Title
Living in a High CO2 World: Aquaculture and Fish that Breathe Air

Author
Jew, Corey J

Publication Date
2019

UNIVERSITY OF CALIFORNIA,
IRVINE

Living in a High CO_2 World:

Aquaculture and Fish that Breathe Air

DISSERTATION

submitted in partial satisfaction of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in Ecology and Evolutionary Biology

BY

Corey J. Jew

Dissertation Committee:
Professor James W. Hicks, Chair
Professor Timothy J. Bradley
Associate Professor Donovan P. German

2019

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

First, I would like to thank Dr. James W. Hicks, for welcoming me into the UCI family, allowing me to turn the reptile lab to another fish lab, extending your "opportunity fund" to peruse my research curiosity, and providing guidance, international connections, and expertise in reviving old equipment and surgical techniques. In the first two years at UCI, your advice was to take a free trip to Scotland, Vietnam, Brazil, Denmark, and Sweden to try to learn something new, and I have never been so grateful and exhausted. I've learned immensely from our numerous one-on-one lab meetings that spanned your "never say no to opportunity" to "learn when to say no" stages of advice giving. Thank you.

Second, thank you to Dr. Donovan German and Dr. Timothy Bradley for serving as my committee member and sitting through my talks for the past five and a half years, providing invaluable guidance and encouragement, and seeing this dissertation to its end.

The highlights of my graduate career were the opportunities to work and live in numerous place across the globe with some of the most welcoming colleagues that made sure I always felt at home away from home. Dr. Mark Bayley, who's laugh could be heard across the entirely of Can Tho University, was not only responsible for making my tour of Vietnam possible, but also extending a generous invitation to spend three months in his home laboratory at Aarhus University in Denmark. Gam Le and Tuong Diem who I cannot thank enough for countless scooter rides and hotpot nights. And Mikkel Thomsen for the countless hours we spent cleaning fish tanks in the vivarium basements and for introducing me to Danish cake diversity.

Thank you to the many undergraduate assistants over the years in taking over fish care duties and even whole experiments in some cases. It would not have been possible to finish over 30 surgeries (3 a day, 3 days a week) if not for the hard work and dedication of Mourad Lamely, Elissa Vong, and Angelina Ball.

In addition, I would like to take friends and family. My parents and brothers and sister in-law, coworkers, old college roommates, new climber friends, and everyone in between, thank you for the support, encouragement, great times, and distractions away from work.

Lastly, I would like to thank my funding sources. The American Physiological Society who covered to the cost of travel for me and many other students to attend the 2014 inaugural course: Physiology of Air-Breathing Fish in the Mekong Delta, Vietnam: Basic, Applied and Conservation in Vietnam. The Journal of Experimental Biology - Company of Biologist Traveling Fellowship (JEBTF-150426) which provided housing and travel funds for my summer at Aarhus University, Aarhus Denmark. The Graduate Dean's Dissertation Fellowship which allowed to focus on finishing my final chapter during Summer and Fall 2018. And of course the Department of Ecology and Evolutionary Biology which provided Teaching Assistantships throughout my tenure at UCI and travel funds to present at conferences across the country.

Thank you all for your contributions to this work and my graduate experience.

CURRICULUM VITAE

Corey J. Jew

2013-2019	PhD. in Ecology and Evolutionary Biology University of California, Irvine
2010-2013	MSc. in Marine Sciences Scripps Institution of Oceanography University of California, San Diego
2005-2009	B.S. in General Biology University of California, San Diego

FIELD OF STUDY

Ecology and Evolutionary Biology

PUBLICATIONS

Graham J.B., Jew C.J., Wegner N.C., Modeling variable Phanerozoic oxygen effects on physiology and evolution. In Roach, R.C., Wagner, P.D., Hackett, P.H. (Eds.) *Hypoxia: Translation in Progress,* Advances in Experimental Medicine and Biology 903.

Graham J., Wegner N., Miller L., Jew C., Lai N., Berquist R., Lawrence F., Long J. (2014) Spiracular air breathing in polypterid fishes and its implications for aerial respiration in stem tetrapods. *Nature Communications* 5: 3022.

Jew, C. J., Wegner, N. C., Yanagitsuru, Y., Tresguerres, M. & Graham, J.B. (2013). Atmospheric oxygen levels affect mudskipper terrestrial performance: Implications for early tetrapods. *Integrative and Comparative Biology* 53: 248–257.

ABSTRACT OF THE DISSERTATION

Living in a High CO_2 World:
Aquaculture and Fish that Breathe Air

By

Corey J. Jew

Doctor of Philosophy in Ecology and Evolutionary Biology

University of California, Irvine, 2019

Professor James W. Hicks, Chair

Over the past two decades, global aquaculture production has seen significant growth, particularly in Vietnam with the use of air-breathing fishes. However, the sudden economic importance of air-breathing species, such as *Pangasianodon hypophthalmus*, has resulted in a significant knowledge gap surrounding their basic biology. A fundamental understanding of how these animals work and how they interact with their environment is vital for the establishment of data-driven policies and best practice and essential for optimizing their growth and production. The aim of this work is to provide the aquaculture industry with the knowledge base necessary to fulfill it's potential in improving food security and meeting the sustainable development goals of our planet in the 21st century and beyond.

The content of this dissertation consists of several studies characterizing different aspects of adapting, both behaviorally and physiologically, to aquatic hypercapnia and are as follows: Chapter 1 explores the effect on pulmonary respiration when brachial CO_2 exchange is disrupted either though aquatic hypercapnia or forced emersion in *Polypterus lapradei*. Chapter 2 compares how air-breathing is used differently in three Vietnamese aquaculture species when encountered with aquatic hypoxia and hypercapnia. Chapter 3 investigates the role of ecophysiology on the use of air-breathing in the avoidance behaviors to either hypoxia and hypercapnia in *Po. lapradei* and *Pa. hypophthalmus*. Chapter 4 uses blood stress bioindicators to assess if hypercapnia levels in *Pa. hypophthalmus* aquaculture ponds

represent a significant physiological challenge. We found *Pa. hypophthalmus* does not respond from a respiratory, behavioral, or blood biomarker perspective when exposed to levels of hypercapnia typically found in aquaculture (34 mmHg CO_2). This demonstrates the extreme tolerance of *Pa. hypophthalmus* to aquatic hypercapnia acquired though millions of years of evolution to its natural environment, as well as suggest a lack of potential benefit for regulating CO_2 in aquaculture ponds.

Much of this research focuses on *P. hypophthalmus* due to its central role in aquaculture, yet several other air-breathing fish species were used as a comparative approach to investigate how uniquely evolved respiratory systems respond differently. My hope is that that is work can provide scientific data informing the impact of hypercapnia in aquaculture practices, and at the same time, demonstrate the diversity of evolutionary solutions to an environmental challenge that exist among a fascinating group of organisms, the air-breathing fishes.

INTRODUCTION

Importance of seafood and fisheries

Seafood and seafood products make up a significant contribution to the global food supply and world economy. According the FAO's State of the World Fisheries and Aquaculture 2018 Report, 171 million tons are produced globally, driving $152 billion in trade and providing income for millions, particularly in developing countries. Of that, human consumption accounts for 151 million tons (88%), which is a per capita seafood consumption of 45 lbs. per year. Our dependence on and demand for seafood continues to grow as the health benefits of seafood are becoming realized, global populations soar, and emerging economies drive the transition from plant to animals-based diets across developing world.

