 103 \pm 5$ Pa P_{CO_2} ; Fig. 2.2) and significantly less HCO_3^- (2.4-fold; hemolymph $[\text{HCO}_3^-] = 5.9 \pm 0.4$ mmol l^{-1} ; urine $[\text{HCO}_3^-] = 2.5 \pm 0.06$ mmol l^{-1} ; Fig. 2.3) than the urine, although the fluids contain similar amounts of ammonia (hemolymph $[\text{T}_{\text{Amm}}] = 208 \pm 21$ $\mu\text{mol l}^{-1}$; urine $[\text{T}_{\text{Amm}}] = 128 \pm 28$ $\mu\text{mol l}^{-1}$; Fig. 2.4). Control crabs maintained constant hemolymph and urine acid-base equilibria throughout their exposure to seawater of pH 8.10 containing 10 mmol l^{-1} HEPES and 200 $\mu\text{mol l}^{-1}$ NH_4Cl averaging 7.96 ± 0.01 and 7.94 ± 0.01 pH after 12-hours, respectively (Fig. 2.1). Although the general differences between the acid-base equivalents of the hemolymph and urine were maintained throughout the exposure, the HCO_3^- levels in the urine increased slightly, albeit significantly from an initial 2.5 ± 0.06 $\mu\text{mol HCO}_3^- \text{l}^{-1}$ to 3.3 ± 0.1 $\mu\text{mol HCO}_3^- \text{l}^{-1}$ after 12-hours of exposure, representing a 1.3-fold increase (Fig. 2.3). Hemolymph ammonia increased gradually over the 12-hours of exposure, shifting from an initial 208 ± 21 $\mu\text{mol T}_{\text{Amm}} \text{l}^{-1}$ to 262 and 293 $\mu\text{mol T}_{\text{Amm}} \text{l}^{-1}$ after 6 and 12-hours, respectively (Fig. 2.4). The ammonia concentration of the urine remained constant throughout the exposure, averaging 133 ± 5 $\mu\text{mol T}_{\text{Amm}} \text{l}^{-1}$ after 12-hours (Fig. 2.4).

2.4.2. Whole animal response to acid-stress and 200 $\mu\text{mol l}^{-1}$ NH_4Cl

Acidified conditions caused a slight but significant respiratory acidosis as characterized by a significant reduction in hemolymph pH ($\Delta\text{pH}_E = -0.095$ units; Fig. 2.1) and a slight, but statistically insignificant 25% increase in hemolymph P_{CO_2} from an initial 149.4 ± 10 Pa P_{CO_2} to 187.1 ± 13 Pa P_{CO_2} after 3-hours of exposure (Fig. 2.2; $p = 0.0927$). The crabs' hemolymph remained at a similar pH throughout the remaining exposure (7.903 ± 0.02 pH; 12-hours); however, urine was maintained in an acidified state ($\Delta\text{pH}_U = -0.71$ units) throughout the entire exposure averaging 7.281 ± 0.01 after 12-hours (Fig. 2.1). Similarly, hemolymph HCO_3^- was unaffected by the acid exposure, averaging 5.2 ± 0.4 mmol l^{-1} after 12-hours, whereas urinary

HCO_3^- concentrations were significantly reduced by 73% throughout the exposure changing from an initial $2.5 \pm 0.04 \text{ mmol l}^{-1}$ to $0.8 \pm 0.08 \text{ mmol l}^{-1}$ after 12-hours (Fig. 2.3). Hemolymph ammonia remained constant throughout the first 3-hours of the exposure, averaging $216 \pm 25 \text{ } \mu\text{mol l}^{-1}$, but was significantly reduced after 6 and 12-hours, averaging 171 ± 13 and $195 \pm 24 \text{ } \mu\text{mol T}_{\text{Amm}} \text{ l}^{-1}$, respectively, despite the elevated levels of ammonia within their ambient environment (Fig. 2.4). Urinary ammonia concentrations were unaffected by the experimental conditions, remaining significantly below those of the hemolymph and averaging $76 \pm 11 \text{ } \mu\text{mol T}_{\text{Amm}} \text{ l}^{-1}$ after 12-hours of exposure (Fig. 2.4).

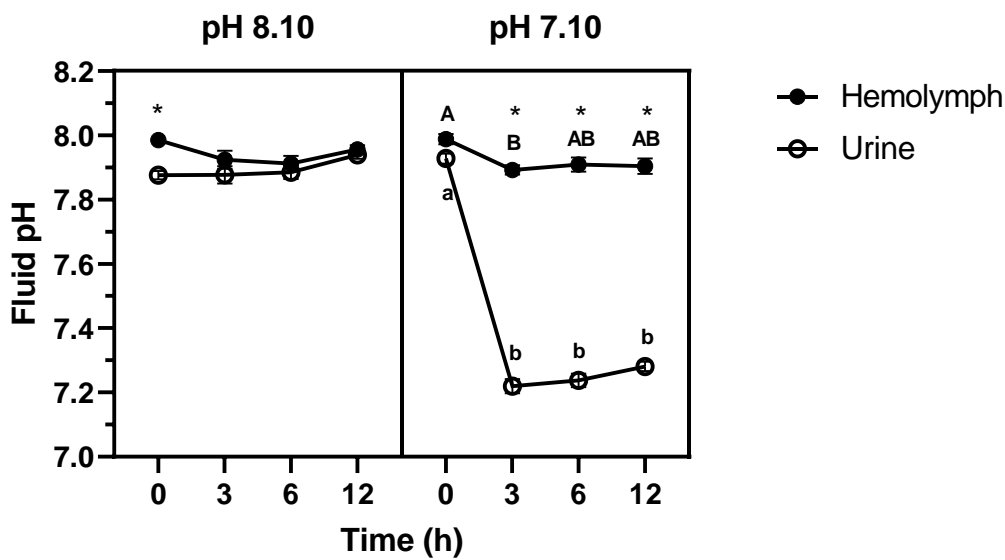


Figure 2.1. Hemolymph and urine pH during 12-hours of exposure to either seawater adjusted to pH 8.10 (N = 7) or pH 7.10 (N = 6-7) containing 10 mmol l⁻¹ HEPES and 200 $\mu\text{mol l}^{-1}$ NH₄Cl. Asterisks indicate a significant difference between the hemolymph and urine pH at the same time point, whereas uppercase and lowercase letters denote significant differences between the pH of hemolymph and urine over time, respectively.

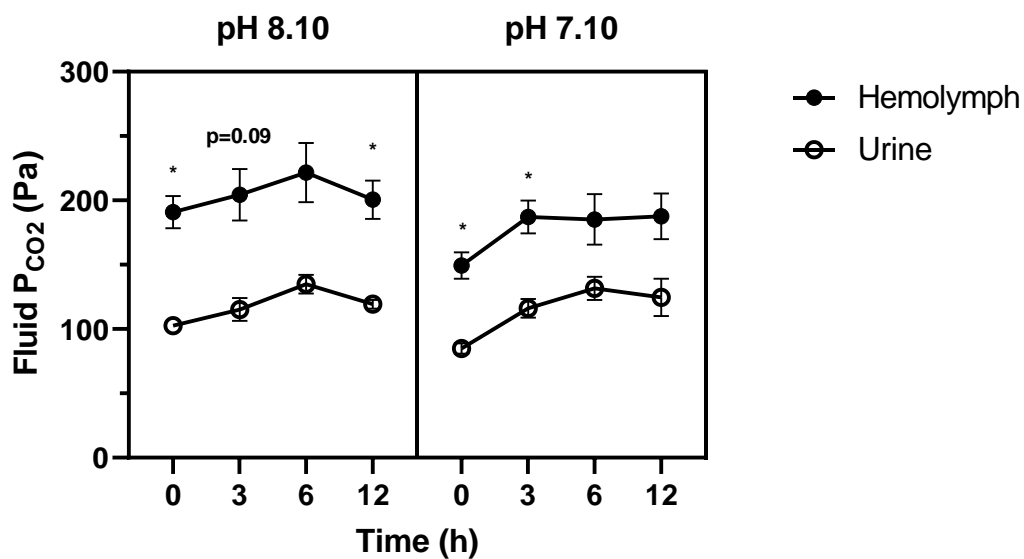


Figure 2.2. Hemolymph and urine P_{CO2} during 12-hours of exposure to either seawater adjusted to pH 8.10 (N = 7) or pH 7.10 (N = 6-7) containing 10 mmol l⁻¹ HEPES and 200 μmol l⁻¹ NH₄Cl. Asterisks or listed p-values indicate a significant difference between the hemolymph and urine P_{CO2} at the same time point.

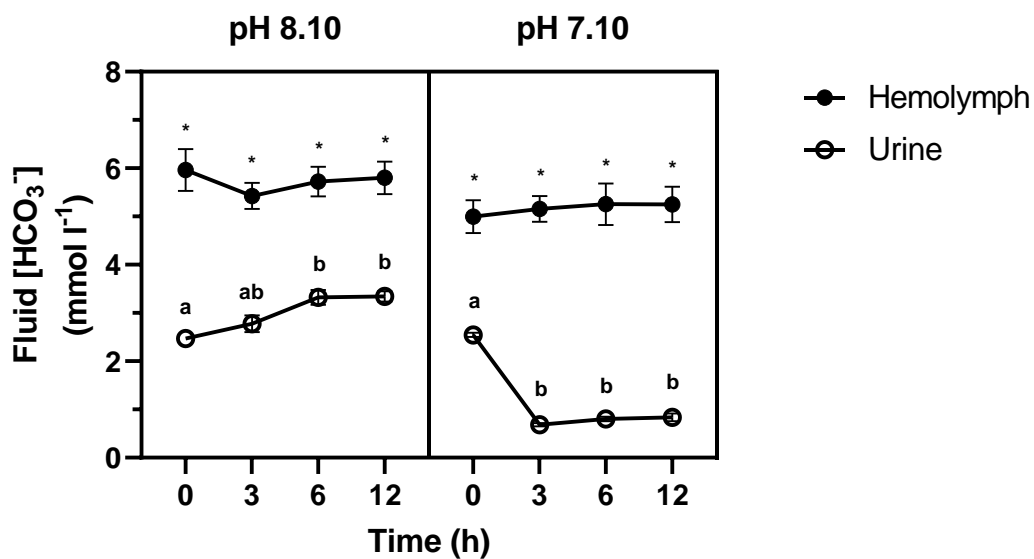


Figure 2.3. Hemolymph and urine [HCO₃⁻] during 12-hours of exposure to either seawater adjusted to pH 8.10 (N = 7) or pH 7.10 (N = 6-7) containing 10 mmol l⁻¹ HEPES and 200 μmol l⁻¹ NH₄Cl. Asterisks indicate a significant difference between the hemolymph and urine [HCO₃⁻] at the same time point, whereas lowercase letters denote significant differences between the [HCO₃⁻] of the urine over time.

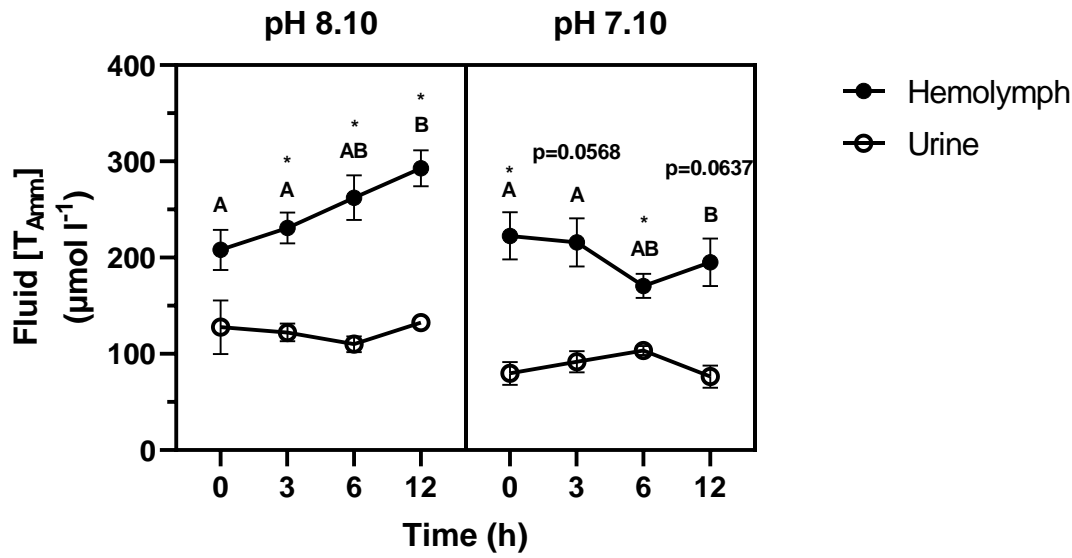


Figure 2.4. Hemolymph and urine [T_{Amm}] during 12-hours of exposure to either seawater adjusted to pH 8.10 (N = 7) or pH 7.10 (N = 3-7) containing 10 mmol l⁻¹ HEPES and 200 µmol l⁻¹ NH₄Cl. Asterisks indicate a significant difference between the hemolymph and urine [T_{Amm}] at the same time point, whereas uppercase letters denote significant differences between the [T_{Amm}] of the hemolymph over time.

2.4.3. Baseline transbranchial transport rates of perfused gills

Since preliminary experiments identified that the H⁺_{Eq}, CO₂, HCO₃⁻, and T_{Amm} transport rates of anterior gill #4 and posterior gills #7-9 (SI Fig. 2.1) were not significantly different, data was collected using perfused posterior gills and pooled together for interpretation. When the control bathing media contained no ammonia, perfused gills alkalized the perfusion saline at a rate of 16.9 ± 2.3 nmol H⁺_{Eq} g_{FW}⁻¹ h⁻¹ (Fig. 2.5A), equating to an approximate 0.1-unit increase in its pH as it passed through the gill. The alkalization was accompanied by a net-efflux of CO₂ (268.6 ± 34.5 Pa CO₂ g_{FW}⁻¹ h⁻¹; Fig. 2.5B) and HCO₃⁻ (7.3 ± 3.2 µmol HCO₃⁻ g_{FW}⁻¹ h⁻¹; Fig. 2.5C) from the perfusion saline across the gill. Transbranchial transport rates of H⁺, CO₂, and HCO₃⁻ were not affected by the presence of an equimolar NH₄Cl concentration between the environment and perfusion saline (Fig. 2.5A-C). Ammonia excretion rates across the gills were significantly reduced from 4.5 ± 0.4 µmol T_{Amm} g_{FW}⁻¹ h⁻¹ under ammonia-free conditions to 3.3 ± 0.4 µmol T_{Amm} g_{FW}⁻¹ h⁻¹ under equimolar NH₄Cl conditions (Fig. 2.5D).

Acidification of the bathing media caused H^+ , CO_2 , and HCO_3^- transport to shift from a net-efflux to net-influx across the perfused gill. When the acidified bathing media contained no ammonia, the perfusion saline was acidified by an influx of acid-equivalents across the gill at a rate of $36.3 \pm 5.2 \text{ nmol } H^+_{Eq} \text{ g}_{FW}^{-1} \text{ h}^{-1}$ equating to an average 0.15-unit reduction in its pH as it passed through the gill (Fig. 2.5A). Accumulation of CO_2 and HCO_3^- within the perfusate occurred at rates of $749.2 \pm 67.4 \text{ Pa } CO_2 \text{ g}_{FW}^{-1} \text{ h}^{-1}$ and $30.4 \pm 3.1 \text{ } \mu\text{mol } \text{g}_{FW}^{-1} \text{ h}^{-1}$, respectively (Fig. 2.5BC). Transbranchial ammonia excretion rates under all conditions were significantly higher in an acidified environment than control environment (Fig. 2.5D). Equimolar NH_4Cl concentrations between the acidified environment and the perfusion saline did not affect transbranchial ammonia excretion rates ($5.8 \text{ } \mu\text{mol } T_{Amm} \text{ g}_{FW}^{-1} \text{ h}^{-1}$ compared to $5.3 \pm 0.4 \text{ } \mu\text{mol } T_{Amm} \text{ g}_{FW}^{-1} \text{ h}^{-1}$ in ammonia-free and equimolar NH_4Cl conditions, respectively; Fig. 2.5D).

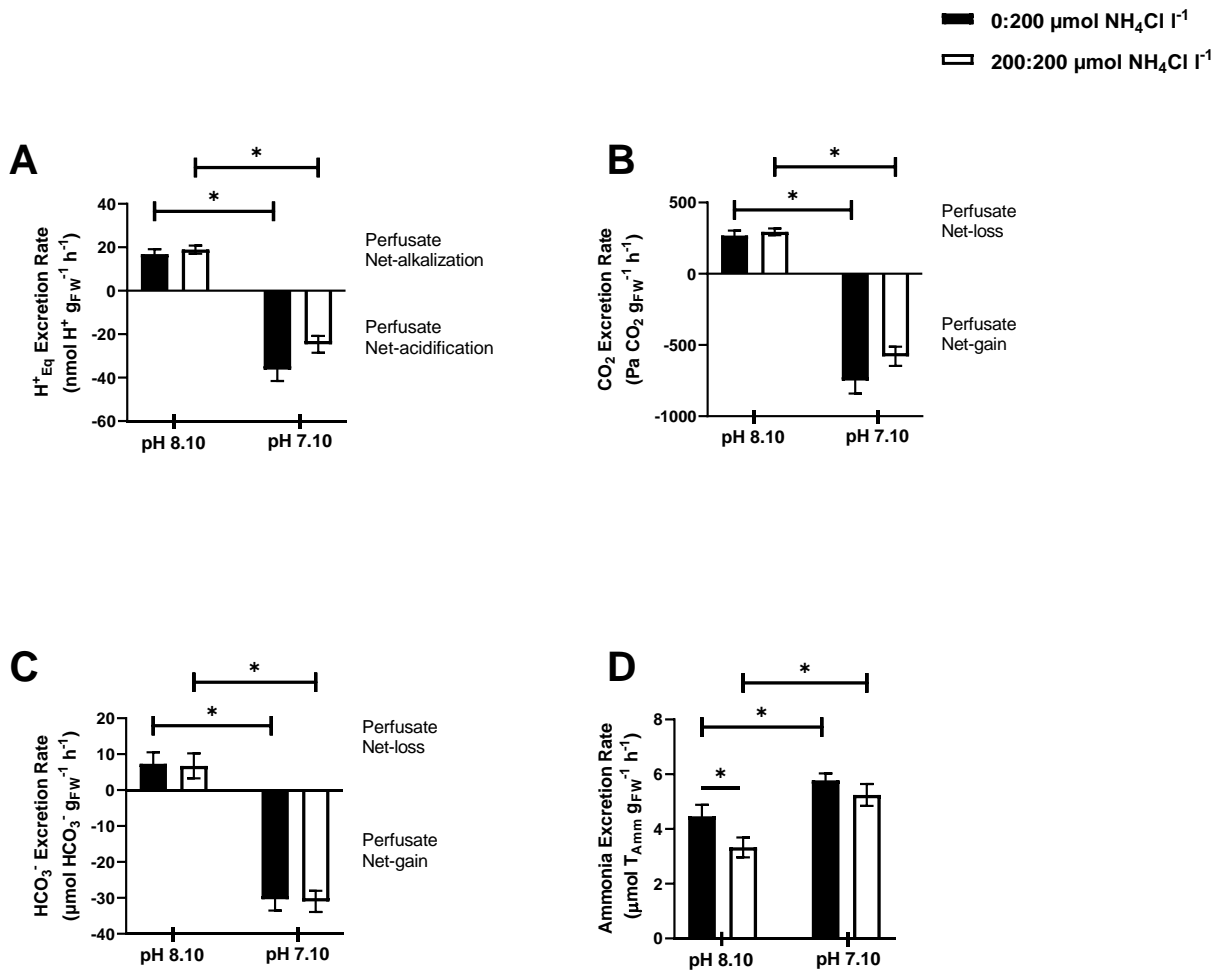


Figure 2.5. Transport rates of H^+_{Eq} (A), CO_2 (B), HCO_3^- (C), and T_{AmM} (D) across the perfused gills of *M. magister* bathed in HEPES-enriched seawater of either pH 8.10 or 7.10 containing either 0 or 200 $\mu\text{mol l}^{-1}$ NH_4Cl . Asterisks denote significant differences between the transport processes. Data pertaining to crabs exposed to pH 8.10 conditions is based on 29-31 crabs whereas those pertaining to pH 7.10 are based on 36-39 crabs, with only one gill being used per crab per replicate.

2.4.4. Effects of pharmaceutical exposure on perfused gills

Under control conditions, inhibition of carbonic anhydrase through the basolateral application of acetazolamide significantly increased the gill's ability to alkalize the perfusion saline by 74% as it passed through the gill (Fig. 2.6A). Disruption of the cytoskeletal system by

colchicine tended to increase the perfused gill's ability to excrete CO_2 (Fig. 2.6B; 69% increase, $p = 0.055$). Transbranchial HCO_3^- excretion underwent a significant 2.9-fold increase due to ouabain's inhibition of the Na^+/K^+ -ATPase (Fig. 2.6C). Interestingly, inhibition of carbonic anhydrase activity by acetazolamide abolished transbranchial HCO_3^- excretion and instead caused HCO_3^- to accumulate within the perfusate (Fig. 2.6C). The inhibition of the microtubule network by colchicine resulted in a significant 71.8% reduction in transbranchial ammonia excretion rates (Fig. 2.6D). Inhibition of the Na^+/K^+ -ATPase by ouabain abolished ammonia excretion rates and caused ammonia to accumulate within the perfusate (Fig. 2.6D).

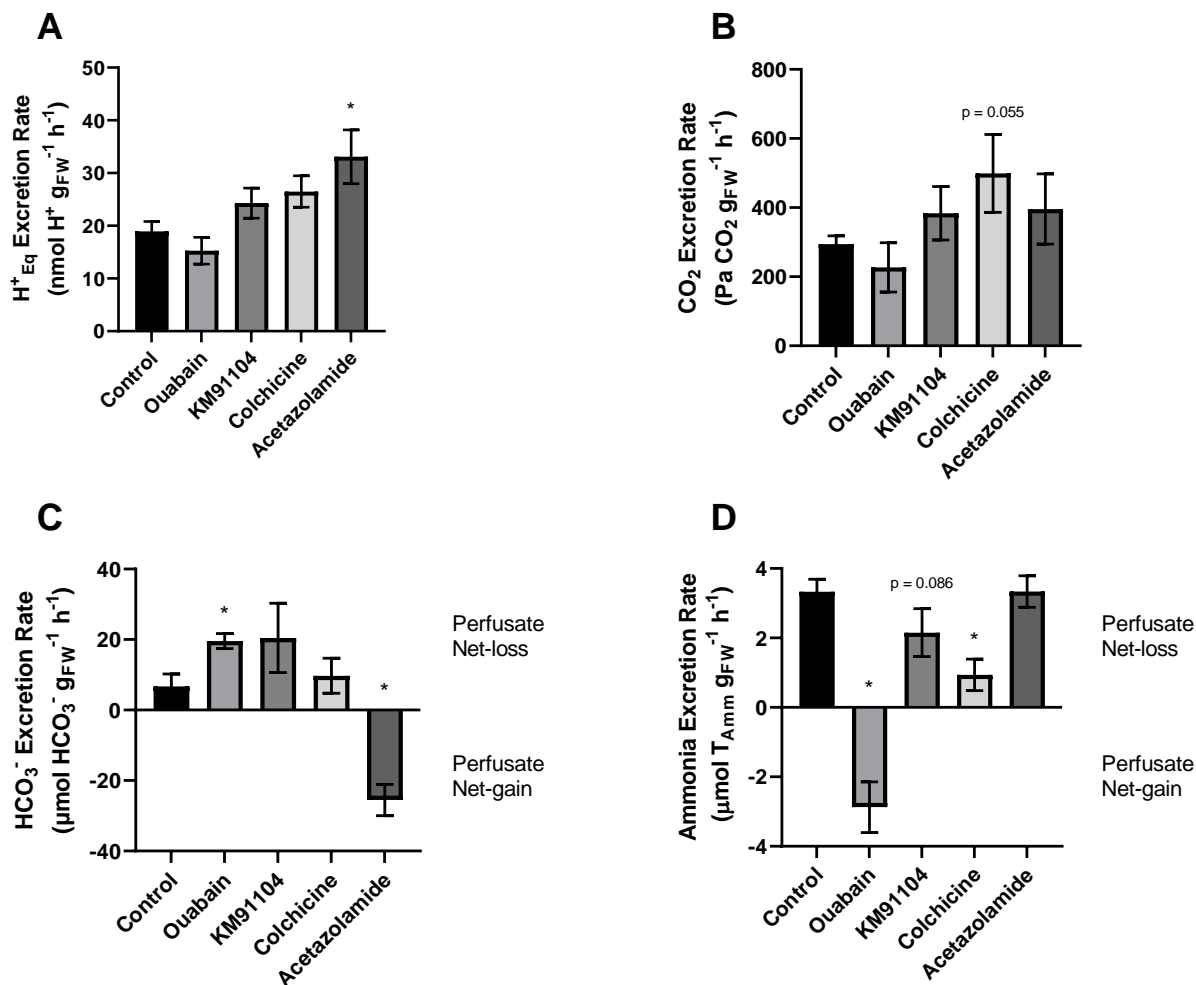


Figure 2.6. Effects of basolaterally applied pharmaceuticals to the transport rates of H⁺_{Eq} (A), CO₂ (B), HCO₃⁻ (C), and T_{amm} (D) across the perfused gills of *M. magister* bathed in HEPES-enriched seawater of pH 8.10 containing 200 μmol l⁻¹ NH₄Cl. Asterisks denote significant differences between the transport rate of pharmaceutically exposed gills and non-exposed gills. N_{Ouabain} = 6, N_{KM91104} = 11, N_{Colchicine} = 6, N_{Acetazolamide} = 6.

Under acidified conditions, ouabain's inhibition of the Na⁺/K⁺-ATPase caused a significant 3.9-fold increase in the rate of perfusate acidification whereas inhibition of carbonic anhydrase by acetazolamide significantly increased the gill's ability to alkalize the perfusion saline due to an impairment of HCO₃⁻ secretion resulting in no net-change between the pH of the perfusate and initial perfusion solution (Fig. 2.7A). Inhibition of the Na⁺/K⁺-ATPase and carbonic anhydrase similarly effected the rate of CO₂ accumulation within the perfusate causing

a significant 2.7-fold increase and 2.8-fold decrease, respectively (Fig. 2.7B). Transbranchial HCO_3^- uptake was significantly reduced by 71% when the Na^+/K^+ -ATPase was inhibited whereas inhibition of the V-type H^+ -ATPase by KM91104 abolished transbranchial HCO_3^- uptake (Fig. 2.7C). Transbranchial ammonia excretion rates underwent a significant 61% reduction upon inhibition of the Na^+/K^+ -ATPase (Fig. 2.7D).

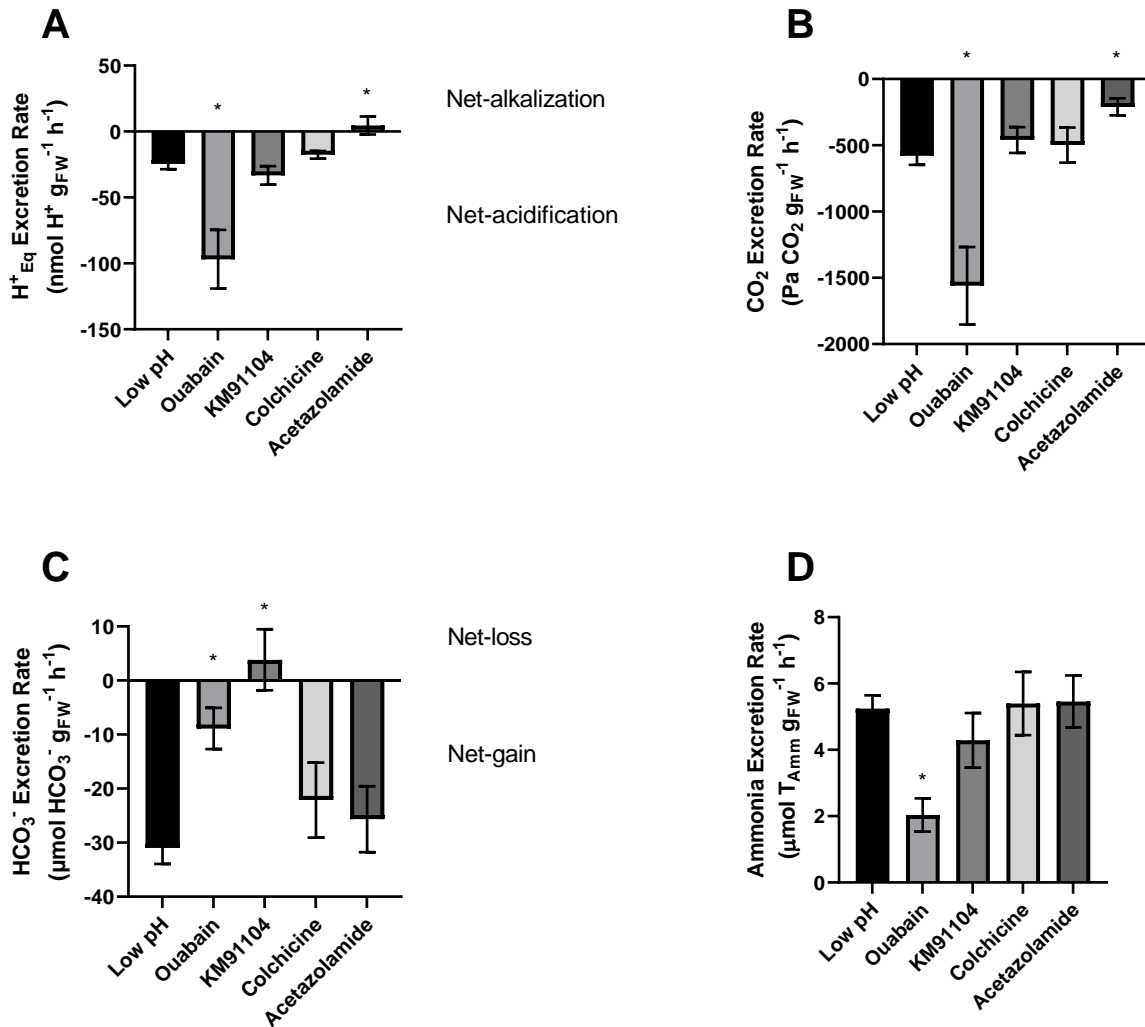


Figure 2.7. Effects of basolaterally applied pharmaceuticals to the transport rates of H^+_{Eq} (A), CO_2 (B), HCO_3^- (C), and T_{Amm} (D) across the perfused gills of *M. magister* bathed in HEPES-enriched seawater of pH 7.10 containing $200 \mu\text{mol l}^{-1} \text{NH}_4\text{Cl}$. Asterisks denote significant differences between the transport rate of pharmaceutically exposed gills and non-exposed gills.

$N_{\text{Ouabain}} = 8$, $N_{\text{KM91104}} = 8$, $N_{\text{Colchicine}} = 8$, $N_{\text{Acetazolamide}} = 9$.

The collective results of the pharmaceutical experiments alongside the hypotheses and results of other studies were used to design hypothetical transbranchial transport mechanisms for the carbonate species (Fig. 2.8) and ammonia (Fig. 2.9) in *M. magister*.

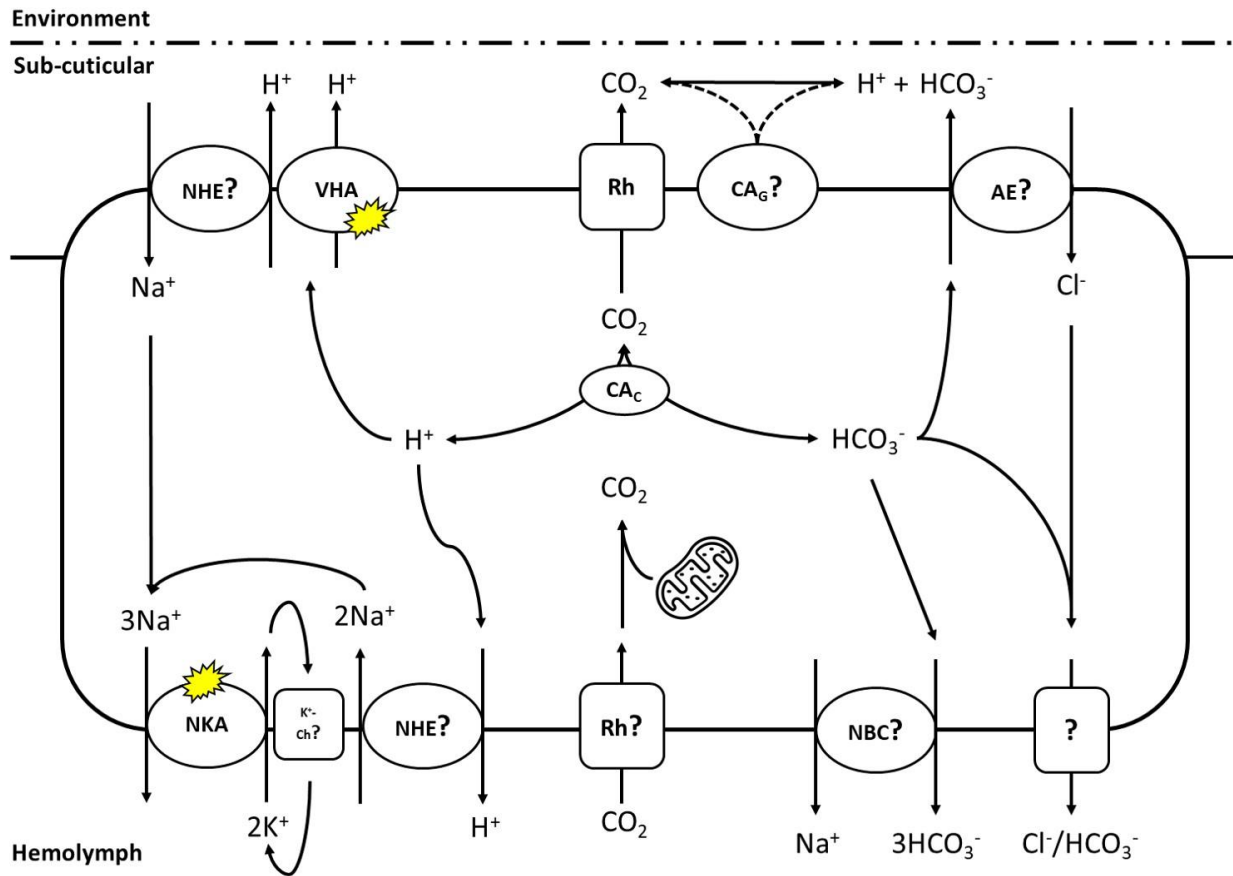


Figure 2.8. Proposed hypothetical model describing the transbranchial transport of H^+ , CO_2 , and HCO_3^- in *M. magister*. Transporters whose presence and/or cellular localization are unconfirmed are indicated by question marks. Active processes requiring the consumption of ATP are denoted by a yellow ‘spark.’ Models are based on results generated from gills 7 – 9 but may apply to gills 1 -6 as the baseline transport rates of these molecules was statistically similar in the anterior gills investigated in this study.

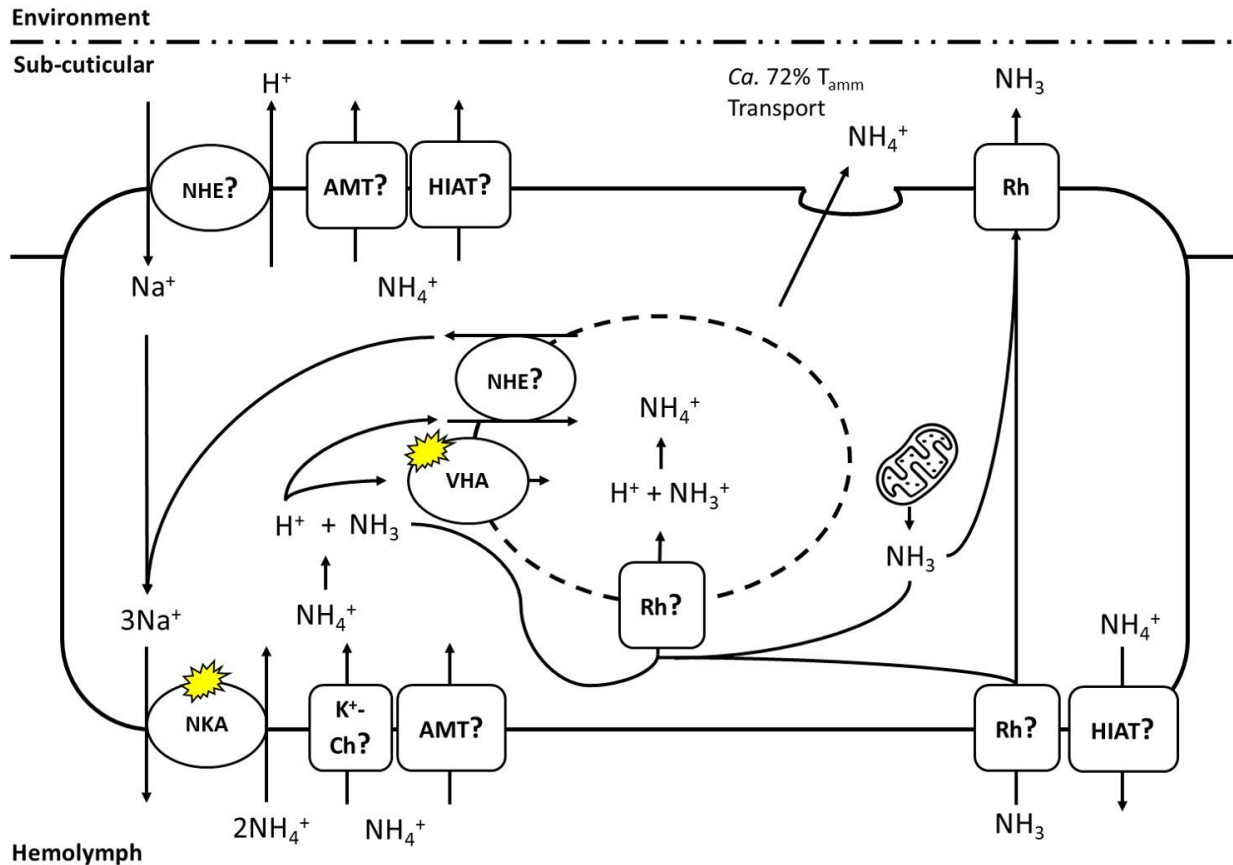


Figure 2.9. Proposed hypothetical model describing the transbranchial transport of ammonia ($\text{NH}_3/\text{NH}_4^+$) in *M. magister*. Transporters whose presence and/or cellular localization are unconfirmed are indicated by question marks. Active processes requiring the consumption of ATP are denoted by a yellow ‘spark.’ Models are based on results generated from gills 7 – 9 but may apply to gills 1 -6 as the baseline transport rates of these molecules was statistically similar in the anterior gills investigated in this study.