As demand for seafood continues to accelerate, the state of the world fisheries has been in decline, according to the FAO's monitoring of marine fish stocks. The fraction of stocks harvested at or below their maximally sustainable rate dove from 90% in 1974 to 66.7% in 2015, while the fraction of stocks classified as overfished rose from 10% to 33.1% over the same period. As a result, world-wide production from wild capture fisheries has plateaued since the 1990s.

Blue revolution

Despite the stagnation of wild capture fisheries, aquaculture production has seen significant and sustained growth in the recent half century, bridging the world's expanding gap in supply and demand for seafood products. This expansion in farming fish and other seafood products beginning in the late 1960s, termed the "Blue Revolution", has continued to accelerate, undergoing double-digit annual growth through the 1980s and 1990s. In 2016, for the first time food fish consumption from aquaculture surpassed global wild capture fisheries, and today aquaculture remains the fasting growing major food production sector (FAO, 2018).

In the West aquaculture is typically thought of as the cultivation of salmonids (trout and salmon), in ocean sea pens and concrete raceways. Salmonids are the dominant crop species of many countries with temperate climates, however the use of these species require cool temperatures and clear water, making them unfeasible for production in tropical regions. Nevertheless, countries with tropical climates, such as Vietnam, have seen recent exponential growth over the past two decades utilizing native species adapted to local conditions. The successful adoption of these unconventional species into the aquaculture industry has driven Vietnam onto the global stage, creating $7.4 billion in exports in 2016. Vietnam is now the world's third largest exporter of fish products, generating more in aquaculture than Europe, Africa, or the Americas combined (FAO, 2016).

Much of Vietnam's export revenue is attributed to the farming of Pangas catfishes (*Pangasianodon spp*.) (FAO, 2016; FAO, 2018). Several members of this group possess the ability to breathe air, such as *Pangasianodon hypophthalmus*, as do a number of other Vietnamese aquaculture species endemic to this region. The use of these air-breathing fishes in aquaculture has risen along with the success of the industry in Southeast Asia due to their ability to thrive in less than optimal aquatic conditions (e.g., low O_2, high CO_2 and nitrogenous waste) (Lefevre et al., 2014). Air-breathing fishes, have evolved under these environmental conditions, typical of tropical freshwaters, and possess adaptations to these marginal aquatic habitats (Graham, 1997). The ability to breath air enables these fishes to access the atmosphere as an auxiliary source of O_2 when encountering environmental hypoxia (low O_2). This allows elevated aerobic activity and habitat use area, for foraging, predator evasion, reproduction, and survival, where other fishes may be more negatively impacted. Aquaculture ponds often mimic these marginal aquatic conditions due to their high stocking densities and intensive feeding rates. Use of these more tolerate fishes are thus naturally ideal for aquaculture, offering higher survivability and lower operational cost in water turnover and aeration of artificial ponds.

The knowledge gap and need for research

Although the recent wave of air-breathing fishes in aquaculture has elevated their importance to both the global food supply and economics, the body of scientific knowledge surrounding these species is still in its infancy. Countries developing the use of these species tend to be emerging economies without long-established research programs like those in the US, Europe, and Canada. To demonstrate this point, a literature search of Pubmed for articles published after 1997 on the genus *Pangasius* returns 127 results, while the same search on rainbow trout *(Oncorhynchus mykiss)*, a single species, returns 8117 articles. This lack of scientific research represents a significant knowledge gap in the understanding of the fundamental biology of these and other air-breathing fishes used in aquaculture. Scientific research is required for the establishment of evidence-based policies and best practices that can drive industry efficiency and productivity.

Case study – Hypoxia and *P. hypophthalmus*

An example of how research as altered the thinking about how environmental conditions effect aquaculture species is the impact of aquatic hypoxia on *P. hypophthalmus.* Starting in the 1980s, rural household scale farming of *P. hypophthalmus* in the Mekong Delta underwent vigorous expansion as a result of government policies aimed at promoting food security and supplemental income for primarily subsistence communities (Khiem et al., 2010). Many of these farms simply cultured juveniles in flooded rice patties and harvested both the fish and rice when draining the patties at the end of the season, a practice known as Rice-fish Culture, dating as far back as 900 AD (Halwart et al.). Despite the inability to monitor and control aquatic O_2 content in these ponds, the success of these systems, and the observation of these animal’s ability to breath air (first published by Browman and Kramer 1985) lead to the notion that aquatic O_2 was not an issue affecting their cultivation. As large scale commercial facilities replaced rural farms, making up 10% aquaculture production 2011 and 70% in 2016, the assumption that oxygenation of these ponds was not beneficial above the level necessary for survival persisted with little scientific investigation (The World Bank, 2016). In 2014, (Lefevre et al., 2014) published findings that showed *P. hypophthalmus* raised in O_2 saturated aquatic conditions resulted in an elevated growth rate and decreased food conversation ratio (less food require to increase body mass). While further laboratory and pond scale studies are necessary to evaluate the potential benefits of regulating aquatic O_2, this example highlights the importance of understanding the fundamental biology of *P. hypophthalmus* and other aquaculture species in establishing optimal growth conditions. With the transition from Vietnam’s rural household ponds to modern industrial aquaculture farms, aquatic conditions can now be monitored, regulated, and standardized, but first these conditions must be defined.

Objective of this research

This research seeks to contribute scientific data toward understanding the environmental effects of CO_2 on air-breathing fishes and provide recommendations on the use of CO_2 monitoring and control in aquaculture. High CO_2 (hypercapnia) in aquatic habitats is created through the same process as hypoxia, biological respiration, typically by a high density of aquatic organisms and decomposition of submerged organic material. Aquatic hypercapnia has been overlooked in the past because of CO_2’s relatively high solubility compared to that of O_2. Because CO_2 is roughly 30 times more soluble in water than O_2, it has been assumed that hypoxic stress will always become limiting before CO_2 can reach harmful levels. However, hypercapnia has been found to accumulate in natural environments, particularly in tropical bodies of freshwater which frequently exhibit turbidity, high organic loading, and stagnation (Ultsch, 1996; Willmer, 1934). These three factors create hypoxic/hypercapnic environments through little photosynthetic activity, high rates of bacterial respiration and anaerobiosis, and low mixing with the atmosphere. Anthropogenic influences can also produce hypoxic/hypercapnic environments by eutrophication from agricultural runoff, burning of fossil fuels, resequestration and injection of atmospheric CO_2 into deep ocean sites, and aquaculture (Brauner and Baker, 2009; Damsgaard et al., 2015; Ishimatsu et al., 2005). Hypercapnia in aquaculture, created by high stocking densities and feeding rates, is often exacerbated by the practice of pure O_2 injection (Hu et al., 2011; Lefevre et al., 2014). In this practice, 100% O_2 gas is infused into the water to meet the aerobic demands of growing fish, yet result in an aeration rate that is insufficient in removing the associated metabolically produced CO_2.

Biological effects of CO_2

In general, physiological effects of hypercapnia in fishes have been well studied. During exposure, the diffusion gradient by which CO_2 is normally released at the gills is reduced (or even reversed if hypercapnia is high enough), and a respiratory acidosis occurs (Brauner and Baker, 2009). To reestablish acid-base homeostasis, bicarbonate is accumulated through branchial ion exchange. The maintenance of new cellular ionic gradients has been shown to be energetically costly and result in long-term consequences associated with chronic stress, effecting growth, metabolism, food conversion, and survival (Crocker and Cech Jr., 1996; Danley et al., 2005; Hu et al., 2011; Lefevre et al., 2014; Petochi et al., 2011; Ross et al., 2001).