2.5. Discussion

2.5.1. Comparative consequences of acid-stress in aquatic crustaceans

Studies focusing on the consequences of non- CO_2 -driven acid-exposure on decapod crustaceans have only previously targeted freshwater crayfish. Within 24-hours of acid-exposure, crayfish experience a severe extracellular acidosis as hemolymph pH falls by 0.4 – 0.6 units (Dejours and Armand, 1983; Ellis and Morris, 1995; Morgan and McMahon, 1982; Wood and

Rogano, 1986). Although a brief respiratory acidosis occurs during this time, most of the acidotic stress is metabolic as indicated by a rapid decline in extracellular HCO_3^- levels which eventually fall below 1 mmol l^{-1} as the animals fail to restore their acid-base equilibrium (Dejours and Armand, 1983; Morgan and McMahon, 1982; Wood and Rogano, 1986). Although the Dungeness crab experienced a brief respiratory acidosis within the first few hours of acid-exposure, its extracellular HCO_3^- concentrations remained constant allowing the crab to stabilize its acid-base equilibrium through the combined compensatory action of its gills and antennal glands (Fig. 2.1). While crayfish possess similar acid-base regulatory organs (Henry et al., 2012; Henry and Wheatly, 1992), they are encumbered by the osmoregulatory burden associated with hyperosmoregulation. Many of the key transporters associated with the transbranchial movement of acid-base equivalents in crustaceans are directly linked to osmoregulation as Na^+ and Cl^- are exchanged for H^+ and HCO_3^- , respectively (Henry and Wheatly, 1992; Wheatly and Henry, 1992). Upon severe acid-base disturbances, hyperosmoregulators must balance their physiological processes carefully or risk cell volume disruption and its associated consequences (Larsen et al., 2014). Although the gills of osmoconformers may possess a lower overall expression of transport machinery (Barra et al., 1983; Compere et al., 1989; Freire et al., 2008), they could be capable of dedicating a greater proportion of transport processes to specific stressors such as acid-base disturbances. Evidence for this can also be seen when considering freshwater brachyurans (Cameron and Batterton, 1978a; Quijada-Rodriguez et al., 2021) appear to require more time and resources to recover from hypercapnic stress than brackish-water dwelling hyperosmoregulators (Appelhans et al., 2012; Fehsenfeld and Weihrauch, 2013) who in turn are slower than stenohaline osmoconformers (Allen et al., 2020; Hans et al., 2014; Pane and Barry, 2007; Small et al., 2010). It should be noted that the difference between HCO_3^- concentrations in these environments may contribute to the ability of their inhabitants to accumulate hemolymph HCO_3^- , although the acidified conditions used in the present study did not appear to impair HCO_3^- transport processes despite the titrated conditions.

Although the Dungeness crab has been previously found to prevent rapid accumulation of ammonia within its hemolymph upon acute exposure to high environmental ammonia (Martin et al., 2011), our results indicate that a simultaneous acid-exposure causes the crabs to slightly reduce their extracellular ammonia concentrations. These results alongside the observed increase in transbranchial ammonia excretion rates under acidified conditions (Fig. 2.5) would tend to

suggest that the crab's ability to actively excrete ammonia may benefit its ability to regulate its systemic acid-base status by eliminating NH_4^+ as an acid-equivalent. This strategy may benefit the animal in its natural environment when buried within the sediment where poor water-circulation may result in the gradual accumulation of ammonia and acidic metabolic wastes.

2.5.2. Involvement of the gills and antennal glands in acid-base regulation of *M. magister*

The ability of the Dungeness crab to stabilize its systemic acid-base status under acid-stress involved a coordinated compensatory response by the gills and antennal glands. Upon acid exposure, perfused gills shifted the direction of their transbranchial HCO_3^- transport, whose hypothetical mechanism (Fig. 2.8) is later discussed, as is believed to be a common acid-base compensatory strategy in aquatic crustaceans (Allen et al., 2020; Fehsenfeld and Weihrauch, 2016c; Hans et al., 2014; Klymasz-Swartz et al., 2019; Quijada-Rodriguez et al., 2021; Tresguerres et al., 2008). While this finding would suggest the Dungeness crab should accumulate extracellular HCO_3^- over time, their antennal glands adjusted their HCO_3^- reabsorption and acid-equivalent secretion rates upon acid-stress resulting in the stabilization of the crab's acid-base equilibrium (Fig. 2.1-2.4) with little risk of overcompensatory alkalization of the hemolymph due to excessive HCO_3^- concentrations similar to emersion-stressed semi-terrestrial crabs that rely on urine reprocessing (Allen et al., 2021). Although the importance of the antennal glands in the acid-base compensatory response of marine and brackish-dwelling crabs has often been deemed negligible (Fehsenfeld and Weihrauch, 2017; Henry et al., 2012; Wheatly and Henry, 1992), few studies have investigated the veracity of this statement and often make assumptions relating to the organ's osmoregulatory potential and production of isotonic urine (Chung and Lin, 2006; Holliday, 1980; Lockwood and Riegel, 1969; Mantel and Farmer, 1983; Smith, 1967; Wheatly, 1985). A previous study by Wheatly (1985) found that the antennal glands of the Dungeness crab are capable of adjusting their HCO_3^- reabsorption and acid-equivalent secretion rates in response to hypoosmotic stress. The American lobster, *Homarus americanus*, has also been found to occasionally produce acidified urine although its context has only received attention in relation to moulting and Ca^{2+} regulation (Wheatly et al., 2002). Given that the Dungeness crab is a stenohaline osmoconformer, it seems that the transport epithelial characteristics of their antennal glands (Holliday, 1980) has a clear role in regulating systemic acid-base status in addition to regulating systemic concentrations of divalent cations. Interestingly, the antennal glands of the Dungeness crab appear to produce urine that contains

nearly half of the ammonia that is found within the hemolymph (Fig. 2.4). While this opposes the nearly equimolar ammonia concentrations found within the hemolymph and urine of *C. sapidus* (Cameron and Batterton, 1978b), it is similar to those of *H. formosensis* upon emersion (Allen et al., 2021). While it is unlikely that the antennal glands of the Dungeness crab limit the concentration of urinary ammonia in preparation for emersion given their lifestyle, osmoconforming crabs urinate infrequently and may hold urine within their bladder for some time before its release (Freire et al., 2008; Holliday, 1980). Limiting the amount of ammonia held within the bladder may reduce the toxic effects associated with ammonia or it may be reabsorbed through the antennal glands' Na⁺/K⁺-ATPase activity (Tseng et al., 2020; Wheatly, 1985) as a result of the long incubation period, although the usefulness of such a process to the animal is unclear. Future studies focusing on the acid-base regulatory potential of the antennal glands of marine osmoconformers would benefit from developing a clearer picture of their anatomy (Tsai and Lin, 2014; Tseng et al., 2020) including immunohistochemical localization of potential acid-base transporters.

2.5.3. Hypothetical mechanism of transbranchial CO₂ transport under control conditions

Transbranchial CO₂ transport occurs through passive diffusion down its P_{CO₂} gradient, resulting in extracellular and metabolic CO₂ being excreted into the environment. Although we have confirmed that one Rhesus protein is localized within the apical epithelium of gill 7 in *M. magister* (Jonathan Wilson, pers. comm.), some aquatic arthropods are now recognized to express two Rhesus protein isoforms within their gills at the mRNA level (Hans et al., 2018; Tripp et al., 2022) that may be localized elsewhere, presumably along the basolateral epithelium. These channels are known to function as CO₂ gas channels in several organisms (Endeward et al., 2008; Soupene et al., 2002; Thies et al., 2022), and likely increase the CO₂ permeability of *M. magister*'s gills. Interestingly, our results found the process to be insensitive to inhibition of carbonic anhydrase activity (Fig. 2.6B) in opposition to other brachyuran studies. Pharmaceutical studies on the perfused gills of seawater acclimated *C. maenas* found that carbonic anhydrase was significantly involved in transbranchial CO₂ transport, as indicated by a 50% inhibition in CO₂ excretion rates following the application of basolateral acetazolamide (Fehsenfeld and Weihrauch, 2016a). A similar dependence on carbonic anhydrase activity has been identified within the gills of *C. sapidus* (Burnett, 1984) and *N. granulata* (Genovese et al., 2005; Tresguerres et al., 2008). These previous studies have suggested that the main reason carbonic

anhydrase activity is involved in transbranchial CO₂ excretion relates to the activity of a hypothetical basolaterally-localized, hemolymph-acting, membrane-bound carbonic anhydrase isoform (CA_G). This isoform is believed to convert hemolymph HCO₃⁻ to CO₂, allowing for the gill to maintain a favorable environmentally directed P_{CO2} gradient once the pre-existing hemolymph CO₂ has already diffused across the gill (Aldridge and Cameron, 1979; Burnett et al., 1985). While the intracellular carbonic anhydrase (CA_C) activity may contribute to transbranchial CO₂ processes, it is believed to play a more important role in maintaining cytosolic concentrations of H⁺ and HCO₃⁻ by hydrating CO₂ (Henry et al., 2003; Henry and Cameron, 1983; Serrano et al., 2007). While pharmaceutical evidence has supported this hypothesis, we have recently identified that CA_G is localized within the apical epithelium of gill 8 in seawater acclimated *C. maenas* (Jonathan Wilson, pers. comms.), providing strong counter-evidence to this hypothesis. This appears to be the case for the gills of *M. magister* where transbranchial CO₂ transport is not enhanced by the conversion of hemolymph HCO₃⁻ into CO₂ by a basolateral CA_G. While the intracellular CA_C does likely contribute to CO₂ excretion rates under certain conditions it does not appear to be significant in comparison to the passive flow of extracellular CO₂ across the branchial epithelium under our control conditions. These results and hypotheses are visualized in figure 2.8 alongside the proposed transport mechanism of the other carbonate system's components.

2.5.4. Hypothetical mechanism of transbranchial HCO₃⁻ transport under control conditions

As hypothesized in other brachyurans (Allen et al., 2020; Fehsenfeld and Weihrauch, 2017, 2016c; Hans et al., 2014; Quijada-Rodriguez et al., 2021), the transport of HCO₃⁻ across the gills of *M. magister* is bi-directional with a net-efflux from the gill occurring under control conditions and a net-influx of HCO₃⁻ occurring under acidotic stress. Efflux of HCO₃⁻ from the perfused gill was promoted upon inhibition of the Na⁺/K⁺-ATPase, suggesting the transporter provides an electrochemical gradient (low intracellular [Na⁺], hyperpolarized epithelia) that either promotes the apical expulsion of cytosolic HCO₃⁻ or reduce the ability of basolateral HCO₃⁻ transporters to secrete HCO₃⁻ into the extracellular space. Although branchial HCO₃⁻ transporters are largely uncharacterized in crustaceans, pharmaceutical, electrophysiological, and transcriptional evidence supports at least one apical Cl⁻/HCO₃⁻ anion exchanger and at least one basolateral Na⁺/HCO₃⁻ cotransporter is involved in acid-base regulation (Fehsenfeld and Weihrauch, 2016c; Tresguerres et al., 2008). Transcriptional evidence has also suggested a

basolateral HCO_3^- -accepting anion channel such as bestrophin is also involved in *H. araneus* (Harms et al., 2014). Apical $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers are likely electroneutral and therefore not affected by the reduced epithelial hyperpolarization or intracellular Na^+ concentrations upon inhibition of the Na^+/K^+ -ATPase, although they do likely contribute to net HCO_3^- transport. A basolateral $\text{Na}^+/\text{HCO}_3^-$ cotransporter may transport its substrates either into the cytosol or into the extracellular space, as occurs in NBC1 within the proximal tubule of mammalian nephrons. When unphosphorylated, NBC1 relies on its strongly electrogenic transport ($1\text{Na}^+:3\text{HCO}_3^-$) to move its substrates against their chemical gradients (low intracellular $[\text{Na}^+]$ and $[\text{HCO}_3^-]$ compared to the blood; Boron et al., 1997). Upon phosphorylation, NBC1 transport is less electrogenic ($1\text{Na}^+:2\text{HCO}_3^-$) resulting in the movement of cytosolic substrates into the blood (Gross et al., 2001). If a similar $\text{Na}^+/\text{HCO}_3^-$ cotransport is found in the gills of *M. magister*, bi-directional transport of HCO_3^- would be possible and may not require the cell to alter its protein expression profile to acutely do so. While the current study does not provide direct evidence that this transporter is present within the gills, inhibition of the Na^+/K^+ -ATPase would reduce the tissue's hyperpolarization and increase intracellular Na^+ concentrations, limiting the favourability of a $\text{Na}^+/\text{HCO}_3^-$ cotransporter to secrete cytosolic HCO_3^- into the extracellular space and thereby increasing the rate of HCO_3^- excreted across the gill into the environment as we have observed. Reduced hyperpolarization would similarly reduce the favourability of cytosolic HCO_3^- entering the extracellular space through potential basolateral anion channels, causing a similar increase in apical efflux of HCO_3^- .

Inhibition of carbonic anhydrase activity caused the net-efflux of HCO_3^- out of the gill to a net-influx of HCO_3^- within the extracellular space. Under uninhibited conditions, intracellular HCO_3^- concentrations are continuously maintained through the hydration of intracellular CO_2 by intracellular carbonic anhydrase activity (Böttcher et al., 1991; Burnett et al., 1985; Henry and Cameron, 1983; Whiteley et al., 2001). Inhibition likely caused intracellular HCO_3^- concentrations to fall, potentially reducing or even redirecting the activity of electroneutral $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers that are believed to be the main transporters involved in the movement of HCO_3^- across the gill's apical epithelium (Fehsenfeld and Weihrauch, 2016a; Genovese et al., 2005; Tresguerres et al., 2008). Although the activity of the hypothesized basolateral $\text{Na}^+/\text{HCO}_3^-$ cotransporter and anion channels may be negatively affected by reduced intracellular HCO_3^- concentrations, inhibition of carbonic anhydrase should not affect the tissue's hyperpolarization

that drives these transporters. These results and hypotheses are visualized in figure 2.8 alongside the proposed transport mechanism of the other carbonate system's components.

2.5.5. Hypothetical mechanism of transbranchial ammonia transport under control conditions

Under equimolar ammonia conditions, ammonia excretion across the perfused gills occurred through an acidified vesicular trafficking mechanism that depended on Na^+/K^+ -ATPase activity, similar to mechanisms proposed for the gills of *C. maenas* (Fehsenfeld and Weihrauch, 2016a; Weihrauch et al., 2002) and *Portunus trituberculatus* (Ren et al., 2015). In fact, inhibition of the Na^+/K^+ -ATPase caused ammonia to accumulate within the perfusate as has been previously observed in another stenohaline osmoconforming crab, *Cancer pagarus* (Weihrauch et al., 1999). In both cases, the net-accumulation of ammonia within the perfusate is likely caused by a severe reduction in ammonia excretion across the apical epithelium as well as the deposition of ammonia produced metabolically within the gill into the extracellular space. Under normal conditions, approximately 10% of the ammonia produced by the gills of *M. magister* is secreted into the extracellular space (Martin et al., 2011). This proportion may increase upon inhibition of the Na^+/K^+ -ATPase, resulting in ammonia's net-accumulation within the perfusate. These results, alongside the 72% inhibition of ammonia excretion following the inhibition of the microtubule network, demonstrate most of the components of the proposed transbranchial ammonia transport mechanisms of *C. maenas* are similar to that of *M. magister*. Ammonia is actively transported across the basolateral branchial epithelium by the Na^+/K^+ -ATPase with NH_4^+ being accepted as a mimic of K^+ (Chew et al., 2014; Towle and Holleland, 1987). Once within the cytosol, the slight portion of intracellular gaseous NH_3 enters acidified vesicles that may express Rhesus proteins to facilitate the process where it is trapped as NH_4^+ until its excretion *via* exocytosis. Given that inhibition of the V-type H^+ -ATPase tended to reduce transbranchial ammonia excretion rates under control conditions, it may be responsible for the acidification of these vesicles (Fig. 2.8D). Although not investigated by the present study, Na^+/H^+ exchangers could also be expressed within these vesicles to aid in their acidification. Given that transbranchial excretion rates were only 25% lower under equimolar ammonia conditions when compared to ammonia excretion rates in an ammonia-free environment (Fig. 2.5D), this active mechanism could be responsible for the majority of total ammonia excretion even when the passive excretion of NH_3 down a P_{NH_3} gradient is favorable. Alternative

transporters such as the ammonium transporters (AMTs; Chasiotis et al., 2016; Durant and Donini, 2018; Thiel et al., 2017) or the hippocampus abundant transcript (HIAT) protein (Dirk Weihrauch, pers. comm.) may also be involved in the transbranchial ammonia transport mechanism; however, their role in brachyurans remains unstudied. These results and hypotheses are visualized in figure 2.9.

2.5.6. Effects of acute acid-stress on transbranchial CO₂ and HCO₃⁻ transport

Acute acid-exposure caused perfused gills to accumulate CO₂ and HCO₃⁻ within the perfusate (Fig. 2.5). The influx of CO₂ is likely due to changes in the carbonate equilibrium of the bathing solution upon acidification, causing a slight hemolymph directed P_{CO2} gradient to be developed across the gill. In the intact animal, this hemolymph directed P_{CO2} gradient is likely a short-lived consequence of acid-stress as their extracellular fluid slightly increased (Fig. 2.2) allowing the typical passive diffusion of CO₂ to occur down an environmentally directed P_{CO2} gradient. Inhibition of carbonic anhydrase activity reduced the rate that CO₂ accumulated within the gills, suggesting that the enzyme favoured the protonation of HCO₃⁻ under uninhibited conditions (Fig. 2.7B). While this may seem counterproductive to the accumulation of extracellular HCO₃⁻, it would consume an intracellular H⁺ and may diminish the effects of the acute acid-stress on intracellular pH. Interestingly, inhibition of the Na⁺/K⁺-ATPase caused a nearly 3-fold increase in the rate that CO₂ accumulated within the perfusate; however, it is likely related to the inhibition's reductive effect on the accumulation of extracellular HCO₃⁻ (Fig. 2.7B). Given the acute nature of the acid-stress, the previously hypothesized basolateral Na⁺/HCO₃⁻ cotransporter and anion channel would still be the primary means of secreting cytosolic HCO₃⁻ into the hemolymph. Inhibition of the Na⁺/K⁺-ATPase reduces these transporter's favourability, reducing the rate of HCO₃⁻ accumulation that also appears to greatly increase the rate of perfusate acidification (Fig. 2.7A, C). As a result, the carbonate equilibrium of the perfusate would shift to the left increasing its P_{CO2}. Interestingly, the V-type H⁺-ATPase became a significant factor in the accumulation of extracellular HCO₃⁻ under acid-stress. Due to its apical localization (Jonathan Wilson, pers. comm.), the V-type H⁺-ATPase acts as a direct means to actively counteract the acute acid-stress but also a means to further hyperpolarize the epithelia to enhance transporters responsible for secreting cytosolic HCO₃⁻ into the extracellular space.

2.5.7. Effects of acute acid-stress on transbranchial ammonia transport

Acidified environmental conditions enhanced transbranchial ammonia excretion rates which may function to elevate the elimination of acid-equivalents from the hemolymph (Fig. 2.4 and 2.5). An acidified environment could elevate ammonia excretion rates by creating a greater outwardly-directed P_{NH_3} gradient operating in the same fashion as the acidified boundary layer along the gills that most water-breathing freshwater animals create (Nawata et al., 2007; Weihrauch et al., 2009). This does not seem to be the case in the gills of the Dungeness crab, as elevating environmental ammonia concentrations and thereby reducing the magnitude of an outwardly directed P_{NH_3} gradient did not affect transbranchial ammonia excretion rates (Fig. 2.5). The aforementioned vesicular trafficking mechanism that was a central component of transbranchial ammonia excretion mechanisms under control conditions appears to cease function under acidified conditions and therefore cannot explain the increase in branchial ammonia excretion rates (Fig. 2.7D). The only transporter that we identified as being significantly important to transbranchial ammonia excretion under acidified conditions was the Na^+/K^+ -ATPase (Fig. 2.7D). Unlike control conditions where inhibition of the Na^+/K^+ -ATPase completely abolished transbranchial ammonia transport, inhibition of the Na^+/K^+ -ATPase under acidified conditions only reduces excretion rates by 61%. The aforementioned AMTs (Durant and Donini, 2018; Thiel et al., 2017) and HIAT (Dirk Weihrauch, pers. comm.) may be valuable under such conditions if their activity is positively impacted by acidotic stress and should be considered in future studies.

2.6. Conclusions

Although acid-base regulation is generally viewed as a gill-dominated physiological process in aquatic crustaceans, it appears that the antennal glands of osmoconforming brachyurans may play an important role in acid-secretion and regulation of systemic HCO_3^- concentrations. These organs work together upon acid-exposure in the Dungeness crab allowing the animal to readily counteract acid-base disturbances where the gills begin accumulating environmental HCO_3^- within the hemolymph and the antennal glands limiting the urinary loss of HCO_3^- while simultaneously excreting acid-equivalents. This process may have been largely overlooked in euryhaline species that must be prepared to enter a hyperosmoregulating state thereby limiting their ability to respond to acid-base stress in comparison to an osmoconformer. Although the transepithelial transport processes of the antennal glands remain to be

characterized, pharmaceutical studies have provided evidence that the Na^+/K^+ -ATPase, carbonic anhydrase, and several currently uncharacterized HCO_3^- transporters are crucial in the branchial acid-base regulatory mechanisms.

Transition to chapter 3

Although most marine osmoconforming brachyurans experience slight bouts of environmental stress, their environment is relatively stable due to the sheer volume of the ocean. Expulsion of volcanic emissions from vents along the ocean floor create extremely hazardous niche environments that are often deep, hot, and extremely acidic. Although most of the biodiversity in vent environments consists of microbes, annelid tube worms, caridean shrimps, mussels, many endemic brachyurans call vents home. While studies have made assumptions of vent-endemic animal physiology, the pressure associated with deep-sea vents prevents physiological experiments to be directly performed on these species. The following study explored the acid-base regulatory capacity of a shallow hydrothermal vent endemic brachyuran, *X. testudinatus*, to identify specialized characteristics that allow them to tolerate extreme hypercapnia.

Chapter 3: Specialized adaptations allow vent-endemic crabs (*Xenograpsus testudinatus*) to thrive under extreme environmental hypercapnia.

Garett Joseph Patrick Allen¹, Pou-Long Kuan², Yung-Che Tseng², Pung-Pung Hwang³, Alex R. Quijada-Rodriguez¹, and Dirk Weihrauch¹

¹Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2

²Institute of Cellular and Organismal Biology's Marine Research Station, Academia Sinica, No. 23-10 Dawen Rd., Jiaoxi, 262, Yilan County, Taiwan

³Institute of Cellular and Organismal Biology, Academia Sinica, No. 128, Section 2, Academia Rd., Nangang District, Taipei City, 11529, Taiwan

Published in: Scientific Reports. 2020. 11720.

Author Contributions:

G.J.P.A. designed the study, performed the experiments, analyzed and interpreted data, and wrote the manuscript. P.-L.K. assisted on experiments. Y.-C.T. aided in the study design and provided funding for experiments and animal collection. P.-P.H. aided in study design and provided equipment. A.R.Q.R. aided in manuscript editing. D.W. helped design the study, preparation of the manuscript, and provided financial support.

3.1. Abstract

Shallow hydrothermal vent environments are typically very warm and acidic due to the mixing of ambient seawater with volcanic gasses (>92% CO₂) released through the seafloor making them potential ‘natural laboratories’ to study long-term adaptations to extreme hypercapnic conditions. *Xenograpsus testudinatus*, the shallow hydrothermal vent crab, is the sole metazoan inhabitant endemic to vents surrounding Kueishantao Island, Taiwan, where it inhabits waters that are generally pH 6.50 with maximum acidities reported as pH 5.50. This study assessed the acid-base regulatory capacity and the compensatory response of *X. testudinatus* to investigate its remarkable physiological adaptations. Hemolymph parameters (pH, [HCO₃⁻], P_{CO2}, [NH₄⁺], and major ion compositions) and the whole animal’s rates of oxygen consumption and ammonia excretion were measured throughout a 14-day acclimation to pH 6.5 and 5.5. Data revealed that vent crabs are exceptionally strong acid-base regulators capable of maintaining homeostatic pH against extreme hypercapnia (pH 5.50, 24.6 kPa P_{CO2}) *via* HCO₃⁻/Cl⁻ exchange, retention, and utilization of extracellular ammonia. Maintenance of hemolymph P_{CO2} tensions below environmental levels in the whole animal were measured and found to be paralleled in isolated perfused gills, suggesting gills can excrete CO₂ against a hemolymph directed P_{CO2} gradient. Species that can utilize similar mechanisms should be less at risk in future oceanic conditions.

3.2. Introduction

Hydrothermal vents produce some of the most challenging environmental conditions found throughout the world's oceans – often being considered the “edge of life”. As such, vent endemic animals are believed to be well-adapted to stressors such as extreme temperatures, acidities, hypoxia or anoxia, pressures, and the presence of toxic compounds (*i.e.* sulfur, metals) driven by the cooling of magma below the oceanic floor (Spiess et al., 1980). Shallow vent systems have recently been described as natural laboratories to study CO₂ perturbations related to predicted future ocean acidification scenarios (Hall-Spencer et al., 2008; Lin et al., 2019; Lu et al., 2014); particularly concerning adaptations of vent-endemic animals who were essentially born into conditions similar to projected future oceanic acidities and temperatures. Climate change projections suggest that by the year 2300 ocean surface waters could warm by approximately 5 °C (Nicholls et al., 2018) while increasing partial pressure of CO₂ (P_{CO2}) simultaneously reduces oceanic pH as much as 0.7 – 0.8 units (Caldeira and Wickett, 2003; Hartin et al., 2016; IPCC, 2013). Experimental exposure of marine organisms to similar or milder conditions has demonstrated that marine organisms experience a breadth of physiological responses spanning structural, physiological, and chemosensory challenges (Wittmann and Pörtner, 2013). Heightened environmental P_{CO2} tensions cause an even greater increase in an organism's extracellular and intracellular P_{CO2}, as animals are generally believed to rely upon diffusive loss of metabolic CO₂ from the cellular level to the extracellular space and eventual excretion to the environment (Melzner et al., 2009; Truchot, 1975a). Elevated arterial P_{CO2} promotes acidification of extracellular fluids, forcing organisms to either compensate or succumb to the disturbance should enzyme/protein, and thus cellular, function fail (Riggs, 1988; Somero, 1986).

Acid-base compensation is typically achieved *via* accumulation of HCO₃⁻ within the extracellular fluid of an organism; however, the source of HCO₃⁻ may be environmental (*i.e.* branchial HCO₃⁻/Cl⁻ exchange; Baker et al., 2015; Goss et al., 1992) or mobilized from stored sources and calcified structures (*i.e.* bones or the haemocoel and shell of calcified invertebrates; Defur et al., 1980; McMahon et al., 1978; Truchot, 1975a). Non-bicarbonate buffers such as ammonia, proteins/amino acids, and phosphates generally play an important, but lesser role depending on species (Appelhans et al., 2012; Weihrauch and Allen, 2018). These compensatory

mechanisms are becoming increasingly important to aquatic organisms as the dissolution of CO₂ into the world's waterways continues to decrease environmental pH – a process commonly termed ocean acidification (IPCC, 2013). While most animals fully compensate for environmental hypercapnia related extracellular acid-base disturbances, the mechanism and the necessary energy reallocation to do so are believed to cause a variety of downstream consequences (Heuer and Grosell, 2014). Several studies have identified an overarching consequence of future ocean conditions to be caused by energetic reallocation to promote maintenance of acid-base homeostasis and survival – reducing the capacity of organisms to grow, reproduce, and potentially survive (Doney et al., 2009; Kroeker et al., 2013; Orr et al., 2005; Portner et al., 2004; Xu et al., 2016). In the Tanner crab, *Chionoecetes bairdi*, energy used to restore acid-base status comes at the cost of hemocyte mortality reducing the crab's ability to fight off pathogens (Meseck et al., 2016). Additionally, marine calcifying organisms, particularly those who are sessile such as bivalves and corals, generally experience reduced growth with the latter remaining functional yet 'naked,' as it loses its exoskeletal support (Medina et al., 2006). Calcifying sea urchin larvae, *Strongylocentrotus droebachiensis*, experience reduced growth and development even under mild acidification (Dupont et al., 2010; Kurihara and Shirayama, 2004) due to loss of energy reserves following maintenance of intracellular pH (Stumpp et al., 2012).

Behavioral consequences of ocean acidification have also been documented in vertebrate and invertebrate species. Fishes and elasmobranchs may experience detrimental effects including reduced olfactory sensation and increased predation (Dixson et al., 2015; Doney et al., 2009). In hermit crabs, *Pagurus bernhardus*, exposure to acidified conditions reduces their capacity to assess shell quality and takes them more time to switch shells due to an overall reduction in movement (de la Haye et al., 2011). In the mud crabs, *Panopeus herstii*, acidified conditions result in a reduction in their foraging ability, experiencing a reduced capacity to locate, handle, and consume oysters (Dodd et al., 2015). While these studies provide insights and potential physiological effects of global change, they struggle to provide comprehensive information as to an adapted animal's physiological state due to the fact that in these studies, animals have not experienced generations of such exposure.

Vent endemic organisms represent one of few natural scenarios to investigate how chronic stress such as hypercapnia may drive physiological adaptations. Research has largely

focused on adaptations deep-sea vent endemic animals have developed in relation to temperature, oxygen-uptake, and sulfide/hydrogen sulfide toxicity with few studies considering pH or hypercapnia. Deep-sea vent endemic species such as the gutless and mouthless vestimentiferan tubeworm, *Riftia pachyptila*, often rely on symbiotic microbial relationships to thrive under stressful conditions (Childress et al., 1993). *Riftia pachyptila* take advantage of the high environmental concentrations of CO₂, sulfide, and nitrates within the vent and redirect these molecules from their extracellular space into their trophosome, a highly vascularized organ housing symbionts that produce nutrients and detoxify sulfide for the host (Childress et al., 1993). Although blood and coelomic fluid of *R. pachyptila* contains up to 4.4 kPa P_{CO2} due to diffusion from the vent environment, it maintains a relatively constant blood and coelomic pH suggesting the tubeworms effectively compensate for acid disturbances related to their symbiotic relationship (Childress et al., 1993; Goffredi et al., 1997b); however, their means of CO₂ transport is poorly understood and is not linked to respiratory pigment or protein-interactions (Childress et al., 1993; Toulmond et al., 1994). Unlike their deep-sea counterparts, shallow vent endemic animals are not generally symbiotrophic (Tarasov et al., 2005) and may instead have to rely on their own physiological adaptations to thrive under extreme conditions. Unfortunately, these adaptations have received minimal attention despite the ease of obtaining shallow animals compared to deep-sea inhabitants.

Kueishantao Islet (121°57' E, 24°50' N) off the northeastern coast of Taiwan houses a series of shallow (10 to 30-meter depth) hydrothermal vents whose discharged fluids are amongst the most acidic of the known oceanic systems (Minimum pH 1.54; Chen et al., 2005). Surrounding waters within this 500,000 m² shallow vent system are maintained at *ca.* 30 °C and approximately pH 6.50, although some reports have measured waters as acidic as pH 5.50 (Han et al., 2014; Yang et al., 2012), due in part to the abundance of CO₂ within discharged fluids (> 90% CO₂ (Chen et al., 2005; Yang et al., 2012). *Xenograpsus testudinatus*, the shallow hydrothermal vent crab, is the sole endemic metazoan found within Kuseishantao Islet's vent system and thrives in high density (364 individuals/m²; Ng et al., 2000) where ambient pH is between 6.5-5.5, becoming increasingly acidic when feeding near the mouths of vent chimneys (Jeng et al., 2004). While they can survive typical oceanic conditions (Hu et al., 2016; Wang et al., 2014), they are believed to only do so during trans-vent migration where they serve as a key

species to the vent systems' energy flow (Chang et al., 2018) and are practically absent from surrounding non-vent fisheries as by-catch despite the area's economic activity (Wang et al., 2013). While Hu and colleagues (2016) identified the physiological response of normal-ocean acclimated *X. testudinatus* (pH 8.0) exposed for 2-days to waters acidified to pH 6.5, they did not investigate the animal's native state under chronic acidification, nor the potentially exacerbated acidification experienced while feeding (Jeng et al., 2004).

To date, few studies have investigated physiological adaptations of vent endemic animals to acid-base disturbances despite pH challenge being a common environmental stress of vent environments. Here we investigated the physiological plasticity of *X. testudinatus* in extreme hypercapnic vent-like environments by creating pseudo-vent conditions in the lab *via* CO₂-injection into seawater (pH 6.5 with 2.8 kPa P_{CO2} or pH 5.5 with 24.6 kPa P_{CO2}). To maintain a vent-like state, crabs were moved directly from capture at the vent site into pre-acidified aquaria. Physiological strategies used by *X. testudinatus* to regulate extracellular acid-base homeostasis were investigated at the whole animal level by quantification of hemolymph acid-base status and ionic composition, metabolic rate and ammonia excretion in animals acclimated at two ends of their reported vent pH range (pH 6.5 and 5.5). Additionally, at the isolated gill level, perfusions were used to assess the gill's ability to manipulate hemolymph-like salines under vent-like hypercapnic conditions. The results of this study highlight that even though vent endemic species are less common amongst shallow vent systems, the selective pressure of intense environmental stressors have driven animals such as *X. testudinatus* to evolve unique physiological adaptations akin to those of deep-sea vent endemic species.

3.3. Materials and methods

3.3.1. Animals and acclimatory conditions

Adult *X. testudinatus* were captured from Kueishantao's vent field (121°57' E, 24°50' N) and either directly sampled for hemolymph parameters or transported to pre-acidified acclimation tanks within the Institute of Cellular and Organismal Biology's Marine Research Centre. Water samples collected in parallel indicated the native environment of these crabs was similar to experimental pH 6.50 conditions (Table 3.1). Water samples were obtained in duplicate by divers using sealed vessels with one sample being measured for pH immediately upon resurfacing. Upon returning to the research station, both the previously measured and un-

opened remaining samples were measured for pH, total carbon, and ammonia (see methods below).

Six aquaria (3x pH 6.50 and 3x pH 5.50; 75 L, 20-24 crabs per tank) operated as flow-through systems (flow of *ca.* 3 l h⁻¹) fed directly with natural seawater (35 ppt). Animals were selected at random from tanks for all experiments and permanently removed from the experimental tanks after sampling. Aquaria were set to mimic the normal-to-highest reported acidities the crabs normally inhabit - namely pH 6.5 and 5.5, respectively - as adjusted by CO₂-injection (pH controller, MACRO) for either long-term acclimation (pH 6.5) or 14-days in elevated P_{CO2} (pH = 5.5), as detailed in Table 3.1. Temperature was maintained constant at 30 °C throughout all trials as was a 12:12h light:dark cycle. All crabs were given an initial 14-days to pre-acclimate to the control conditions and to adjust following removal from their native environment. A total of 143 crabs were used throughout the experiment, 61 of which were used exclusively for hemolymph measurements (30 crabs in pH 6.5 and 31 crabs in pH 5.5) and 82 of which were used for whole animal experiments (43 crabs in pH 6.5 and 39 crabs in pH 5.5).

Table 3.1. Water physiochemical parameters of acclimatory pH 6.50 (2.7 kPa P_{CO2}) and pH 5.50 (24.6 kPa P_{CO2}) tanks over the 14-day acclimatory periods as well as water samples obtained during animal collection at the vent site (N = 8). Acclimatory tank conditions were monitored every 1 – 2 days throughout experimentation. Data presented as means ± s.e.m; ‘N/a’ represents unmeasured parameters.

Environment	pH	Temperature (°C)	Total alkalinity (μmol kg ⁻¹ l ⁻¹)	C _T (μmol l ⁻¹)	P _{CO2} (kPa)	[HCO ₃ ⁻] (μmol l ⁻¹)	T _{AMM} (μmol l ⁻¹)
Control	6.489 ± 0.004	30.15 ± 0.07	2361 ± 42	3020 ± 48	2.765 ± 0.056	2331 ± 40	N/a
High P _{CO2}	5.549 ± 0.006	30.62 ± 0.05	2373 ± 25	8379 ± 53	24.581 ± 0.187	2374 ± 25	N/a
Vent Site	6.40 ± 0.02	N/a	2475 ± 45	3365 ± 59	3.624 ± 0.135	2462 ± 44	≤ 5

Whole animal experiments and hemolymph sampling were not assessed using repeated measures. Crabs were measured for metabolic rate and ammonia excretion rate, and then returned to their acclimatory conditions for approximately 3-hours prior to hemolymph sampling to allow hemolymph to stabilize due to handling stress. Sampled crabs were then removed from the pool of experimental animals and not reused at additional time points. Crabs were fed diced squid *ad libitum* every two days but were fasted for two days prior to all experimentation.

3.3.2. Hemolymph and water sampling

Hemolymph was sampled and analyzed following methods described by Hu *et al.* (2016) where samples were maintained at 30 °C for all measurements using a temperature-controlled water bath. Extracellular pH was measured immediately using an InLab Micro Combination pH electrode (Mettler-Toledo, Greisensee, Switzerland) attached to a pH-ISE meter model 225 (Denver Instruments, Gottingen, Germany). The pH was measured in the NBS scale and

electrodes calibrated with pH buffers (4.01, 7.00, 10.01) traceable to NIST standard reference material (Thermofisher Orion). Sub-samples were then diluted 1:2 with crab ringer without HCO_3^- (see gill perfusion for ion composition) and their total dissolved inorganic carbon (C_T) was measured in duplicate using a Corning 965 carbon dioxide analyzer (Olympic Analytical Services, England). Hemolymph P_{CO_2} was mathematically determined using a rearrangement of the Henderson-Hasselbalch equation (Eq. 3.1) and *C. maenas*' carbonate system pK_{a1} and αCO_2 constants derived by Truchot (1976) adjusted for temperature and osmolality of the hemolymph ($pK_{a1} = 5.807273697$, $\alpha\text{CO}_2 = 0.000254931 \text{ mmol l}^{-1} \text{ Pa}^{-1}$).

Equation 3.1.
$$P_{\text{CO}_2} = \text{TCO}_2 / [(1 + \text{antilog}(pH - pK_{a1})) * \alpha\text{CO}_2]$$

Hemolymph [HCO_3^- ; mmol l^{-1}] was then further calculated using equation 3.2:

Equation 3.2.
$$[\text{HCO}_3^-] = \text{TCO}_2 - (P_{\text{CO}_2} * \alpha\text{CO}_2)$$

The remaining undiluted hemolymph samples were frozen and stored at -80°C for the measurement of ammonia concentration and major ion composition. Measurement of pH and C_T , as well as storage of water samples, was similarly performed and analyzed by the same methods as described above, however, C_T was determined using a Corning 965 total carbon dioxide analyzer as well as a Dissolved Inorganic Carbon Analyzer (Model AS-C3; Apollo SciTech) and a Li-7000 $\text{CO}_2/\text{HCO}_3^-$ Analyzer (LiCOR)) for verification. Seawater HCO_3^- and P_{CO_2} was calculated directly inputting measured C_T , pH, temperature, and salinity into CO2SYS software (Pierrot et al., 2006) using dissociation constants of Mehrbach *et al.* (1973) refit by Dickson and Millero (1987). Ammonia concentrations of hemolymph and water samples were determined using the amino acid and protein insensitive p-orthophthaldialdehyde method described by Holmes *et al.* (1999) using a microplate reader (Molecular Device, SpectraMax, M5). Hemolymph and water major cation composition (Na^+ , K^+ , Ca^{2+} , Mg^{2+}) was determined *via* flame absorption spectrophotometry (Polarized Zeeman Atomic Absorption Spectrophotometer Z-5000, Hitachi High-Technologies, Tokyo, Japan), whereas Cl^- concentration was determined

spectrophotometrically using the mercury (II) thiocyanate method adjusted for microplate use (Florence and Farrar, 1971).

3.3.3. Whole animal ammonia excretion

Whole animal ammonia excretion rates were determined as the change in ambient ammonia concentration following a 1.5-hour incubatory period to account for the stress-invoked release of ammonia. Water was carefully drained and refilled after the incubatory period *via* suction as to minimize animal stress and mitigate the influence of incubatory-period ammonia accumulation on ammonia excretion rates. Crabs were individually isolated inside beakers containing 75 ml of 3X-filtered (0.45 μM) seawater, pre-adjusted to the appropriate pH *via* CO_2 -injection. Beakers were connected to two series of polyethylene tubing capable of delivering either CO_2 or air in order to roughly control pH and oxygenation throughout the exposure using an additional ‘proxy’ beaker fitted with a pH-probe. CO_2 or air was manually injected if the seawater’s pH changed by *ca.* 0.3 pH units above or below acclimation conditions, respectively. Samples were collected 30, 60, and 90 minutes following the incubatory period and used to determine the excretion rate per crab mass as $\mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$.

3.3.4. Calculation of P_{NH_3}

Partial pressure of NH_3 (P_{NH_3}) was calculated by determining the speciation of NH_3 and NH_4^+ from total ammonia (T_{Amm} ; $\mu\text{mol l}^{-1}$) using a rearrangement of the Henderson-Hasselbalch equation (Wright and Wood, 1985; Eq. 3.3 and 3.4) where pH of either water or hemolymph and an appropriate pKa was selected based on salinity and temperature using nomograms by Cameron and Heisler (1983). As ambient ammonia within vent fields was below detectable methods (Table 3.1), calculations were completed assuming 1 $\mu\text{mol l}^{-1}$ T_{Amm} within the acidified waters and 1500 $\mu\text{mol l}^{-1}$ within hemolymph of *X. testudinatus*.

Equation 3.3:
$$[\text{NH}_4^+] = T_{\text{Amm}} / [1 + \text{antilog}(\text{pH} - \text{pK})]$$

Speciation of NH_3 was then determined using the following equation:

Equation 3.4: $[NH_3] = T_{Amm} - [NH_4^+]$

P_{NH_3} was then calculated by dividing the concentration of NH_3 by αNH_3 using the following equation in conjunction with 30 °C NH_3 solubility coefficients (αNH_3 ; $\mu mol l^{-1} torr^{-1}$) derived by Boutilier *et al.* (1984):

Equation 3.5: $P_{NH_3} = [NH_3]/\alpha NH_3$

The differential P_{NH_3} gradient between the hemolymph and environment (ΔP_{NH_3}) determined using equation 3.6 where a positive value infers an environmentally directed gradient favouring excretion of NH_3 along its diffusion gradient.

Equation 3.6: $\Delta P_{NH_3} = P_{\alpha} NH_3 - P_w NH_3$

3.3.5. Resting metabolic rate

Metabolic rate was measured using closed system respirometry techniques where crabs were isolated in darkened airtight glass chambers filled with 350 ml of 3X-filtered seawater (0.45 μm) pre-adjusted to the appropriate temperature and pH *via* CO_2 -injection. Crabs in the pH 5.50 exposure group were removed from control acclimatory tanks and immediately exposed to pH 5.50 during these experiments. Chambers were submerged in a temperature-controlled water bath to maintain a constant temperature of 30 °C. Oxygen concentrations were continuously measured using fiber optic oxygen sensors (PreSens oxygen micro optode, type PSt1; PreSens Precision Sensing GmbH, Regensburg, Germany) connected to an OXY-4 Mini multichannel fiber optic oxygen transmitter (PreSens Precision Sensing GmbH). Ventilation rate of the crabs was found to sufficiently mix water within the chamber as indicated by a linear decline in oxygen bioavailability. Oxygen consumption was determined until oxygen was approximately 60% of air saturation levels at which point crabs were weighed. Background respiration was measured in parallel to all animal measurements by measuring oxygen consumption in a similar fashion in the absence of crabs to account for any microbial activity that may be present. Oxygen consumption rates were calculated as the linear decline in oxygen concentration throughout the

duration of the experiment, after accounting for bacterial influence, per gram of crab per hour ($\mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$).

3.3.6. Gill perfusions

Crabs acclimated to pH 6.5 were terminated at the end of the time-course trial and their fifth gill, selected due to its feasible size. Excised gills were then submerged in hemolymph-like perfusion saline based on ion composition of their hemolymph (in mmol l^{-1} : 400 Na^+ , 17 K^+ , 36 Mg^{2+} , 24 Ca^{2+} , 518 Cl^- , 20 HCO_3^- , 1.5 NH_4^+ ; pH 7.48). Perfusion saline was further supplemented with glucose (0.3 mM) and L-glutamine (0.5 mM) as nutrient sources as well as glutathione (0.1 mM) to promote gill viability. Bathing solution was prepared as artificial seawater (in mmol l^{-1} : 402 Na^+ , 10 K^+ , 10 Ca^{2+} , 55 Mg^{2+} , 547 Cl^- , 2 HCO_3^-) in order to better control the ambient conditions.

Under a dissecting microscope, the afferent vessel was cannulated with FEP-tubing led into the gill by a Microloader pipette tip whereas the efferent vessel was directly cannulated with a Microloader pipette tip and collected as perfusate. Gills and the cannula were sealed using modified neoprene clamps fitted with an outer neoprene layer to ensure the gill floated without contacting surfaces. Gills were perfused in glass petri-plates containing 15 ml of pH-adjusted and filtered seawater (0.45 μM). The pH of the bathing solution was continuously monitored using a micro pH-electrode and manually adjusted by injecting either CO_2 or air to increase or decrease acidity, respectively. Temperature was maintained at approximately 30 °C using the light source of the microscope.

Gills were allowed 15 minutes to equilibrate in the setup at pH 6.5 and then perfusate was collected for 20 minutes for the sampling period. Following measurements at pH 6.5, the gills were acutely exposed to pH 5.5 *via* CO_2 injection into the bathing solution. After equilibrating for 15 minutes at pH 5.5, the perfusate was collected for 20 minutes. Perfusate pH and C_T was measured and P_{CO_2} and $[\text{HCO}_3^-]$ calculated as described for hemolymph. The capacity of the gill to alter initial perfusion saline during the passage through the gill was used to determine the acid-base transport capacities of the tissue. While perfusion describes the capacity of the gill to alter P_{CO_2} and HCO_3^- of artificial hemolymph-like saline in a single passage, results are reported as rates that were determined assuming equal transport per hour, per tissue mass.

3.3.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.2.1 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Data were tested for normality using the Shapiro-Wilk test and tested for homogeneity of variance using either an F-test or Levene's test. A two-way ANOVA was performed to analyze hemolymph acid-base parameters, whole animal ammonia excretion, and resting metabolic rate with time and pH as variables and subsequently tested using the *post hoc* Holm-Sidak's multiple comparison tests. Paired *t*-tests were used to compare perfused gills ability to manipulate pH, HCO₃⁻, and CO₂ as well as differences between ion concentrations of hemolymph and/or seawater. Data within all texts and figures are presented as the mean ± s.e.m where significance was denoted as $p < 0.05$.

3.4. Results

3.4.1. Hemolymph compensatory acid-base response

Crabs exposed to *ca.* pH 6.5 (pH 6.49 ± 0.004 , 2.77 ± 0.06 kPa P_{CO2}) maintained an extracellular pH of 7.42 ± 0.03 (n = 6) throughout the time-course exposure (Fig. 3.1A) which is well within the normal physiological range of aquatic species. Upon exposure to *ca.* pH 5.5 (pH 5.55 ± 0.01 , 24.58 ± 0.19 kPa P_{CO2}), the minimum vent pH normally experienced, crabs underwent a significant respiratory acidosis (two-way ANOVA, n=6, $p = 0.002$) marked by a 0.16 ± 0.03 (n = 6) reduction in extracellular pH (Fig. 3.1A) alongside a 6.88 ± 0.34 kPa (n = 6) increase in arterial P_{CO2} (two-way ANOVA, n=6, $p < 0.0001$; Fig. 3.1C). Acute compensation appears to partially rely on an increased excretion of NH₄⁺ as a H⁺-equivalent (Fig. 1D) occurring in parallel to the accumulation of HCO₃⁻ until the hemolymph buffering capacity is sufficient to compensate the influx of H⁺ from the environment (Fig. 3.1B). Extracellular pH is restored within 7-days and maintained after 14-days of acclimation (Fig. 3.1A) at which point the investigated hemolymph parameters stabilize – most notably when HCO₃⁻ accumulates to 88 ± 2.2 mmol l⁻¹ (n = 6) in an apparent exchange for Cl⁻ (Table 3.2) and as hemolymph ammonia concentrations are partially restored to control values representing a new homeostatic plateau (Fig. 1D).

Table 3.2. Major hemolymph cation and anion composition of freshly captured wild *X. testudinatus* (N = 6) as well as crabs exposed to either pH 6.50 (2.7 kPa P_{CO2}; N = 6) or pH 5.50 (24.6 kPa P_{CO2}; N = 6-7) for 14-days. Data presented as mean concentrations (mmol l⁻¹) ± s.e.m; ‘N/a’ represents unmeasured parameters.

Exposure	Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺	Cl ⁻	HCO ₃ ⁻	T _{AMM}
pH 6.50	440 ± 10	16.7 ± 0.8	24 ± 2	36 ± 1	480 ± 10	19 ± 3	1.4 ± 0.2
pH 5.50	348 ± 7	10.1 ± 0.2	15.4 ± 0.9	41 ± 2	426 ± 5	88 ± 5	1.2 ± 0.2
Wild caught (pH 6.40)	410 ± 10	16.0 ± 0.9	14.2 ± 0.9	36 ± 1	490 ± 8	N/a	N/a

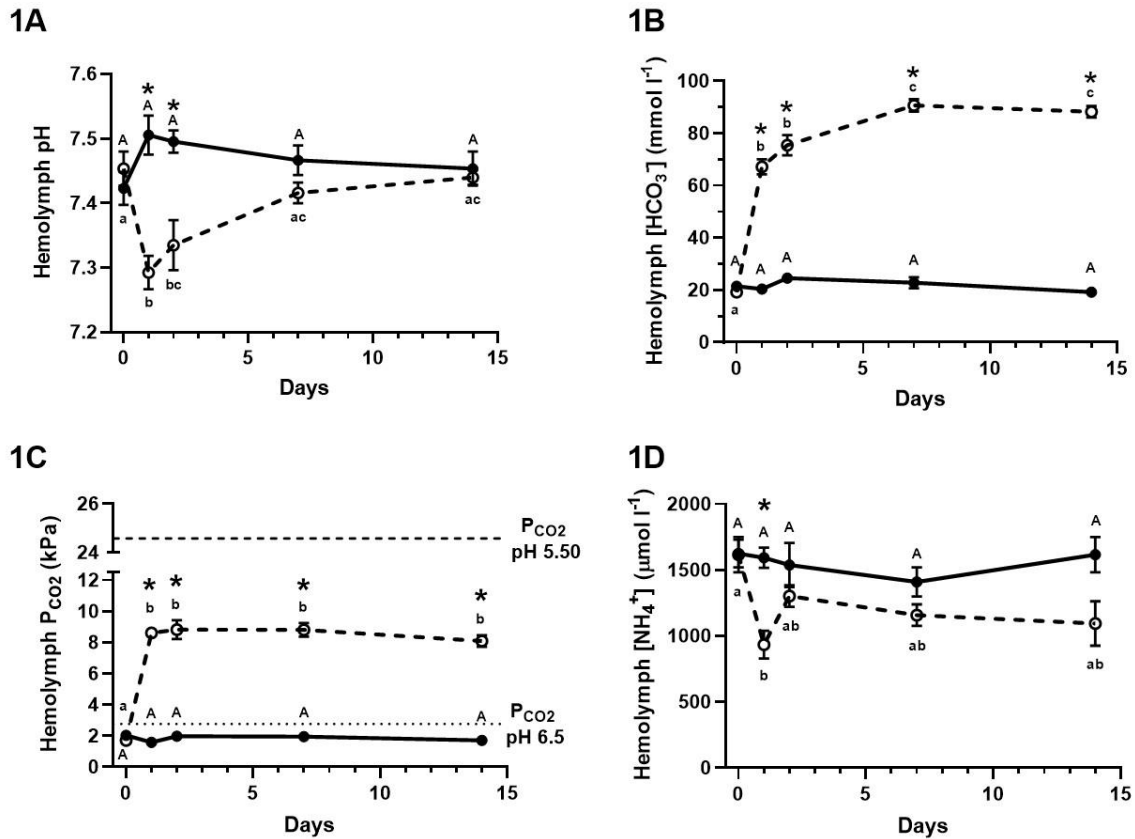


Figure 3.1. Hemolymph acid-base parameters of *X. testudinatus* acclimated over a 14-day period to seawater acidified to either pH 6.50 (2.7 kPa P_{CO2}; closed circles) or pH 5.50 (24.6 kPa P_{CO2}; open circles). Changes in extracellular pH (A; N = 6), HCO₃⁻ (B; N = 6), P_{CO2} (C; N = 6), and [NH₄⁺] (D; N = 5-7) were measured from pre-branchial hemolymph after 0, 1, 2, 7, and 14 days of acclimation. Environmental P_{CO2} levels are indicated by dashed lines to indicate the presence of inwardly directed P_{CO2} gradients (C). Asterisks denote significance based upon acclimation pH. Upper-case letters denote time-dependent differences within pH 6.50 exposed crabs whereas lower-case letters denote time-dependent differences within pH 5.50 exposed crabs. Data presented as means ± s.e.m, p < 0.05.

3.4.2. Isolated gill acid-base regulatory capacity under exposure to chronic (pH 6.50) and acute (pH 5.50) acidification

Gills of crabs fully acclimated to pH 6.5 conditions were isolated and perfused under their acclimated conditions as well as an acute exposure to increased hypercapnia. Perfusion of the fifth gill showed that the branchial tissue carries out a significant alkalinisation ($\Delta\text{pH} = 0.34$

± 0.01 ; $n = 6$, paired two-tailed t-test, $p < 0.0001$; Fig. 3.2A) of the artificial hemolymph-like saline following a single gill passage under exposure to their acclimation conditions (pH 6.49 ± 0.004 , 2.77 ± 0.06 kPa P_{CO_2}). Increasing ambient H^+ abundance by 10-fold (pH 5.55 ± 0.01 , 24.58 ± 0.19 kPa P_{CO_2}) reduced alkalisation of artificial hemolymph-like saline ($\Delta pH = 0.036 \pm 0.019$; $n = 6$, paired two-tailed t-test, $p = 0.11$; Fig. 3.2A); however, the gill maintained a physiological pH of the perfusate (Fig. 3.1A). Interestingly, under both levels of acidification, gills were found to excrete HCO_3^- (Fig. 3.2B) in a fashion that increased in response to acidification ($n = 6$, paired two-tailed t-test, $p < 0.0001$). Under acclimation conditions (*ca.* pH 6.5, P_{CO_2} 2.8 kPa) it is conceivable that the gill produces excess base which is excreted or exchanged for counterions (*i.e.* Cl^-). Under acute acidification (*ca.* pH 5.5, P_{CO_2} 24.6 kPa), it was expected that HCO_3^- would be accumulated as a compensatory mechanism which may instead be a delayed response. Excretion of CO_2 (Fig. 3.2C) was maintained by the isolated gill despite a massive inward hemolymph directed P_{CO_2} gradient, suggesting an active excretory mechanism exists within the branchial tissue. Gills acutely exposed to *ca.* 24.6kPa P_{CO_2} (pH 5.5) experienced a *ca.* 4-fold reduction in CO_2 excretion rate ($n = 6$, pair two-tailed t-test, $p = 0.0026$) as compared to their exposure to *ca.* 2.8 kPa P_{CO_2} (pH 6.5).

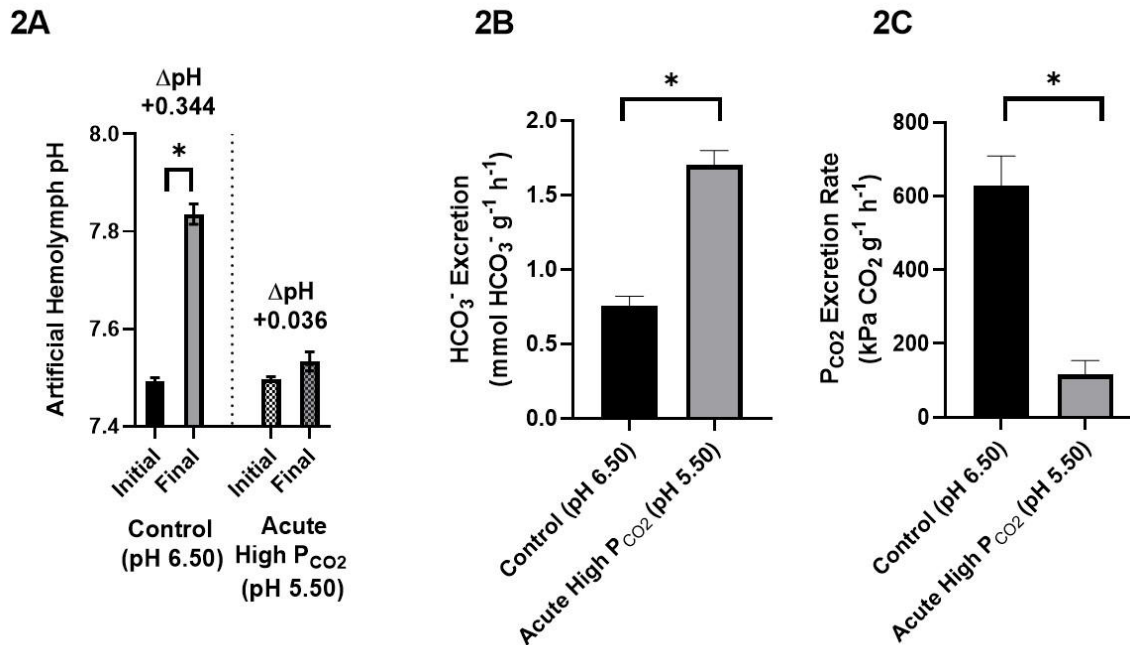


Figure 3.2. Capacity of isolated perfused gill 5 of pH 6.50 acclimated *X. testudinatus* to alter pH (A), P_{CO2} (B), and HCO₃⁻ (C) of artificial hemolymph-like saline following a single gill passage. Gills were first exposed to pH 6.50 (2.7 kPa P_{CO2}) to determine their transport capacity under the acclimated condition. Gills were subsequently exposed acutely to pH 5.50 (24.6 kPa P_{CO2}) to observe changes in transport capacity. Degree of hemolymph alkalization by the gill is represented as Δ pH (A). Positive HCO₃⁻ and P_{CO2} excretion rates infer the molecule is lost to the environment as indicated by a lesser presence in the perfusate as compared to initial amounts within perfusion saline. Asterisks denote significance between initial and final pH or significant differences in transport rates depending on pH exposure. Data presented as means \pm s.e.m, $p < 0.05$, N = 6 for all data points.

3.4.3. Whole animal response to acidification

Metabolic rate was maintained throughout the 14-day time-course exposure to *ca.* pH 6.5 (2.8 kPa P_{CO2}) at 1.87 ± 0.08 (n = 8-9) mg O₂ g⁻¹ h⁻¹, which was immediately reduced by nearly 64 ± 4 % (two-way ANOVA, n = 8-9, $p < 0.0001$) upon exposure to elevated hypercapnia (*ca.* pH 5.5, 24.6 kPa P_{CO2}). Restoration of metabolic rate occurred over the 14-day exposure to *ca.* pH 5.5 and was statistically the same as the rate of pH 6.5-exposed crabs after 14-days (Fig.

3.3A). Whole animal NH_4^+ excretion was found to be independent of crabs' acclimation to environmental pH (two-way ANOVA, $n = 6-9$, $p = 0.38$; Fig. 3.3B).

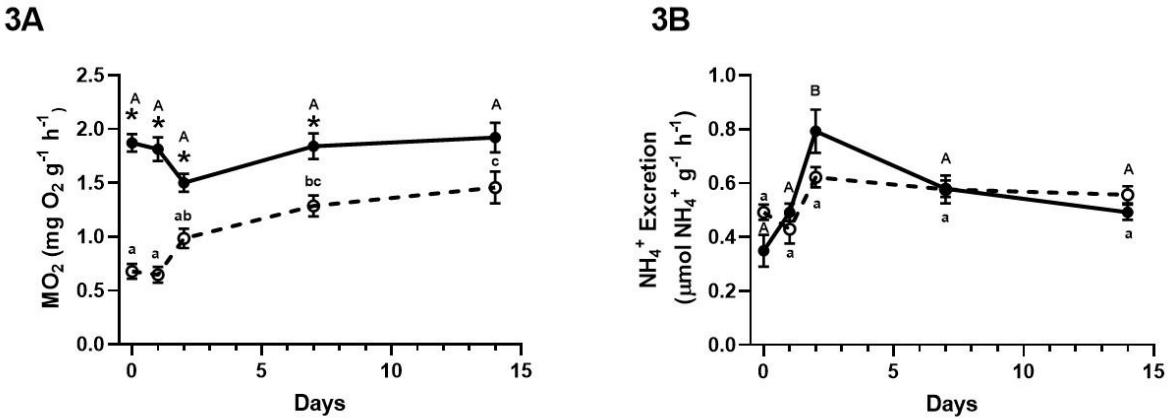


Figure 3.3. Whole animal metabolic rate (A) and ammonia excretion rates (B) were measured to indicate shifts in the crab's metabolism in response to acidification over a 14-day time course acclimation to either pH 6.50 (2.7 kPa P_{CO_2} ; open circles) or pH 5.50 (24.6 kPa P_{CO_2} ; closed circles). Metabolic rate (A) was determined as the rate of oxygen consumption per body mass per time ($\mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$) using closed-system respirometry ($N = 6-9$). Ammonia excretion rates (B) were determined as based on accumulation of ammonia within ambient water per body mass per time ($\mu\text{mol NH}_4^+ \text{ g}^{-1} \text{ h}^{-1}$; $N = 6-9$). Asterisks denote significance based upon acclimation pH. Upper-case letters denote time-dependent differences within pH 6.50 exposed crabs whereas lower-case letters denote time-dependent differences within pH 5.50 exposed crabs. Data presented as means \pm s.e.m, $p < 0.05$.

3.5. Discussion

3.5.1. The compensatory accumulation of extracellular HCO_3^-

Under typical-vent conditions for *X. testudinatus*' habitat (P_{CO_2} 2.7 kPa, pH 6.5), crabs maintain extracellular acid-base homeostasis (Fig. 3.1A) by maintaining *ca.* 20 mmol l^{-1} hemolymph HCO_3^- concentration and responds to acute hypercapnia by further accumulating hemolymph HCO_3^- (Fig 3.1B). While this study and the sole previous study regarding acid-base capabilities of *X. testudinatus* both found that *X. testudinatus* maintains its hemolymph pH at approximately 7.40 - 7.50, which is considerably lower than most marine decapod crustaceans (*M. magister* $\text{pH}_e = 7.93$ (Hans et al., 2014); *C. maenas* $\text{pH}_e = 7.87$ (Fehsenfeld and Weihrauch,

2016a); *N. puber* $pH_e = 7.90$ (Spicer et al., 2007); *C. sapidus* $pH_e = 8.00$ (Cameron, 1985a)), the crabs resting HCO_3^- differed (Hu et al., 2016). The resting concentration of hemolymph HCO_3^- of crabs acclimated to pH 6.50 in this study is about 10 mmol l^{-1} lower than those reported by Hu and coworkers. However, in the previous study, crabs were moved from long-term holding in normal seawater (pH 8.10) and moved for up to 48 hours to acidified conditions (pH 6.5). If crabs had been given longer to reach a steady-state in pH 6.5 it is likely that their hemolymph HCO_3^- would slightly decrease and be within the range reported in this study. This type of initial over-compensatory HCO_3^- accumulation followed by a reduced plateau has been demonstrated in *N. puber* exposed to hypercapnic seawater of pH 6.74 (Spicer et al., 2007). *Necora puber*, much like *X. testudinatus*, responds to acute hypercapnia by accumulating $29.3 \text{ mmol l}^{-1} HCO_3^-$ within its hemolymph after 48 hours of exposure; however, prolonged acclimation causes the crabs to reduce circulating $[HCO_3^-]$ to 22 mmol l^{-1} (Spicer et al., 2007). Acute exposure to a 10-fold increase in acidity ($P_{CO_2} = 24.6 \text{ kPa}$, pH 5.5) caused a respiratory acidosis (Fig. 3.1 A, C) that induced rapid accumulation of hemolymph bicarbonate (*ca.* 350% increase) within 24-hours (Fig. 3.1B), amongst the highest hemolymph HCO_3^- concentration ever reported for aquatic species including that of the Pacific hagfish at $6 \text{ kPa } P_{CO_2}$ for 96 hours $78.2 \pm 4.5 \text{ mmol l}^{-1}$ plasma HCO_3^- (Baker et al., 2015) or *N. puber* exposed to $6.04 \text{ kPa } P_{CO_2}$ that accumulated $55.9 \text{ mmol l}^{-1} HCO_3^-$ after 24 hours of exposure, although this exposure reported 100% mortality to the crabs after 4 days (Spicer et al., 2007). This hyperbolic increase of hemolymph HCO_3^- is nearly identical to that observed by Hu and coworkers (2016) when *X. testudinatus* were acutely challenged by exposure of pH 6.5 suggesting the crab is using a similar mechanism at a greater magnitude. Hemolymph pH was fully restored after 7-days of acclimation to pH 5.5 ($24.6 \text{ kPa } P_{CO_2}$; Fig. 3.1A) at which point $[HCO_3^-]$ exceeded 90 mmol l^{-1} , a response that was maintained throughout the remainder of the 14-day acclimation (Fig. 3.1B). The origin of HCO_3^- accumulated by aquatic crustaceans has received some debate over recent years whether it is environmentally sourced, the result of carapace dissolution, or a mixture of the two. Dissolution of the carapace has been largely disproven to be a major source of HCO_3^- in crabs exposed to hypercapnia as studies indicate extracellular concentrations of Ca^{2+} and Mg^{2+} , the main divalent components of the exoskeleton, do not match the rise in extracellular HCO_3^- concentration as would be expected (Spicer et al., 2007). Studies on *C. sapidus* favour the hypothesis that accumulated HCO_3^- is due to ion exchange within the gill epithelia during hypercapnic exposure

based on reduced chloride uptake rates (Cameron, 1981). Similarly, *Riftia pachyptila* are believed to rely on the diffusion of CO₂ gas into their extracellular fluid in order to sequester carbon into their trophosome for symbionts (Childress et al., 1993). In this study, hemolymph ion composition indicated that HCO₃⁻ accumulation was equally matched by the disappearance of hemolymph chloride ions (Cl⁻; Table 3.2) suggesting the observed accumulation of HCO₃⁻ is from the environment as either HCO₃⁻ directly or by rapid hydration of CO₂ following its diffusion into the tissues. Presence of an unidentified HCO₃⁻/Cl⁻ exchanger within the branchial tissue of *X. testudinatus* was previously hypothesized to be a major factor contributing to the crab's strong acid-base regulatory capacity (Hu et al., 2016). The previous study hypothesized that an increase in the enzyme activity and mRNA expression of basolateral Na⁺/K⁺-ATPase within the gills of *X. testudinatus* helped fuel HCO₃⁻ uptake similar to that of *N. granulata* (Tresguerres et al., 2008) and the tenidap-sensitivity of *C. maenas*' branchial CO₂ and H⁺ flux (Fehsenfeld and Weihrauch, 2016a). Pacific hagfish, referred to as the champions of CO₂-tolerance, are the only other known water-breathing organism capable of accumulating and sustaining nearly 100 mmol l⁻¹ HCO₃⁻ within their extracellular space in response to hypercapnic exposure and is believed to do so *via* HCO₃⁻/Cl⁻ exchange (Baker et al., 2015). Despite their high capacity for CO₂-tolerance, hagfish experienced nearly one full unit reduction in extracellular pH upon exposure to approximately 6 kPa P_{CO2} (Baker et al., 2015) whereas *X. testudinatus* experiences only a maximum 0.2 pH unit reduction of extracellular pH (Fig. 3.1A) upon exposure to 24.6 kPa P_{CO2} inferring the crab's extraordinary acid-base regulatory capacity. Given that hagfish are evolutionarily 'ancient' (Baker et al., 2015) and vent environments are 'origin of life' environments (Martin et al., 2008); this high capacity of HCO₃⁻ accumulation and/or excess Cl⁻ exchange may have been a mechanism shared amongst organisms that was later reduced in capacity as typical aquatic environments do not require such a degree of specialization.

3.5.2. Excretion of CO₂ against an inwardly directed P_{CO2} gradient

Following the central paradigm behind animals' respiratory physiology, CO₂ is excreted to the ambient environment down a P_{CO2} diffusion gradient, where tensions decrease from the cellular level to the extracellular space, and eventually the environment through an excretory epithelium (*i.e.* lungs, gills, skin). Failure to excrete metabolic and/or environmentally sourced CO₂ will cause animals' bodily fluids to continuously acidify until their demise. Under both hypercapnic vent conditions, environmental P_{CO2} of 2.8 kPa and 24.6 kPa, *X. testudinatus*'

hemolymph P_{CO_2} was found to be maintained consistently and dramatically below environmental levels by *ca.* 0.9 kPa and 16 kPa, respectively (Fig. 3.1C), representing an inwardly-directed P_{CO_2} gradient. The presence of an inwardly directed P_{CO_2} gradient infers excretion of CO_2 occurs either (a) across an organ that is not exposed to the environment (*i.e.* antennal gland) or (b) *via* active-transport of CO_2 or an equivalent molecule such as HCO_3^- or CO_3^{2-} . Perfusion of isolated gills excised from crabs acclimated to pH 6.5 revealed that the branchial tissue alone excretes $628 \text{ kPa g gill}^{-1} \text{ h}^{-1} \text{ CO}_2$ against a 2.7 kPa environmental P_{CO_2} as well as $116 \text{ kPa g gill}^{-1} \text{ h}^{-1}$ upon acute exposure to 24.6 kPa P_{CO_2} (Fig. 3.2C), suggesting the requirement of a specialized ‘concentrating’ organ unexposed to the environmental conditions, such as the antennal gland, is not necessarily required. This branchial CO_2 -excretory system, while mechanistically unclear, alkalizes hemolymph-like saline by *ca.* 0.344 pH units in a single gill passage in pH 6.5-acclimated animals while maintaining physiological pH despite acute exposure to a 10-fold increase in H^+ abundance and 22 kPa P_{CO_2} (Fig. 3.2A). Interestingly, acute exposure to pH 5.5 resulted in an increased rate of HCO_3^- loss to the environment but did not result in failure of the gill to restore homeostatic pH (Fig. 3.2B), suggesting an alternative acid-base equivalent, e.g., ammonium, may be important in acute response to acidification.

Active transport of CO_2 represents a puzzling energetic challenge as the production of energy required to continually excrete mass-amounts of CO_2 will also result in the production of metabolic CO_2 . In parallel, branchial epithelia must be either highly gas-selective or semi-impermeable to gases to prevent the influx of environmental P_{CO_2} as well as to prevent the back-flow of excreted carbon, as the release of ionic CO_2 equivalents (HCO_3^- or CO_3^{2-}) will revert to CO_2 in the acidic environment ($\text{pK}_a \text{ HCO}_3^- = 10.3$). While unspecific to gases, gills of marine crustaceans are typically ion ‘leaky’ (Weihrauch et al., 1999) which further raises the required effectiveness of *X. testudinatus*’ branchial capacity to maintaining physiological pH during isolated and acute exposure to a 10-fold increase in H^+ abundance and 22 kPa increase in ambient P_{CO_2} (Fig. 3.2A). Branchial gas-selectivity or impermeability is a tempting hypothesis, while uninvestigated, it would be an asset to survival in vent-environments as it would not only explain some degree of the continual excretion of CO_2 despite the presence of an inwardly-directed P_{CO_2} gradient but also improve *X. testudinatus*’ hydrogen sulfide resistance alongside bacterial symbiosis (Yang et al., 2016). Gas permeability in hydrothermal vent species has only been investigated in *Riftia pachyptila* in regard to the diffusion of both H_2S and CO_2 into the

animal's extracellular space. Although CO₂ was shown to freely diffuse into the extracellular fluid of *Riftia pachyptila* (Goffredi et al., 1997b), the major uptake of sulfides by the tubeworm has been identified as uptake of HS⁻ and that diffusion of H₂S into the animal does not occur freely (Goffredi et al., 1997a). Selective pressure on vent endemic species that are continuously exposed to high levels of CO₂ and H₂S gases may have caused some to develop a sort of semi-impermeable or selectively permeable gas epithelia; however, there has yet to be experiments to directly prove this can occur let alone a descriptive mechanism of how it could occur. If this hypothesis was true, the animal would require a means of efficient oxygen uptake from a relatively hypoxic or anoxic vent environment to support the animals' metabolic rate (Fig. 3.3A), which is 2.5-fold higher than those of reasonably active aquatic crustaceans such as *Penaeus japonicus* (0.753 mg O₂ g⁻¹ h⁻¹, 30 °C, 35 ppt; Chen and Lai, 1993) and up to 14-fold higher than some brachyuran crabs namely *N. granulata* (0.136 mg O₂ g⁻¹ h⁻¹, 21.6 °C, 2 ppt; Bianchini and Carvalho De Castilho, 1999) and *C. sapidus* (0.129 mg O₂ g⁻¹ h⁻¹, 30 ppt, 24 °C; Laird and Haefner, 1976). Furthermore, normally negligible manipulation of extracellular non-bicarbonate buffering systems may be invaluable for these animals as transport of acid-base equivalents, such as ammonium (NH₄⁺) that is easily excreted in acidic ammonia-free waters, may be more effective than directly manipulating components of the carbonate system. While these membrane properties are unlikely to exist in non-vent endemic species, maintenance of an inwardly directed P_{CO2} gradient while excreting CO₂, prevention of sulfur toxicity, as well as specialized O₂-uptake are physiological processes that would seem favorable for animals that are endemic to vent environments.

3.5.3. The role of ammonia in acid-base regulation

While the carbonate system is undoubtedly the major buffering agent within *X. testudinatus*' hemolymph, crabs were found to maintain hemolymph ammonia *ca.* 8 to 15-fold higher (Fig. 3.1D) than most aquatic crustaceans (Weihrach et al., 2017, 2004b), despite ammonia's immense toxic potential (Larsen et al., 2014). Undetectable concentrations of ambient ammonia ($\leq 5 \mu\text{mol l}^{-1}$) within the vents as well as their acidic nature (Table 3.1; pK_a ammonia *ca.* 9.25) should facilitate diffusion of NH₃ to the environment given the presence of a *ca.* 798 Pa P_{NH3} outwardly-directed diffusion gradient and that acid-trapping of NH₄⁺ is possible within vents, unlike slightly-alkaline marine environments whose buffering potential is believed to counteract maintenance of an acidic zone across the branchial epithelia (Garrels and

Thompson, 1962; Weihrauch and Allen, 2018). In fact, whole animal ammonia excretion rates (Fig. 3.2b) were unaltered under increasingly acidified conditions as compared to control measurements and are within the range of non-vent dwelling aquatic crustaceans, such as *C. maenas* (187 nmol gFW⁻¹ h⁻¹), *M. magister* (367 nmol gFW⁻¹ h⁻¹), or *E. sinensis* (123 nmol gFW⁻¹ h⁻¹) with circulating ammonia that typically do not exceed 160 μmol l⁻¹ (Martin et al., 2011; Weihrauch et al., 2017, 1999). Retention of ammonia and potential ‘ammonia homeostasis’ has been hypothesized as an important acid-base regulatory process that occurs in parallel to HCO₃⁻ accumulation in some invertebrates such as the American horseshoe crab (Hans et al., 2018) and the common octopus (Hu et al., 2017). Selective retention or excretion of ammonia would require a bi-directional mechanism to be present such as the vesicular trafficking mechanism believed to be generally employed by other marine invertebrates and animals inhabiting buffered environments (Adlimoghaddam et al., 2015; Ren et al., 2015; Weihrauch et al., 2002; Weihrauch and Allen, 2018). The vesicular trafficking mechanism hypothesizes that ammonia becomes trapped as NH₄⁺ in vesicles acidified by either V⁺-type H⁺-ATPases (HAT) or Na⁺/H⁺ exchangers and moved within the cell by the microtubular network (Fehsenfeld and Weihrauch, 2016a; Weihrauch et al., 2002). While this study did not test the presence of this mechanism, previous studies have localized HAT within the cytosol with minor presence along the apical membrane of *X. testudinatus*’ branchial epithelium (Hu et al., 2016). Furthermore, HAT mRNA expression as well as its enzyme activity increases upon *X. testudinatus*’ exposure to acute hypercapnia as would be expected if the crab was to mobilize retained NH₄⁺ as an excretable H⁺-equivalent (Hu et al., 2016). Reliance on a typically negligible extracellular buffering agent, such as ammonia, may become more critical under situations where the movement of H⁺ or HCO₃⁻ is less favorable as may occur when animals are abruptly challenged by a 10-fold increase in ambient protons as presented in this study. Furthermore, it has been suggested that under severe acid-stress aquatic animals may preferentially degrade HCO₃⁻ yielding amino acids such as asparagine, glutamine, and their dicarboxylic acids (Langenbuch and Pörtner, 2003, 2002). Such a reliance would cause an increase in ammonia and HCO₃⁻ that could be used by *X. testudinatus* to regulate the organism’s acid-base status by retention of HCO₃⁻ and excretion of NH₄⁺ as an acid-equivalent.

3.5.4. Vent-endemic adaptations

Although this study identifies the surface of what physiological adaptations can be present in shallow hydrothermal vent endemic animals, the study of solely hypercapnia cannot be used as an explicative alone. While temperature could affect the observations made in this study, vent inhabitants may correct for variations by repositioning their proximity to the vents. Some stressors, however, are more difficult for animals to deal with. For example, high environmental sulfide levels at Kuieshantao Island's vent fields may have a substantial impact on their metabolic rate and thus their ability to recover from environmental challenges, particularly acidification due to the pH-sensitivity of sulfide toxicity. Sulfide's expected inhibition of cytochrome C oxidase and resultant disruption of ATP synthesis (Jiang et al., 2016) would be expected to severely impact the energetic demand *X. testudinatus* is expected to require in order to rapidly accumulate HCO_3^- . Further investigations into the individual effects of sulfur as well as observing the crab's response to a combination of hypercapnia and sulfides would be of interest, especially given the permanent presence of both in their native environment.