Relatively less work however has been done on the effects of hypercapnia in air-breathing fishes or their tolerance thresholds (Shartau and Brauner, 2014). Air-breathing fishes are inherently different because of their dual respiratory system and natural inhabitance of aquatic hypercapnic environments. For example, air-breathing fishes generally rely less on aquatic respiration, and as a result, their blood is considered chronically hypercapnic compared to their solely water breathing counterparts. Air-breathing fish have also been observed to insulate themselves from exposure to aquatic conditions with reduced gill surface area and by decreasing branchial ventilation (Ch.1, (Burggren and Haswell, 1979; Graham, 1997;

Milsom, 2012; Sanchez et al., 2005). Reduce gill surface area has also been suggested to impact the rate of ion exchange that can occur during hypercapnic acid-base compensation (Brauner and Baker, 2009). The response of hypercapnia on different species of air-breathing fishes are quite diverse as this group represent 65 independent evolutions of air-breathing systems spanning a spectrum of air and water breathing and amphibious capacities. Further, while thresholds of CO_2 tolerance have been determined for species such as rainbow trout, the same have not been established for any air-breathing species. According to Ross et al. 2001, aquatic PCO_2 should remain at 2 mmHg for maintaining healthy populations of rainbow trout (*Oncorhynchus mykiss*), while above 8 mmHg becomes harmful for survival. In contrast, (Damsgaard et al., 2015) found commercial *P. hypophthalmus* aquaculture ponds regularly experience 34 mmHg CO_2. While the productivity of these commercial aquaculture ponds illustrates the extreme hypercapnic tolerance of *P. hypophthalmus*, whether these fish are merely tolerating or thriving under these conditions remains unknown, as well as if regulating PCO_2 at a lower level has the potential to provide additional benefits to the health of these animals.

Summary of chapters

The content of this dissertation consists of several studies characterizing different aspects of adapting, both behaviorally and physiologically, to aquatic hypercapnia and are as follows: Chapter 1 explores the effect on pulmonary respiration when brachial CO_2 exchange is disrupted either though aquatic hypercapnia or forced emersion in *Polypterus lapradei*. Chapter 2 compares how air-breathing is used differently in three Vietnamese aquaculture species when encountered with aquatic hypoxia and hypercapnia. Chapter 3 investigates the role of ecophysiology on the use of air-breathing in the avoidance behaviors to either hypoxia and hypercapnia in *Polypterus* and *P. hypophthalmus*. Chapter 4 uses blood stress bioindicators to assess if hypercapnia levels in *P. hypophthalmus* aquaculture ponds represent a significant physiological challenge. Much of this research focuses on *P. hypophthalmus* due to its central role in aquaculture, yet several other air-breathing fish species were used as a comparative approach to investigate how uniquely evolved respiratory systems respond differently. My hope is that that is work can provide scientific data informing the impact of hypercapnia in aquaculture practices, and at the same time, demonstrate the diversity of evolutionary solutions to an environmental challenge that exist among a fascinating group of organisms, the air-breathing fishes.

Brauner, C. J. and Baker, D. W. (2009). Patterns of acid–base regulation during exposure to hypercarbia in fishes. In *Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects*, pp. 43–63.

Burggren, W. and Haswell, S. (1979). Aerial CO2 excretion in the obligate air breathing fish *Trichogaster trichopterus*: A role for carbonic anhydrase. *J. Exp. Biol.* **82**, 215–225.

Crocker, C. E. and Cech Jr., J. J. (1996). The effects of hypercapnia on the growth of juvenile white sturgeon, *Acipenser transmontanus*. *Aquaculture* **147**, 293–299.

Damsgaard, C., Gam, L. T. H., Dang, D. T., Van Thinh, P., Huong, D. T. T., Wang, T. and Bayley, M. (2015). High capacity for extracellular acid-base regulation in the air-breathing fish *Pangasianodon hypophthalmus*. *J. Exp. Biol.* **218**, 1290–1294.

Danley, L., Kenney, P. B. and Hankins, A. (2005). Effects of carbon dioxide exposure on intensively cultured rainbow trout *Oncorhynchus mykiss*: Physiological responses and fillet attributes. *J. World Aquac. Soc.* **36**, 249–261.

FAO (2016). *FAO yearbook. Fisheries and Aquaculture Statistics 2016*.

FAO (2018). The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. *THE STATE OF THE WORLD* **Rome**,.

Graham, J. B. (1997). *Air-breathing Fishes - Evolution, diversity and adaptation*. San Diego, CA: Academic Press.

Halwart, M., Halwart, M., Gupta, M. V and Gupta, M. V *Culture of Fish Rice Fields*.

Hu, Y., Ni, Q., Wu, Y., Zhang, Y. and Guan, C. (2011). Study on CO2 removal method in recirculating aquaculture waters. *Procedia Eng.* **15**, 4780–4789.

Ishimatsu, A., Hayashi, M. and Lee, K. (2005). Physiological effects on fishes in a high CO2 world. *J. Geophys. Res.* **110**, 1–8.

Khiem, N. T., Bush, S. R., Nguyen, M. C. and Loc, V. T. T. (2010). Upgrading small-holders in the Vietnamese *Pangasius* value chain: Final Report. *Final Report, ODI grant number RO334* 158.

Lefevre, S., Wang, T., Jensen, A., Cong, N. V., Huong, D. T. T., Phuong, N. T. and Bayley, M. (2014). Air-breathing fishes in aquaculture. What can we learn from physiology? *J. Fish Biol.* **84**, 705–731.

Milsom, W. K. (2012). New insights into gill chemoreception: Receptor distribution and roles in water and air breathing fish. *Respir. Physiol. Neurobiol.* **184**, 326–339.

Petochi, T., Di Marco, P., Priori, A., Finoia, M. G., Mercatali, I. and Marino, G. (2011). Coping strategy and stress response of European sea bass *Dicentrarchus labrax* to acute and chronic environmental hypercapnia under hyperoxic conditions. *Aquaculture* **315**, 312–320.

Ross, R. M., Krise, W. F., Redell, L. A. and Bennett, R. M. (2001). Effects of dissolved carbon dioxide on the physiology and behavior of fish in artificial streams. *Environ. Toxicol.* **16**, 84–95.

Sanchez, A. P., Giusti, H., Bassi, M. and Glass, M. L. (2005). Acid-base regulation in the South American lungfish *Lepidosiren paradoxa*: Effects of prolonged hypercarbia on blood gases and pulmonary ventilation. *Physiol. Biochem. Zool.* **78**, 908–915.

Shartau, R. B. and Brauner, C. J. (2014). Acid-base and ion balance in fishes with bimodal respiration. *J. Fish Biol.* **84**, 682–704.

The World Bank (2016). *Transforming Vietnamese Agriculture : Gaining More from Less*. Hanoi, Vietnam.

Ultsch, G. R. (1996). Gas exchange, hypercarbia and acid-base balance, paleoecology, and the evolutionary transition from water-breathing to air-breathing among vertebrates. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **123**, 1–27.

Willmer, E. N. (1934). Some observations on the respiration of certain tropical fresh-water fishes. *J.Exp.Biol.* **11**, 283–306.