While it is clear that *X. testudinatus* has developed unique adaptations to thrive in its environment, it remains unclear what evolutionary history the crab may have experienced in order for them to develop. While it may be appealing to hypothesize that the physiology of *X. testudinatus*, and other vent endemic species, is due to it being an 'antique' species or 'living fossil,' molecular evidence has indicated that modern vent endemic fauna is relatively recently diversified as recently as the last 100 million years (see review by Van Dover and coworkers (2002) and Little and Vrijenhoek (2003)). In fact, modern deep-sea and seep fauna are believed to have originated from environments such as shallow hydrothermal vents (Little and Vrijenhoek, 2003). This raises the question of whether the physiological achievements of deep-sea fauna, approximately 10% of which are decapod crustaceans (Martin and Haney, 2005), are similar or based upon those of species like *X. testudinatus* that have since taken refuge in deeper environments over generations. Unfortunately, studies of shallow vent endemic fauna have not received the attention that deep-sea vents have regarding crustaceans. Transcriptomic information has only recently begun to exist for vent endemic crustaceans in regards to how physiological adaptations have originated, although only in deep-sea species (Hui et al., 2017). Further evolutionary investigation into shallow vent endemic fauna and its pairing to

physiological measurements may help answer questions related to hard-to-obtain deep-sea counterparts.

Cumulatively, the reported physiological results may reflect the physiological plasticity developed by *X. testudinatus* in response to how their feeding behaviour relates to harsh conditions created by vent emissions (Jeng et al., 2004). At high and low tides during feeding, *X. testudinatus* approaches the mouths of vents to feed upon zooplankton killed by the hot, sulfuric, and acidic vent emissions, exposing the animals to an acute increase in acidity, hypoxia, hypercapnia, and high levels of sulfides. The observed immediate 64% reduction in metabolic rate upon exposure to pH 5.5 (24.6 kPa P_{CO_2}) may indicate that crabs reduce their ventilatory rate upon exposure to minimize the exchange of hazardous waters over the gills – similar to how terrestrial and semi-terrestrial crabs may retain fluids within their branchial chambers (Eliss, 1968). As the metabolic rate and cardiac output positively correlate in some animals (Currie et al., 2014; Farrell, 2016, 2009; Goodman et al., 1978), hemolymph flow rates throughout the crab may slow upon acute exposure to hypercapnia. Reduced branchial perfusion rates may allow for a more thorough exchange of HCO_3^-/Cl^- to occur across the gills, forming post-branchial hemolymph with a greater buffer capacity to wash over the animals' tissue. Furthermore, while feeding *X. testudinatus* should experience an increase in hemolymph ammonia due to protein catabolism. Experimental results indicate that following 24-hours of exposure to increased acidity, hemolymph ammonia is reduced by 41% (Fig. 3.1D) but was in the following days restored as acid-base homeostasis returned. As a marine crustacean, *X. testudinatus*' gill epithelia are expected to be ion “leaky” (Weihrauch et al., 1999), likely facilitating the influx of H^+ through paracellular junctions upon acute exposure to increasing environmental acidity. Paracellular ion fluxes may then be reduced by increasing epithelial tightness following sufficient acclimation time, similar to how tissue conductance changes under salinity stress in crustaceans (Henry et al., 2012; McNamara and Faria, 2012). Vent mouths, where emitted fluids may be pH 1.52 (Chen et al., 2005), may create inwardly directed H^+ gradients too large to effectively rely on H^+ extrusion or HCO_3^- accumulation alone. Dietary ammonia, as well as stores within the hemolymph, may be protonated forming ammonium and actively excreted within proximity to vents along the ammonia concentration and P_{NH_3} gradients (Weihrauch et al., 1999). While unmeasured, transbranchial NH_3 and/or NH_4^+ excretion and retention may be partially responsible for the restoration of hemolymph pH (Fig. 3.1A), despite increased

transbranchial HCO_3^- excretion in perfused gills acutely exposed to pH 5.5 (Fig. 3.2B), a pH regime crabs could experience during feeding at closer proximities to the vent. In addition to the increased acidity associated with feeding, *X. testudinatus* may experience acid-stress due to the effects of ocean acidification. Many of the aquatic animals expected to be heavily impacted by ocean acidification are those that inhabit niche zones as they may already be operating at or near maximal physiological capacity. While *X. testudinatus* does inhabit a niche zone, it is capable of surviving a broad pH range (pH 8.2–5.5) based on results of this study as well as experiments by Hu et al. (2016) and its known migration through non-vent zones while seeking neighboring vent fields (Wang et al., 2013). Furthermore, vent conditions near Kuieshantao already surpass the maximal projected P_{CO_2} and pH values given by the IPCC even if considering the worst case ‘business-as-usual’ scenario (IPCC, 2013). As such, it seems illogical that the vent environment would become more acidic, as an increase in the P_{CO_2} of surrounding surface waters would only reduce the rate that CO_2 diffuses from the vent site. In essence, the normal pH zone *X. testudinatus* inhabits, ca. pH 6.5, would become larger in diameter but not become more acidic. Given the animal’s strong acid–base regulatory capacity and the described assumptions regarding ocean acidification’s effect on Kueishantao Island’s vent fields it seems *X. testudinatus* will not experience great consequences due to ocean acidification.

3.6. Conclusions

Investigating the physiological states and responses of vent-endemic species offers valuable information as to how animals may be biologically challenged in response to future changes in ocean physiochemistry. Maintenance of extracellular acid-base homeostasis within *X. testudinatus* appears to rely on conventional compensatory methods that operate at a vastly amplified capacity. These amplified compensatory methods, and the animal’s ability to maintain an extracellular P_{CO_2} less than its ambient environment, would be beneficial when crabs are in close proximity to the vent emissions as may occur when feeding (Jeng et al., 2004). While unlikely that the majority of animals are capable of maintaining an inwardly directed P_{CO_2} gradient, measuring the ability of aquatic species to perform $\text{HCO}_3^-/\text{Cl}^-$ exchange and their capacity to manipulate non-bicarbonate buffering systems holds value in assessing their future status. In *X. testudinatus*, a large degree of extracellular chloride is replaced with accumulated HCO_3^- (Fig. 3.1B and table 3.2) exceeding the apparent limits of the bicarbonate concentration

threshold described by Heisler (1986), potentially indicating that vent endemic animals cope with acid-stress by carrying an expendable excess of Cl^- . Non-bicarbonate buffering systems, such as ammonia, may become more crucial to maintaining physiological pH due to their independence from the carbonate equilibrium and P_{CO_2} .

Transition to chapter 4

As discussed in chapter 1, many brachyurans face emersion due to tidal shifts or behavioural choices. While it is well understood that emersion poses many physiological challenges to aquatic crabs, our understanding of its effects is largely built on the study of intertidal species that usually emerge only at low tide. The few studies on semi-terrestrial species that undergo prolonged emersion indicate highly specialized urine reprocessing mechanisms that allow for the concentration of ammonia and the potential volatilization of acid-equivalents (De Vries et al., 1994; Wolcott, 1991). The following chapter undertook an effort to provide basic physiological parameters of a largely uncharacterized semi-terrestrial mangrove crab, *H. formosensis*, and investigated urinary and systemic acid-base equilibria over prolonged emersion. Interest was given to the role of nitrogenous wastes in the animal as their expected accumulation while emersed may increase their contribution to systemic acid-base status.

Chapter 4: Effects of emersion on acid-base regulation, osmoregulation, and nitrogen physiology in the semi-terrestrial mangrove crab, *Helice formosensis*

Garett Joseph Patrick Allen¹, Min-Chen Wang², Yung-Che Tseng², and Dirk Weihrauch¹

¹Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2

²Institute of Cellular and Organismal Biology's Marine Research Station, Academia Sinica, No. 23-10 Dawen Rd., Jiaoxi, 262, Yilan County, Taiwan

Published in: *Journal of Comparative Physiology B*. 2021. 191.

Author contributions:

G.J.P.A. designed the study, performed the experiments, analyzed and interpreted the results, and wrote the manuscript. M.-C.W. performed some cation and amino acid measurements. Y.-C.T. and D.W. helped design the study, revise the manuscript, and provided financial assistance.

4.1. Abstract

Emersion limits water availability and impairs the gill function of water-breathing animals resulting in a reduced capacity to regulate respiratory gas exchange, acid–base balance, and nitrogenous waste excretion. Semi-terrestrial crustaceans such as *H. formosensis* mitigate these physiological consequences by modifying and recycling urine and branchial water shifting some branchial workload to the antennal glands. To investigate how this process occurs, *H. formosensis* were emersed for up to 160 h and their hemolymph and urinary acid–base, nitrogenous waste, free amino acids, and osmoregulatory parameters were investigated. Upon emersion, crabs experienced a respiratory acidosis that is restored by bicarbonate accumulation and ammonia reduction within the hemolymph and urine after 24 h. Prolonged emersion caused an overcompensatory metabolic alkalosis potentially limiting the crab's ability to remain emersed. During the alkalosis, hemolymph ammonia was maintained at control levels while urinary ammonia remained reduced by 60% of control values. During emersion, ammonia may be temporarily converted to alanine as part of the Cahill cycle until re-immersion when crabs can revert alanine to ammonia for excretion coinciding with the crabs' observed delayed ammonia excretion response. The presence of high hemolymph alanine concentrations even when immersed may indicate this cycle's use outside of emersion or in preparation for emersion. Furthermore, *H. formosensis* appears to be uniquely capable of actively suppressing its rate of desiccation in absence of behavioral changes, in part by creating hyperosmotic urine that mitigates evaporative water loss.

4.2. Introduction

Maintenance of acid-base homeostasis and minimizing the toxic potential of ammonia are fundamental physiological processes of all organisms. Incoming acid or alkali loads affect the function of intra- and extracellular proteins due to their pH sensitivity (Somero, 1986) and may have substantial consequences when compensatory action cannot maintain homeostasis. The majority of extracellular compensatory action relies on the carbonate equilibrium and carbonic anhydrase activity (Gilmour and Perry, 2009). Carbon dioxide (CO_2) is hydrated by the catalytic action of carbonic anhydrase forming carbonic acid (H_2CO_3) that readily dissociates into a H^+ and HCO_3^- in solutions of physiological pH (*ca.* pH 7.2 -7.8; $\text{pK}_a = 6.35$) and may further dissociate into another H^+ and CO_3^- in alkaline solutions ($\text{pK}_a = 10.33$). Exhalation of CO_2 during respiratory gas exchange and the renal system's ability to resorb and secrete HCO_3^- allows the carbonate equilibrium to be manipulated at the respiratory and ionoregulatory levels, respectively. In a simplified example, a hyperventilating animal that is rapidly exhaling CO_2 will cause the carbonate equilibrium to shift leftward favouring the formation of CO_2 and H_2O and causing extracellular fluid to become alkaline. As the animal reduces its ventilation rate, CO_2 excretion rates are reduced, and the equilibrium shifts to the right restoring homeostasis by forming HCO_3^- that can accept excess H^+ . Generation of ammonia (*i.e.* combined ammonia gas (NH_3) and ionic ammonium (NH_4^+) as a metabolic by-product of amino acid catabolism can cause deleterious effects such as seizures, disrupted branchial gas exchange and ionoregulation (Wilkie, 1997) and swimming capabilities (McKenzie et al., 2009; McKenzie et al., 2003). The accumulation of ammonia within the bodily fluids of aquatic species reduces branchial excretory capacity (Martin et al., 2011) and immune function (Le Moullac and Haffner, 2000; Young-Lai et al., 1991), eventually becoming lethal if not excreted or detoxified (Larsen et al., 2014). As an amphoteric molecule (pK_a *ca.* 9.3), ammonia also contributes to acid-base regulation acting by accepting or donating a H^+ as NH_3 becomes NH_4^+ and vice-versa or by excretion of NH_4^+ as an H^+ -equivalent. While this non-carbonate buffering system is considered to be a negligible component in many organisms' acid-base compensatory response, some invertebrates have been identified to maintain distinct concentrations of extracellular ammonia and use bi-directional ammonia transport across the gill epithelia, presumably to be used as an acid-base equivalent (Allen et al., 2020; Hans et al., 2018; Hu et al., 2017; Weihrauch and Allen, 2018).

Crustacean gills are multifunctional organs that perform the majority of respiratory gas exchange, maintenance of acid-base status, ionoregulation, and ammonia excretion (Henry et al., 2012). In comparison to aerial habitats, water has relatively low oxygen bioavailability meaning that most water-breathing species must maintain high ventilation rates to extract enough oxygen to survive (Claiborne et al., 2002; Gilmour and Perry, 2009). Since water-breathers must rapidly ventilate, they do not have the choice to rely upon changes in ventilation rate to adjust acid-base status through ventilatory loss of CO₂ (Claiborne et al., 2002; Gilmour and Perry, 2009). Instead, these animals largely rely on ion-exchange processes such as H⁺-excretion by Na⁺/H⁺ exchangers or HCO₃⁻ excretion by Cl⁻/HCO₃⁻ exchangers to regulate acid-base homeostasis (Claiborne et al., 2002; Fehsenfeld and Weihrauch, 2016a; Gilmour and Perry, 2009; Goss et al., 1992; Perry et al., 2003). Although NH₃ may be diffusively excreted along the partial pressure gradient of NH₃ (ΔP_{NH_3}) aided by Rhesus-proteins across the gill epithelium and into the environment (Nawata et al., 2010), most (ca. 95-99.5%) of systemic and cellular ammonia exists as NH₄⁺ due to the pH of biological systems. NH₄⁺ transport is typically performed by K⁺-accepting transporters (Na⁺/K⁺-ATPase, K⁺-channels, cation/H⁺ exchangers) due to the similar hydration radii and the same molecular charge of the two ions, by ammonium transporters (AMTs) in invertebrates (Chasiotis et al., 2016; Durant and Donini, 2018; Thiel et al., 2017), or by vesicular trafficking, where NH₄⁺ accumulates in acidified vesicles that can be excreted independent of electrochemical gradients and environmental factors (Weihrauch et al., 2002, 1998; Weihrauch and Allen, 2018). In some cases, ammonia excretion over the apical epithelial membrane of the branchial tissue is suggested to be Na⁺-dependent such as in freshwater fishes and is potentially related to Na⁺/H⁺-exchanger activities (Wright and Wood, 2012). Thus, environmental conditions that impair gill function or affect ionoregulatory activity may also affect the organism's acid-base and nitrogen homeostasis.

Mangroves are harsh coastal environments whose inhabitants must be capable of surviving dramatic shifts in salinities, temperature, oxygen bioavailability, waste accumulation, and aerial exposure (Abel et al., 1987; Kathiresan and Bingham, 2001). Crabs are key-stone mangrove inhabitants and act as ecosystem engineers whose dense populations and burrowing behavior play a crucial role in the environment's nutrient cycling (Kristensen, 2008; Penha-Lopes et al., 2009). Semi-terrestrial crabs spend extended periods of time emersed while performing mating rituals and foraging for food, with some species developing lung-like

structures and trapping air within their burrows at high tides (Burggren, 1992; Eshky, 1992; Jimenez and Bennett, 2015; Teal and Carey, 1967). As gills require moisture to properly function, emersion stress causes impairment of respiratory gas exchange and transport mechanisms, reducing the animal's overall ability to excrete wastes such as CO₂ and ammonia (Morris, 2002). Therefore, animals must not only combat acute loss of water availability and search for high humidities (Adamczewska and Morris, 2000), but are also required to counteract physiological consequences related to osmotic and pH disruption.

While some terrestrial crabs have generally developed lung-like epibranchial structures (Morris, 2002), most semi-terrestrial species continue to rely on trapped moisture within the gill chamber to eliminate CO₂. Select species such as *Scopimera* and *Dotilla* sand-bubbler crabs use respiratory 'windows' on their legs to perform gas exchange either in place or in addition to their gill function (Maitland, 1986). Nitrogen eliminating strategies in terrestrial and semi-terrestrial crabs differ; however, most species are believed to accumulate or detoxify ammonia into temporary stores of less toxic metabolites until the animal can bathe itself in a water-source (Linton et al., 2017). Some semi-terrestrial species use their antennal glands to concentrate urinary ammonia that may be degassed as the urine passes over their mouth and into their branchial chamber as a portion of urine recycling (De Vries et al., 1994). This strategy is exploited by Ocypodoidea fiddler crabs, whereas Grapsoidea crabs are generally unable to shift their reliance from the gills to antennal glands to handle wastes (Tseng et al., 2020). Inability to rely on antennal glands and reduced gill function should cause most *Grapsoidea* crabs to accumulate nitrogenous wastes as ammonia or urea; however, several studies have noted that while the wastes are not detectably excreted it also does not accumulate in the body fluids as expected, although urinary contribution has often been excluded from studies (Wood et al., 1986; Wood and Boutilier, 1985).

Helice formosensis, the thick crab, is a burrowing mangrove species native to South-East Asia that undergoes emersion during low tides to forage for food. Unlike other members of the *Helice* family, *H. formosensis* is carnivorous and primarily feeds on other crabs (Mia et al., 2001) inferring that it faces a greater burden of nitrogenous waste excretion than its relatives. When not foraging, the crabs retreat into water-filled burrows where poor water-circulation exposes the animal to its own ammonia rich waste products (Mchenga et al., 2007). Thick crabs

do not possess epibranchial lung-like structures or respiratory ‘windows’ adaptations that would facilitate their lifestyle, making them an interesting case for developing adaptive strategies used by general crab species. *Helice formosensis* has been noted to rapidly recycle urine, a process used by most terrestrial and semi-terrestrial crabs to mitigate physiological consequences related to reduced gill function and dehydration (De Vries et al., 1994). Urine produced by the antennal glands is released from antennal pores and allowed to flow across the anterior surface of the crab where it is either swallowed or recollected into the water filled branchial chamber. The following study aims to characterize how emersion affects the thick crab’s acid-base, osmotic, and nitrogen physiology with a focus on the relative importance of recycled urine to the hemolymph in a crab that is unusually carnivorous amongst semi-terrestrial species.

4.3. Materials and methods

4.3.1. Animal husbandry and experimental conditions

Adult male thick crabs (*H. formosensis*; 16.5 ± 0.4 g) were purchased from local bait shops in Chiayi (嘉義縣) county, Taiwan and transported to the Institute of Cellular and Organismal Biology’s Marine Research Station (Yilan (宜蘭縣) county, Taiwan). Crabs were housed in four aquaria (50 gallons, 15–20 individuals/tank) containing constantly aerated brackish water (15.5 ± 0.1 ppt. salinity, pH 7.99 ± 0.01 , 21.1 ± 0.1 °C, $P_{CO_2} = 52.3 \pm 4.8$ Pa CO₂) maintained on a 12:12-h light:dark cycle and fed cuttlefish three times a week ad libitum. Brackish water was prepared by mixing dechlorinated freshwater with natural seawater. Accumulation of CO₂ within aquaria was monitored daily (see below) and complete water changes in aquaria were performed three times per week 3 h post-feeding to minimize the build-up of ammonia within the tanks. Animals were fasted for 3 days prior to experimentation to minimize the potential effects of dietary nitrogen on results.

Crabs were selected from the housing aquaria and immediately sampled representing immersed control animals. Treatment crabs were randomly selected from the control group, their bodies blotted dry, weight recorded, and stored in water-free containers for 6, 12, 24, 48, or 160 h at 21 °C temperature and *ca.* 90–95% relative humidity. Different crabs were used for either whole animal experiments or hemolymph and urine sampling—animals were not repeatedly measured. The mass of crabs after being blotted dry was compared to the mass of the

animal at the end of the emersion exposure to determine changes in body mass which were assumed to be primarily caused by evaporative water loss.

4.3.2. Hemolymph and urine carbonate equilibrium

Hemolymph was sampled by puncturing the arthroal membrane with a 25G needle and gas-tight Hamilton syringe. Urine was collected using a syringe following deflection of the antennal pore cover (Wolcott, 1991). Sample pH was immediately measured using a micro-pH electrode (InLab-Micro, Mettler-Toledo) attached to a pH-ISE meter model 225 (Denver Instruments, Gottingen, Germany) followed by measurement of total CO₂ using methods described by Lee et al. (2018) and the remaining samples were frozen for further analysis. In brief, 5 µL of the sample was injected through a septum into a glass chamber containing 0.1 mmol l⁻¹ HCl to transform dissolved inorganic carbon within the sample to CO₂. CO₂ was degassed by the continuous input of N₂-gas (60—80 ml N₂ gas min⁻¹, GFC17, Aalborg, Orangeburg, NY, USA) and passed into a total CO₂ detector (LI-850 infra-red CO₂/H₂O gas analyzer, LI-COR, Lincoln, NE, USA). LI-8 × 0 software (v1.0.2, LI-COR) was used to collect data. Total CO₂ within the samples was determined by comparing data to that of NaHCO₃ standards. The partial pressure of CO₂ (P_{CO2}) within the samples were mathematically determined by inputting the measured pH and total CO₂ into a rearrangement of the Henderson-Hasselbalch equation (Eq. 4.1) where the solubility of CO₂ ($\alpha = 0.045 \text{ mmol Torr}^{-1}$) and first dissociation constant of carbonic acid (pK_{a1} = 6.05) were experimentally determined in *C. maenas* and extrapolated from nomograms supplied by Truchot (1976) at 21 °C and 15 ppt. salinity

Equation 4.1.
$$P_{CO_2} = TCO_2 / [(1 + \text{antilog}(pH - pK_{a1})) * \alpha CO_2]$$

The concentration of HCO₃⁻ within the samples was then determined using Eq. 4.2 applying the same constants

Equation 4.2.
$$[HCO_3^-] = TCO_2 - (P_{CO_2} * \alpha CO_2)$$

The total CO₂ within water samples was similarly determined; however, the carbonate equilibrium was determined using the CO₂SYS software (Pierrot et al., 2006) using dissociation constants of Mehrbach et al. (1973) refit by Dickson and Millero (1987).

4.3.3. Ammonia measurements

Ammonium concentrations in hemolymph, urine, and water samples were determined fluorometrically (360 and 442 nm excitation and emission wavelengths, respectively; Spectramax M5, Molecular Devices) using a borate buffered ortho-phthalaldehyde plate assay that is insensitive to amino acids and proteins (Holmes et al., 1999), the latter being confirmed by cross-analyzing results of deproteinized and non-deproteinized samples (data not shown; deproteinizing sample preparation kit—TCA, ab204708, AbCam, Toronto, Canada). Osmolality of the NH₄Cl standards for each sample type were adjusted with NaCl to account for potential osmotic sensitivity in the assay.

4.3.4. Major ion composition

The major cation composition (Na⁺, K⁺, Mg²⁺, Ca²⁺) of samples was determined by flame absorption spectroscopy (Polarized Zeeman Atomic Absorption Spectrophotometer ZA3000 series, Hitachi High-Technologies, Tokyo, Japan) by comparing sample readings to a calibration curve made with standard solutions of individual metal ions. Concentration of HCO₃⁻ was determined as previously described whereas the mercuric-ferrous cyanide assay was used to spectrophotometrically detect the concentration of Cl⁻ within the samples (Florence and Farrar, 1971). Osmolality of samples was measured using a vapour pressure osmometer (VAPRO Model 5520, Wescor, UT, USA).

4.3.5. Free amino acid composition

Free amino acids from 20 µL samples of hemolymph and urine were extracted in ice cold 50% (v/v) methanol:water solutions spiked with isotopically labeled norvaline internal standards (4 µg/mL) and subsequently derivatized with AccQ•Tag Ultra Derivatization Kit (Waters, MA., U.S.A.; Armenta et al., 2010). The UPLC (Acquity UPLC H-Class System, Waters) was equipped with a BEH C18 column and a FLU detector was used to detect the derivatized amino

acids. The free amino acids within samples were quantified by comparison to known standards (WAT088122, Waters).

4.3.6. Whole animal ammonia excretion upon re-immersion

Ammonia excretion rates were determined by adding 300 mL of filtered ($0.45 \mu\text{mol l}^{-1}$) 15 ppt. salinity brackish water into the jars holding animals and measuring the accumulation of ammonia after 30, 60, 90, and 120 min. To account for ammonia that may have been adhered to the jar during the crab's air exposure, samples were also taken immediately after water was added and subtracted from the final data (time = 0 min). Ammonia concentrations were determined fluorometrically as previously described and excretion rates determined as $\mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$.

4.3.7. Statistical analysis

Data analysis and statistics were performed using GraphPad Prism 8.4.3 software. Normal distribution and variance in collected data were investigated using the Shapiro–Wilk and Brown-Forsythe tests, respectively. Non-normal data were normalized using a box-cox transformation. Outliers were detected using Grubb's outlier test ($p < 0.05$) and removed from data sets prior to analysis. Normally distributed time-course data with equal variance was tested for statistical significance using one-way ANOVAs and Tukey's post hoc test. Statistical differences between the hemolymph and urine samples at each respective time point were assessed using a two-way ANOVA where time and fluid type (hemolymph or urine) were set as factors and Tukey's post hoc test. If data could not be normalized by transformation, statistical significance was determined using Mann–Whitney U rank comparison tests. Data are presented as the mean \pm S.E.M. Statistical significance was denoted a $p < 0.05$.

4.4. Results

Hemolymph of immersed crabs was 7.78 ± 0.02 pH (Fig. 4.1) with extracellular P_{CO_2} levels of 401 ± 13 Pa (Fig. 4.2) and 7.38 ± 0.26 mmol l^{-1} HCO_3^- (Fig. 4.3). The immersed crabs' hemolymph ammonium concentrations were 381 ± 27 $\mu\text{mol l}^{-1}$ NH_4^+ (Fig. 4.4). Urinary pH (7.83 ± 0.02 ; Fig. 4.1), P_{CO_2} (360 ± 13 Pa; Fig. 4.2), $[\text{HCO}_3^-]$ (7.37 ± 0.31 mmol l^{-1} ; Fig. 4.3), and NH_4^+ (320 ± 45 $\mu\text{mol l}^{-1}$; Fig. 4.4) were similar to that of the immersed crabs' hemolymph.

Crabs experienced a slight respiratory acidosis after 6 h of emersion as indicated by a 0.84 pH unit reduction in hemolymph pH (Fig. 4.1, $p = 0.0651$) and 77% increase in hemolymph P_{CO_2} (Fig. 4.2) that is compensated within 24 h by the accumulation of extracellular HCO_3^- (Fig. 4.3a). Urine parallels the hemolymph changes in pH, P_{CO_2} , and HCO_3^- (Figs. 4.1, 4.2, 4.3) indicating the fluids remain in equilibrium upon immersion. Prolonged emersion caused animals to apparently over-compensate, resulting in a metabolic alkalosis indicated by alkalization of hemolymph and urine (Fig. 4.1) caused by the accumulation of HCO_3^- (Fig. 4.3) while maintaining constant P_{CO_2} (Fig. 4.2). Ammonia concentrations within the hemolymph were reduced by 29% after 6 h of emersion ($p = 0.0685$) but were restored to control levels after 24 h (Fig. 4.4). Urinary ammonia concentrations were immediately reduced by 60% upon acute emersion and remained constant throughout prolonged emersion (Fig. 4.4). Whole animal ammonia excretion upon re-immersion was measured in crabs that were emersed for 160 h (Fig. 4.5) indicating that the crabs progressively increase their ammonia excretion rate rather than immediately releasing ammonia accumulated over time. Excretion rates beginning at $0.57 \pm 0.04 \mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$ upon immediate re-immersion significantly increased by 3.6-fold reaching $2.04 \pm 0.37 \mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$ after 2 h of immersion (Fig. 4.5).

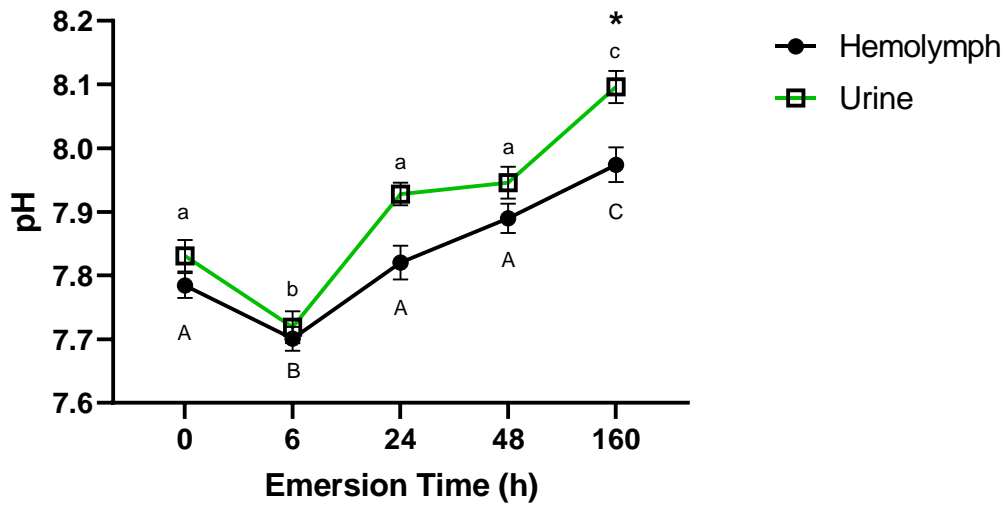


Figure 4.1. pH of hemolymph and urine of immersed ($N_H = 22$, $N_U = 12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H = 18$, $N_U = 18$), 24 ($N_H = 12$, $N_U = 12$), 48 ($N_H = 6$, $N_U = 5$), and 160 ($N_H = 12$, $N_U = 12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M.

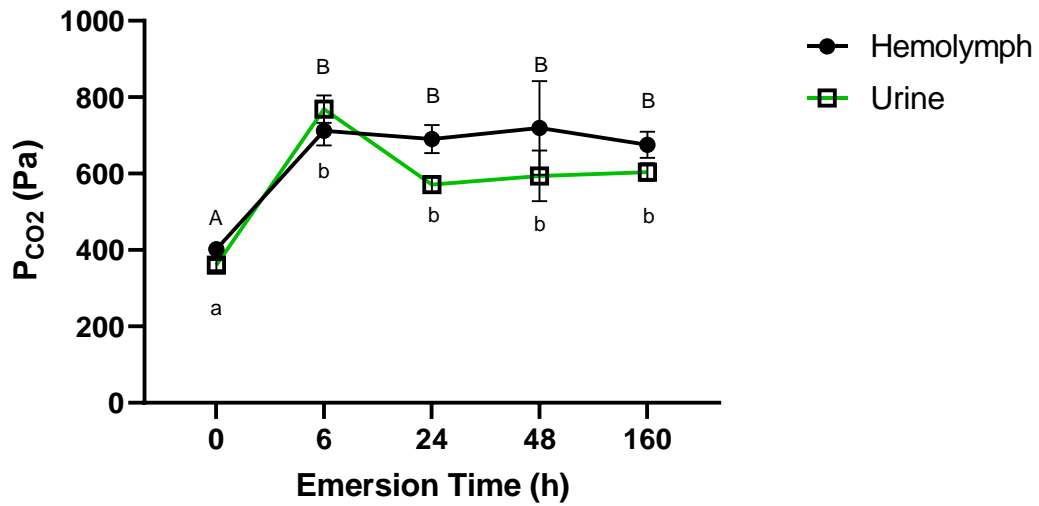


Figure 4.2. Partial pressure of CO₂ (P_{CO_2}) within the hemolymph and urine of immersed ($N_H = 22$, $N_U = 12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H = 18$, $N_U = 18$), 24 ($N_H = 12$, $N_U = 12$), 48 ($N_H = 6$, $N_U = 5$), and 160 ($N_H = 12$, $N_U = 12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M.

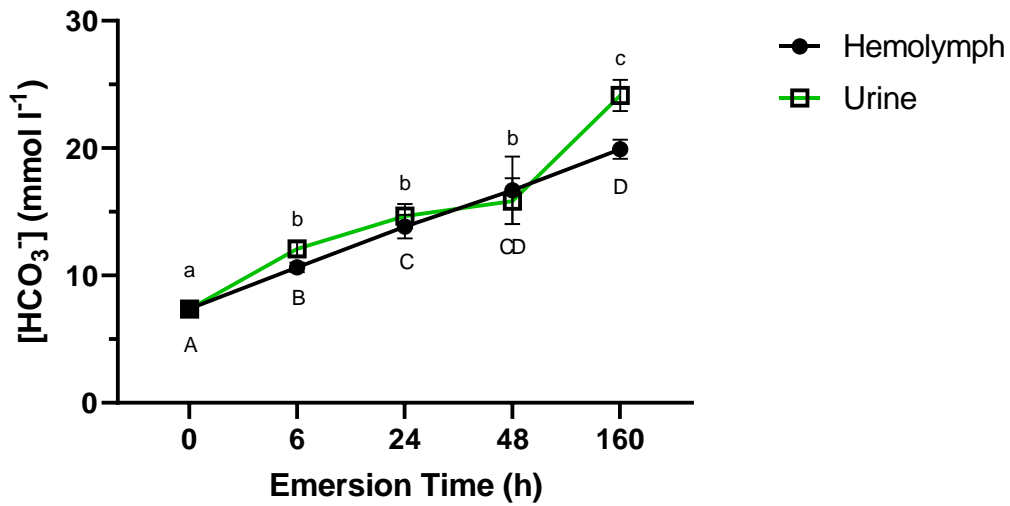


Figure 4.3. Concentrations of HCO_3^- within the hemolymph and urine of immersed ($N_H = 22$, $N_U = 12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H = 18$, $N_U = 18$), 24 ($N_H = 12$, $N_U = 12$), 48 ($N_H = 6$, $N_U = 5$), and 160 ($N_H = 12$, $N_U = 12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M.

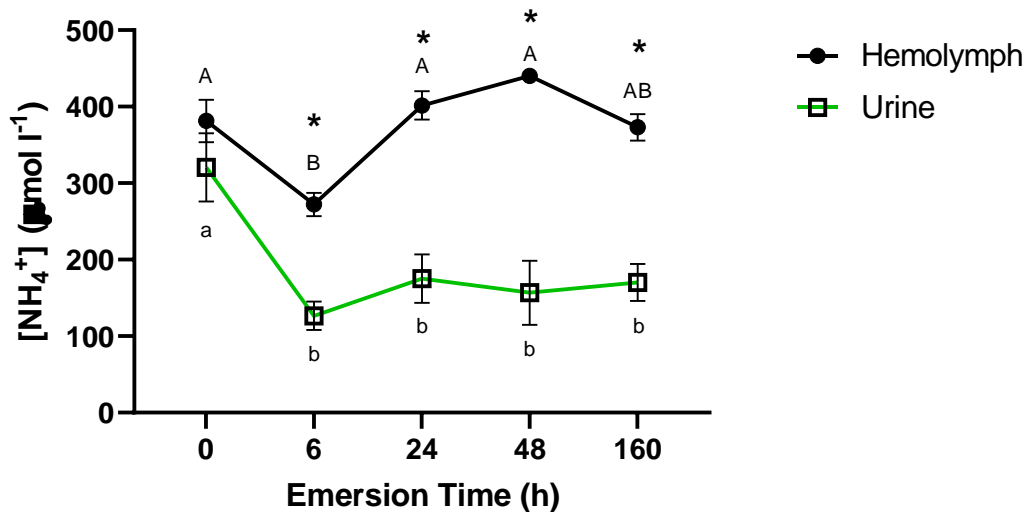


Figure 4.4. Concentrations of NH_4^+ within the hemolymph and urine of immersed ($N_H = 12$, $N_U = 12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H = 12$, $N_U = 12$), 24 ($N_H = 12$, $N_U = 6$), 48 ($N_H = 6$, $N_U = 5$), and 160 ($N_H = 12$, $N_U = 12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M.

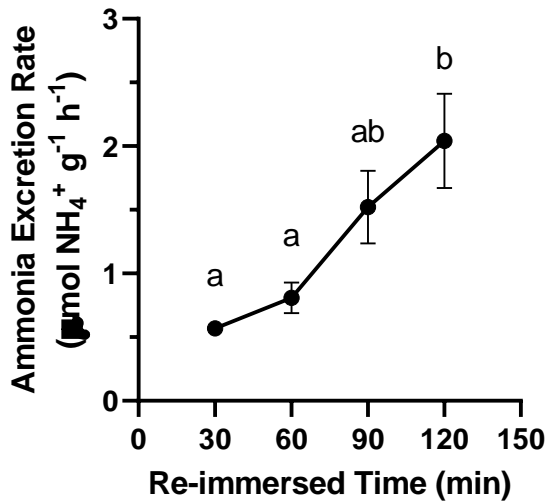


Figure 4.5. Whole animal ammonia excretion of crabs (N = 5) emersed for 160-hours immediately following re-immersion. Values are represented as the mean \pm S.E.M.

Hemolymph and urine of immersed and emersed crabs were assessed for 21 amino acids via UPLC (Fig. 4.6 and supplemental Figs. 4.1–3). Hemolymph contained detectable concentrations of alanine, cysteine, glutamate, glutamine, glycine, proline, taurine, arginine, asparagine, methionine, valine, isoleucine, serine, threonine, and leucine while histidine, lysine, tyrosine, aspartate, and phenylalanine were undetectable. Urine contained fewer detectable amino acids and lower concentrations of amino acids overall compared to the hemolymph. Alanine was the predominant free amino acid detected in hemolymph and urine, accounting for most of the total free amino acids in both fluids (Fig. 4.6a). High alanine concentrations of 30–40 and 20 mmol l⁻¹ indicate that the amino acid is the third most abundant osmolyte in immersed and emersed crab’s hemolymph and urine, respectively, only being surpassed by Na⁺ and Cl⁻ concentrations (Figs. 4.6a and 4.8). Crabs reduced hemolymph alanine concentrations upon acute emersion for up to 24 h reaching 78% of control levels (Fig. 4.6a). Prolonged emersion (> 24 h) is accompanied by a 22% increase in alanine concentrations returning to control levels. Urinary alanine concentrations remained stable throughout the experiment, consistently containing 10–15 mmol l⁻¹ less alanine than hemolymph (Fig. 4.6a). Cysteine was the second most abundant free amino acid in the hemolymph, remaining stable throughout all time-points at 1.6–1.8 mmol l⁻¹, but was undetectable in urine (Fig. 4.6d). Hemolymph glutamate and glutamine levels were unchanged throughout the experiments; however, the urine was devoid of glutamine

and glutamate concentrations were significantly reduced upon emersion (Fig. 4.6b, c). Detected free essential amino acids decreased in samples as crabs were unfed throughout the experiment and fasted for 3 days prior to experimentation (Fig. 4.7e and supplemental Fig. 4.1).

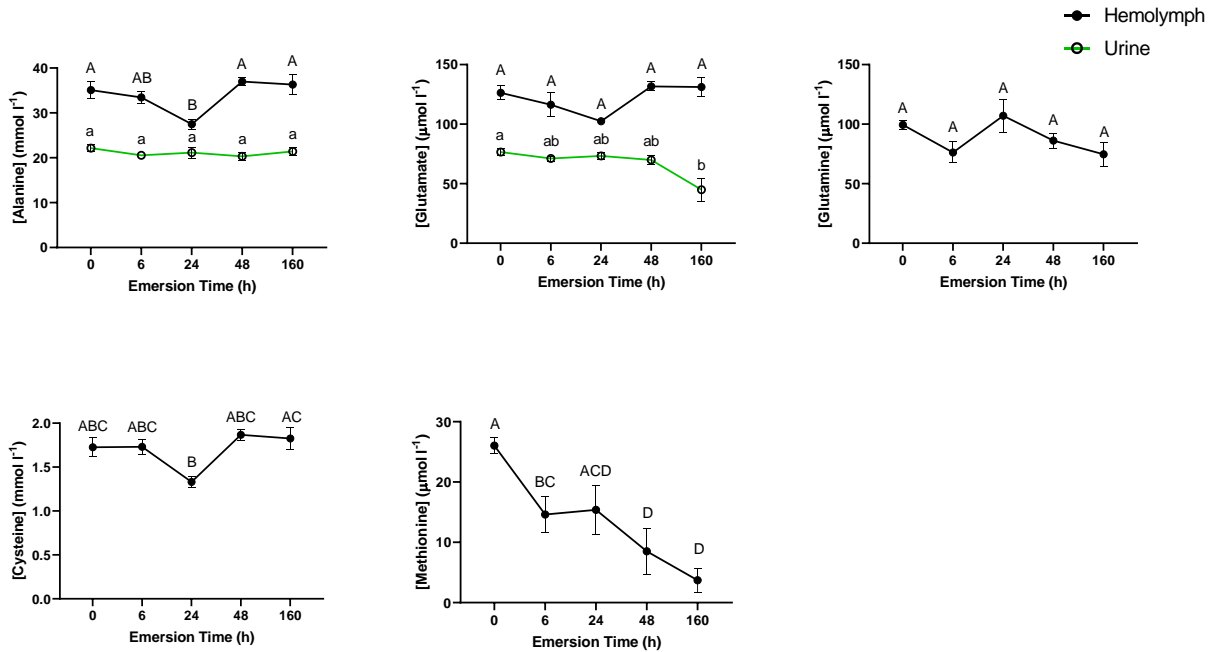


Figure 4.6. Predominant free amino acids in the hemolymph (N = 6–12) and urine (N = 4–12) of immersed (0-h) or emersed crabs over a 160-hour time-course exposure. Where data is not available for urinary concentrations, no detectable amino acid presence was found. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M.

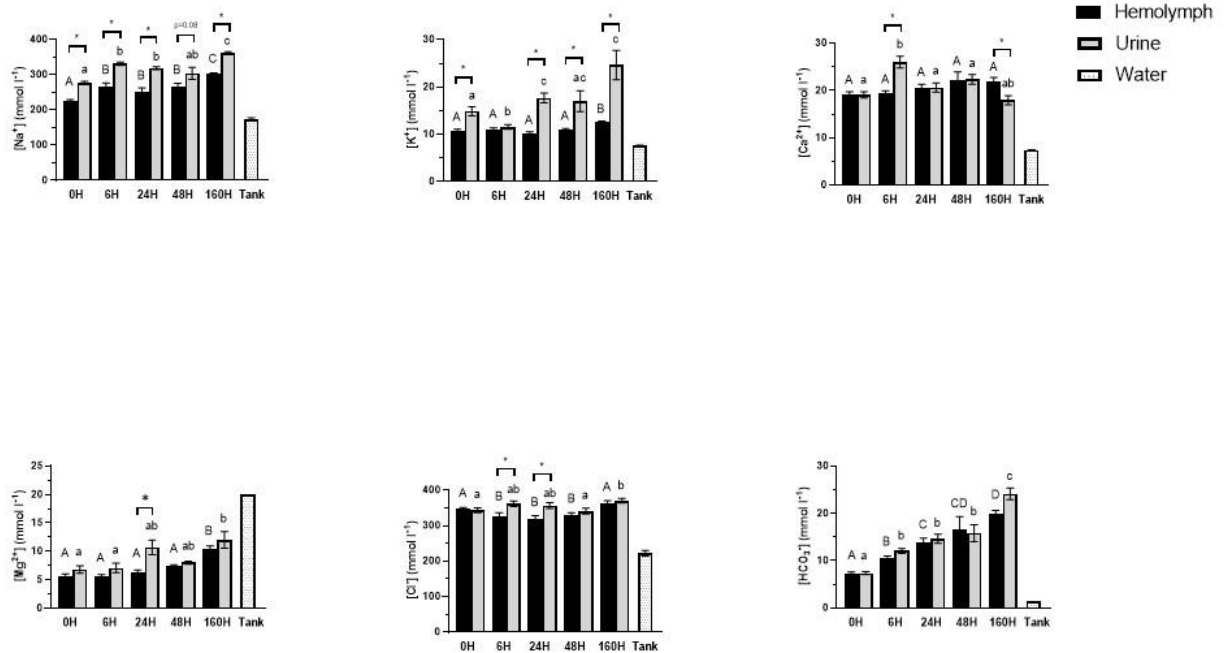


Figure 4.7. Major ion composition of immersed (0-h) and emersed crabs' hemolymph (N= 6–12 for all ions) and urine (N = 5–12 for all ions) over a 160-hour time-course exposure. Tank parameters are included as comparison to the immersed crabs' environmental conditions at 15 ppt salinity. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M.

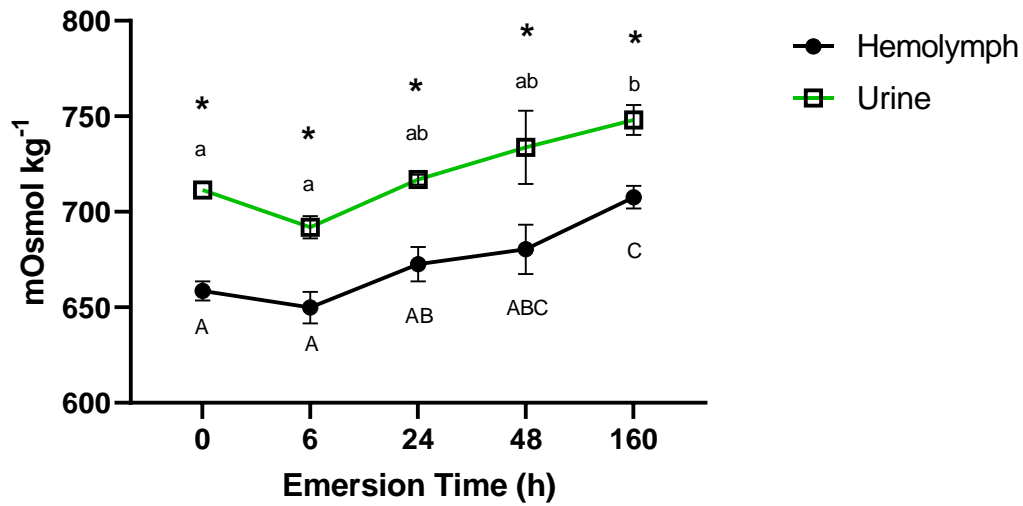


Figure 4.8. Osmolality of the hemolymph and urine of immersed ($N_H = 22$, $N_U = 12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H = 18$, $N_U = 12$), 24 ($N_H = 12$, $N_U = 6$), 48 ($N_H = 6$, $N_U = 5$), and 160 ($N_H = 12$, $N_U = 11$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M.

Helice formosensis emersed in 15-ppt. brackish water displayed characteristics of strong hyperosmoregulators maintaining 658 ± 5 mOsm kg^{-1} hemolymph against 410 mOsm kg^{-1} ambient water (Fig. 4.8). Urine is maintained hyperosmotic by 40 – 53 mOsm kg^{-1} in relation to the hemolymph (Fig. 4.8) due to increased Na^+ , K^+ , and Mg^{2+} concentrations but not Ca^{2+} or Cl^- (Fig. 4.6). Emersion caused both hemolymph and urine osmolality to increase over time; however, the osmotic difference between the fluids is maintained (Fig. 4.8). Crabs were able to significantly reduce their rate of evaporative water loss over time from an initial rate of $0.2 \pm 0.02\%$ of their body mass per hour after 6 h of emersion to $0.05 \pm 0.003\%$ of their body mass per hour after 160 h of emersion (Fig. 4.9).

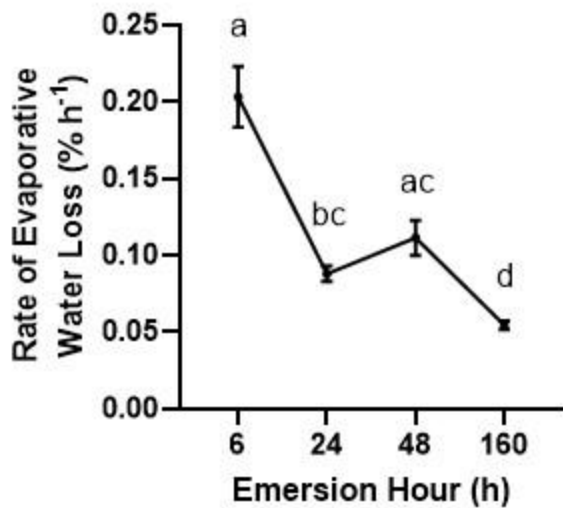


Figure 4.9. Rate of evaporative water loss as measured by changes in emersed crabs’ total mass over a time course exposure. Emersed measurements were made after 6 (N = 12), 24 (N = 12), 48 (N = 12), and 160 (N = 24) hours of emersion. Values are represented as the mean ± S.E.M.

4.5. Discussion

4.5.1. Immersed physiology

As a relatively unstudied species, there is an absence of information on hemolymph and urine compositions of members of the *Helice* crab family. The hemolymph of immersed *H. formosensis* measured 7.78 ± 0.02 (Fig. 4.1) and was similar to those of other crab species (e.g., *C. maenas* 7.82 pH and 150 Pa P_{CO2} (Truchot, 1975a); *M. magister* 7.93 pH and 132.9 Pa P_{CO2} (Hans et al., 2014); *C. sapidus* 7.78 pH and 270 Pa P_{CO2} (Henry and Cameron, 1982b), although the resting levels of *H. formosensis*’ P_{CO2} were considerably higher (401 ± 13 Pa P_{CO2}; Fig. 4.2). The comparably high resting levels of P_{CO2} are balanced by the presence of 7.38 ± 0.26 mmol l⁻¹ HCO₃⁻ (Fig. 4.3) and 381 ± 27 μmol l⁻¹ NH₄⁺ (Fig. 4.4) within the crab’s hemolymph. Apart from the uniquely specialized ghost crab, *Ocypode quadrata* (De Vries et al., 1994), few brachyuran crustaceans have had their urine characterized in terms of acid–base and nitrogen composition. Urine collected from immersed *H. formosensis* was similar in pH, P_{CO2}, HCO₃⁻, and NH₄⁺ to the hemolymph (pH 7.83 ± 0.02 ; Fig. 4.1; 360 ± 13 Pa P_{CO2}; Fig. 4.2; 7.37 ± 0.31 mmol HCO₃⁻ l⁻¹; Fig. 4.3; 320 ± 45 μmol NH₄⁺ l⁻¹; Fig. 4.4), suggesting the fluids are in equilibrium and that wastes are not concentrated in urine prior to emersion; however, the

apparently high urinary flow-rate was not determined and could play an important factor in its overall excretory importance.

Helice formosensis remains immersed within its burrows beneath the sediment of the mangrove floor. Studies of the physiochemical properties of the burrow shafts and chambers indicate that *H. formosensis* significantly reduces the salinity of the chamber's water (Mchenga et al., 2007). The current study demonstrates that the thick crab has characteristics of strong hyperosmoregulators (Fig. 4.8) and can maintain its extracellular osmolality well above ambient levels, likely due to inhabiting environments that naturally fluctuate between 10 and 35 ppt. salinity (Tseng et al., 2020). While most of the crab's hemolymph ion composition reflects that of other hyperosmoregulating crabs, its extracellular K^+ (Fig. 4.7b) is maintained at $10.7 \pm 0.4 \text{ mmol l}^{-1}$, exceeding the concentrations typically observed even in seawater-dwelling crabs such as *M. magister* ($6.4 \text{ mmol l}^{-1} K^+$; Hans et al., 2014). Additionally, *H. formosensis*' extracellular NH_4^+ concentrations ($381 \pm 27 \text{ } \mu\text{mol l}^{-1} NH_4^+$ (Fig. 4.4a)) are also above the average of most brachyuran species *ca.* 150—200 $\mu\text{mol l}^{-1} NH_4^+$ (Fehsenfeld and Weihrauch, 2017; Weihrauch et al., 2017). Given that the urine of immersed *H. formosensis* seems to concentrate Na^+ and K^+ while leaving divalent cations (Ca^{2+} , Mg^{2+}) and anions (Cl^- , and HCO_3^-) at the hemolymph level, it is conceivable that the antennal glands may promote the excretion of excess K^+ and is involved in the crabs' osmoregulation as occurs in other crustaceans (Tsai and Lin, 2014; Tseng et al., 2020). Future studies would benefit from determining the flow rate of the urine in immersed crabs to calculate the true amounts of ions and osmolytes expelled over time.

While filtrating organs, including antennal glands, generally reabsorb useful ions and molecules it seems that alanine and glutamate are only partially reabsorbed during primary urine production by *H. formosensis* (Fig. 4.6 and supplemental information). Alanine and glutamate remain present at 22 mmol l^{-1} (Fig. 4.6a) and $76 \text{ } \mu\text{mol l}^{-1}$ (Fig. 4.6b) within the urine, respectively, accounting for 63% and 61% of the respected amino acid within the hemolymph while other amino acids are effectively reabsorbed. While the actual amount of alanine and glutamate lost through urine expulsion would depend on the urinary flow rate of immersed crabs, it seems unfavorable for an animal to lose large millimolar levels of potentially useful amino acids. One hypothesis is, that since the crabs are generally immersed within the confines of their burrow chamber, they may be able to reabsorb at least some of the amino acids across their

branchial epithelia (Blewett and Goss, 2017). Water in the burrow chambers of *H. formosensis* is significantly higher in organic matter than their surroundings, presumably due to detritus and excretion by the crab (Mchenga et al., 2007). While Mchenga et al. (2007) did not measure the presence of amino acids within the burrows, the current study does suggest the crabs can excrete large levels of amino acids through urine. If excess ammonia is detoxified as alanine, its release into the burrow chamber waters would mitigate the build-up of toxic ammonia while also allowing the crab to reabsorb some degree of the amino acid from the water across its branchial chamber, especially considering amino acid uptake across the gills of brachyuran crabs has recently been discovered (Blewett and Goss, 2017). Non-absorbed amino acids may still benefit the crabs and mangrove environment by providing nutrients to the microbes within their burrow chambers. While this study does not provide hard evidence that this process does occur, *H. formosensis* would require a means to reabsorb urinary amino acids through their branchial chamber as part of their urine reprocessing mechanism during emersion (see below). Additionally, maintaining high alanine concentrations within the urine may simply be a strategy used by the crab to be continuously prepared for emersion following tidal movements.

4.5.2. Emerged acid-base regulation

Mangrove crabs are regularly air-exposed during low-tide while they perform energetically demanding tasks such as feeding, digging burrows, or performing mating dances/rituals. While it is unknown how long *H. formosensis* specifically remains emerged, the crabs demonstrated the ability to readily compensate for acid–base disturbances. Despite a significant increase in hemolymph P_{CO_2} (Fig. 4.2), crabs managed to mitigate a statistically relevant change in hemolymph pH (Fig. 4.1) through rapid HCO_3^- accumulation (Fig. 4.3) after only 6-h of emersion. After 24 h of emersion, crabs restored hemolymph pH to near-exact control values, demonstrating a clear ability to fully compensate the observed respiratory acidosis within 24 h. Like immersed crabs, the pH, P_{CO_2} , and HCO_3^- of hemolymph and urine remain in equilibrium when crabs are emerged; however, the urine is no longer lost to the ambient environment and is recycled into the branchial chamber for reprocessing. Urinary CO_2 may be volatilized as urine passes across the ventral body of the crab representing an effective and energetically cheap means of excreting CO_2 for crabs during emersion. Since crabs experience a progressive metabolic alkalosis once emersion passes 24 h, this excretory method

may be capable of maintaining constant hemolymph P_{CO_2} (Fig. 4.2) but is insufficient to prevent the build-up of total carbon and protons resulting in a subsequent alkalosis due to HCO_3^- accumulation (Fig. 4.3).

Upon emersion, changes in the hemolymph of *H. formosensis* are quite different than that of *Cardisoma carnifex*, an air breathing terrestrial lunged crab, that experiences an acute metabolic alkalosis and a delayed respiratory acidosis in absence of water (Wood et al., 1986). Upon desiccation, the hemolymph of *C. carnifex* is maintained at about 0.05–0.10 pH units above its hydrated levels while the crabs manage to prevent over-alkalization of its hemolymph by continuing to gain P_{CO_2} until rehydrated (Wood et al., 1986). The delayed accumulation of CO_2 in *C. carnifex* is likely due to its lungs permitting a greater excretion of CO_2 than the gills of *H. formosensis*. If urinary CO_2 volatilization is sufficient to stabilize hemolymph P_{CO_2} but the crabs do not have an effective means of excreting protons, HCO_3^- accumulation should eventually result in the observed metabolic alkalosis. Given that *H. formosensis* is more likely to experience tidal emersion rather than > 24 h of emersion by choice, the crabs may lack a means of counteracting the process. In the intertidal green shore crab, *C. maenas*, emersion causes an acute respiratory acidosis that requires approximately 115 h to be nearly restored to control levels by the accumulation of HCO_3^- (Truchot, 1975a). In this case, *C. maenas* also maintains a constant level of P_{CO_2} (ca. 660 Pa) while emersed, which is similar to that of *H. formosensis* in this study (Fig. 4.2a). The more rapid accumulation of HCO_3^- and restoration of hemolymph pH in *H. formosensis* compared to *C. maenas* is presumably due to their degree of terrestrialization that is based on their independence from water (Greenaway, 1999; Hartnoll, 1988). Crustaceans can be classified between five tiers of terrestrialization (T_1 to T_5) where *C. maenas*, for example, is a T_1 crab that is relatively quiescent upon emersion that survives brief periods of emersion. While *H. formosensis* has not been previously classified, its lifestyle is better described as a T_2 or T_3 crab as it voluntarily emerges for prolonged periods of time but immerses itself in burrows and requires water to breathe and reproduce (Greenaway, 1999; Hartnoll, 1988; Mchenga et al., 2007; Mia et al., 2001; Mia and Shokita, 1997).

4.5.3. Emerged nitrogen physiology

Despite being studied in several species, the handling of nitrogenous wastes by semi-terrestrial and terrestrial crustaceans during emersion or desiccative stress has remained poorly understood. With the exception of the uricotelic Robber crab (*Birgo latro*; Morris and Greenaway, 1990), all semi-terrestrial and terrestrial brachyuran crustaceans described so far are ammonotelic and cannot rely on branchial excretion of ammonia without access to water. Interestingly, chronically emerged/desiccation stressed crabs manage to prevent the accumulation of hemolymph ammonia despite continued protein catabolism. Therefore, crabs must be capable of either unconventional excretion, such as volatilization of NH_3 , or through temporary detoxification of ammonia through transformation into less toxic metabolites (*e.g.*, urea, uric acid, amino acids) in absence of water.

Volatilization of NH_3 is a prominent strategy used predominately by T₄ terrestrial crustaceans such as the Atlantic ghost crab (*Ocypode quadrata*) and the purple-backed shore crab (*Geograpsus grayi*) whom either form urine with extremely concentrated ammonia ($100 + \text{mmol l}^{-1}$; De Vries et al., 1994) or alkalizes the ammonia from branchial chamber water (Varley and Greenaway, 1994), respectively. These species typically maintain high ($0.9\text{--}1.9 \text{mmol l}^{-1}$; Linton et al., 2017) concentrations of hemolymph ammonia to develop high NH_3 partial pressure gradients that are not observed in the hemolymph of *H. formosensis* (Fig. 4.4a). In NH_3 volatilizing isopods, high hemolymph levels of ammonia are mitigated by temporary detoxification to glutamine that is later catabolized by glutaminase to release ammonia into the pleon fluid for volatilization (Wieser et al., 1969; Wright and O'Donnell, 1993; Wright and Peña-Peralta, 2005). Given that hemolymph glutamine/glutamate and ammonia levels in *H. formosensis* were not elevated at any point of this study (Figs. 4.4 and 4.6b) it is unlikely this crab uses methods similar to isopods or NH_3 volatilizing T₄ crustaceans despite alkalization of its urine (Fig. 4.1) which would otherwise promote volatilization of NH_3 . With some speculation, it can be reasoned that NH_3 volatilization from within the branchial chamber is also unlikely. The fluid within the branchial chamber will presumably become increasingly similar in composition to the urine as emersion continues since recollected urine is the sole fluid source available to the crab. This means that while branchial chamber fluid will also be slightly alkaline, it will probably be low in ammonia concentration which would not favour volatilization of NH_3 . While

these findings do not suggest volatilization occurs to a significant degree, it cannot be completely ruled out without direct measurement, especially considering that *C. maenas* (T₁) has been found to be unexpectedly capable of volatilizing small amounts of NH₃ during bouts of brief emersion (6 h; Simonik and Henry, 2014)

An alternative to volatilization is the temporary storage and detoxification of ammonia as non-essential amino acids, urea, or crystalline urate deposits (Linton et al., 2017). Most investigations have failed to identify significant accumulation or excretion of urea or uric acid in crustaceans (Linton et al., 2017; Martin et al., 2011; Weihrauch et al., 2017; Wood et al., 1986); however, several chronically emersed T₁₋₃ crabs are capable of temporarily storing nitrogenous wastes as amino acids although the methods differ by species. For example, *C. maenas* was found to maintain control levels of hemolymph ammonia even after 72 h of emersion (Durand and Regnault, 1998) but instead accumulate non-essential amino acids (glycine, alanine, and glutamine) in muscle tissue that are reverted to ammonia and released upon return to water (Durand et al., 1999). *Austrothelphusa transversa* (T₃), the Australian arid-zone crab, lives in absence of water for years due to droughts and stores nitrogenous wastes that are gradually re-converted to ammonia once the crab finds water (Linton and Greenaway, 1995; Macmillen and Greenaway, 1978). While the specific storage molecule has not been found, *C. carnifex* returns its hemolymph ammonia levels to non-desiccated control levels for up to 192 h without water following a transient doubling in hemolymph ammonia after the first 36 h (Wood et al., 1986; Wood and Boutilier, 1985). The fact that *H. formosensis* does not accumulate ammonia within its urine or hemolymph while emersed and even reduces urinary ammonia while maintaining control hemolymph levels (Fig. 4.4) suggests nitrogenous wastes are stored. This hypothesis is supported by an observed gradually increasing rate of ammonia excretion by the animal following re-immersion (Fig. 4.5) suggesting the animal requires time to reconvert the storage compounds into ammonia. This counters the usual ‘panic pee’ response associated with crabs where handling stress results in an immediate increase in ammonia excretion rate that decreases over time (Hunter and Kirschner, 1986; Martin et al., 2011; Weihrauch et al., 1999). This process is observed in *A. transversa* (Linton and Greenaway, 1995) and to a lesser degree *C. carnifex* (Wood et al., 1986), where excretion increases gradually upon re-immersion but it differs fundamentally from crabs like *Cancer pagurus* that are rarely emersed (Regnault, 1994).

Collectively, it seems that amphibious crabs that temporarily store nitrogen rather than volatilize it, gradually increase ammonia excretion rates upon re-immersion fitting the observations made for *H. formosensis* in this study.

If ammonia detoxification does occur through temporary storage as amino acids in *H. formosensis*, it appears to occur, at least partially, during the production of urine. While hemolymph ammonia remains constant following 24 h of emersion, urinary ammonia is maintained at 40% of immersed levels (approximately $150 \mu\text{mol l}^{-1}$, Fig. 4.4), indicating the antennal glands reabsorb ammonia. Interestingly, the antennal glands effectively reabsorb free amino acids apart from alanine and glutamate that are only partially reabsorbed (Figs. 4.6a, b). Reabsorbed ammonia and glutamate could be used by the antennal gland to synthesize glutamine through glutamine synthetase to mitigate ammonia accumulation. This process is used by the mammalian kidney when a metabolic alkalosis occurs (Weiner and Verlander, 2013), similar to the physiological state of emerged *H. formosensis*. The peak reabsorption of urinary glutamate occurs after 160 h when the metabolic alkalosis is most severe. Unfortunately, this study did not investigate intracellular amino acid levels and cannot confirm this hypothesis without further investigation. If this process does occur in a similar fashion to the late proximal tubule of the mammalian nephron, it may occur through a similar fashion of K^+ substitution for NH_4^+ through $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters and may be useful to target in future studies (Weiner and Verlander, 2013, 2011).

Alanine was found to be present at several magnitudes higher than all other free amino acids (Fig. 4.6a) in both immersed and emerged crabs reaching up to 37 and 22 mmol l^{-1} in the hemolymph and urine, respectively. During the first 24 h of emersion, hemolymph alanine concentrations decreased from an initial 35 mmol l^{-1} while immersed to 27.5 mmol l^{-1} after 24 h (Fig. 4.6a). Under desiccating conditions, the osmolality of an animal's bodily fluids will gradually increase as water evaporates over time putting greater osmotic stress on the cells. Alanine is a relatively non-toxic amino acid that can be collected from the extracellular space by tissues to counteract osmotic stress. Since emersion is a regular daily stressor for mangrove crabs, this may be another reason that alanine concentrations remain high in immersed crabs. Emersion beyond 24 h caused hemolymph alanine concentrations to increase by 9.5 mmol l^{-1} to remain at about 36–37 mmol l^{-1} after 48 and 160 h of emersion (Fig. 4.6a), potentially as a

temporary store of nitrogenous wastes. When ammonia production increases or its excretion/detoxification is impaired (limited water availability, anaerobic metabolism, etc.), many vertebrates produce alanine within extrahepatic tissues as an NH_4^+ carrier that is consumed within the liver as part of the alanine-glucose cycle (also referred to as the Cahill cycle; Felig, 1973; Felig et al., 1970). During this cycle, extrahepatic tissues collect and oxidize blood glucose forming pyruvate, which is then transaminated by alanine aminotransferase to form alanine (Felig, 1973; Felig et al., 1970). Newly synthesized alanine is exported from the tissue and recirculated until taken up by the hepatic tissue and catabolized as part of gluconeogenesis while the released ammonia is detoxified by the urea cycle (Felig, 1973; Felig et al., 1970). The use of this metabolic pathway by aquatic animals is largely unclear and is of minimal importance or absent in most fishes (Hemre et al., 2002; Mommsen and Walsh, 1991). This is largely due to the low rate of hepatic gluconeogenesis in most fishes and the low ratio of alanine to ammonia release from fish muscle upon exercise (reviewed by Mommsen and Walsh (1991)). To the author's knowledge, this cycle's presence and importance in invertebrates are unknown. If a functional alanine-glucose cycle is present, ammonia produced in non-excretory tissues could be detoxified as alanine prior to entering the extracellular space and later collected by tissues as an osmolyte or be catabolized and the ammonia subsequently released across the branchial epithelium. When the animals are re-immersed, the alanine can be catabolized and the ammonia released into circulation to be excreted across the branchial epithelia after a short delay, as appears to occur in *H. formosensis* (Fig. 4.5). Investigations into the potential use of alanine as a temporary nitrogenous waste storage molecule under environmental conditions limiting ammonia excretion in other crustaceans may help identify the mysterious destination of ammonia as occurs during prolonged exposure of *M. magister* to high environmental ammonia (Martin et al., 2011) or desiccation of *C. carnifex* (Wood et al., 1986).

Regardless of how *H. formosensis* temporarily stores nitrogenous wastes while emersed, the crab is capable of regulating hemolymph and urinary ammonia levels to a fairly constant degree (Fig. 4.4). Regulation of hemolymph ammonia concentrations has recently been documented in several invertebrate species including the American horseshoe crab (Hans et al., 2018), the common octopus (Hu et al., 2017), and the shallow hydrothermal vent crab (Allen et al., 2020). It has been hypothesized that this 'ammonia homeostasis' may be valuable to animals

that are somewhat tolerant to ammonia toxicity as an acid–base equivalent as NH_4^+ may be excreted as a H^+ equivalent or more easily manipulated than free H^+ under some circumstances (Allen et al., 2020; Weihrauch and Allen, 2018). This process becomes more complicated in an air-exposed animal such as *H. formosensis* as ammonia no longer can be freely excreted as NH_4^+ to the surrounding water and could only be used to reduce the number of free protons if NH_4^+ is incorporated into a metabolite (*e.g.*, production of alanine or glutamine). Future studies investigating whether alanine may be used as an alternative to more documented nitrogen detoxification through urea or uric acid may wish to also consider whether this pathway is linked to terrestrialization in crustaceans.

4.5.4. Emerged water conservation

Water conservation is another major concern for emerged crustaceans, especially in species such as *H. formosensis* that lack lung-like structures and rely on moistening gills to retain their function. Animals that are poor water-conservers would be expected to lose body water due to evaporation at either a constant or increasing rate during prolonged emersion. *Helice formosensis* decreases its rate of evaporative water loss the longer it is emersed (Fig. 4.9) indicating that the crab is well prepared to deal with prolonged emersion. The capability of *H. formosensis* to suppress their rate of evaporative water loss is interesting given that they were incapable of altering behavioral aspects whilst trapped in jars and would therefore require an active capacity to do so. To the author's knowledge, amphibious and even true terrestrial crabs (T_{4.5}) are believed to suppress evaporative water loss from their body through behavioral shifts (*i.e.*, aestivation, seeking shade, burrowing, metabolic shifts; (Bliss et al., 1978; McGaw et al., 2019; Simonik and Henry, 2014; Wolcott, 1992; Wood et al., 1986), warranting further investigation into the potential of *H. formosensis* to actively suppress evaporative water loss.

It is possible that the reduced rate of evaporative water loss is due in part to the crab's ability to tolerate elevated osmolality in its extracellular fluid, which increased by 49 mOsmol kg^{-1} in the hemolymph and 37 mOsmol kg^{-1} in the urine after 160 h of emersion (Fig. 4.8). Elevation of the urine osmolality may be particularly important in *H. formosensis*' emersion response as the urine is directly exposed to the ambient atmosphere. A high osmolality of the urine would reduce or potentially neutralize the evaporative water loss upon contact with the

humid environments found in the subtropics (e.g., Taiwan) while still permitting any potential passive loss of waste NH_3 and CO_2 gases to the environment. The process of elevating the urine's osmolality appears to occur through concentration of Na^+ , K^+ , and Mg^{2+} concentrations but not Ca^{2+} or Cl^- (Fig. 4.7). These mechanisms are crucial in terrestrial isopods such as *Porcellio scaber* and are connected to their previously described mechanism of ammonia volatilization (Wieser et al., 1969; Wright and O'Donnell, 1993). As the isopods prepare for NH_3 volatilization, the fluid within pores of the pleon is filled with ions to increase the osmolality up to $8.2 \text{ Osmol kg}^{-1}$ to allow for water vapour absorption to occur (Wright and O'Donnell, 1992). Using formulas described by O'Donnell and Machin (1988) and data collected in this study, it is unlikely that *H. formosensis* is capable of water vapour absorption; however, the osmolyte-rich urine may be capable of mitigating evaporative water loss while permitting some minor degree of off-gassing of waste NH_3 and CO_2 . This may be part of the reason that the antennal glands of *H. formosensis* poorly resorb Na^+ and have reduced Na^+/K^+ -ATPase activity compared to other semi-terrestrial crustaceans (Tseng et al., 2020). Future studies focusing on elucidating the regulatory mechanisms of crustacean antennal glands are required to more fully interpret the potential of crustaceans to mitigate evaporative water loss.

4.6. Conclusions

Understanding physiological adaptations to emersion stress in aquatic species not only helps better understand an animal's fitness, but also provides insights into the biological challenges that apply selective pressure on a species physiology as they become further terrestrialized. Crustaceans are one of the few animal families that have independently terrestrialized several times (Lozano-Fernandez et al., 2016) having found several different strategies to a similar set of physiological challenges. *Helice formosensis* uses a combination of urine recycling and ammonia detoxification to successfully compensate its acid–base and nitrogen homeostasis while emersed. Unlike other investigated crustaceans, it appears that *H. formosensis* may use alanine as a key component to this compensatory response, potentially indicating that at least some crustaceans make use of the alanine-glucose cycle setting their strategies apart from aquatic fishes.

Chapter 5: General discussion and thesis summary

It is truly fascinating how organisms have found ways to survive and maintain physiological homeostasis under such a broad variety of challenges. While we all can appreciate our own ability to not undergo systemic disruption after enjoying a cold drink, the fact that some organisms thrive in conditions that we consider extremely hazardous can be mind-boggling. Brachyurans are interesting from this standpoint and, anecdotally speaking, the public at least in Canada are quite surprised to find out crabs do not just live in the ocean and find it so strange that some crabs can drown let alone survive in hydrothermal vents. The results of this dissertation have only started to open the discussion as to how different the acid-base regulatory processes of brachyuran species may be in terms of their raw tolerance to stress and even how they use of branchial and antennal glands. While it would be unwise to directly compare the results of *M. magister*, *X. testudinatus*, *H. formosensis* and species present in the literature given their different backgrounds, a general discussion concerning their physiology is presented below.

5.1. Comparing acid-base regulation of osmoregulatory-capable and incapable *Brachyura*

The results of this study support the findings of previous literature that suggest stenohaline marine osmoconforming brachyurans are capable of rapidly and fully counteracting acid-base disturbances brought forth by hypercapnia and acid-mediated environmental acidifications. As a representative of a typical stenohaline marine crab, *M. magister* has now been found to fully compensate against slight (329 Pa P_{CO2}, pH 7.4; Hans et al., 2014) and moderate hypercapnic stress (1013 Pa P_{CO2}, pH 7.10; Pane and Barry, 2007) and HCl-mediated acidification as identified in chapter 2 of this dissertation (pH 7.10). Not only can this crab fully compensate its system acid-base status against such stress, it does so within 24-hours under moderate hypercapnia (Pane and Barry, 2007) and 12-hours for acid-mediated conditions. Small et al. (2010) identified a similarly complete compensatory mechanism under several hypercapnic conditions in the osmoconformer *N. puber*, that maintained constant hemolymph pH for 30-days in conditions containing 1250 Pa P_{CO2} (pH 6.7). Although a likely exceptional case, we identified that *X. testudinatus* rapidly restores their acid-base equilibrium in less than 7-days despite a net-increase in environmental P_{CO2} of nearly 22,000 Pa P_{CO2} (Allen et al., 2020). To date, the only stenohaline osmoconformers that can be deemed poor acid-base regulators are deep-sea cold-water dwelling species such as the grooved tanner crab, *Chionoecetes tanneri* (Pane and Barry, 2007), and *H. araneus* (Harms et al., 2014).

Osmoregulatory-capable species are occasionally found to fully counteract hypercapnia-driven acid-disturbances; however, the studies that present these findings usually use slight stress and long acclimation times. For example, seawater acclimated *C. maenas* maintain a constant hemolymph pH after 4-weeks of exposure to 184 Pa P_{CO2} (pH 7.50; Maus et al., 2018) whereas brackish-acclimated specimens perform similarly upon 10-weeks of exposure to 405 Pa P_{CO2} (pH 7.36; Appelhans et al., 2012). Shorter-term acclimations show mixed results with brackish-acclimated *C. maenas* showing full compensation after 7-days of exposure to 324 Pa P_{CO2} (pH 7.0; Fehsenfeld and Weihrauch, 2013), although this study had a relatively low control pH of 7.7 compared to a more typical pH ~8.10, whereas seawater-acclimated *C. maenas* and *C. sapidus* both experience a sustained reduction in hemolymph pH (-0.25 units) following 48 hours of exposure to 1013 Pa P_{CO2} and 24 hours 2026 Pa P_{CO2}, respectively (Cameron, 1985a; Fehsenfeld and Weihrauch, 2016a). Freshwater-acclimated *E. sinensis* fully restore their hemolymph pH after 7-days of exposure to 516 Pa P_{CO2}; however, no signs of compensatory action occur within the first 48 hours (Quijada-Rodriguez et al., 2021). Conversely, freshwater-acclimated *C. sapidus* only partially compensate their systemic acid-base status within 48-hours of exposure to 1013 Pa P_{CO2}, experiencing a sustained -0.25 reduction in hemolymph pH (Cameron, 1978). While it is difficult to come to a clear consensus on these species given the variety of P_{CO2} levels and durations used by these studies, there appears to be a more mixed response in osmoregulatory capable species compared to those that are stenohaline osmoconformers.

Considering that osmoregulation and acid-base regulation share the same regulatory organs and many of the same transporters, the different acid-base regulatory capacities of osmoregulatory capable and incapable species may relate to euryhaline adaptations. In chapter 2 this hypothesis is discussed and referred to as an ‘osmoregulatory burden,’ suggesting that as species became increasingly euryhaline they may have lost some of the freedom to dedicate transport processes to acid-base disturbances, even when they themselves are in an osmoconforming state. Such a process could be related to differences in hormonal regulation (Detaille et al., 1992; Genovese et al., 2006; Halperin et al., 2004; Mo et al., 1998; Sommer and Mantel, 1988) or through a different signal cascade that promotes a shift in the directionality of a basolateral Na⁺/HCO₃⁻ cotransporter; however, these aspects of brachyuran acid-base regulation are beyond the current scope of the field.

5.2. Co-ordination between the gills and antennal glands

While the gills are undeniably crucial to acid-base regulation in brachyurans (Fehsenfeld and Weihrauch, 2017; Henry et al., 2012; Henry and Wheatly, 1992), the antennal glands played an important role in counteracting acid-stress in the Dungeness crab. The results discussed in chapter 2 identified that the antennal gland readily adjusts their HCO_3^- reabsorption rates and presumably acid-secretion rates allowing the crab to maintain a constant extracellular pH and HCO_3^- concentration. The glands responded immediately under acid-stress, unlike the delayed adjustment in HCO_3^- reabsorption rates that occur following hypoosmotic stress (Wheatly, 1985), suggesting they serve a more direct role in systemic acid-base regulation. Together, the gills appear to act as the site of HCO_3^- uptake whereas the antennal glands adjust the rate that HCO_3^- is lost. Neither organ is negligible, rather they play an important role that complements the other, at least under acid-stress.

In a different sense this complementation was observed in *H. formosensis* where urine is recycled into the branchial chamber during emersion allowing the crab to undergo prolonged emersion. Although this species' antennal glands do not seem to adjust HCO_3^- levels, urine that passes over the crabs' anterior body may allow some degree of CO_2 to volatilize. A more significant role for the antennal gland relates to their apparent ability to reduce circulating ammonia concentrations, potentially through the Cahill cycle (Felig, 1973; Felig et al., 1970), or a similar process, where nitrogenous wastes are temporarily detoxified into alanine until they can be safely reconverted and excreted while immersed. Such a cycle was once hypothesized to be useful to aquatic species; however, until now it had not been detected in such animals.

While their importance is different in *M. magister* and *H. formosensis*, the antennal glands are clearly not negligible in the acid-base regulatory processes of all aquatic brachyurans. The gills and antennal glands appear to work together, at least in some instances, to maintain systemic acid-base regulation. Given that studies focusing on osmoregulatory-capable species were largely the reason for their suggested negligible importance (Cameron and Batterton, 1978b; Truchot, 1979), perhaps the aforementioned concept of an osmoregulatory burden extends to the organ level resulting in marine species having a greater freedom to contribute to acid-base regulation. Unfortunately, the belief that they were negligible regulatory organs has led to a limited understanding of even their basic anatomy in brachyurans (Freire et al., 2008),

making it difficult to explain their involvement beyond comparing the composition of the urine and hemolymph.