CHAPTER 1

CO_2 Exchange During Forced Emersion and Aquatic Hypercapnia in Polypterids

Introduction

Bimodal breathers, animals that breathe both air and water and typically consume O_2 from the atmosphere and release CO_2 into the aquatic environment. This decoupling of O_2 and CO_2 exchange across respiratory organs and media, revealed by analyzing the respiratory exchange ratio (R: VCO_2/VO_2) of expired air and water, is well established in the air-breathing fishes (Graham,1997; Martin,1995). An R between 0.7-1.0 is used as an indicator of an animal's ability to maintain CO_2 exchange rates that equal metabolic production. However, when broken down by respiratory organ (gills and air-breathing organ), R can indicate a preferential exchange of O_2 or CO_2. For the air-breathing organ of a fish in water, R is typically between 0.01- 0.3, while for the gills, R is commonly greater than 1.0 indicating, aerial O_2 uptake and aquatic CO_2 release.

When removed from water, aquatic exchange of CO_2 is no longer available. Under these conditions maintaining CO_2 exchange is vital for regulating blood PCO_2 and pH out of water. However, the mechanisms are likely to differ between fishes with independently evolved air-breathing systems. While some fishes possess modified gills for exchange in air, without these, gills coalesce, and CO_2 exchange must be redistributed to the other respiratory surface. Intertidal amphibious fishes maintain an overall R between 0.7 - 1.0 as do some freshwater fishes (*Channa*, *Anabas*, *Trichogaster*), but in other species (*Hoplerythrinus*, *Heteropneustes*, *Clarias*, *Neochanna*) R falls below 0.7 indicating CO_2 accumulation. While many studies have focused on how O_2 exchange is repartitioned during emersion in air-breathing fishes, few studies have investigated the repartitioning CO_2. The repartitioning of CO_2 exchange between the gills and air-breathing organs during emersion is likely to pose a larger physiological challenge than for O_2 because bimodal breathers must adapt with both a change in the respiratory organ and respiratory medium for CO_2 release.

Aquatic hypercapnia represents another environmental challenge to CO_2 homeostasis. In aquatic hypercapnia, the blood-water gradient by which CO_2 is outward diffused from the gills is reduced or can even reversed. Consequently, blood PCO_2 will continue to rise and pH fall until the exchange of CO_2 can be restored either by elevated release via the gills and/or through a transition air breathing. Branchial CO_2 exchange requires elevating blood PCO_2 above aquatic conditions, reestablishing the outward blood-water diffusion gradient. This entails maintaining a chronic respiratory acidosis, from which acid-base balance must be restored through the accumulation of bicarbonate in exchange for chloride ions at the gills (Brauner and Baker, 2009; Shartau and Brauner, 2014). Aerial CO_2 exchange would also benefit from elevated blood PCO_2 by driving more CO_2 into the air-breathing organ per breath, however, these gains would be minimal as CO_2 exchange in air-breathing systems are typically ventilation limited (DeJours, 1981). Solely air-breathing animals maintain both elevated blood PCO_2s (15-40 mmHg compared to 3 mmHg in water breathers) as well as a CO_2 ventilatory drive (Milsom, 2002; Ultsch, 1996). The ability to detect and increase ventilation in response to CO_2 is likely as important for elevating aerial CO_2 exchange rates in aquatic hypercapnia as it is for animals on land.

Although the effects of aquatic hypoxia have been well studied, how air-breathing fish respond to aquatic hypercapnia has received relatively less attention. This is due to the high solubility of CO_2 in water resulting in an almost infinite sink in which CO_2 can be dissolved in without altering PCO_2. However, aquatic PCO_2 has been found to reach elevated levels due to natural and anthropogenic processes. For example, aquaculture ponds have been measured up to 38 mmHg CO_2 (Damsgaard et al., 2015) and up to 60 mmHg CO_2 in bodies of tropical freshwater (Ultsch, 1996; Willmer, 1934). This range of PCO_2 approaches or exceeds the values measured in the arterial blood of a typical terrestrial animal (40 mmHg). How organisms respond to aquatic CO_2 is not only ecologically important, but evolutionarily as well, as hypercapnia may have played a key role in conditioning early vertebrates for life on land. Ultsch 1996 proposed that aquatic hypercapnia may have preadapted primitive tetrapods to develop a high buffering capacity and blood PCO_2 to levels found in modern terrestrial vertebrates before leaving the water.

The vertebrate water to land transition was a major event in vertebrate evolution and the polypterid family has been used as a model in to investigate air-breathing behavior (Graham et al., 2014) and developmental plasticity (Standen et al., 2014; Wilhelm et al., 2015) in early tetrapods. These heavily scaled African freshwater fishes are the most basal extant actinopterygian group, closest related to the sarcopterygian lineage (Inoue et al., 2003), and possess features similar to tetrapods and lungfish in their ventrally paired lungs and pulmonary circulation (Graham, 1997; Lechleuthner et al., 1989; Perry, 2007). The polypterid family consist of the two genera, *Erpetoichtys*, which is known to be amphibious, making voluntary excursions onto land (Sacca and Burggren, 1982), and *Polypterus*, which although yet to be observed to voluntarily emerse itself, *Polypterus senegalus* has been demonstrated to survive in 3 mm of water for 8 months (Standen et al., 2014). Both species (*P. senegalus* and *E. calabaricus*) are facultative air-breathers, able to rely on full on gill breathing in normoxic water, and also facultative water-breathers, able to rely exclusively on lung breathing (Babiker, 1984; Magid, 1966; Magid et al., 1970; Pettit and Beitinger, 1981; Pettit and Beitinger, 1985; Sacca and Burggren, 1982). However, how air-breathing is influenced by is role in CO_2 exchange is not well understood.

This study uses two members from the polypterid family, *Polypterus lapradei* and *Erpetoichtys calabaricus*, to quantify the partitioning of pulmonary and cutaneous gas exchange when aquatic CO_2 release is restricted during forced emersion and aquatic hypercapnia. Respiratory partitioning between pulmonary and cutaneous exchange were measured during forced emersion by assessing wave forms from unrestricted flow-through respirometry (Jew et al., 2013) and were validated against previous methods measuring of respiratory partitioning using a septum placed between the head and body of the animal. Measurements of expired pulmonary gases and gill and lung breathing frequency demonstrated a transition to air breathing in both ventilatory effort and O_2 and CO_2 exchange in response to progressive aquatic hypercapnic exposure.

Methods

Animal Handling

Polypterus lapradei and *Erpetoichtys calabaricus* were obtained though the aquarium trade and shipped to the University of California, Irvine (UCI), where they were held in aquaria connected in a recirculating system at 25°C. Fish were fed a diet of blood worms and chopped shrimp and with a 12/12h light/dark cycle. All experiments were approved by UCI's Institution for Animal Care and Use Committee (protocol # 2013-3105).

Forced Emersion

7 *P. lapradei* (m=107±5 g, mean±s.e.m) and 7 *E. calabricus* (m=18±1 g) were placed in respirometry chambers made from PVC or acrylic pipping with rubber stoppers and ports for air on each end. Fish were acclimated to the chamber for one hour and measurements were made over the subsequent two hours. Room air was continuously flowed through the chamber and analyzed for O_2 and CO_2 using the same methods described in (Jew et al., 2013). From these continuous measurements, total O_2 and CO_2 exchange ($\dot{M}O_{2\ \mathrm{total}}$ and $\dot{M}CO_{2\ \mathrm{total}}$), air-breathing frequency (F_{AB}), and partitioning of lung and cutaneous respiration were determined. Recordings of CO_2 showed peaks corresponding to air-breathes above an elevated baseline level (and the inverse for O_2). The integration of these peaks was determined to be the rate of pulmonary exchange, while the elevated baseline was the rate of cutaneous respiration. To correct for background respiration, blanks were run after each fish was removed.