Shifting from our current approach of considering acid-base regulation almost entirely from a branchial standpoint to a more holistic systemic approach must consider the probable importance of the crustacean endocrine system. Hormones influence the rate of acid-secretion and HCO_3^- reabsorption/generation within the proximal tubule and distal region of the mammalian nephron, due in part to their ability to regulating the presence of transporters within the apical and basolateral membranes of the epithelia (Lee Hamm et al., 2015). Endocrine influence over crustacean acid-base balance has yet to be investigated; however, some insights can be drawn from the few studies that have connected endocrine factors to the active uptake of Na^+/Cl^- across the gills of osmoregulatory-capable brachyurans. Upon moving from seawater to brackish water, circulating levels of dopamine increase 2-fold within an hour in *C. maenas* (Zatta, 1987), significantly increasing cAMP concentrations within the branchial tissue leading to the downstream phosphorylation of enzymes that alters their activity (Halperin et al., 2004; Sommer and Mantel, 1991). Gills perfused with dopamine, cAMP, or modulators of cAMP levels such as theophylline and forskolin have an increased Na^+ and/or Cl^- uptake capacity in several osmoregulating species including *C. maenas* (Sommer and Mantel, 1991, 1988; Zatta, 1987), *C. sapidus* (Lohrmann and Kamemoto, 1987), *E. sinensis* (Bianchini and Gilles, 1990; Detaille et al., 1992; Mo et al., 2003; Onken et al., 2000), and *N. granulata* (Genovese et al., 2006; Halperin et al., 2004). Similar effects on osmoregulation are also reported for gills perfused with the crustacean hyperglycaemic hormone (Eckhardt et al., 1995; Spanings-Pierrot et al., 2000). Although the precise mechanism as to why these changes happen is not well understood, elevated cAMP generally increases Na^+/K^+ -ATPase activity (Bianchini and Gilles, 1990; Genovese et al., 2006; Halperin et al., 2004; Mo et al., 1998; Sommer and Mantel, 1991). In freshwater acclimated *E. sinensis*, elevated cAMP also increases the activity of apical Na^+ channels, the V-type H^+ -ATPase, and $\text{Cl}^-/\text{HCO}_3^-$ exchangers (Riestenpatt et al., 1994) as well as basolateral Cl^- channels (Bianchini and Gilles, 1990). While it is possible that these responses are conserved across many species, conflicting results have been reported by Lucu and Flik (1999) who found that cAMP levels decreased in *C. maenas* upon acclimation to dilute salinities. Furthermore, the specific role of certain hormones may be limited to certain groups of brachyurans given that serotonin but not dopamine alters the osmoregulatory system of terrestrial

crabs (Morris, 2001). Given that acid-base and osmoregulatory transport mechanisms are directly connected, as discussed throughout this dissertation, endocrine factors are likely a currently unknown but major factor in crustacean acid-base regulation. Unfortunately, endocrine influence over these transporters has not been studied in osmoregulatory-incapable species, or even seawater acclimated osmoregulatory-capable crabs, making it difficult to discuss whether these responses extend outside of osmoregulatory roles.

5.3. Brachyuran diversity extends into their acid-base regulatory strategies

Each experimental chapter of this thesis identified an unexpected aspect of brachyuran acid-base regulation. A typical stenohaline marine osmoconformer with ion-leaky gills (Weihrauch et al., 1999) exhibited a remarkable ability to tolerate acute acid-stress through the combined action of the gills and antennal glands whereas freshwater crustaceans have been found incapable of compensation despite possessing ion-tight epithelia (Ellis and Morris, 1995; Morgan and McMahon, 1982; Patterson and DeFur, 1988; Wood and Rogano, 1986). The hydrothermal vent crab, *X. testudinatus*, is not only highly hypercapnia-tolerant, but appears to possess a means to actively excrete CO₂ defying the otherwise unanimous reliance on passive diffusion observed throughout the animal kingdom (Melzner et al., 2009). *Helice formosensis* was found to possess unprecedented levels of alanine within its hemolymph that is selectively not filtered by the antennal glands while being immersed or emersed suggesting it may rely on a metabolic process (Felig, 1973; Felig et al., 1970) that has not been identified in other aquatic species. While the excretion of alanine from the immersed animal may seem counterproductive, the relationship between its immersion and its burrowing lifestyle may allow for some beneficial relationship with microbial life in the mangrove sediments (Mchenga et al., 2007).

Such specialized adaptations presumably developed over the course of the species' different life histories, shaping common essential aspects of animal physiology such as acid-base regulation into unique strategies fit for their lifestyle or rather they may have always existed but were not lost. The latter possibility being an interesting consideration of the existence of hydrothermal vent species where some believe life to have originated (Baross, 1985; Martin et al., 2008; Sojo et al., 2016). Assuming these beliefs hold some merit, are the strong acid-base regulatory strategies of *X. testudinatus* a strategy other species lost once species expansion moved beyond hydrothermal vents? Did osmoregulatory-capable brachyurans sacrifice a portion

of their acid-base regulatory capacities to become euryhaline? Did the lifestyle of *H. formosensis* and the unique niche environments within mangroves and burrows allow for a unique use of alanine in nitrogenous waste management and acid-base regulation? While I cannot answer these questions with my dissertation, broadening our understanding of the physiology of different species may make it possible to at least probe these questions at a deeper level, if not eventually meet a consensus on some topics.

5.4. Conclusions and future directions

The results of this dissertation point to the fact that we cannot generalize the broader physiology of brachyurans by focusing on several species that are useful models for osmoregulation. Three species with drastically different lifestyles investigated throughout this dissertation all possessed acid-base regulatory capacities and strategies with key differences from the species typically used as model brachyurans. As it is unfeasible to study the physiology of every species, models must be used to make generalized statements; however, attempting to model one of the most diverse animal families with species that were mostly researched for their osmoregulatory capabilities is unwise. In the world of acid-base regulation, the accuracy of these models and generalizations are not only important to biologists but also to policymakers and panelists attempting to identify risks associated with anthropogenic activity such as waste-handling and climate change (IPCC, 2019). While my findings do not serve to identify what a ‘correct’ model species is for acid-base regulation, they do provide an argument for diversifying the field.

Aside from expanding species diversity, this dissertation also provides some level of groundwork to shift from studying individual acid-base regulatory organs to understanding how the many different organs operate as a cohesive unit. The indication that the antennal glands play such a pivotal role in acid-base regulation in *M. magister* and a unique role in *H. formosensis* cannot be ignored but cannot be understood without considering their relation to the gills. Even if the relative contribution of the antennal glands is a fraction of that of the gills, they both play an important role in the intact animal by complementing each other. Considering that the brachyuran exoskeleton and gut are also rarely considered from an acid-base standpoint, there are likely several complementary processes that have gone ignored despite their repeated potential importance in the fields most prominent reviews (Henry et al., 2012; Henry and

Wheatly, 1992; Wheatly and Henry, 1992). While it would take considerable efforts to develop an understanding of these organs' transport profiles and response to stressors, it is something that should be undertaken in the future. This sort of research along with the characterization of the numerous essential transporters that are hypothesized but never identified within the gills (*e.g.* $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers, $\text{Na}^+/\text{HCO}_3^-$ cotransporters, and Na^+/H^+ exchangers) should be amongst the next steps in understanding brachyuran acid-base regulation.

Bibliography

- Abel, D.C., Koenig, C.C., Davis, W.P., 1987. Emersion in the mangrove forest fish *Rivulus marmoratus*: a unique response to hydrogen sulfide. *Environ. Biol. Fishes* 18, 67–72.
<https://doi.org/10.1007/BF00002329>
- Adamczewska, A.M., Morris, S., 2000. Respiratory gas transport, metabolic status, and locomotor capacity of the Christmas Island red crab *Gecarcoidea natalis* assessed in the field with respect to dichotomous seasonal activity levels. *J. Exp. Zool.* 286, 552–562.
[https://doi.org/10.1002/\(SICI\)1097-010X\(20000501\)286:6<552::AID-JEZ2>3.0.CO;2-J](https://doi.org/10.1002/(SICI)1097-010X(20000501)286:6<552::AID-JEZ2>3.0.CO;2-J)
- Adlimoghaddam, A., Boeckstaens, M., Marini, A.M., Treberg, J.R., Brassinga, A.K.C., Weihrauch, D., 2015. Ammonia excretion in *Caenorhabditis elegans*: Mechanism and evidence of ammonia transport of the Rhesus protein CeRhr-1. *J. Exp. Biol.* 218, 675–683.
<https://doi.org/10.1242/jeb.111856>
- Aldridge, J.B., Cameron, J.N., 1979. CO₂ exchange in the blue crab, *Callinectes sapidus* (Rathburn). *J. Exp. Zool.* 207, 321–328.
- Allen, G.J.P., Kuan, P.L., Tseng, Y.C., Hwang, P.P., Quijada-Rodriguez, A.R., Weihrauch, D., 2020. Specialized adaptations allow vent-endemic crabs (*Xenograpsus testudinatus*) to thrive under extreme environmental hypercapnia. *Sci. Rep.* 10, 1–13.
<https://doi.org/10.1038/s41598-020-68656-1>
- Allen, G.J.P., Wang, M.-C., Tseng, Y.-C., Weihrauch, D., 2021. Effects of emersion on acid – base regulation, osmoregulation, and nitrogen physiology in the semi-terrestrial mangrove crab, *Helice formosensis*. *J. Comp. Physiol. B* 191, 455–468.
<https://doi.org/10.1007/s00360-021-01354-0>
- Allen, G.J.P., Weihrauch, D., 2021. Exploring the versatility of the perfused crustacean gill as a model for transbranchial transport processes. *Comp. Biochem. Physiol.* 254, 110572.
<https://doi.org/10.1016/j.cbpb.2021.110572>
- Appelhans, Y.S., Thomsen, J., Pansch, C., Melzner, F., Wahl, M., 2012. Sour times: Seawater acidification effects on growth, feeding behaviour and acid-base status of *Asterias rubens* and *Carcinus maenas*. *Mar. Ecol. Prog. Ser.* 459, 85–97. <https://doi.org/10.3354/meps09697>

- Armenta, J.M., Cortes, D.F., Pisciotta, J.M., Shuman, J.L., Rasoloson, D., Ogunbiyi, O., Jr, D.J.S., Shulaev, V., Armenta, A.J.M., Cortes, D.F., Pisciotta, J.M., Shuman, J.L., Rasoloson, D., Ogunbiyi, O., Sullivan, D.J., 2010. A sensitive and rapid method for amino acid quantitation in malaria biological samples using AccQ•Tag UPLC-ESI-MS/MS with multiple reaction monitoring. *Anal. Chem.* 82, 548–558.
<https://doi.org/10.1021/ac901790q.A>
- Avenet, P., Lignon, J.M., 1985. Ionic permeabilities of the gill lamina cuticle of the crayfish, *Astacus leptodactylus* (E). *J. Physiol.* 363, 377–401.
<https://doi.org/10.1113/jphysiol.1985.sp015717>
- Baker, D.W., Sardella, B., Rummer, J.L., Sackville, M., Brauner, C.J., 2015. Hagfish: Champions of CO₂ tolerance question the origins of vertebrate gill function. *Sci. Rep.* 5, 1–8. <https://doi.org/10.1038/srep11182>
- Baptista, A.M., Martel, P.J., Petersen, S.B., 1997. Simulation of protein conformational freedom as a function of pH: Constant-pH molecular dynamics using implicit titration. *Proteins Struct. Funct. Genet.* 27, 523–544. [https://doi.org/10.1002/\(SICI\)1097-0134\(199704\)27:4<523::AID-PROT6>3.0.CO;2-B](https://doi.org/10.1002/(SICI)1097-0134(199704)27:4<523::AID-PROT6>3.0.CO;2-B)
- Baross, J.A., 1985. Submarine hydrothermal vents and associated gradient environments as sites for the origin and evolution of life. *Orig. Life Evol. Biosph.* 15, 327–345.
- Barra, J.A., Pequeux, A., Humbert, W., 1983. A morphological study on gills of a crab acclimated to fresh water. *Tissue Cell* 15, 583–596. [https://doi.org/10.1016/0040-8166\(83\)90009-5](https://doi.org/10.1016/0040-8166(83)90009-5)
- Bernatis, J.L., Gerstenberger, S.L., McGaw, I.J., 2007. Behavioural responses of the Dungeness crab, *Cancer magister*, during feeding and digestion in hypoxic conditions. *Mar. Biol.* 150, 941–951. <https://doi.org/10.1007/s00227-006-0392-3>
- Bianchini, A., Carvalho De Castilho, P., 1999. Effects of zinc exposure on oxygen consumption and gill Na⁺, K⁺- ATPase of the estuarine crab *Chasmagnathus granulata* Dana, 1851 (Decapoda - Grapsidae). *Bull. Environ. Contam. Toxicol.* 62, 63–69.
<https://doi.org/10.1007/s001289900842>

- Bianchini, A., Gilles, R., 1990. Cyclic AMP as a modulator of NaCl transport in gills of the euryhaline Chinese crab *Eriocheir sinensis*. *Mar. Biol.* 104, 191–195.
<https://doi.org/10.1007/BF00367639>
- Blewett, T.A., Goss, G.G., 2017. A novel pathway of nutrient absorption in crustaceans: Branchial amino acid uptake in the green shore crab (*Carcinus maenas*). *Proc. R. Soc. B Biol. Sci.* 284, 1–6. <https://doi.org/10.1098/rspb.2017.1298>
- Bliss, D.E., Van Montfrans, J., Van Montfrans, M., Boyer, J.R., 1978. Behavior and growth of the land crab *Gecarcinus lateralis* (Freminville) in southern Florida. *Bull. Amer. Mus. Nat. Hist.* 160, 113–151.
- Boron, W.F., 2004. Regulation of intracellular pH. *Am. J. Physiol. - Adv. Physiol. Educ.* 28, 160–179. <https://doi.org/10.1152/advan.00045.2004>
- Boron, W.F., Hediger, M.A., Boulpaep, E.L., Romero, M.F., 1997. The renal electrogenic Na⁺:HCO₃⁻ cotransporter. *J. Exp. Biol.* 200, 263–268.
- Böttcher, K., Siebers, D., Becker, W., Petrausch, G., 1991. Physiological role of branchial carbonic anhydrase in the shore crab *Carcinus maenas*. *Mar. Biol.* 110, 337–342.
<https://doi.org/10.1007/BF01344352>
- Boutilier, R.G., Heming, T.A., Iwama, G.K., 1984. Gills - Anatomy, gas transfer, and acid-base regulation, in: Hoar, W.S., Randall, D. (Eds.), *Fish Physiology*. Elsevier, pp. 403–430.
- Braun, M.H., Steele, S.L., Ekker, M., Perry, S.F., 2009. Nitrogen excretion in developing zebrafish (*Danio rerio*): a role for Rh proteins and urea transporters. *Am J Physiol Ren. Physiol* 296, 994–1005. <https://doi.org/10.1152/ajprenal.90656.2008>.
- Bridgman, H.A., 1989. Acid rain studies in Australia and New Zealand. *Arch. Environ. Contam. Toxicol.* 18, 137–146. <https://doi.org/10.1007/BF01056198>
- Bundy, H.F., 1977. Carbonic Anhydrase. *Comp. Biochem. Physiol.* 57B, 1–7.
- Burggren, W.W., 1992. Respiration and circulation in land crabs: Novel variations on the marine design. *Am. Zool.* 32, 417–427. <https://doi.org/10.1093/icb/32.3.417>
- Burnett, L.E., 1997. The challenges of living in hypoxic and hypercapnic aquatic environments.

- Am. Zool. 37, 633–640. <https://doi.org/10.1093/icb/37.6.633>
- Burnett, L.E., 1984. CO₂ excretion across isolated perfused crab gills: Facilitation by carbonic anhydrase. *Integr. Comp. Biol.* 24, 253–264. <https://doi.org/10.1093/icb/24.1.253>
- Burnett, L.E., Dunn, T.N., Infantino, R.L.J., 1985. The function of carbonic anhydrase in crustacean gills, in: Gilles, R., Gilles-Baillien, M. (Eds.), *Transport Processes, Iono- and Osmoregulation*. Springer-Verlag, Berlin Heidelberg, pp. 159–168. <https://doi.org/10.1042/bj0500190>
- Burnett, L.E., Johansen, K., 1981. The role of branchial ventilation in hemolymph acid-base changes in the shore crab *Carcinus maenas* during hypoxia. *J. Comp. Physiol.* 141, 489–494.
- Burnett, L.E., McMahon, B.R., 1987. Gas exchange, hemolymph acid-base status, and the role of branchial water stores during air exposure in three littoral crab species. *Physiol. Zool.* 60, 27–36.
- Burnett, L.E., McMahon, B.R., 1985. Facilitation of CO₂ excretion by carbonic anhydrase located on the surface of the basal membrane of crab gill epithelium. *Respir. Physiol.* 62, 341–348.
- Burnett, L.E., Towle, D.W., 1990. Sodium ion uptake by perfused gills of the blue crab *Callinectes sapidus*: Effects of ouabain and amiloride. *J. Exp. Biol.* 149, 293–305.
- Burton, R.F., 2002. Temperature and acid-base balance in ectothermic vertebrates: The imidazole alaphastat hypotheses and beyond. *J. Exp. Biol.* 205, 3587–3600. <https://doi.org/10.1242/jeb.205.23.3587>
- Caldeira, K., Wickett, M.E., 2003. Anthropogenic carbon and ocean pH. *Nature* 425, 365. <https://doi.org/10.1038/425365a>
- Cameron, J.N., 1989. Acid-base homeostasis : Past and present perspectives. *Physiol. Zool.* 62, 845–865.
- Cameron, J.N., 1985a. Compensation of hypercapnic acidosis in the aquatic blue crab, *Callinectes sapidus*: The predominance of external sea water over carapace carbonate as the

- proton sink. *J. Exp. Biol.* 114, 197–206. <https://doi.org/10.1242/jeb.114.1.197>
- Cameron, J.N., 1985b. Molting in the blue crab. *Sci. Am.* 252, 102–109.
- Cameron, J.N., 1981. The Role of CaCO₃ Dissolution As a Source of HCO₃⁻ For the Buffering of Hypercapnic Acidosis in Aquatic and Terrestrial Decapod Crustaceans. *J. Exp. Biol.* 94, 269–274.
- Cameron, J.N., 1978. Effects of hypercapnia on blood acid-base status, NaCl fluxes, and trans-gill potential in freshwater blue crabs, *Callinectes sapidus*. *J. Comp. Physiol.* □ B 123, 137–141. <https://doi.org/10.1007/BF00687841>
- Cameron, J.N., Batterton, C. V., 1978a. Temperature and blood acid-base status in the blue crab, *Callinectes sapidus*. *Respir. Physiol.* 35, 101–110.
- Cameron, J.N., Batterton, C. V., 1978b. Antennal gland function in the freshwater blue crab, *Callinectes sapidus*: Water, electrolyte, acid-base and ammonia excretion. *J. Comp. Physiol.* 123, 143–148. <https://doi.org/10.1007/BF00687842>
- Cameron, J.N., Heisler, N., 1983. Studies of ammonia in the rainbow trout: Physico-chemical parameters, acid-base behaviour and respiratory clearance. *J. Exp. Biol.* 105, 107–125.
- Cameron, J.N., Wood, C.M., 1985. Apparent H⁺ excretion and CO₂ dynamics accompanying carapace mineralization in the blue crab (*Callinectes sapidus*) following moulting. *J. Exp. Biol.* 114, 181–196. <https://doi.org/10.1242/jeb.114.1.181>
- Cannon, W.B., 1929. Organization for physiological homeostasis. *Physiol. Rev.* 9, 399–431.
- Carrisoza-gaytán, R., Rangel, C., Salvador, C., Saldaña-meyer, R., Escalona, C., Satlin, L.M., Liu, W., Zamilowicz, B., Trujillo, J., Bobadilla, N.A., Escobar, L.I., 2011. The hyperpolarization-activated cyclic nucleotide-gated HCN2 channel transports ammonium in the distal nephron. *Kidney Int.* 80, 832–840. <https://doi.org/10.1038/ki.2011.230>
- Chang, N.N., Lin, L.H., Tu, T.H., Jeng, M.S., Chikaraishi, Y., Wang, P.L., 2018. Trophic structure and energy flow in a shallow-water hydrothermal vent: Insights from a stable isotope approach. *PLoS One* 13, e0204753. <https://doi.org/10.1371/journal.pone.0204753>
- Chasiotis, H., Ionescu, A., Misyura, L., Bui, P., Fazio, K., Wang, J., Patrick, M., Weihrauch, D.,

- Donini, A., 2016. An animal homolog of plant Mep/Amt transporters promotes ammonia excretion by the anal papillae of the disease vector mosquito *Aedes aegypti*. *J. Exp. Biol.* 219, 1346–1355. <https://doi.org/10.1242/jeb.134494>
- Chen, C.T.A., Zeng, Z., Kuo, F.W., Yang, T.F., Wang, B.J., Tu, Y.Y., 2005. Tide-influenced acidic hydrothermal system offshore NE Taiwan. *Chem. Geol.* 224, 69–81. <https://doi.org/10.1016/j.chemgeo.2005.07.022>
- Chen, J.C., Lai, S.H., 1993. Effects of temperature and salinity on oxygen consumption and ammonia-N excretion of juvenile *Penaeus japonicus* Bate. *J. Exp. Mar. Bio. Ecol.* 165, 161–170. [https://doi.org/10.1016/0022-0981\(93\)90103-U](https://doi.org/10.1016/0022-0981(93)90103-U)
- Chew, S.F., Hiong, K.C., Lam, S.P., Ong, S.W., Wee, W.L., Wong, W.P., Ip, Y.K., 2014. Functional roles of Na⁺/K⁺-ATPase in active ammonia excretion and seawater acclimation in the giant mudskipper, *Periophthalmodon schlosseri*. *Front. Physiol.* 5, 1–17. <https://doi.org/10.3389/fphys.2014.00158>
- Childress, J.J., Lee, R.W., Sanders, N.K., Felbeck, H., Oros, D.R., Toulmond, A., Desbruyeres, D., Kennicutt, M.C., Brooks, J., 1993. Inorganic carbon uptake in hydrothermal vent tubeworms facilitated by high environmental pCO₂. *Nature* 362, 147–149. <https://doi.org/10.1038/362147a0>
- Childress, J.J., Seibel, B.A., 1998. Life at stable low oxygen levels: Adaptations of animals to oceanic oxygen minimum layers. *J. Exp. Biol.* 201, 1223–1232. <https://doi.org/10.1039/c7cc09389f>
- Chung, K.F., Lin, H.C., 2006. Osmoregulation and Na,K-ATPase expression in osmoregulatory organs of *Scylla paramamosain*. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 144, 48–57. <https://doi.org/10.1016/j.cbpa.2006.02.003>
- Claiborne, J.B., Edwards, S.L., Morrison-Shetlar, A.I., 2002. Acid-base regulation in fishes: Cellular and molecular mechanisms. *J. Exp. Zool.* 293, 302–319. <https://doi.org/10.1002/jez.10125>
- Compère, P., Thorez, A., Goffinet, G., 1998. Fine structural survey of old cuticle degradation during pre-ecdysis in two European Atlantic crabs. *Tissue Cell* 30, 41–56.

[https://doi.org/10.1016/S0040-8166\(98\)80005-0](https://doi.org/10.1016/S0040-8166(98)80005-0)

Compere, P., Wanson, S., Pequeux, A., Gilles, R., Goffinet, G., 1989. Ultrastructural changes in the gill epithelium of the green crab *Carcinus maenas* in relation to the external salinity.

Tissue Cell 21, 299–318. [https://doi.org/10.1016/0040-8166\(89\)90073-6](https://doi.org/10.1016/0040-8166(89)90073-6)

Copeland, D.E., Fitzjarrell, A.T., 1968. The salt absorbing cells in the gills of the blue crab (*Callinectes sapidus* rathbun) with notes on modified mitochondria. *Zeitschrift für Zellforsch.* 92, 1–22. <https://doi.org/10.1007/BF00339398>

Currie, S.E., Körtner, G., Geiser, F., 2014. Heart rate as a predictor of metabolic rate in heterothermic bats. *J. Exp. Biol.* 217, 1519–1524. <https://doi.org/10.1242/jeb.098970>

Dahms, H.U., Schizas, N. V., James, R.A., Wang, L., Hwang, J.S., 2018. Marine hydrothermal vents as templates for global change scenarios. *Hydrobiologia* 818, 1–10.

<https://doi.org/10.1007/s10750-018-3598-8>

Davis, J.C., 1975. Minimal dissolved oxygen requirements of aquatic life with emphasis on Canadian species: A review. *J. Fish. Res. Board Canada* 32, 2295–2332.

<https://doi.org/10.1139/f75-268>

de Duve, C., 1983. Lysosomes revisited. *Eur. J. Biochem.* 137, 391–397.

<https://doi.org/10.1111/j.1432-1033.1983.tb07841.x>

de la Haye, K.L., Spicer, J.I., Widdicombe, S., Briffa, M., 2011. Reduced sea water pH disrupts resource assessment and decision making in the hermit crab *Pagurus bernhardus*. *Anim. Behav.* 82, 495–501. <https://doi.org/10.1016/j.anbehav.2011.05.030>

<https://doi.org/10.1016/j.anbehav.2011.05.030>

De Vries, M.C., Wolcott, D.L., Holliday, C.W., 1994. High ammonia and low pH in the urine of the ghost crab, *Ocypode quadrata*. *Biol. Bull.* 186, 342–348.

<https://doi.org/10.2307/1542280>

DeFur, P.L., McMahon, B.R., 1984. Physiological compensation to short-term air exposure in red rock crabs, *Cancer productus* Randall, from littoral and sublittoral habitats. II. Acid-base balance. *Physiol. Zool.* 57, 151–160.

DeFur, P.L., McMahon, B.R., Booth, C.E., 1983. Analysis of hemolymph oxygen levels and

- acid-base status during emersion “in situ” in the red rock crab, *Cancer productus*. *Biol. Bull.* 165, 582–590.
- DeFur, P.L., Wilkes, P.R.H., McMahon, B.R., 1980. Non-equilibrium acid-base status in *C. productus*: Role of exoskeletal carbonate buffers. *Respir. Physiol.* 42, 247–261.
[https://doi.org/10.1016/0034-5687\(80\)90118-8](https://doi.org/10.1016/0034-5687(80)90118-8)
- Dejours, P., 1975. *Principles of comparative respiratory physiology*. North-Holland Publ. Co., Amsterdam.
- Dejours, P., Armand, J., 1983. Acid-base balance of crayfish hemolymph : Effects of simultaneous changes of ambient temperature and water oxygenation. *J. Comp. Physiol.* 149, 463–468.
- Detaille, D., Trausch, G., Devos, P., 1992. Dopamine as a modulator of ionic transport and Na⁺/K⁺-ATPase activity in the gills of the Chinese crab *Eriocheir sinensis*. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* 103, 521–526. <https://doi.org/10.2307/1549409>
- Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep Sea Res. Part A, Oceanogr. Res. Pap.* 34, 1733–1743. [https://doi.org/10.1016/0198-0149\(87\)90021-5](https://doi.org/10.1016/0198-0149(87)90021-5)
- Dixson, D.L., Jennings, A.R., Atema, J., Munday, P.L., 2015. Odor tracking in sharks is reduced under future ocean acidification conditions. *Glob. Chang. Biol.* 21, 1454–1462.
<https://doi.org/10.1111/gcb.12678>
- Dodd, L.F., Grabowski, J.H., Piehler, M.F., Westfield, I., Ries, J.B., 2015. Ocean acidification impairs crab foraging behavior. *Proc. R. Soc. B Biol. Sci.* 282, 20150333.
<https://doi.org/10.1098/rspb.2015.0333>
- Doney, S.C., Fabry, V.J., Feely, R.A., Kleypas, J.A., 2009. Ocean acidification: The other CO₂ problem. *Ann. Rev. Mar. Sci.* 1, 169–192.
<https://doi.org/10.1146/annurev.marine.010908.163834>
- Dupont, S., Ortega-Martínez, O., Thorndyke, M., 2010. Impact of near-future ocean acidification on echinoderms. *Ecotoxicology* 19, 449–462. <https://doi.org/10.1007/s10646-010-0463-6>

- Durand, F., Chausson, F., Regnault, M., 1999. Increases in tissue free amino acid levels in response to prolonged emersion in marine crabs: An ammonia-detoxifying process efficient in the intertidal *Carcinus maenas* but not in the subtidal *Necora puber*. *J. Exp. Biol.* 202, 2191–2202.
- Durand, F., Regnault, M., 1998. Nitrogen metabolism of two portunid crabs, *Carcinus maenas* and *Necora puber*, during prolonged air exposure and subsequent recovery: A comparative study. *J. Exp. Biol.* 201, 2515–2528.
- Durant, A.C., Donini, A., 2018. Ammonia excretion in an osmoregulatory syncytium is facilitated by AeAmt2, a novel ammonia transporter in *aedes aegypti* larvae. *Front. Physiol.* 9, 1–16. <https://doi.org/10.3389/fphys.2018.00339>
- Eckhardt, E., Pierrot, C., Thuet, P., Van Herp, F., Charmantier-Daures, M., Trilles, J.-P., Charmantier, G., 1995. Stimulation of osmoregulating processes in the perfused gill of the crab *Pachygrapsus marmoratus* (Crustacea, Decapoda) by a sinus gland peptide. *Gen. Comp. Endocrinol.* 99, 160–177.
- Eliss, D.E., 1968. Transition from water to land in decapod crustaceans. *Am. Zool.* 8, 355–392. <https://doi.org/10.1093/icb/8.3.355>
- Ellis, B.A., Morris, S., 1995. Effects of extreme pH on the physiology of the Australian “Yabby” *Cherax destructor*: Acute and chronic changes in haemolymph carbon dioxide, acid-base and ionic status. *J. Exp. Biol.* 198, 395–407.
- Endeward, V., Cartron, J., Ripoche, P., Gros, G., 2008. RhAG protein of the Rhesus complex is a CO₂ channel in the human red cell membrane. *FASEB J.* 22, 64–73. <https://doi.org/10.1096/fj.07-9097com>
- Eshky, A.A., 1992. Evidence of additional functions of the pericardial sacs in the bronchial ventilation in the grapsid crab *Grapsus tenuicrustatus*. *J. King Abdulaziz Univ. (Marine Sci.* 3, 91–104.
- Farrell, A.P., 2016. Pragmatic perspective on aerobic scope: Peaking, plummeting, pejus and apportioning. *J. Fish Biol.* 88, 322–343. <https://doi.org/10.1111/jfb.12789>
- Farrell, A.P., 2009. Environment, antecedents and climate change: Lessons from the study of

- temperature physiology and river migration of salmonids. *J. Exp. Biol.* 212, 3771–3780.
<https://doi.org/10.1242/jeb.023671>
- Fehsenfeld, S., Kiko, R., Appelhans, Y., Towle, D.W., Zimmer, M., Melzner, F., 2011. Effects of elevated seawater pCO₂ on gene expression patterns in the gills of the green crab, *Carcinus maenas*. *BMC Genomics* 12, 488. <https://doi.org/10.1186/1471-2164-12-488>
- Fehsenfeld, S., Weihrauch, D., 2017. Acid-base regulation in aquatic decapod crustaceans, in: Weihrauch, D., O'Donnell, M.J. (Eds.), *Acid-Base Balance and Nitrogen Excretion in Invertebrates*. Springer International Publishing, Switzerland, pp. 152–185.
- Fehsenfeld, S., Weihrauch, D., 2016a. Mechanisms of acid-base regulation in seawater-acclimated green crabs, *Carcinus maenas*. *Can. J. Zool.* 94, 95–107.
- Fehsenfeld, S., Weihrauch, D., 2016b. The role of an ancestral hyperpolarization-activated cyclic + nucleotide-gated K channel in branchial acid-base regulation in the green crab, *Carcinus maenas*. *J. Exp. Biol.* 219, 887–896. <https://doi.org/10.1242/jeb.134502>
- Fehsenfeld, S., Weihrauch, D., 2016c. Mechanisms of acid–base regulation in seawater-acclimated green crabs (*Carcinus maenas*). *Can. J. Zool.* 94, 95–107.
<https://doi.org/10.1139/cjz-2015-0132>
- Fehsenfeld, S., Weihrauch, D., 2013. Differential acid-base regulation in various gills of the green crab *Carcinus maenas*: Effects of elevated environmental pCO₂. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 164, 54–65. <https://doi.org/10.1016/j.cbpa.2012.09.016>
- Felig, P., 1973. The glucose-alanine cycle. *Metabolism* 22, 179–207.
[https://doi.org/10.1016/0026-0495\(73\)90269-2](https://doi.org/10.1016/0026-0495(73)90269-2)
- Felig, P., Pozefsk, T., Marlis, E., Cahill, G.F., 1970. Alanine: Key role in gluconeogenesis. *Science* (80-.). 167, 1003–1004.
- Florence, T.M., Farrar, Y.J., 1971. Spectrophotometric determination of chloride at the parts-per-billion level by the mercury(II) thiocyanate method. *Anal. Chim. Acta* 54, 373–377.
[https://doi.org/10.1016/S0003-2670\(01\)82142-5](https://doi.org/10.1016/S0003-2670(01)82142-5)
- Forgue, J., Massabuau, J.C., Truchot, J.-P., 1992. When are resting water-breathers lacking

- oxygen arterial oxygen? Portal pressure at the anaerobic threshold in crab. *Respir. Physiol.* 88, 247–256.
- Freire, C.A., Onken, H., McNamara, J.C., 2008. A structure-function analysis of ion transport in crustacean gills and excretory organs. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 151, 272–304. <https://doi.org/10.1016/j.cbpa.2007.05.008>
- Fruton, J.S., 2002. A history of pepsin and related enzymes. *Q. Rev. Biol.* 77, 124–147. <https://doi.org/10.1086/703580>
- Garrels, R.M., Thompson, M.E., 1962. A chemical model for sea water at 25 degrees C and one atmosphere total pressure. *Am. J. Sci.* 260, 57–66. <https://doi.org/10.2475/ajs.260.1.57>
- Genovese, G., Ortiz, N., Urcola, M.R., Luquet, C.M., 2005. Possible role of carbonic anhydrase, V-H⁺-ATPase, and Cl⁻/HCO₃⁻ exchanger in electrogenic ion transport across the gills of the euryhaline crab *Chasmagnathus granulatus*. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 142, 362–369. <https://doi.org/10.1016/j.cbpa.2005.08.024>
- Genovese, G., Senek, M., Ortiz, N., Regueira, M., Towle, D.W., Tresguerres, M., Luquet, C.M., 2006. Dopaminergic regulation of ion transport in gills of the euryhaline semiterrestrial crab *Chasmagnathus granulatus*: Interaction between D1- and D2-like receptors. *J. Exp. Biol.* 209, 2785–2793. <https://doi.org/10.1242/jeb.02308>
- Geyer, R.R., Musa-Aziz, R., Qin, X., Boron, W.F., 2013. Relative CO₂/NH₃ selectivities of mammalian aquaporins 0-9. *Am. J. Physiol. - Cell Physiol.* 304, C985–C994. <https://doi.org/10.1152/ajpcell.00033.2013>
- Gilles, R., Péqueux, A., 1981. Cell volume regulation in crustaceans: Relationship between mechanisms for controlling the osmolality of extracellular and intracellular fluids. *J. Exp. Zool.* 215, 351–362. <https://doi.org/10.1002/jez.1402150312>
- Gilmour, K.M., Perry, S.F., 2009. Carbonic anhydrase and acid-base regulation in fish. *J. Exp. Biol.* 212, 1647–1661. <https://doi.org/10.1242/jeb.029181>
- Goffredi, S.K., Childress, J.J., Desaulniers, N.T., Lallier, F.H., 1997a. Sulfide acquisition by the vent worm *Riftia pachyptila* appears to be via uptake of HS⁻, rather than H₂S. *J. Exp. Biol.* 200, 2609–2616. <https://doi.org/10.1242/jeb.200.20.2609>

- Goffredi, S.K., Childress, J.J., Desaulniers, N.T., Lee, R.W., Lallier, F.H., Hammond, D., 1997b. Inorganic carbon acquisition by the hydrothermal vent tubeworm *Riftia pachyptila* depends upon high external P(CO₂) and upon proton-equivalent ion transport by the worm. *J. Exp. Biol.* 200, 883–896. <https://doi.org/10.1242/jeb.200.5.883>
- Goodman, A.H., Einstein, R., Granger, H.J., 1978. Effect of changing metabolic rate on local blood flow control in the canine hindlimb. *Circ. Res.* 43, 769–776. <https://doi.org/10.1161/01.RES.43.5.769>
- Goodman, S.H., Cavey, M.J., 1990. Organization of a phyllobranchiate gill from the green shore crab *Carcinus maenas* (Crustacea, Decapoda). *Cell Tissue Res.* 260, 495–505. <https://doi.org/10.1007/BF00297229>
- Goss, G.G., Perry, S.F., Wood, C.M., Laurent, P., 1992. Mechanisms of ion and acid-base regulation at the gills of freshwater fish. *J. Exp. Zool.* 263, 143–159. <https://doi.org/10.1002/jez.1402630205>
- Greenaway, P., 1999. Physiological diversity and the colonization of land, in: Schram, F.R., von Vaupel Klein, J.C. (Eds.), *Crustaceans and the Biodiversity Crisis*. Koninklijke Brill NV, Leiden, pp. 823–842.
- Greenaway, P., MacMillen, R.E., 1978. Salt and water balance in the terrestrial phase of the inland crab *Holthusiana* (*Austrohelphusa*_ *transversa* Martens (Parathelphusoidea: Sundathelphusidae). *Physiol. Zool.* 51, 217–229.
- Greenaway, P., Taylor, H.H., Morris, S., 1990. Adaptations to a terrestrial existence by the robber crab, *birgus latro*, VI. The role of the excretory system in fluid balance. *J. Exp. Biol.* 152, 505–519.
- Gross, E., Hawkins, K., Pushkin, A., Sassani, P., Dukkupati, R., Abuladze, N., Hopfer, U., Kurtz, I., 2001. Phosphorylation of Ser982 in the sodium bicarbonate cotransporter kNBC1 shifts the HCO₃⁻ : Na⁺ stoichiometry from 3 : 1 to 2 : 1 in murine proximal tubule cells. *J. Physiol.* 537, 559–665. <https://doi.org/10.1111/j.1469-7793.2002.t01-2-01003.x>
- Hagerman, L., Uglow, R.F., 1984. The influence of hypoxia on the blood regulation of the brackish water shrimp *Palaemonetes varians* Leach. *J. Exp. Mar. Bio. Ecol.* 76, 157–165.

[https://doi.org/10.1016/0022-0981\(84\)90063-7](https://doi.org/10.1016/0022-0981(84)90063-7)

Haldane, J.S., Priestley, J.G., 1905. The regulation of the lung-ventilation. *J Physiol* 32, 225–266.

Hall-Spencer, J.M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S.M., Rowley, S.J., Tedesco, D., Buia, M.C., 2008. Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature* 454, 96–99.
<https://doi.org/10.1038/nature07051>

Halperin, J., Genovese, G., Tresguerres, M., Luquet, C.M., 2004. Modulation of ion uptake across posterior gills of the crab *Chasmagnathus granulatus* by dopamine and cAMP. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 139, 103–109.
<https://doi.org/10.1016/j.cbpb.2004.07.001>

Han, C., Ye, Y., Pan, Y., Qin, H., Wu, G., Chen, C.T.A., 2014. Spatial distribution pattern of seafloor hydrothermal vents to the southeastern Kueishan Tao offshore Taiwan Island. *Acta Oceanol. Sin.* 33, 37–44. <https://doi.org/10.1007/s13131-014-0405-x>

Hans, S., Fehsenfeld, S., Treberg, J.R., Weihrauch, D., 2014. Acid-base regulation in the Dungeness crab (*Metacarcinus magister*). *Mar. Biol.* 161, 1179–1193.
<https://doi.org/10.1007/s00227-014-2409-7>

Hans, S., Quijada-Rodriguez, A.R., Allen, G.J.P., Onken, H., Treberg, J.R., Weihrauch, D., 2018. Ammonia excretion and acid-base regulation in the American horseshoe crab, *Limulus polyphemus*. *J. Exp. Biol.* 221. <https://doi.org/10.1242/jeb.151894>

Harms, L., Frickenhaus, S., Schiffer, M., Mark, F.C., Storch, D., Held, C., Pörtner, H.O., Lucassen, M., 2014. Gene expression profiling in gills of the great spider crab *Hyas araneus* in response to ocean acidification and warming. *BMC Genomics* 15, 789.
<https://doi.org/10.1186/1471-2164-15-789>

Hartin, C.A., Bond-Lamberty, B., Patel, P., Mundra, A., 2016. Ocean acidification over the next three centuries using a simple global climate carbon-cycle model: Projections and sensitivities. *Biogeosciences* 13, 4329–4342. <https://doi.org/10.5194/bg-13-4329-2016>

Hartnoll, R.G., 1988. Evolution, systematics, and geographical distribution, in: Burggren, W.W.,

- McMahon, B.R. (Eds.), *Biology of the Land Crabs*. Cambridge University Press, Cambridge, U.K., pp. 6–54.
- Hasler, C.T., Butman, D., Jeffrey, J.D., Suski, C.D., 2016. Freshwater biota and rising pCO₂? *Ecol. Lett.* 19, 98–108. <https://doi.org/10.1111/ele.12549>
- Heisler, N., 1986. Comparative aspects of acid-base regulation, in: Heisler, N. (Ed.), *Acid-Base Regulation in Animals*. Elsevier, Amsterdam, pp. 397–450.
- Hemre, G.-I., Mommsen, T.P., Krogdahl, A., 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquac. Nutr.* 8, 175–194. <https://doi.org/10.17660/ActaHortic.2005.686.72>
- Henry, R.P., Cameron, J.N., 1983. The role of carbonic anhydrase in respiration, ion regulation and acid-base balance in the aquatic crab *Callinectes sapidus* and the terrestrial crab *Gecarcinus lateralis*. *J. Exp. Biol.* 103, 205–223.
- Henry, R.P., Cameron, J.N., 1982a. The distribution and partial characterization of carbonic anhydrase in selected aquatic and terrestrial decapod crustaceans. *J. Exp. Zool.* 221, 309–321. <https://doi.org/10.1002/jez.1402210306>
- Henry, R.P., Cameron, J.N., 1982b. Acid-base balance in *Callinectes sapidus* during acclimation from high to low salinity. *J. Exp. Biol.* 101, 255–264.
- Henry, R.P., Gehnrich, S., Weihrauch, D., Towle, D.W., 2003. Salinity-mediated carbonic anhydrase induction in the gills of the euryhaline green crab, *Carcinus maenas*. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 136, 243–258. [https://doi.org/10.1016/S1095-6433\(03\)00113-2](https://doi.org/10.1016/S1095-6433(03)00113-2)
- Henry, R.P., Lucu, Č., Onken, H., Weihrauch, D., 2012. Multiple functions of the crustacean gill: Osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. *Front. Physiol.* 3, 1–33. <https://doi.org/10.3389/fphys.2012.00431>
- Henry, R.P., Wheatly, M.G., 1992. Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. *Integr. Comp. Biol.* 32, 407–416. <https://doi.org/10.1093/icb/32.3.407>

- Heuer, R.M., Grosell, M., 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 307, R1061–R1084. <https://doi.org/10.1152/ajpregu.00064.2014>
- Holliday, C.W., 1980. Magnesium transport by the urinary bladder of the crab, *Cancer magister*. *J. Exp. Biol.* 85, 187–201. <https://doi.org/10.1242/jeb.85.1.187>
- Holmes, R.M., Aminot, A., K erouel, R., Hooker, B.A., Peterson, B.J., 1999. A simple and precise method for measuring ammonium in marine and freshwater ecosystems. *Can. J. Fish. Aquat. Sci.* 56, 1801–1808. <https://doi.org/10.1139/f99-128>
- Howell, B.J., Rahn, H., Goodfellow, D., Herreid, C., 1973. Acid-base regulation and temperature in selected invertebrates as a function of temperature. *Am. Zool.* 13, 557–563. <https://doi.org/10.1093/icb/13.2.557>
- Hu, M.Y., Guh, Y.J., Shao, Y.T., Kuan, P.L., Chen, G.L., Lee, J.R., Jeng, M.S., Tseng, Y.C., 2016. Strong ion regulatory abilities enable the crab *xenograpsus testudinatus* to inhabit highly acidified marine vent systems. *Front. Physiol.* 7, 1–11. <https://doi.org/10.3389/fphys.2016.00014>
- Hu, M.Y., Sung, P.H., Guh, Y.J., Lee, J.R., Hwang, P.P., Weihrauch, D., Tseng, Y.C., 2017. Perfused gills reveal fundamental principles of pH regulation and ammonia homeostasis in the cephalopod *Octopus vulgaris*. *Front. Physiol.* 8. <https://doi.org/10.3389/fphys.2017.00162>
- Hui, M., Song, C., Liu, Y., Li, C., Cui, Z., 2017. Exploring the molecular basis of adaptive evolution in hydrothermal vent crab *Austino-graea alayseae* by transcriptome analysis. *PLoS One* 12, e0178417. <https://doi.org/10.1371/journal.pone.0178417>
- Hunter, K.C., Kirschner, L.B., 1986. Sodium absorption coupled to ammonia excretion in osmoconforming marine invertebrates. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 251. <https://doi.org/10.1152/ajpregu.1986.251.5.r957>
- Ip, Y.K., Chew, S.F., 2010. Ammonia production, excretion, toxicity, and defense in fish: A review. *Front. Physiol.* 1, 1–20. <https://doi.org/10.3389/fphys.2010.00134>
- IPCC, 2019. IPCC special report on the ocean and cryosphere in a changing climate.

- IPCC, 2013. Summary for policymakers, in: Stocke, T.F., Qin, D., K., P.G. (Eds.), *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, New York, p. 1535.
- Jeffries, D.S., Cox, C.M., Dillon, P.J., 1979. Depression of pH in lakes and streams in central Ontario during snowmelt. *J. Fish. Res. Board Canada* 36, 640–646.
<https://doi.org/10.1139/f79-093>
- Jeng, M.S., Ng, N.K., Ng, P.K.L., 2004. Hydrothermal vent crabs feast on sea “snow.” *Nature* 432, 969. <https://doi.org/10.1038/432969a>
- Jiang, J., Chan, A., Ali, S., Saha, A., Haushalter, K.J., Lam, W.L.M.R., Glasheen, M., Parker, J., Brenner, M., Mahon, S.B., Patel, H.H., Ambasadhan, R., Lipton, S.A., Pilz, R.B., Boss, G.R., 2016. Hydrogen sulfide-mechanisms of toxicity and development of an antidote. *Sci. Rep.* 6, 20831. <https://doi.org/10.1038/srep20831>
- Jimenez, A.G., Bennett, W.A., 2015. Respiratory physiology of three Indo-Pacific fiddler crabs : Metabolic responses to intertidal zonation patterns. *Crustaceana* 78, 965–974.
- Johnson, I., Uglow, R.F., 1987. The effects of hypoxia on ion regulation and acid-base balance in *Carcinus maenas* (L.). *Comp. Biochem. Physiol. -- Part A Physiol.* 86, 261–267.
[https://doi.org/10.1016/0300-9629\(87\)90328-8](https://doi.org/10.1016/0300-9629(87)90328-8)
- Kartner, N., Yao, Y., Li, K., Crasto, G.J., Datti, A., Manolson, M.F., 2010. Inhibition of osteoclast bone resorption by disrupting vacuolar H⁺-ATPase α 3-B2 subunit interaction. *J. Biol. Chem.* 285, 37476–37490. <https://doi.org/10.1074/jbc.M110.123281>
- Kathiresan, K., Bingham, B.L., 2001. Biology of mangroves and mangrove ecosystems. *Adv. Mar. Biol.* 40, 81–251.
- Klocke, R.A., 1978. Catalysis of CO₂ reactions by lung carbonic anhydrase. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* 44, 882–888. <https://doi.org/10.1152/jappl.1978.44.6.882>
- Klymasz-Swartz, A.K.A.K., Allen, G.J.P., Treberg, J.R., Yoon, G.R., Tripp, A., Quijada-Rodriguez, A.R., Weihrauch, D., 2019. Impact of climate change on the American lobster (*Homarus americanus*): Physiological responses to combined exposure of elevated

- temperature and pCO₂. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* 235, 202–210. <https://doi.org/10.1016/j.cbpa.2019.06.005>
- Knapp, J.L., Bridges, C.R., Krohn, J., Hoffman, L.C., Auerswald, L., 2016. The effects of hypercapnia on the West Coast rock lobster (*Jasus lalandii*) through acute exposure to decreased seawater pH - Physiological and biochemical responses. *J. Exp. Mar. Bio. Ecol.* 476, 58–64. <https://doi.org/10.1016/j.jembe.2015.12.001>
- Koch, H.J., Evans, J., Schicks, E., 1954. The active absorption of ions by the isolated gills of the crab *Eriocheir sinensis* (M. Edw.). *Meded. Vlaam. Acad. K. Wet.* 16, 3–16.
- Koeppen, B.M., 2009. The kidney and acid-base regulation. *Am. J. Physiol. - Adv. Physiol. Educ.* 33, 275–281. <https://doi.org/10.1152/advan.00054.2009>
- Kormanick, G.A., Cameron, J.N., 1981. Ammonia excretion in animals that breathe water: A review. *Mar. Biol. Lett.* 2, 11–23.
- Kozłowski, J., 1999. Adaptation: A life history perspective. *Oikos* 86, 185–194.
- Kramer, D.L., 1987. Dissolved oxygen and fish behavior. *Environ. Biol. Fishes* 18, 81–92. <https://doi.org/10.1007/BF00002597>
- Krapf, R., 1988. Basolateral membrane H/OH/HCO₃ transport in the rat cortical thick ascending limb. *J. Clin. Invest.* 82, 234–241.
- Kristensen, E., 2008. Mangrove crabs as ecosystem engineers; with emphasis on sediment processes. *J. Sea Res.* 59, 30–43. <https://doi.org/10.1016/j.seares.2007.05.004>
- Kroeker, K.J., Kordas, R.L., Crim, R., Hendriks, I.E., Ramajo, L., Singh, G.S., Duarte, C.M., Gattuso, J.-P., 2013. Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Glob. Chang. Biol.* 19, 1884–1896. <https://doi.org/10.1111/gcb.12179>
- Krogh, A., 1938. The active absorption of ions in some freshwater animals. *Z. Vgl. Physiol.* 25, 335–350. <https://doi.org/10.1007/BF00339641>
- Kurihara, H., Shirayama, Y., 2004. Effects of increased atmospheric CO₂ on sea urchin early development. *Mar. Ecol. Prog. Ser.* 274, 161–169. <https://doi.org/10.3354/meps274161>

- Laird, C.E., Haefner, P.A., 1976. Effects of intrinsic and environmental factors on oxygen consumption in the blue crab, *Callinectes sapidus* Rathbun. *J. Exp. Mar. Bio. Ecol.* [https://doi.org/10.1016/0022-0981\(76\)90093-9](https://doi.org/10.1016/0022-0981(76)90093-9)
- Lang, F., Paulmichl, M., 1995. Properties and regulation of ion channels in MDCK cells. *Kidney Int.* 48, 1200–1205. <https://doi.org/10.1038/ki.1995.403>
- Langenbuch, M., Pörtner, H.O., 2003. Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempfi*) as a function of ambient CO₂: pH-dependent limitations of cellular protein biosynthesis? *J. Exp. Biol.* 206, 3895–3903. <https://doi.org/10.1242/jeb.00620>
- Langenbuch, M., Pörtner, H.O., 2002. Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: Identifying effective acid-base variables. *J. Exp. Biol.* 205, 1153–1160.
- Larsen, E.H., Deaton, L.E., Onken, H., O'Donnell, M., Grosell, M., Dantzler, W.H., Weihrauch, D., 2014. Osmoregulation and excretion. *Compr. Physiol.* 4, 405–573. <https://doi.org/10.1002/cphy.c130004>
- Le Chatelier, H., 1884. A general statement of the laws of chemical equilibrium. *C. R. Acad. Sci. Paris* 99, 786–789.
- Le Moullac, G., Haffner, P., 2000. Environmental factors affecting immune responses in Crustacea. *Aquaculture* 191, 121–131. [https://doi.org/10.1016/S0044-8486\(00\)00422-1](https://doi.org/10.1016/S0044-8486(00)00422-1)
- Lee, D.J., Gutbrod, M., Ferreras, F.M., Matthews, P.G.D., 2018. Changes in hemolymph total CO₂ content during the water-to-air respiratory transition of amphibiotic dragonflies. *J. Exp. Biol.* 221, 181438. <https://doi.org/10.1242/jeb.181438>
- Lee Hamm, L., Nakhoul, N., Hering-Smith, K.S., 2015. Acid-base homeostasis. *Clin. J. Am. Soc. Nephrol.* 10, 2232–2242. <https://doi.org/10.2215/CJN.07400715>
- Leone, F.A., Lucena, M.N., Garçon, D.P., Pinto, M.R., McNamara, J.C., 2017. Gill Ion Transport ATPases and Ammonia Excretion in Aquatic Crustaceans, in: Weihrauch, D., O'Donnell, M.J. (Eds.), *Acid-Base Balance and Nitrogen Excretion in Invertebrates*. Springer Nature, Gewerbestrasse, pp. 61–107. https://doi.org/10.1007/978-3-319-39617-0_3

- Lignon, J.M., 1987. Ionic permeabilities of the isolated gill cuticle of the shore crab *Carcinus maenas*. *J. Exp. Biol.* 131, 159–174. <https://doi.org/10.1242/jeb.131.1.159>
- Lignon, J.M., 1986. Structure and permeability of decapod crustacea cuticle, in: Lahlou, K. (Ed.), *Comparative Physiology of Environmental Adaptations*. Strasbourg, pp. 178–187. <https://doi.org/10.1159/000413947>
- Lin, Y.S., Lui, H.K., Lee, J., Chen, C.T.A., Burr, G.S., Chou, W.C., Kuo, F.W., 2019. Fates of vent CO₂ and its impact on carbonate chemistry in the shallow-water hydrothermal field offshore Kueishantao Islet, NE Taiwan. *Mar. Chem.* 210, 1–12. <https://doi.org/10.1016/j.marchem.2019.02.002>
- Linton, S.M., Greenaway, P., 1995. Nitrogenous excretion in the amphibious crab *Holthuisana transversa* under wet and dry conditions. *J. Crustac. Biol.* 15, 633–644.
- Linton, S.M., Wright, J.C., Howe, C.G., 2017. Nitrogenous waste metabolism within terrestrial crustacea, with special reference to purine deposits and their metabolism, in: Weihrauch, D., O'Donnell, M.J. (Eds.), *Acid-Base Balance and Nitrogen Excretion in Invertebrates*. Springer International Publishing, Gewerbestrasse, pp. 27–40.
- Little, C.T.S., Vrijenhoek, R.C., 2003. Are hydrothermal vent animals living fossils? *Trends Ecol. Evol.* 18, 582–588. <https://doi.org/10.1016/j.tree.2003.08.009>
- Lockwood, A.P.M., Riegel, J.A., 1969. The excretion of magnesium by *Carcinus maenas*. *J. Exp. Biol.* 51, 575–589.
- Lohrmann, D.M., Kamemoto, F.I., 1987. The effect of dibutyryl cAMP on sodium uptake by isolated perfused gills of *Callinectes sapidus*. *Gen. Comp. Endocrinol.* 65, 300–305. [https://doi.org/10.1016/0016-6480\(87\)90177-8](https://doi.org/10.1016/0016-6480(87)90177-8)
- Lozano-Fernandez, J., Carton, R., Tanner, A.R., Puttick, M.N., Blaxter, M., Vinther, J., Olesen, J., Giribet, G., Edgecombe, G.D., Pisani, D., 2016. A molecular palaeobiological exploration of arthropod terrestrialization. *Philos. Trans. R. Soc. B Biol. Sci.* 371. <https://doi.org/10.1098/rstb.2015.0133>
- Lu, S.Y., Shen, C.H., Chiau, W.Y., 2014. Zoning strategies for marine protected areas in Taiwan: Case study of Gueishan Island in Yilan County, Taiwan. *Mar. Policy* 48, 21–29.

<https://doi.org/10.1016/j.marpol.2014.03.001>

- Lucu, Č., Flik, G., 1999. Na⁺-K⁺-ATPase and Na⁺/Ca²⁺ exchange activities in gills of hyperregulating *Carcinus maenas*. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 276, R490-499.
- Lucu, Č., Siebers, D., 1986. Amiloride-sensitive sodium flux and potentials in perfused *Carcinus* gill preparations. *J. Exp. Biol.* 122, 25–35.
- Luquet, C.M., Ansaldo, M., 1997. Acid-base balance and ionic regulation during emersion in the estuarine intertidal crab *Chasmagnathus granulata* dana (Decapoda Grapsidae). *Comp. Biochem. Physiol. - A Physiol.* 117, 407–410. [https://doi.org/10.1016/S0300-9629\(96\)00282-4](https://doi.org/10.1016/S0300-9629(96)00282-4)
- Luquet, C.M., Genovese, G., Rosa, G.A., Pellerano, G.N., 2002a. Ultrastructural changes in the gill epithelium of the crab *Chasmagnathus granulatus* (Decapoda: Grapsidae) in diluted and concentrated seawater. *Mar. Biol.* 141, 753–760. <https://doi.org/10.1007/s00227-002-0860-3>
- Luquet, C.M., Postel, U., Halperin, J., Urcola, M.R., Marques, R., Siebers, D., 2002b. Transepithelial potential differences and Na⁺ flux in isolated perfused gills of the crab *Chasmagnathus granulatus* (Grapsidae) acclimated to hyper- and hypo-salinity. *J. Exp. Biol.* 205, 71–77.
- Macklem, P.T., Seely, A., 2010. Towards a definition of life. *Perspect. Biol. Med.* 53, 330–340. <https://doi.org/10.1353/pbm.0.0167>
- Macmillen, R.E., Greenaway, P., 1978. Adjustments of energy and water metabolism to drought in an Australian arid-zone crab. *Physiol. Zool.* 51, 230–240.
- Maitland, D.P., 1986. Crabs that breathe air with their legs-scopimera and dotilla. *Nature* 319, 493–495. <https://doi.org/10.1038/319493a0>
- Mangum, C.P., McMahon, B.R., DeFur, P.L., Wheatly, M.G., 1985. Gas exchange, acid-base balance, and the oxygen supply to the tissues during a molt of the blue crab *Callinectes sapidus*. *J. Crustac. Biol.* 5, 188–206. <https://doi.org/10.2307/1547866>

- Mangum, C.P., Silverthorn, S.U., Harris, J.L., Towle, D.W., Krall, A.R., 1976. The relationship between blood pH, ammonia excretion and adaptation. *J. Exp. Zool.* 195, 129–136.
- Mantel, L.H., Farmer, L.L., 1983. Osmotic and ionic regulation, in: Bliss, D.E. (Ed.), *The Biology of Crustacea*. Academic Press, New York, pp. 53–161.
- Martin, J.W., Davis, G.E., 2001. An updated classification of the recent crustacea, in: *Science Series*. Natural History Museum of Los Angeles County: Los Angeles, Los Angeles, p. 123.
- Martin, J.W., Haney, T.A., 2005. Decapod crustaceans from hydrothermal vents and cold seeps: A review through 2005. *Zool. J. Linn. Soc.* 145, 445–522.
- Martin, M., Fehsenfeld, S., Sourial, M.M., Weihrauch, D., 2011. Effects of high environmental ammonia on branchial ammonia excretion rates and tissue Rh-protein mRNA expression levels in seawater acclimated Dungeness crab *Metacarcinus magister*. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 160, 267–277. <https://doi.org/10.1016/j.cbpa.2011.06.012>
- Martin, W., Baross, J., Kelley, D., Russell, M.J., 2008. Hydrothermal vents and the origin of life. *Nat. Rev. Microbiol.* 6, 805–814. <https://doi.org/10.1038/nrmicro1991>
- Maus, B., Bock, C., Pörtner, H.O., 2018. Water bicarbonate modulates the response of the shore crab *Carcinus maenas* to ocean acidification. *J. Comp. Physiol. B* 188, 749–764. <https://doi.org/10.1007/s00360-018-1162-5>
- McGaw, I.J., 2005. Burying behaviour of two sympatric crab species: *Cancer magister* and *Cancer productus*. *Sci. Mar.* 69, 375–381. <https://doi.org/10.3989/scimar.2005.69n3375>
- McGaw, I.J., Reiber, C.L., 2015. Circulatory Physiology, in: Chang, S.E., Thiel, M. (Eds.), *The Natural History of Crustaceans*. Oxford University Press, pp. 199–246.
- McGaw, I.J., Reiber, C.L., 2002. Cardiovascular system of the blue crab *Callinectes sapidus*. *J. Morphol.* 251, 1–21. <https://doi.org/10.1002/jmor.1071>
- McGaw, I.J., Van Leeuwen, T.E., Trehern, R.H., Bates, A.E., 2019. Changes in precipitation may alter food preference in an ecosystem engineer, the black land crab, *Gecarcinus ruricola*. *PeerJ* 7, e6818. <https://doi.org/10.7717/peerj.6818>
- Mchenga, I.S.S., Mfilinge, P.L., Tsuchiya, M., 2007. Bioturbation activity by the grapsid crab

- Helice formosensis* and its effects on mangrove sedimentary organic matter. *Estuar. Coast. Shelf Sci.* 73, 316–324. <https://doi.org/10.1016/j.ecss.2007.01.016>
- Mckenzie, D.J., Shingles, A., Claireaux, G., Domenici, P., 2009. Sublethal concentrations of ammonia impair performance of the teleost fast-start escape response. *Physiol. Biochem. Zool.* 82, 353–362. <https://doi.org/10.1086/590218>
- McKenzie, D.J., Shingles, A., Taylor, E.W., 2003. Sub-lethal plasma ammonia accumulation and the exercise performance of salmonids. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 135, 515–526. [https://doi.org/10.1016/S1095-6433\(03\)00116-8](https://doi.org/10.1016/S1095-6433(03)00116-8)
- McMahon, B., Butler, P.J., Taylor, E.W., 1978. Acid base changes during recovery from disturbance and during long term hypoxic exposure in the lobster *Homarus vulgaris*. *J. Exp. Zool.* 205, 361–370. <https://doi.org/10.1002/jez.1402050304>
- McMahon, B.R., 1988. Physiological responses to oxygen depletion in intertidal animals. *Integr. Comp. Biol.* 28, 39–53. <https://doi.org/10.1093/icb/28.1.39>
- McNamara, J.C., Faria, S.C., 2012. Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: A review. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 182, 997–1014. <https://doi.org/10.1007/s00360-012-0665-8>
- Medina, M., Collins, A.G., Takaoka, T.L., Kuehl, J. V., Boore, J.L., 2006. Naked corals: Skeleton loss in Scleractinia. *Proc. Natl. Acad. Sci. U. S. A.* 103, 9096–9100. <https://doi.org/10.1073/pnas.0602444103>
- Mehrbach, C., Culbertson, C.H., Hawley, J.E., Pytkowicz, R.M., 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* 18, 897–907. <https://doi.org/10.4319/lo.1973.18.6.0897>
- Melzner, F., Gutowska, M.A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M.C., Bleich, M., Pörtner, H.O., 2009. Physiological basis for high CO₂ tolerance in marine ectothermic animals: Pre-adaptation through lifestyle and ontogeny? *Biogeosciences* 6, 2313–2331. <https://doi.org/10.5194/bg-6-2313-2009>
- Meseck, S.L., Alix, J.H., Swiney, K.M., Long, W.C., Wikfors, G.H., Foy, R.J., 2016. Ocean acidification affects hemocyte physiology in the tanner crab (*Chionoecetes bairdi*). *PLoS*

- One 11, 1–23. <https://doi.org/10.1371/journal.pone.0148477>
- Mia, Y., Shokita, S., 1997. Optimal salinity required for the larval development of two grapsid crabs, *Helice leachi* Hess and *H. formosensis* Rathbun. *Crustac. Res.* 26, 70–74.
- Mia, Y., Shokita, S., Watanabe, S., 2001. Stomach contents of two grapsid crabs, *Helice formosensis* and *Helice leachi*. *Fish. Sci.* 67, 173–175. <https://doi.org/10.1046/j.1444-2906.2001.00216.x>
- Michaelidis, B., Ouzounis, C., Paleras, A., Portner, H.O., 2005. Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Mar. Ecol. Prog. Ser.* 293, 109–118.
- Mills, B.J., Geddes, M.C., 1980. Salinity tolerance and osmoregulation of the Australian freshwater crayfish *Cherax destructor* Clark (Decapoda: Parastacidae). *Mar. Freshw. Res.* 31, 667–676. <https://doi.org/10.1071/MF9800667>
- Mo, J.L., Devos, P., Trausch, G., 2003. Active absorption of Cl⁻ and Na⁺ in posterior gills of chinese crab *Eriocheir sinensis*: Modulation by dopamine and cAMP. *J. Crustac. Biol.* 23, 505–512. <https://doi.org/10.1651/c-2295>
- Mo, J.L., Devos, P., Trausch, G., 1998. Dopamine as a modulator of ionic transport and Na⁺/K⁺-ATPase activity in the gills of the Chinese crab *Eriocheir sinensis*. *J. Crustac. Biol.* 18, 442–448.
- Mommsen, T.P., Walsh, P.J., 1991. Urea synthesis in fishes: evolutionary and biochemical perspectives, in: Hochachka, P.W., Mommsen, T.P. (Eds.), *Biochemistry and Molecular Biology of Fishes*. Elsevier Science Publishers, pp. 137–163. <https://doi.org/10.1016/B978-0-444-89124-2.50010-5>
- Morgan, D.O., McMahon, B.R., 1982. Acid tolerance and effects of sublethal acid exposure on iono-regulation and acid-base status in two crayfish *Procambarus clarki* and *Orconectes rusticus*. *J. Exp. Biol.* 97, 241–252.
- Morris, S., 2002. The ecophysiology of air-breathing in crabs with special reference to *Gecarcoidea natalis*. *Comp. Biochem. Physiol. B* 131, 559–570. [https://doi.org/10.1016/S1096-4959\(02\)00011-8](https://doi.org/10.1016/S1096-4959(02)00011-8)

- Morris, S., 2001. Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. *J. Exp. Biol.* 204, 979–989.
- Morris, S., Butler, S.L., 1996. Hemolymph respiratory gas, acid-base, and ion status of the amphibious purple shore crab *Leptograpsus variegatus* (Fabricus) during immersion and environmental hypoxia. *J. Crustac. Biol.* 16, 253–266.
- Morris, S., Greenaway, P., 1990. Adaptations to a terrestrial existence by the robber crab, *Birgus latro* L. - V. The activity of carbonic anhydrase in gills and lungs. *J. Comp. Physiol. B* 160, 217–221. <https://doi.org/10.1007/BF00300957>
- Nakada, T., Westhoff, C.M., Kato, A., Hirose, S., 2007. Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *The FASEB* 21, 1067–1074. <https://doi.org/10.1096/fj.06-6834com>
- Nawata, C.M., Hung, C.C.Y., Tsui, T.K.N., Wilson, J.M., Wright, P.A., Wood, C.M., 2007. Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H⁺-ATPase involvement. *Physiol. Genomics* 31, 463–474. <https://doi.org/10.1152/physiolgenomics.00061.2007>.
- Nawata, M., Wood, C.M., O'Donnell, M.J., 2010. Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. *J. Exp. Biol.* 213, 1049–1059. <https://doi.org/10.1242/jeb.038752>
- Ng, P.K.L., Huang, J.F., Ho, P.-O., 2000. Description of a new species of hydrothermal crab, *Xenograpsus testudinatus* (Crustacea: Decapoda: Brachyura: Grapsidae) from Taiwan. *Natl. Taiwan Museum Spec. Publ. Ser.* 191–199.
- Nicholls, R.J., Brown, S., Goodwin, P., Wahl, T., Lowe, J., Solan, M., Godbold, J.A., Haigh, I.D., Lincke, D., Hinkel, J., Wolff, C., Merckens, J.-L., 2018. Stabilization of global temperature at 1.5°C and 2.0°C: implications for coastal areas. *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.* 376, 20160448.
- O'Donnell, M.J., Machin, J., 1988. Water vapor absorption by terrestrial organisms, in: *Advances in Comparative and Environmental Physiology*. Springer-Verlag, Heidelberg, pp. 47–87.

- Onken, H., Graszynski, K., 1989. Active Cl⁻ absorption by the Chinese crab (*Eriocheir sinensis*) gill epithelium measured by transepithelial potential difference. *J. Comp. Physiol. B* 159, 21–28. <https://doi.org/10.1007/BF00692679>
- Onken, H., McNamara, J.C., 2002. Hyperosmoregulation in the red freshwater crab *Dilocarcinus pagei* (Brachyura, Trichodactylidae): Structural and functional asymmetries of the posterior gills. *J. Exp. Biol.* 205, 167–175.
- Onken, H., Putzenlechner, M., 1995. A V-ATPase drives active, electrogenic and Na⁺-independent Cl⁻ absorption across the gills of *Eriocheir sinensis*. *J. Exp. Biol.* 198, 767–774.
- Onken, H., Riestenpatt, S., 2002. Ion transport across posterior gills of hyperosmoregulating shore crabs (*Carcinus maenas*): amiloride blocks the cuticular Na⁺ conductance and induces current-noise. *J. Exp. Biol.* 205, 523–531.
- Onken, H., Schöbel, A., Kraft, J., Putzenlechner, M., 2000. Active NaCl absorption across split lamellae of posterior gills of the Chinese crab *Eriocheir sinensis*: Stimulation by eyestalk extract. *J. Exp. Biol.* 203, 1373–1381.
- Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G.K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M.F., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* 437, 681–686. <https://doi.org/10.1038/nature04095>
- Pane, E.F., Barry, J.P., 2007. Extracellular acid – base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. *Mar. Ecol. Prog. Ser.* 334, 1–9.
- Parks, S.K., Tresguerres, M., Goss, G.G., 2008. Theoretical considerations underlying Na⁺ uptake mechanisms in freshwater fishes. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* 148, 411–418. <https://doi.org/10.1016/j.cbpc.2008.03.002>
- Parks, S.K., Tresguerres, M., Goss, G.G., 2007. Interactions between Na⁺ channels and Na⁺-

- HCO₃⁻ cotransporters in the freshwater fish gill MR cell: A model for transepithelial Na⁺ uptake. *Am. J. Physiol. - Cell Physiol.* 292, 935–944.
<https://doi.org/10.1152/ajpcell.00604.2005>
- Patterson, N.E., DeFur, P.L., 1988. Ventilatory and circulatory responses of the crayfish *Procambarus clarki* to low environmental pH. *Physiol. Zool.* 61, 396–406.
- Penha-Lopes, G., Bartolini, F., Limbu, S., Cannicci, S., Kristensen, E., Paula, J., 2009. Are fiddler crabs potentially useful ecosystem engineers in mangrove wastewater wetlands? *Mar. Pollut. Bull.* 58, 1694–1703. <https://doi.org/10.1016/j.marpolbul.2009.06.015>
- Perry, S.F., Braun, M.H., Noland, M., Dawdy, J., Walsh, P.J., 2010. Do zebrafish Rh proteins act as dual ammonia – CO₂ channels? *J. Exp. Zool. Part A Ecol. Genet. Physiol.* 313A, 618–621. <https://doi.org/10.1002/jez.631>
- Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S.L.Y., 2003. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: Their role in ionic and acid-base regulation. *J. Exp. Zool. Part A Comp. Exp. Biol.* 300, 53–62.
<https://doi.org/10.1002/jez.a.10309>
- Peterson, D.R., Loizzi, R.F., 1974. Ultrastructure of the crayfish kidney - Coelomosac, labyrinth, nephridial canal. *J. Morphol.* 142, 241–263.
- Pfister, C.A., 2007. Intertidal invertebrates locally enhance primary production. *Ecology* 88, 1647–1653. <https://doi.org/10.1890/06-1913.1>
- Pierrot, D., Lewis, E., Wallace, D., 2006. MS Excel program developed for CO₂ system calculations, ORNL/CDIAC-105. Oak Ridge, TN.
- Poléo, A.B.S., Østbye, K., Øxnevad, S.A., Andersen, R.A., Heibo, E., Vøllestad, L.A., 1997. Toxicity of acid aluminium-rich water to seven freshwater fish species: A comparative laboratory study. *Environ. Pollut.* 96, 129–139. [https://doi.org/10.1016/S0269-7491\(97\)00033-X](https://doi.org/10.1016/S0269-7491(97)00033-X)
- Portner Hans O, Langenbuch Martina, Reipschlagel Anke, 2004. Biological impact of elevated ocean CO₂ concentrations: lessons from animal physiology and earth history. *J. Oceanogr.* 60, 705–718.

- Postel, U., Petrausch, G., Riestenpatt, S., Weihrauch, D., Malykh, J., Becker, W., Siebers, D., 1998. Inhibition of Na⁺/K⁺-ATPase and of active ion-transport functions in the gills of the shore crab *Carcinus maenas* induced by cadmium. *Mar. Biol.* 130, 407–416.
<https://doi.org/10.1007/s002270050261>
- Qadri, S.A., Camacho, J., Wang, H., Taylor, J.R., Grosell, M., Worden, M.K., 2007. Temperature and acid–base balance in the American lobster *Homarus americanus*. *J. Exp. Biol.* 210, 1245–1254. <https://doi.org/10.1242/jeb.02709>
- Qu, Z., Hartzell, H.C., 2008. Bestrophin Cl⁻ channels are highly permeable to HCO₃⁻. *Am. J. Physiol. - Cell Physiol.* 294, C1371–C1377. <https://doi.org/10.1152/ajpcell.00398.2007>
- Quijada-Rodriguez, A.R., Allen, G.J.P., Nash, M.T., Weihrauch, D., 2022. Postprandial nitrogen and acid-base regulation in the seawater acclimated green crab, *Carcinus maenas*. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 267, 111171.
- Quijada-Rodriguez, A.R., Kuan, P., Sung, P., Hsu, M., Allen, G.J.P., Hwang, P.P., Tseng, Y.-C., Weihrauch, D., 2021. Anthropogenic CO₂-mediated freshwater acidification limits survival, calcification, metabolism, and behaviour in stress-tolerant freshwater crustaceans. *Biogeosciences* 18, 6287–6300.
- Rabalais, N.N., Turner, R.E., Wiseman, W.J.J., 2001. Hypoxia in the Gulf of Mexico. *J. Environ. Qual.* 30, 320–329. <https://doi.org/10.2134/jeq2001.302320x>
- Ran, L., Lu, X.X., Liu, S., 2017. Dynamics of riverine CO₂ in the Yangtze River fluvial network and their implications for carbon evasion. *Biogeosciences* 14, 2183–2198.
<https://doi.org/10.5194/bg-14-2183-2017>
- Ran, L., Lu, X.X., Richey, J.E., Sun, H., Han, J., Yu, R., Liao, S., Yi, Q., 2015. Long-term spatial and temporal variation of CO₂ partial pressure in the Yellow River, China. *Biogeosciences* 12, 921–932. <https://doi.org/10.5194/bg-12-921-2015>
- Rasmussen, A.D., Andersen, O., 1996. Apparent water permeability as a physiological parameter in crustaceans. *J. Exp. Biol.* 199, 2555–2564. <https://doi.org/10.1242/jeb.199.12.2555>
- Reeves, R.B., 1977. The interaction of body temperature and acid-base balance in ectothermic vertebrates. *Annu. Rev. Physiol.* 39, 559–586.