Aquatic Hypercapnia

P. lapradei (n=10, m=114 ± 4 g) was placed in a custom acrylic tank consisting of a lower compartment filled with water in which the fish was held and an upper enclosed compartment, allowing the fish to air breath *ad libitum*. Water was gravity fed into the front of the tank by elevated reservoirs. The flow rate was held constant by maintain a steady head pressure in these reservoirs using a custom-built float switch and pump system. The reservoirs were aerated with different gas mixtures, 0, 15, 40, and 60 mmHg CO_2 with O_2 held at 155 mmHg and balanced by N_2. Mixtures were controlled using a Gas Mixing Flowmeter (GF-3, Cameron Instruments Co., Port Aransas, Texas, USA) which were then bubbled in the reservoirs via air stones. Exposure to progressive hypercapnia was controlled by opening respective reservoir valves which fed into the tank. To confirm that tank was in equilibrium with the aerated CO_2 concentration, a pH probe was placed in the back of the tank and the pH was checked against a pH/CO_2 calibration curve. The air phase was continuously flushed with room air and the exiting gases were dried using an air sample dehydrator (ND-2, Sable Systems, Las Vegas NV) and measured for O_2 and CO_2 content (Oxzilla II and CA-10, Sable Systems, Las Vegas NV). These continuous measurements revealed dips and peaks in O_2 and CO_2 concentrations corresponding to air-breathing events. The integration of these dips and peaks were used to calculate aerial O_2 consumption ($\dot{M}O_{2\ air}$) and aerial CO_2 excretion ($\dot{M}CO_{2\ air}$). Air-breathing frequency was also measured from these recordings and R was calculated using the equation:

$$R = \frac{VCO_{2\ air}}{VO_{2\ air}}$$

Gill ventilation frequency (F_G) was recorded using a video camera. Fish were acclimated to the tank for an hour in air equilibrated water, exposed to each CO_2 concentration for one hour, and then followed by returning to air equilibrated water for one hour.

Data sampling and analysis

Data was recorded using a MP 100 BIOPAC system (Biopac Systems Inc., Santa Barbara CA, USA) and stored in AcqKnowledge. To test for statistical differences between treatments, one-way repeated-measures ANOVAs and post-hoc Tukey Tests were run using SigmaPlot ($p<0.05$).

Results

Forced Emersion

Gas exchange rates for *P. lapradei* and *E. calabaricus* are show in Figure 1. No statistical significance was found between the two species for cutaneous, pulmonary, and total R values, and in CO_2 and O_2 partitioning. In both species, pulmonary exchange made up a large majority of VO_2, while less so for VCO_2, although still more than half. The resulting R values were also similar in both species, being high for cutaneous exchange (1.75) and low for pulmonary exchange (0.57). Total R for both respiratory organs combined were at the expected RQ value of 0.8.

Aquatic hypercapnia

In *P. lapradei*, gill ventilation (F_G) decreased throughout progressive hypercapnia and returned to pre-exposure levels during recovery, while air breathing frequency (F_{AB}), and aerial O_2 and CO_2 exchange ($VO_{2\ air}$ and $VCO_{2\ air}$) and R all showed the opposite trend (Figure 2). While $VCO_{2\ air}$ returned to pre-exposure levels when recovered in normal CO_2 conditions, $VO_{2\ air}$ decreased below pre-exposure levels. The resulting R during recovery was also elevated above pre-exposure levels. R values ranged from 0.1 in normocapnia to 1.6 in the highest hypercapnia level.

Mean O_2 content per breath remained constant though out all conditions ($p>0.05$), while CO_2 content increased in progressive hypercapnia exposure and returned to pre-exposure conditions on recovery in normocapnia (Figure 3).

Discussion

Air exposure

During emersion, gills can no longer function to aquatically eliminate CO_2 and blood PCO_2 rises to a new equilibrium in air-breathing fishes (Daxboeck et al., 1981; DeLaney et al., 1977; Ishimatsu and Itazawa, 1983; Pelster et al., 1988). Blood PCO_2 was not measured in this study, however elevation in blood PCO_2 likely occurred since polypterids lack gill specializations for branchial exchange out of water (Sacca and Burggren, 1982). An elevated blood-air CO_2 gradient in the lungs and skin, as well as a respiratory drive sensitive to CO_2, as see in hypercapnic exposure (Fig. 2), function together to maintain PCO_2 homeostasis during emersion. The resulting overall R of 0.75 in *P. lapradei* and *E. calabaricus*, indicate that polypterids can completely offload metabolically produced CO_2 when out of water and maintain PCO_2 homeostasis.

Both *P. lapradei* and *E. calabaricus* rely heavily on lungs for O_2 exchange when on land. The majority of CO_2 exchange also occurred at the lungs, however, cutaneous respiration also plays a significant role accounting for 40% of $\dot{M}CO_{2\ \mathrm{total}}$ compared to only 18% of $\dot{M}O_{2\ \mathrm{total}}$ (Fig. 1D). This divergent partitioning of O_2 and CO_2 exchange between respiratory organs is reflected in R values being high for the skin (1.75) and low for the lungs (0.57) (Table 1, Fig. 1).

$\dot{M}CO_{2\ \mathrm{total}}$ for *E. calabaricus* (0.073 +/- 0.004 ml O_2 g^{-1} hr^{-1}) match values reported by (Pettit and Beitinger, 1985) but are less than those reported by (Sacca and Burggren, 1982) for submerged (0.088 ml

O_2 g^{-1} h^{-1}) and forcibly emersed (0.117 ml O_2 g^{-1} h^{-1}). In the latter study, O_2 respiratory partition was measured using a rubber septum separating the gases exposed to the head and body, and reported the lungs 40%, gills 28%, and skin 32% of $\dot{M}O_{2\ \mathrm{total}}$ when submerged, and lungs 63% and body 37% when out of water. Similar values for $\dot{M}O_{2\ \mathrm{total}}$ and respiratory partitioning validate these methods for *E. calabaricus*. However, methods involving septums have been shown to underestimate cutaneous respiration if significant gas exchange occurs though the skin covering the head region. (Urbina et al., 2014) addressed this issue with septums in *Neochanna burrowsius*, calculating as high as 8% of branchial $\dot{M}O_2$ could be attributed to cutaneous respiration from skin covering the head region. Measurements of cutaneous respiratory partitioning could be underestimated further in cases where gas exchange along the body is not uniform, such as in the densely capillerized cephalic epidermis of some mudskippers (Zhang et al., 2003). Methods for measuring respiratory partitioning provided in this study avoid these issues with septum respirometry, however, they do require the subject species have a distinct intermittent ventilatory pattern. (Jew et al., 2013) was only able to measure partitioning in the mudskipper *Periophthalmus modestus* post-exercise when buccopharyngeal ventilation resembled a “gulp and hold” pattern, and not at rest when the opercula were held open and “fluttering” was observed. No studies on emersion in *Polypterus* are available for comparison.