- Reeves, R.B., 1972. An imidazole alaphstat hypothesis for vertebrate acid-base regulation: Tissue carbon dioxide content and body temperature in bullfrogs. *Respir. Physiol.* 14, 219–236. [https://doi.org/10.1016/0034-5687\(72\)90030-8](https://doi.org/10.1016/0034-5687(72)90030-8)
- Regnault, M., 1994. Effect of air exposure on ammonia excretion and ammonia content of branchial water of the crab *Cancer pagurus*. *J. Exp. Zool.* 268, 208–217. <https://doi.org/10.1002/jez.1402680305>
- Ren, Q., Pan, L., Zhao, Q., Si, L., 2015. Ammonia and urea excretion in the swimming crab *Portunus trituberculatus* exposed to elevated ambient ammonia-N. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* 187, 48–54. <https://doi.org/10.1016/j.cbpa.2015.04.013>
- Ricklefs, R., Wikelski, M., 2002. The physiology/life-history nexus. *Trends Ecol. Evol.* 17, 462–468.
- Riegel, J.A., Cook, M.A., 1975. Recent studies of excretion in crustacea. *Fortschr. Zool.* 23, 48–75.
- Riestenpatt, S., Petrausch, G., Siebers, D., 1995. Cl⁻ influx across posterior gills of the Chinese crab (*Eriocheir sinensis*): potential energization by a V-type H⁺-ATPase. *Comp. Biochem. Physiol. -- Part A Physiol.* 110, 235–241. [https://doi.org/10.1016/0300-9629\(94\)00169-T](https://doi.org/10.1016/0300-9629(94)00169-T)
- Riestenpatt, S., Zeiske, W., Onken, H., 1994. Cyclic AMP stimulation of electrogenic uptake of Na⁺ and Cl⁻ across the gill epithelium of the Chinese crab *Eriocheir sinensis*. *J. Exp. Biol.* 188, 159–174.
- Riggs, A., 1988. The Bohr Effect. *Annu. Rev. Physiol.* 50, 181–204. <https://doi.org/10.1146/annurev.physiol.50.1.181>
- Roer, R., 1980. Mechanisms of resorption and deposition of calcium in the carapace of the crab *Carcinus maenas*. *J. Exp. Biol.* 88, 205–218.
- Roer, R., Dillaman, R., 1984. The structure and calcification of the crustacean cuticle. *Integr. Comp. Biol.* 24, 893–909. <https://doi.org/10.1093/icb/24.4.893>
- Rogers, A.D., Tyler, P.A., Connelly, D.P., Copley, J.T., James, R., Larter, R.D., Linse, K., Mills,

- R.A., Garabato, A.N., Pancost, R.D., Pearce, D.A., Polunin, N.V.C., German, C.R., Shank, T., Boersch-Supan, P.H., Alker, B.J., Aquilina, A., Bennett, S.A., Clarke, A., Dinley, R.J.J., Graham, A.G.C., Green, D.R.H., Hawkes, J.A., Hepburn, L., Hilario, A., Huvenne, V.A.I., Marsh, L., Ramirez-Llodra, E., Reid, W.D.K., Roterman, C.N., Sweeting, C.J., Thatje, S., Zwirgmaier, K., 2012. The discovery of new deep-sea hydrothermal vent communities in the Southern ocean and implications for biogeography. *PLoS Biol.* 10. <https://doi.org/10.1371/journal.pbio.1001234>
- Schindler, D.W., 1988. Effects of acid rain on freshwater ecosystems. *Science* (80-.). 239, 149–157. <https://doi.org/10.1126/science.239.4836.149>
- Serrano, L., Halanych, K.M., Henry, R.P., 2007. Salinity-stimulated changes in expression and activity of two carbonic anhydrase isoforms in the blue crab *Callinectes sapidus*. *J. Exp. Biol.* 210, 2320–2332. <https://doi.org/10.1242/jeb.005041>
- Shartau, R.B., Baker, D.W., Crossley, D.A., Brauner, C.J., 2016. Preferential intracellular pH regulation: Hypotheses and perspectives. *J. Exp. Biol.* 219, 2235–2244. <https://doi.org/10.1242/jeb.126631>
- Si, L., Pan, L., Wang, H., Zhang, X., 2018. Identification of the role of Rh protein in ammonia excretion of the swimming crab *Portunus trituberculatus*. *J. Exp. Biol.* 221, 1–12. <https://doi.org/10.1242/jeb.184655>
- Siebers, D., Leweck, K., Markus, H., Winkler, A., 1982. Sodium regulation in the shore crab *Carcinus maenas* as related to ambient salinity. *Mar. Biol.* 69, 37–43. <https://doi.org/10.1007/BF00396958>
- Siebers, D., Lucu, Č., Böttcher, K., Jürss, K., 1994. Regulation of pH in the isolated perfused gills of the shore crab *Carcinus maenas*. *J. Comp. Physiol. B* 164, 16–22. <https://doi.org/10.1007/BF00714566>
- Siebers, D., Winkler, A., Lucu, Č., Thedens, G., Weichart, D., 1985. Na-K-ATPase generates an active transport potential in the gills of the hyperregulating shore crab *Carcinus maenas*. *Mar. Biol.* 87, 185–192.
- Simonik, E., Henry, R.P., 2014. Physiological responses to emersion in the intertidal green crab,

- Carcinus maenas* (L.). *Mar. Freshw. Behav. Physiol.* 47, 101–115.
<https://doi.org/10.1080/10236244.2014.905001>
- Small, D., Calosi, P., White, D., Spicer, J.I., Widdicombe, S., 2010. Impact of medium-term exposure to CO₂ enriched seawater on the physiological functions of the velvet swimming crab *Necora puber*. *Aquat. Biol.* 10, 11–21. <https://doi.org/10.3354/ab00266>
- Smith, R.I., 1967. Osmotic regulation and adaptive reduction of water-permeability in a brackish-water crab, *Rhithropanopeus harrisi* (Brachyura, Xanthidae). *Biologic* 133, 10–27.
- Sojo, V., Herschy, B., Whicher, A., Camprubí, E., Lane, N., 2016. The origin of life in alkaline hydrothermal vents. *Astrobiology* 16, 181–197. <https://doi.org/10.1089/ast.2015.1406>
- Somero, G.N., 1986. Protons, osmolytes, and fitness of internal milieu for protein function. *Am. J. Physiol.* 251. <https://doi.org/10.1152/ajpregu.1986.251.2.r197>
- Sommer, M.J., Mantel, L.H., 1991. Effects of dopamine and acclimation to reduced salinity on the concentration of cyclic amp in the gills of the green crab, *Carcinus maenas* (L.). *Gen. Comp. Endocrinol.* 82, 364–368. [https://doi.org/10.1016/0016-6480\(91\)90311-S](https://doi.org/10.1016/0016-6480(91)90311-S)
- Sommer, M.J., Mantel, L.H., 1988. Effects of dopamine, cyclic AMP, and pericardial organs on sodium uptake and Na/K-ATPase activity in gills of the green crab, *Carcinus maenas* (L.). *J. Exp. Zool.* 248, 272–277.
- Soupene, E., King, N., Feild, E., Liu, P., Niyogi, K.K., Huang, C.-H., Kustu, S., 2002. Rhesus expression in a green alga is regulated by CO₂. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7769–7773.
- Spanings-Pierrot, C., Soyeux, D., Van Herp, F., Gompel, M., Skaret, G., Grousset, E., Charmantier, G., 2000. Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. *Gen. Comp. Endocrinol.* 119, 340–350. <https://doi.org/10.1006/gcen.2000.7527>
- Spicer, J.I., Raffo, A., Widdicombe, S., 2007. Influence of CO₂-related seawater acidification on extracellular acid-base balance in the velvet swimming crab *Necora puber*. *Mar. Biol.* 151, 1117–1125. <https://doi.org/10.1007/s00227-006-0551-6>

- Spiess, F.N., Macdonald, K.C., Atwater, T., Ballard, R., Carranza, A., Cordoba, D., Cox, C., Diaz Garcia, V.M., Francheteau, J., Guerrero, J., Hawkins, J., Haymon, R., Hessler, R., Juteau, T., Kastner, M., Larson, R., Luyendyk, B., Macdougall, J.D., Miller, S., Normark, W., Orcutt, J., Rangin, C., 1980. East Pacific rise: Hot springs and geophysical experiments. *Science* (80-.). 207, 1421–1433.
- Stewart, P.A., 1983. Modern quantitative acid-base chemistry. *Can J Physiol Pharmacol* 61, 1444–1461.
- Stumpff, M., Hu, M.Y., Melzner, F., Gutowska, M.A., Dorey, N., Himmerkus, N., Holtmann, W.C., Dupont, S.T., Thorndyke, M.C., Bleich, M., 2012. Acidified seawater impacts sea urchin larvae pH regulatory systems relevant for calcification. *Proc. Natl. Acad. Sci. U. S. A.* 109, 18192–18197. <https://doi.org/10.1073/pnas.1209174109>
- Tarasov, V.G., Gebruk, A. V., Mironov, A.N., Moskalev, L.I., 2005. Deep-sea and shallow-water hydrothermal vent communities: Two different phenomena? *Chem. Geol.* 224, 5–39. <https://doi.org/10.1016/j.chemgeo.2005.07.021>
- Taylor, H.H., Taylor, E.W., 1986. Observations of valve-like structures and evidence for rectification of flow within the gill lamellae of the crab *Carcinus maenas* (Crustacea, Decapoda). *Zoomo* 106, 1–11.
- Teal, J.M., Carey, F.G., 1967. The metabolism of marsh crabs under conditions of reduced oxygen pressure. *Physiol. Zool.* 40, 83–91.
- Thiel, D., Hugenschutt, M., Meyer, H., Paululat, A., Quijada-Rodriguez, A.R., Purschke, G., Weihrauch, D., 2017. Ammonia excretion in the marine polychaete *Eurythoe complanata* (Annelida). *J. Exp. Biol.* 220, 425–436. <https://doi.org/10.1242/jeb.145615>
- Thies, A.B., Quijada-rodriguez, A.R., Zhouyao, H., Weihrauch, D., Tresguerres, M., 2022. A Rhesus channel in the coral symbiosome membrane suggests a novel mechanism to regulate NH₃ and CO₂ delivery to algal symbionts. *Sci. Adv.* 8, 1–11.
- Thurber, A.R., Jones, W.J., Schnabel, K., 2011. Dancing for food in the deep sea: Bacterial farming by a new species of Yeti crab. *PLoS One* 6, 1–12. <https://doi.org/10.1371/journal.pone.0026243>

- Torday, J.S., 2015. Homeostasis as the mechanism of evolution. *Biology (Basel)*. 4, 573–590.
<https://doi.org/10.3390/biology4030573>
- Toulmond, A., Lallier, F.H., de Frescheville, J., Childress, J.J., Lee, R., Sanders, N.K.,
Desbruyères, D., 1994. Unusual carbon dioxide-combining properties of body fluids in the
hydrothermal vent tubeworm *Riftia pachyptila*. *Deep. Res. Part I Oceanogr. Res. Pap.* 41,
1447–1456. [https://doi.org/10.1016/0967-0637\(94\)90054-X](https://doi.org/10.1016/0967-0637(94)90054-X)
- Towle, D.W., Holleland, T., 1987. Ammonium ion substitutes for K⁺ in ATP-dependent Na⁺
transport by basolateral membrane vesicles. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.*
252. <https://doi.org/10.1152/ajpregu.1987.252.3.r479>
- Towle, D.W., Rushton, M.E., Heidysch, D., Magnani, J.J., Rose, M.J., Amstutz, A., Jordan,
M.K., Shearer, D.W., Wu, W.S., 1997. Sodium/proton antiporter in the euryhaline crab
Carcinus maenas: Molecular cloning, expression and tissue distribution. *J. Exp. Biol.* 200,
1003–1014.
- Towle, D.W., Weihrauch, D., 2001. Osmoregulation by gills of euryhaline crabs: Molecular
analysis of transporters. *Am. Zool.* 41, 770–780. <https://doi.org/10.1093/icb/41.4.770>
- Tresguerres, M., Parks, S.K., Sabatini, S.E., Goss, G.G., Luquet, C.M., 2008. Regulation of ion
transport by pH and [HCO₃⁻] in isolated gills of the crab *Neohelice (Chasmagnathus)*
granulata. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 294, 1033–1043.
<https://doi.org/10.1152/ajpregu.00516.2007>
- Tripp, A., Allen, G.J.P., Quijada-Rodriguez, A.R., Yoon, G.R., Weihrauch, D., 2022. Effects of
single and dual-stressor elevation of environmental temperature and PCO₂ on metabolism
and acid-base regulation in the Louisiana red swamp crayfish, *Procambarus clarkii*. *Comp.*
Biochem. Physiol. Part A Mol. Integr. Physiol. 266, 111151.
- Truchot, J.-P., 1992. Acid-base changes on transfer between sea- and freshwater in the Chinese
crab, *Eriocheir sinensis*. *Respir. Physiol.* 87, 419–427.
- Truchot, J.-P., 1988. Problems of acid-base balance in rapidly changing intertidal environments.
Am. Zool. 28, 55–64. <https://doi.org/10.1093/icb/28.1.55>
- Truchot, J.-P., 1981. The effect of water salinity and acid-base status on the blood acid-base

- balance in the euryhaline crab, *Carcinus maenas* (L.). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 68, 555–561.
- Truchot, J.-P., 1975a. Blood acid-base changes during experimental emersion and reimmersion of the intertidal crab *Carcinus maenas* (L.). *Respir. Physiol.* 23, 351–360.
[https://doi.org/10.1016/0034-5687\(75\)90086-9](https://doi.org/10.1016/0034-5687(75)90086-9)
- Truchot, J.-P., 1975b. Effects of hypercapnia on the acid base states of the blood in the crab *Carcinus maenas* (L.) (Crustacea - Decapoda). *C. R. Acad. Hebd. Seances Acad. Sci D.* 280, 311–314.
- Truchot, J.-P., Duhamel-Jouve, A., 1980. Oxygen and carbon dioxide in the marine intertidal environment: Diurnal and tidal changes in rockpools. *Respir. Physiol.* 39, 241–254.
[https://doi.org/10.1016/0034-5687\(80\)90056-0](https://doi.org/10.1016/0034-5687(80)90056-0)
- Truchot, J., 1986. Changes in the hemolymph acid-base state of the shore crab, *Carcinus maenas*, exposed to simulated tidepool conditions. *Biol. Bull.* 170, 506–518.
- Truchot, J., 1983. Regulation of acid-base balance, in: Bliss, D.E. (Ed.), *The Biology of Crustacea*. Academic Press, New York, pp. 431–457.
- Truchot, J., 1979. Mechanisms of the compensation of blood respiratory acid-base disturbances in the shore crab, *Carcinus maenas* (L.). *J. Exp. Zool.* 210, 407–416.
<https://doi.org/10.1002/jez.1402100305>
- Truchot, J., 1978. Mechanisms of extracellular acid-base regulation as temperature changes in decapod crustaceans. *Respir. Physiol.* 33, 161–176.
- Truchot, J., 1976. Carbon dioxide combining properties of the blood of the shore crab *Carcinus maenas* (L.): carbon dioxide solubility coefficient and carbonic acid dissociation constants. *J. Exp. Biol.* 64, 45–57.
- Truchot, J., 1975. Changements De L'Etat Acide Base Du Sang En Fonction De L'Oxygenation De L'Eau Chez Le Crabe, *Carcinus Maenas* (L.). *J. Physiol. (Paris)*. 70, 583–592.
- Truchot, J., 1973. Temperature and acid-base regulation in the shore crab *Carcinus maenas* (L.). *Respir. Physiol.* 17, 11–20. [https://doi.org/10.1016/0034-5687\(73\)90106-0](https://doi.org/10.1016/0034-5687(73)90106-0)

- Tsai, J.R., Lin, H.C., 2014. Functional anatomy and ion regulatory mechanisms of the antennal gland in a semi-terrestrial crab, *Ocypode stimpsoni*. *Biol. Open* 3, 409–417.
<https://doi.org/10.1242/bio.20147336>
- Tsai, J.R., Lin, H.C., 2007. V-type H⁺-ATPase and Na⁺,K⁺-ATPase in the gills of 13 euryhaline crabs during salinity acclimation. *J. Exp. Biol.* 210, 620–627.
<https://doi.org/10.1242/jeb.02684>
- Tseng, K.Y., Tsai, J.R., Lin, H.C., 2020. Ion regulation in the antennal glands differs among Ocypodoidea and Grapsoidea crab species. *Comp. Biochem. Physiol. A* 248, 110753.
<https://doi.org/10.1016/j.cbpa.2020.110753>
- Van Dover, C.L., German, C.R., Speer, K.G., Parson, L.M., Vrijenhoek, R.C., 2002. Evolution and biogeography of deep-sea vent and seep invertebrates. *Science* (80-.). 295, 1253–1257.
<https://doi.org/10.1126/science.1067361>
- Varley, D.G., Greenaway, P., 1994. Nitrogenous excretion in the terrestrial carnivorous crab *Geograpsus grayi*: Site and mechanism of excretion. *J. Exp. Biol.* 190, 179–193.
- Veilleux, E., De Lafontaine, Y., 2007. Biological synopsis of the Chinese mitten crab (*Eriocheir sinensis*). *Can. Manusc. Rep. Fish. Aquat. Sci.* 2812, vi–45.
- Wang, T.W., Chan, T.Y., Chan, B.K.K., 2014. Trophic relationships of hydrothermal vent and non-vent communities in the upper sublittoral and upper bathyal zones off Kueishan Island, Taiwan: a combined morphological, gut content analysis and stable isotope approach. *Mar. Biol.* 161, 2447–2463. <https://doi.org/10.1007/s00227-014-2479-6>
- Wang, T.W., Chan, T.Y., Chan, B.K.K., 2013. Diversity and community structure of decapod crustaceans at hydrothermal vents and nearby deep-water fishing grounds off Kueishan Island, Taiwan: A high biodiversity deep-sea area in the NW Pacific. *Bull. Mar. Sci.* 89, 505–528. <https://doi.org/10.5343/bms.2012.1036>
- Watson-Zink, V.M., 2021. Making the grade: Physiological adaptations to terrestrial environments in decapod crabs. *Arthropod Struct. Dev.* 64, 101089.
<https://doi.org/10.1016/j.asd.2021.101089>
- Weihrauch, D., Allen, G.J.P., 2018. Ammonia excretion in aquatic invertebrates: New insights

- and questions. *J. Exp. Biol.* 221. <https://doi.org/10.1242/jeb.169219>
- Weihrauch, D., Becker, W., Postel, U., Luck-Kopp, S., Siebers, D., 1999. Potential of active excretion of ammonia in three different haline species of crabs. *J. Comp. Physiol. - B Biochem. Syst. Environ. Physiol.* 169, 25–37. <https://doi.org/10.1007/s003600050190>
- Weihrauch, D., Becker, W., Postel, U., Riestenpatt, S., Siebers, D., 1998. Active excretion of ammonia across the gills of the shore crab *Carcinus maenas* and its relation to osmoregulatory ion uptake. *J. Comp. Physiol. B* 168, 364–376. <https://doi.org/10.1007/s003600050156>
- Weihrauch, D., Fehsenfeld, S., Quijada-Rodriguez, A.R., 2017. Nitrogen Excretion in Aquatic Crustaceans, in: Weihrauch, D., O'Donnell, M.J. (Eds.), *Acid-Base Balance and Nitrogen Excretion in Invertebrates*. Springer, Gewerbestrasse, pp. 2–21.
- Weihrauch, D., McNamara, J.C., Towle, D.W., Onken, H., 2004a. Ion-motive ATPases and active, transbranchial NaCl uptake in the red freshwater crab, *Dilocarcinus pagei* (Decapoda, Trichodactylidae). *J. Exp. Biol.* 207, 4623–4631. <https://doi.org/10.1242/jeb.01333>
- Weihrauch, D., Morris, S., Towle, D.W., 2004b. Ammonia excretion in aquatic and terrestrial crabs. *J. Exp. Biol.* 207, 4491–4504. <https://doi.org/10.1242/jeb.01308>
- Weihrauch, D., Wilkie, M.P., Walsh, P.J., 2009. Ammonia and urea transporters in gills of fish and aquatic crustaceans. *J. Exp. Biol.* 212, 1716–1730. <https://doi.org/10.1242/jeb.024851>
- Weihrauch, D., Ziegler, A., Siebers, D., Towle, D.W., 2002. Active ammonia excretion across the gills of the green shore crab *Carcinus maenas*: Participation of Na⁺/K⁺-ATPase, V-type H⁺-ATPase and functional microtubules. *J. Exp. Biol.* 205, 2765–2775.
- Weihrauch, D., Ziegler, A., Siebers, D., Towle, D.W., 2001. Molecular characterization of V-type H⁺-ATPase (B-subunit) in gills of euryhaline crabs and its physiological role in osmoregulatory ion uptake. *J. Exp. Biol.* 204, 25–37.
- Weiner, I.D., Verlander, J.W., 2013. Renal ammonia metabolism and transport. *Compr. Physiol.* 3, 201–220. <https://doi.org/10.1002/cphy.c120010>

- Weiner, I.D., Verlander, J.W., 2011. Role of NH₃ and NH₄⁺ transporters in renal acid-base transport. *Am. J. Physiol. - Ren. Physiol.* 300, F11–F23.
<https://doi.org/10.1152/ajprenal.00554.2010>
- Wheatly, M.G., 1987. Physiological responses of the rock crab *Cancer irroratus* (Say) to environmental hyperoxia. I. Acid-base regulation. *Physiol. Zool.* 60, 398–405.
<https://doi.org/10.1086/physzool.60.4.30157901>
- Wheatly, M.G., 1985. The role of the antennal gland in ion and acid-base regulation during hyposaline exposure of the Dungeness crab *Cancer magister* (Dana). *J. Comp. Physiol.* 155, 445–454.
- Wheatly, M.G., Henry, R.P., 1992. Extracellular and Intracellular Acid-Base Regulation in Crustaceans. *J. Exp. Zool.* 263, 127–142.
- Wheatly, M.G., Zanotto, F.P., Hubbard, M.G., 2002. Calcium homeostasis in crustaceans: Subcellular Ca dynamics. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* 132, 163–178.
[https://doi.org/10.1016/S1096-4959\(01\)00520-6](https://doi.org/10.1016/S1096-4959(01)00520-6)
- Whiteley, N.M., 2011. Physiological and ecological responses of crustaceans to ocean acidification. *Mar. Ecol. Prog. Ser.* 430, 257–271. <https://doi.org/10.3354/meps09185>
- Whiteley, N.M., Naylor, J.K., Taylor, E.W., 1995. Extracellular and intracellular acid-base status in the freshwater crayfish *Austropotamobius pallipes* between 1 and 12°C. *J. Exp. Biol.* 198, 567–576.
- Whiteley, N.M., Scott, J.L., Breeze, S.J., McCann, L., 2001. Effects of water salinity on acid-base balance in decapod crustaceans. *J. Exp. Biol.* 204, 1003–1011.
<https://doi.org/10.1242/jeb.204.5.1003>
- Wieser, W., Schweizer, G., Hartenstein, R., 1969. Patterns in the release of gaseous ammonia by terrestrial isopods. *Oecologia* 3, 390–400. <https://doi.org/10.1007/BF00390384>
- Wilkie, M.P., 1997. Mechanisms of ammonia excretion across fish gills. *Comp. Biochem. Physiol. A* 118, 39–50. [https://doi.org/10.1016/S0300-9629\(96\)00407-0](https://doi.org/10.1016/S0300-9629(96)00407-0)
- Wilson, R.W., Wright, P.M., Munger, S., Wood, C.M., 1994. Ammonia excretion in freshwater

- rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: Lack of evidence for Na⁺/NH₄⁺ exchanger. *J. Exp. Biol.* 191, 37–58.
- Wittmann, A.C., Pörtner, H.O., 2013. Sensitivities of extant animal taxa to ocean acidification. *Nat. Clim. Chang.* 3, 995–1001. <https://doi.org/10.1038/nclimate1982>
- Wolcott, D.L., 1991. Nitrogen excretion is enhanced during urine recycling in two species of terrestrial crab. *J. Exp. Zool.* 259, 181–187. <https://doi.org/10.1002/jez.1402590206>
- Wolcott, T.G., 1992. Water and solute balance in the transition to land. *Am. Zool.* 32, 428–437. <https://doi.org/10.1093/icb/32.3.428>
- Wolfe, J.M., Breinholt, J.W., Crandall, K.A., Lemmon, A.R., Lemmon, E.M., Timm, L.E., Siddall, M.E., Bracken-Grissom, H.D., 2019. A phylogenomic framework, evolutionary timeline and genomic resources for comparative studies of decapod crustaceans. *Proc. R. Soc. B Biol. Sci.* 286, 20190079. <https://doi.org/10.1098/rspb.2019.0079>
- Wood, C.M., Boutilier, R.G., 1985. Osmoregulation, ionic exchange, blood chemistry, and nitrogenous waste excretion in the land crab *Cardisoma carnifex*: A field and laboratory study. *Biol. Bull.* 169, 267–290. <https://doi.org/10.2307/1541403>
- Wood, C.M., Boutilier, R.G., Randall, D.J., 1986. The physiology of dehydration stress in the land crab, *Cardisoma carnifex*: Respiration, ionoregulation, acid-base balance and nitrogenous waste excretion. *J. Exp. Biol.* 126, 271–296.
- Wood, C.M., Cameron, J.N., 1985. Temperature and the physiology of intracellular and extracellular acid-base regulation in the blue crab *Callinectes sapidus*. *J. Exp. Biol.* 114, 151–179. <https://doi.org/10.1242/jeb.114.1.151>
- Wood, C.M., Rogano, M.S., 1986. Physiological responses to acid stress in crayfish (*Orconectes*): Haemolymph ions, acid-base status, and exchanges with the environment. *Can. J. Fish. Aquat. Sci.* 43, 1017–1026.
- Woodward, G., Perkins, D.M., Brown, L.E., 2010. Climate change and freshwater ecosystems: Impacts across multiple levels of organization. *Philos. Trans. R. Soc. B Biol. Sci.* 365, 2093–2106. <https://doi.org/10.1098/rstb.2010.0055>

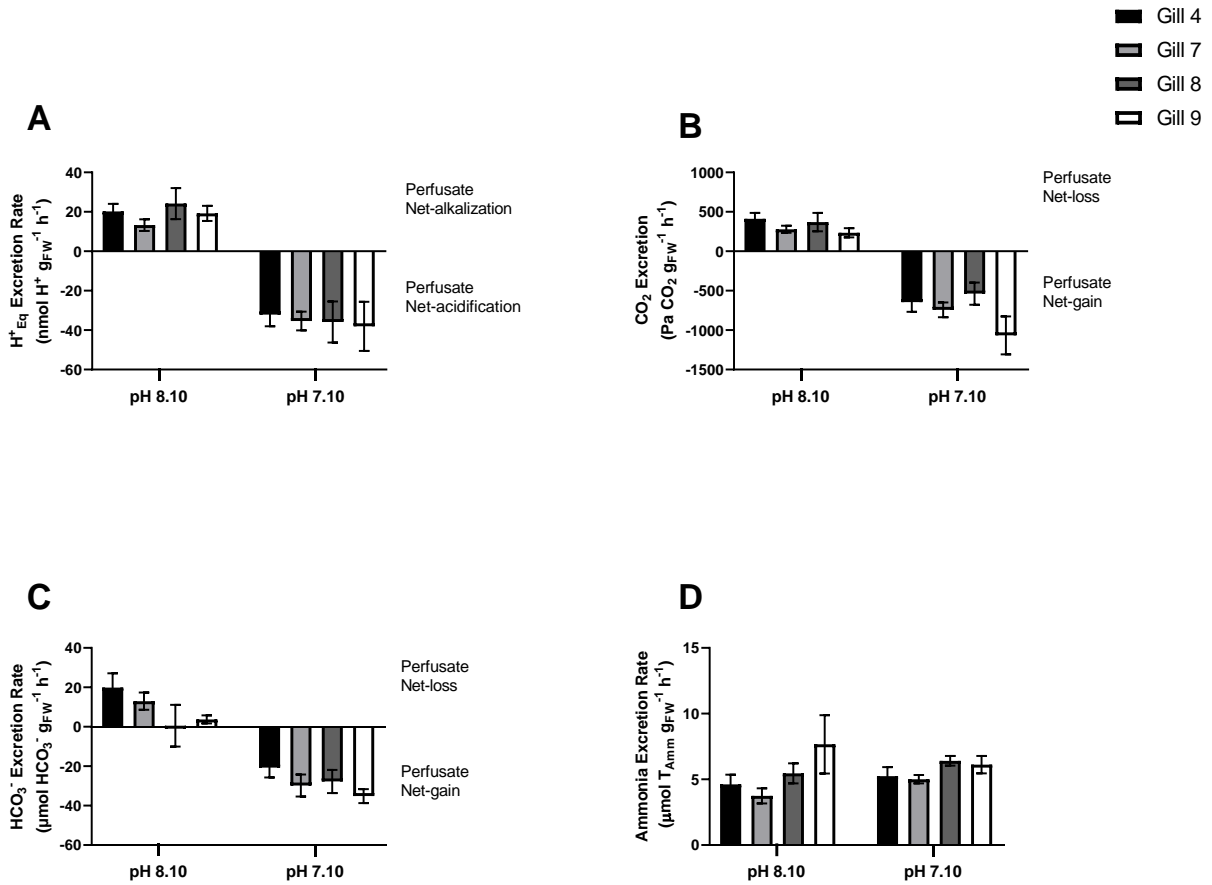
- Wright, J.C., O'Donnell, M.J., 1993. Total ammonia concentration and pH of haemolymph, pleon fluid and maxillary urine in *Porcellio scaber* Latreille (Isopoda, Oniscidea): Relationships to ambient humidity and water vapour uptake. *J. Exp. Biol.* 176, 233–246.
- Wright, J.C., O'Donnell, M.J., 1992. Osmolality and electrolyte composition of pleon fluid in *Porcellio scaber* (Crustacea, Isopoda, Oniscidea): Implications for water vapour absorption. *J. Exp. Biol.* 164, 189–203.
- Wright, J.C., Peña-Peralta, M., 2005. Diel variation in ammonia excretion, glutamine levels, and hydration status in two species of terrestrial isopods. *J. Comp. Physiol. B* 175, 67–75.
<https://doi.org/10.1007/s00360-004-0463-z>
- Wright, P.A., Wood, C.M., 2012. Seven things fish know about ammonia and we don't. *Respir. Physiol. Neurobiol.* 184, 231–240. <https://doi.org/10.1016/j.resp.2012.07.003>
- Wright, P.A., Wood, C.M., 2009. A new paradigm for ammonia excretion in aquatic animals : role of Rhesus (Rh) glycoproteins. *J. Exp. Biol.* 212, 2303–2312.
<https://doi.org/10.1242/jeb.023085>
- Wright, P.A., Wood, C.M., 1985. An analysis of branchial ammonia excretion in the freshwater rainbow trout: Effects of environmental pH change and sodium uptake blockade. *J. Exp. Biol.* 114, 329–353. <https://doi.org/10.1242/jeb.114.1.329>
- Xu, X., Yang, F., Zhao, L., Yan, X., 2016. Seawater acidification affects the physiological energetics and spawning capacity of the Manila clam *Ruditapes philippinarum* during gonadal maturation. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* 196, 20–29.
<https://doi.org/10.1016/j.cbpa.2016.02.014>
- Yang, L., Hong, H., Guo, W., Chen, C.T.A., Pan, P.I., Feng, C.C., 2012. Absorption and fluorescence of dissolved organic matter in submarine hydrothermal vents off NE Taiwan. *Mar. Chem.* 128–129, 64–71. <https://doi.org/10.1016/j.marchem.2011.10.003>
- Yang, S.H., Chiang, P.W., Hsu, T.C., Kao, S.J., Tang, S.L., 2016. Bacterial community associated with organs of shallow hydrothermal vent crab *Xenograpsus testudinatus* near Kuishan Island, Taiwan. *PLoS One* 11, 1–13. <https://doi.org/10.1371/journal.pone.0150597>
- Young-Lai, W., Charmantier-Daures, M., Charmantier, G., 1991. Effect of ammonia on survival

and osmoregulation in different life stages of the lobster *Homarus americanus*. *Mar. Biol.* 110, 293–300. <https://doi.org/10.1007/BF01313716>

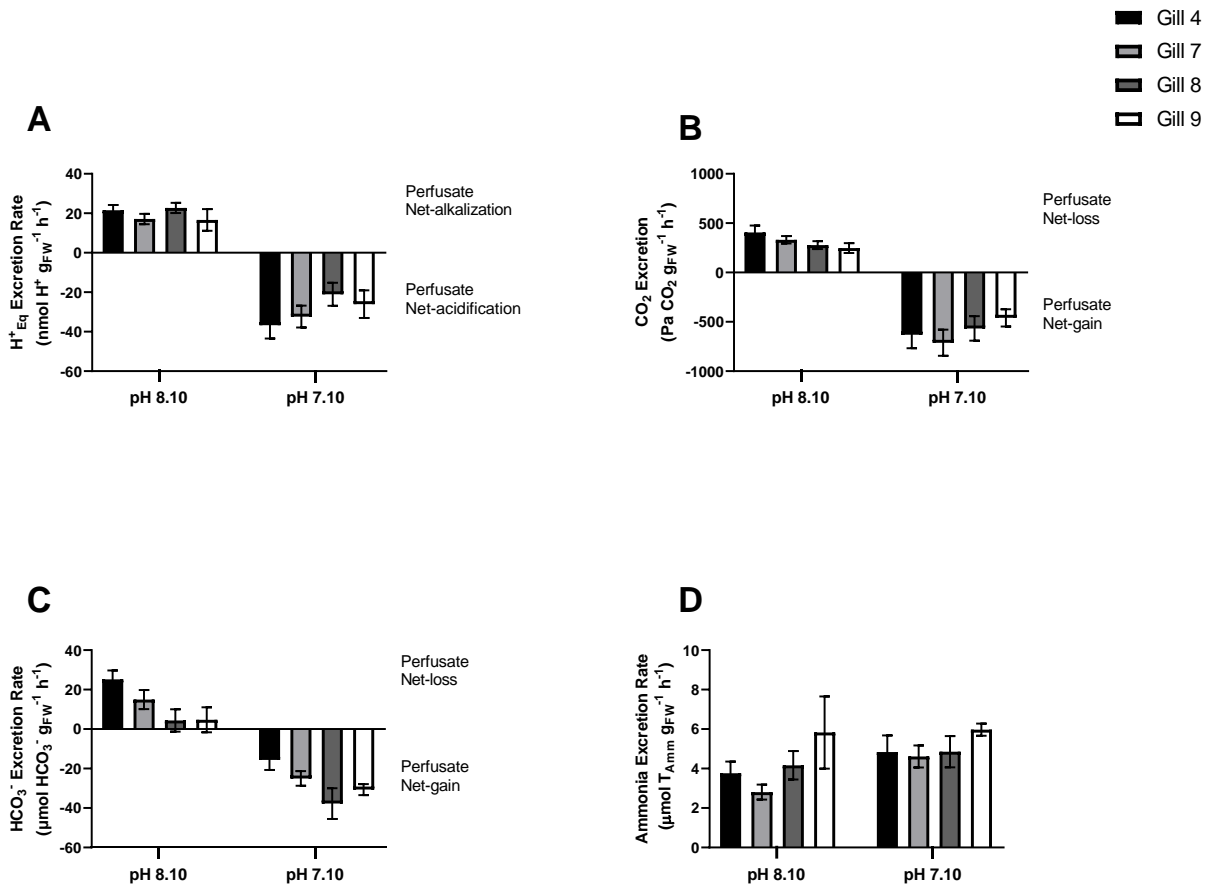
Zanotto, F.P., Wheatly, M.G., 1993. The effect of ambient pH on electrolyte regulation during the postmoult period in freshwater crayfish *Procambarus clarkii*. *J. Exp. Biol.* 178, 1–19.

Zatta, P., 1987. Dopamine, noradrenaline and serotonin during hypo-osmotic stress of *Carcinus maenas*. *Mar. Biol.* 96, 479–481. <https://doi.org/10.1007/BF00397965>

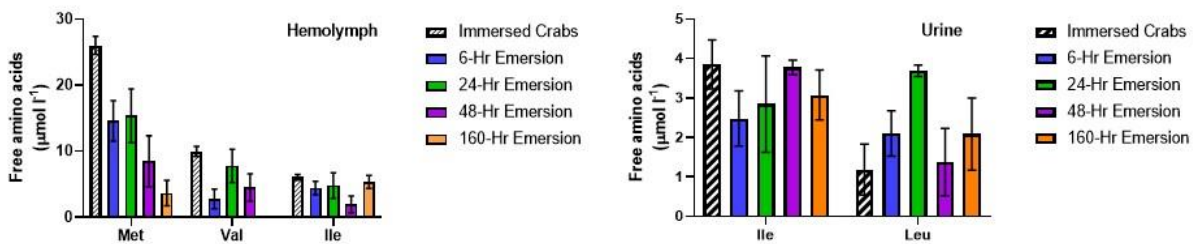
Appendix and Supplemental Information



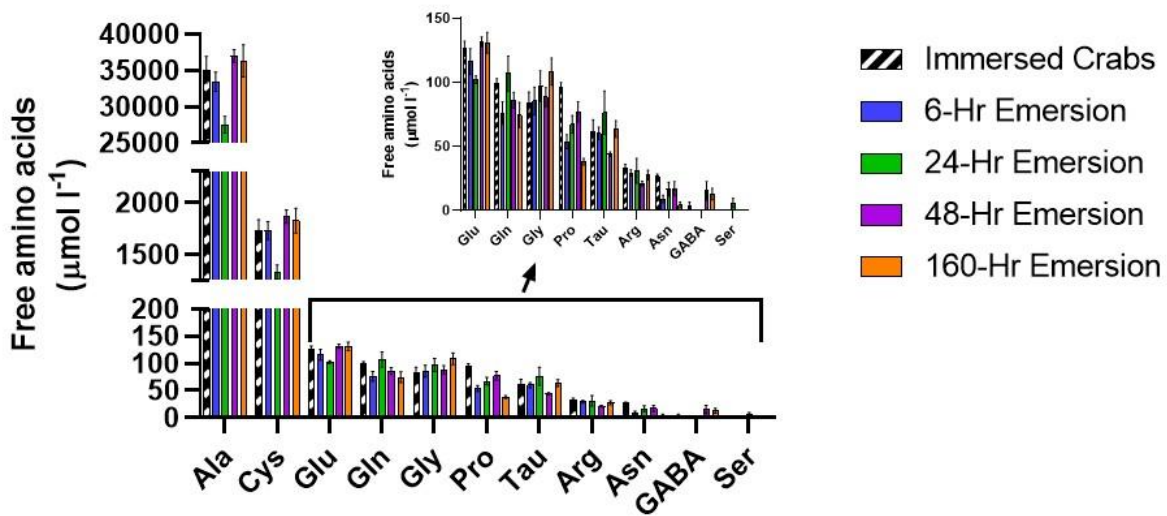
S.I. Figure 2.1. Transport rates of H⁺_{Eq} (A), CO₂ (B), HCO₃⁻ (C), and T_{Amm} (D) across the perfused gills 4, 7, 8, and 9 of *M. magister* bathed in HEPES-enriched seawater of either pH 8.10 or 7.10 containing 0 μmol l⁻¹ NH₄Cl.



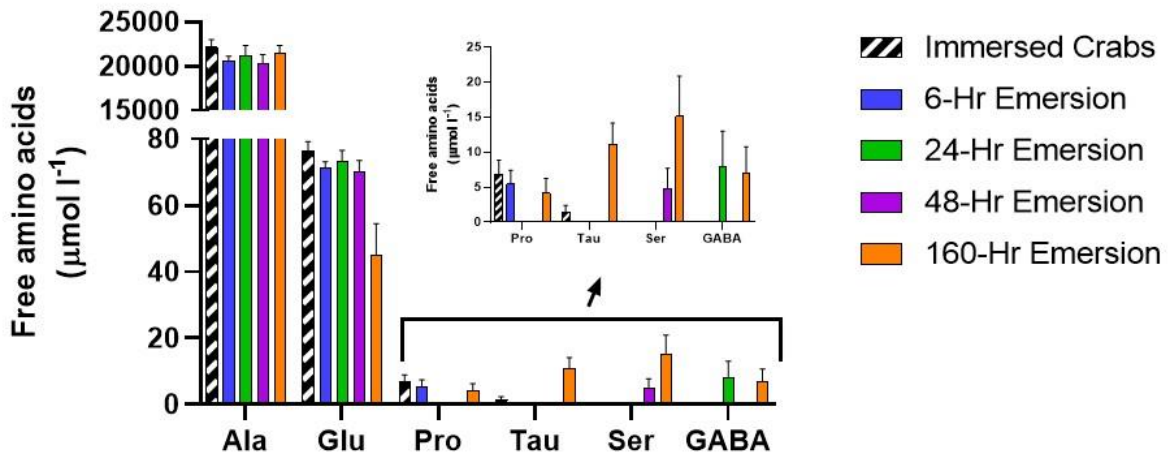
S.I. Figure 2.2. Transport rates of H⁺Eq (A), CO₂ (B), HCO₃⁻ (C), and T_{Amn} (D) across the perfused gills 4, 7, 8, and 9 of *M. magister* bathed in HEPES-enriched seawater of either pH 8.10 or 7.10 containing 200 μmol l⁻¹ NH₄Cl.



S.I. Figure 4.1. Hemolymph (N = 6–12) and urinary (N = 4–12) essential amino acid composition of immersed (0-h) and emersed crabs over a 160-hour time-course exposure. Where data is not available for urinary concentrations no detectable amino acid presence was found. Values are represented as the mean ± S.E.M.



S.I. Figure 4.2. Hemolymph (N = 6–12) non-essential amino acid composition of immersed (0-h) and emersed crabs over a 160-hour time-course exposure. Where data in not available for urinary concentrations no detectable amino acid presence was found. Values are represented as the mean \pm S.E.M.



S.I. Figure 4.3. Urinary (N = 4–12) non-essential amino acid composition of immersed (0-h) and emersed crabs over a 160-hour time-course exposure. Where data in not available for urinary concentrations no detectable amino acid presence was found. Values are represented as the mean \pm S.E.M.