Cutaneous respiration during emersion is common adaptation in air-breathing fishes ranging from 10% in the non-amphibious *Trichogaster trichopterus* to 40-70% in amphibious intertidal fishes depending on species (Graham, 1997; Martin, 1995; Sayer, 2005). Fewer studies, however, have measured the effects of emersion on CO_2 partitioning in air-breathing fishes. Table 1 is an exhaustive list of species in which both O_2 and CO_2 partitioning were measured between the head and body compartments in air-breathing fish during emersion. Cutaneous respiration makes up a significant portion of gas exchange either favoring CO_2 exchange or having a similar proportion to O_2 exchange in most cases. *T. trichopterus* (non-amphibious) again is on the low range of cutaneous respiration for CO_2 (10%) with the Japanese mudskipper, *P. modestus*, known for its amphibious behavior, on the high end (94%). Some studies have proposed that fish that breath air using organs derived from gills are able to maintain high rates of CO_2 release in air due to the presence of carbonic anhydrase, however no strong correlation has been found across species supporting this theory (Burggren and Haswell, 1979; Graham, 1997). While *P. modestus* is likely dependent on cutaneous respiration for CO_2 offloading on land due the high proportion of total respiration, in contrast, *Polypterus* is likely to be able to survive without cutaneous respiration. *Polypterus* has a complex set of lungs effective at meeting its entire metabolic demands for O_2 (Babiker, 1984), and it is thus likely the lungs can function to meet all CO_2 demands as well.

Species	**%$\dot{M}CO_{2\ body}$**	**%$\dot{M}O_{2\ body}$**	**R $_{head}$**	**R $_{body}$**	**Reference**
Blennie pholis	49	51	0.79	0.93	(Pelster et al., 1988)
Clinocottus analis	22	29	0.92	0.66	(Martin, 1991)
Neochanna burrowsius	58	43	0.69	1.25	(Urbina et al., 2014)
Galaxias maculatus	68	64	0.67	0.82	(Urbina et al., 2014)
Lepidogalaxias salmandroides	69	83	1.2	0.57	(Martin et al., 1993)
Trichogaster trichopterus	12	12	0.75	0.75	(Burggren and Haswell, 1979)
[1]*P. modestus* (post-exercise)	93	87	0.67	1.22	(Jew et al., 2013)
[1]*P. lapradei*	38	16	0.58	1.78	This study
[1]*E. calabaricus*	44	20	0.57	1.83	This study

Table 1.1. Contribution to Cutaneous Respiration in Air-breathing Fishes.
Contribution of cutaneous respiration to total $\dot{M}CO_{2\ total}$ and $\dot{M}O_{2\ total}$ between head and body compartments (%$\dot{M}CO_{2\ body}$, %$\dot{M}O_{2\ body}$) and associated respiratory exchange ratio (R) during forced emersion. [1]Respiratory partitioning measured by flow-through respirometry without a septum.

Cutaneous respiration in polypterids and most other air-breathing fishes show a preference for CO_2 over O_2 exchange with R values above 0.7 (Table 1), similar to patterns found other vertebrates that utilize cutaneous respiration. In anurans, cutaneous R values are typically greater than 2.0 and make up 70-80% of $\dot{M}CO_{2\ \mathrm{total}}$ but only 30-40% of $\dot{M}O_{2\ \mathrm{total}}$ (Hutchinson et al., 1968). In urodela, cutaneous exchange exhibits R values between 1.6-2.0 and comprise 80-90% of $\dot{M}CO_{2\ \mathrm{total}}$ and 30-50% of $\dot{M}O_{2\ \mathrm{total}}$ (Whitford and Hutchison, 1960). A large proportion of CO_2 exchange occurs at the skin compared to O_2 due to the difference in gas diffusion constants. CO_2 has much higher solubility but similar molecular weight as O_2 leading to CO_2 diffusing 20 times more rapidly though a respiratory surface than O_2 (West, 2012). In respiratory organs that require active ventilation (e.g., lung, gas, bladders, buccopharyngeal pouches), CO_2 quickly diffuses in and reaches equilibrium with lung gases. In contrast, during apnea the O_2 content and R slowly decline. This was observed in *Synbranchus marmoratus* during normal and artificial ventilation of the air-breathing organ (Graham and Baird, 1984a; Graham et al., 1995), in *P. senegalus* with semi-continuous sampling of lung gases (Magid et al., 1970), and similar studies with *Arapima* and *Channa* (Ishimatsu and Itazawa, 1981; Randall et al., 1978a). In air-breathing organs requiring ventilation, CO_2 offloading is limited by the rate of ventilation, while in cutaneous respiration, the skin is constantly ventilated by the external environment. Although when in water, bounder layers can slow the rate of diffusion, their effect in air is small due to air's low viscosity not hinder cutaneous ventilation (Feder and Burggren, 1985). Thus, conductance and not convention is the limiting factor in cutaneous systems explaining why CO_2 is favored over O_2 exchange.

In contrast, an air-breathing organ requiring ventilation, such as lungs, create ventilatory limited system favoring O_2 over CO_2 exchange. O_2 exchange is maximized by containing a large diffusive capacity through high surface area with thin air-blood distances, while internalizing these delicate surfaces provides protection from physical trauma and desiccation. Changes in lung ventilation have a large impact in CO_2 exchange while leaving O_2 exchange unchanged. Cutaneous respiration's role in CO_2 is therefore expected to have a larger influence on lowering ventilation rates and associated energetic savings than its use for O_2 exchange. Thus, cutaneous respiration in *Polypterus* may provide an auxiliary route for gas exchange, particularly for CO_2, lowering the pulmonary ventilatory requirement for CO_2 homeostasis.

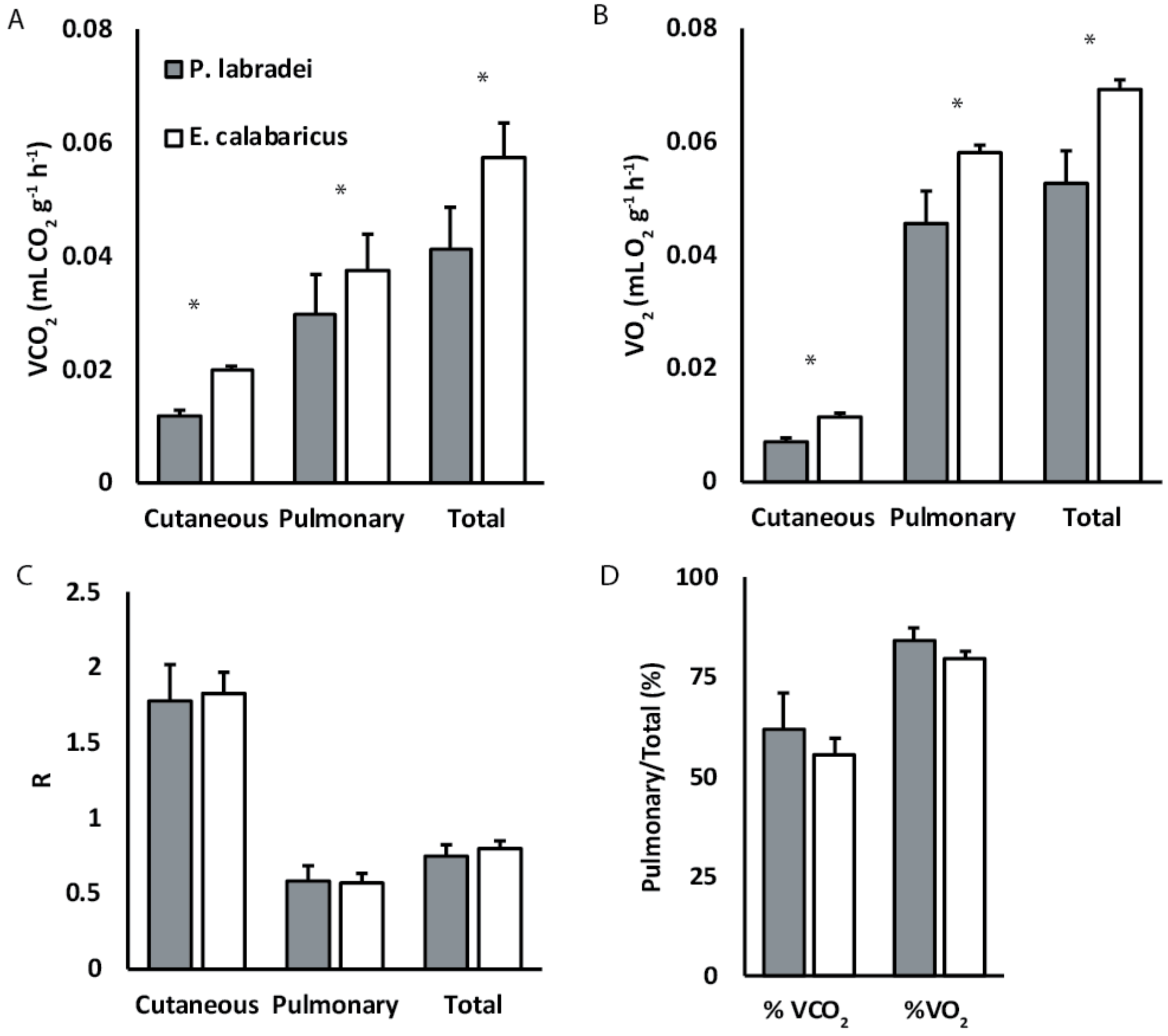

Figure 1.1. CO_2 and O_2 Exchange by Respiratory Organ in *P. lapradei* and *E. calabricus*.

(A) CO_2 and **(B)** O_2 exchange and **(C)** respiratory exchange ratio (R) by respiratory organ, and **(D)** pulmonary fraction of total exchange for CO_2 and O_2 in *P. lapradei* (n=7) and *E. calabricus* (n=10) during forced emersion. An * indicates statistical significance between species as determined by unpaired t-test. No statistical difference was found between the two species for each R value or partitioning of CO_2 or O_2. Results are shown as mean ± s.e.m.

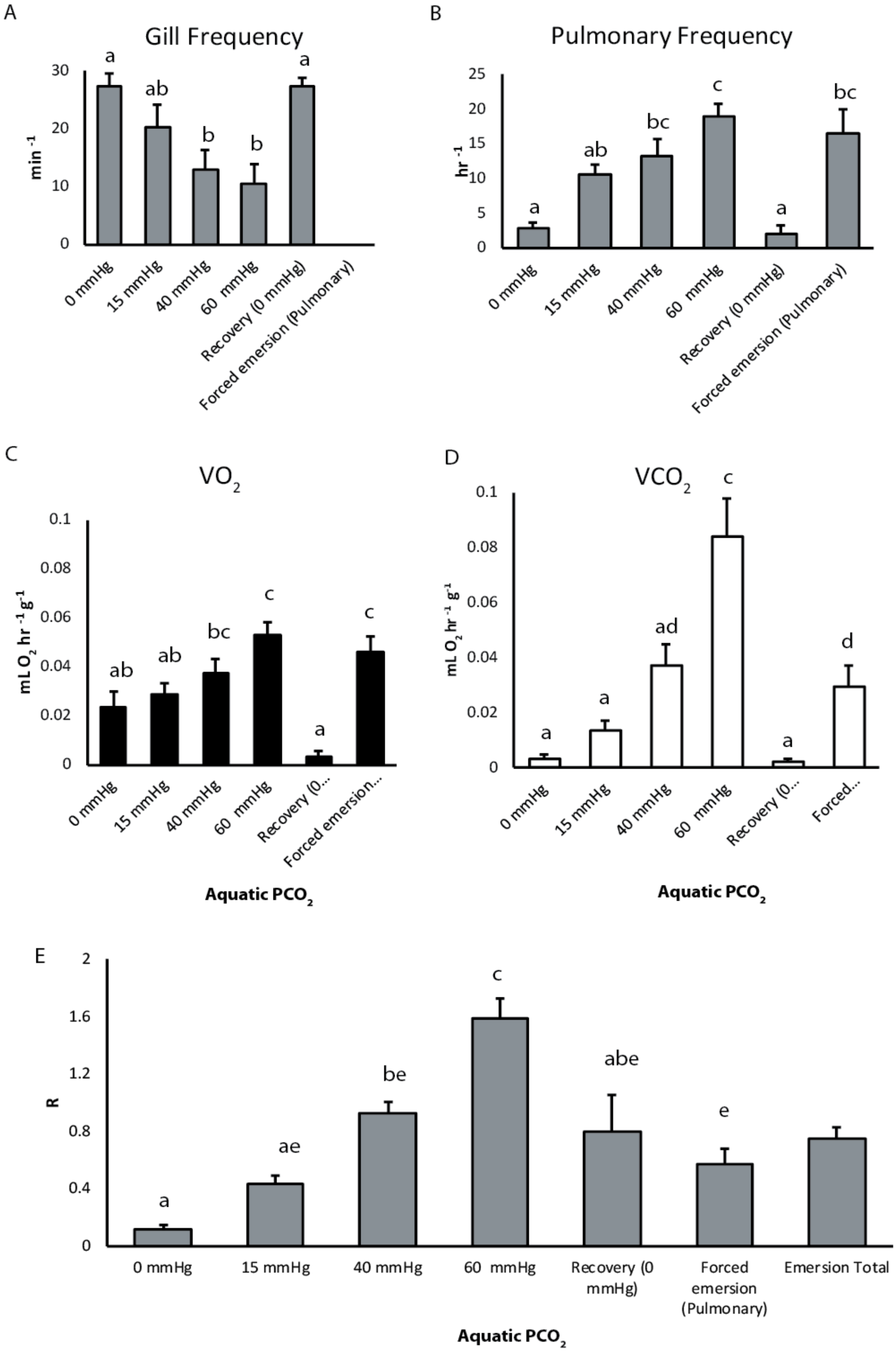
A
Gill Frequency
min -1
0
10
20
30
a
ab
b
b
a
0 mmHg
15 mmHg
40 mmHg
60 mmHg
Recovery (0 mmHg)
Forced emersion (Pulmonary)
B
Pulmonary Frequency
hr -1
0
5
10
15
20
25
a
ab
bc
c
a
bc
0 mmHg
15 mmHg
40 mmHg
60 mmHg
Recovery (0 mmHg)
Forced emersion (Pulmonary)
C
VO_2
$mL\ O_2\ hr^{-1}\ g^{-1}$
0
0.02
0.04
0.06
0.08
0.1
ab
ab
bc
c
a
c
0 mmHg
15 mmHg
40 mmHg
60 mmHg
Recovery (0...
Forced emersion...
Aquatic PCO_2
D
VCO_2
$mL\ O_2\ hr^{-1}\ g^{-1}$
0
0.02
0.04
0.06
0.08
0.1
a
a
ad
c
a
d
0 mmHg
15 mmHg
40 mmHg
60 mmHg
Recovery (0...
Forced...
Aquatic PCO_2
E
R
0
0.4
0.8
1.2
1.6
2
a
ae
be
c
abe
e
0 mmHg
15 mmHg
40 mmHg
60 mmHg
Recovery (0 mmHg)
Forced emersion (Pulmonary)
Emersion Total
Aquatic PCO_2

Figure 1.2. Respiratory Parameters During Progressive Aquatic Hypercapnia in *P. lapradei*.

A) Gill ventilations frequency (F_G), **(B)** air-breathing frequency (F_{AB}), **(C)** O_2 and **(D)** CO_2 exchange, and **(E)** respiratory exchange ratio (R) for *P. labradei* during exposure to various levels of aquatic hypercapnia. Treatments sharing letters are not statistical different as determined using a one-way repeated-measures ANOVA and post hoc Tukey Test. Results are shown as mean ± s.e.m. n=10 for aquatic hypercapnia exposures, n=7 for emersion.

Aquatic Hypercapnia

Aquatic hypercapnia reduces or even reverses the blood-water brachial gradient at the gills creating a respiratory acidosis until rates of CO_2 efflux that match CO_2 production can be reestablished. *P. lapradei* elevates CO_2 efflux by increasing pulmonary ventilation and reduces aquatic exposure by decreasing branchial ventilation (Fig. 2). Despite normoxic conditions, O_2 and CO_2 exchange are recoupled as both sites of gas exchange transition to from the water-breathing to air-breathing organs when exposed to progressive aquatic hypercapnia. These findings are similar to those reported in *P. senegalus* in which O_2 consumption from air and gill ventilation progressively decreased in aquatic hypercapnia (1-12% CO_2) (Babiker, 1984). However, in *P. senegalus* pulmonary ventilation increased up to 1% CO_2 and was subsequently depressed between 1.5-12% CO_2 although still above normocapnic levels.

CO_2 respiratory drive

The same transition of ventilatory effort from water to air breathing in response to aquatic hypercapnia has been observed in other air-breathing fishes, with increases in air breathing in some (*Synbranchus, Lepisosteus, Trichogaster*) and increases in air breathing coupled with reductions in gill ventilation in others (*Amia, Ancistrus, Hypostomus, Hoplerynthinus, Lepdoserin, Protopterus, Neoceratodus*) (Burggren, 1979; Graham, 1997; Milsom, 2012; Sanchez et al., 2005). Respiratory changes in *P. lapradei* are likely driven by external facing CO_2 chemoreceptors located on the gills found in water-breathing and air-breathing fishes (reviewed in Milsom, 2012). Although central chemoreception, shown to be present in the sarcopterygian lungfish and possibly the basal actinopterygians (*Amia, Lepisosteus*), no data is available on this topic for *P. lapradei.*

Hypercapnia's effect on gas exchange

Exposure to aquatic hypercapnia can increase $\dot{M}CO_{2\,air}$ (and R) in two ways, through elevated pulmonary ventilation and through elevated blood PCO_2 increasing the blood-air diffusion gradient. Although we were not able to directly measure the latter in this study, the increased volume of CO_2 exchanged with each breath is an indicator of elevated blood PCO_2 (Fig 3). Because lung PCO_2 quickly equilibrates with the pulmonary blood supply, an increased F_{AB} is expected to have no change (or decrease) in the per breath CO_2 volume. This rise in per breath CO_2 volume despite heightened F_{AB} indicate higher lung PCO_2 equilibrium driven by elevated blood PCO_2.

$\dot{M}O_{2\,air}$ increased in progressive hypercapnia as inhibition of branchial ventilation reduced O_2 uptake from water. O_2 exchange per breath stayed constant though progressive hypercapnia exposure despite elevated F_{AB}, indicating an elevated rate of aerial O_2 extraction. *T. trichopterus* and *Clarias batrachus* were also found to increased $\dot{M}CO_{2\,air}$ and $\dot{M}O_{2\,air}$ in response to aquatic hypercapnia (Burggren, 1979, Ch. 2). (Graham and Baird, 1984b) reported that $\dot{M}CO_{2\,air}$ in *S. marmoratus* was a function of aquatic $PCO_{2,}$ however found $\dot{M}O_{2\,air}$ was independent of $\dot{M}CO_{2\,air}$ and instead a function of breath hold duration. Similar findings were reported in *Monopterus albus*, a close relative to *S. marmoratus* (Ch. 2).

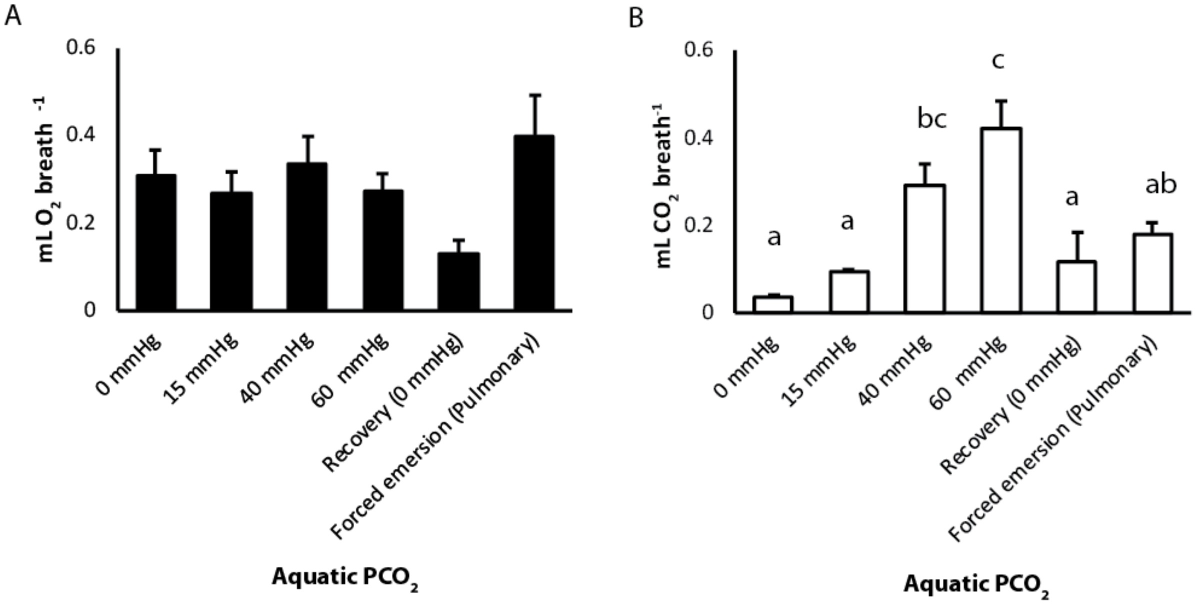

Figure 1.3. Volume of O_2 and CO_2 Exchanged per Breath During Progressive Aquatic Hypercapnia Exposure in *P. lapradei*.
Volume of **(A)** O_2 and **(B)** CO_2 exchanged per breath during exposure to progressive aquatic hypercapnia in *P. labradei*. Treatments sharing letters are not statistical different as determined using a one-way repeated-measures ANOVA and post hoc Tukey Test. Results are shown as mean ± s.e.m. n=10 for aquatic hypercapnia exposures, n=7 for emersion.

Blood PCO_2

Blood PCO_2 may be elevated though transbranchial loading of CO_2, when external PCO_2 is above blood PCO_2 and CO_2 diffuses inward. Many of the adaptations found in air-breathing fish proposed to reduce transbranchial O_2 loss in aquatic hypoxia, would also function to reduce transbrachial CO_2 loading. These include low gill surface area, thick diffusion distance, and reduced gill ventilation. *Polyperids* possess many of these features, yet there are no measurements of blood PCO_2 during aquatic hypercapnia to verify their transbrachial CO_2 load or O_2 loss. The South American lungfish, *Lepidosiren paradoxa*, and the African lungfish, *Protopterus dolloi*, also have highly reduced gill area and are obligate air breathers. However, when exposed to hypercapnia, a respiratory acidosis was found to occur in *L. paradoxa* (Sanchez et al., 2005), while *Protopterus dolloi* experienced no change in acid-base balance (Perry et al., 2005). *Protopterus dolloi* was proposed to maintain constant blood status though increased pulmonary CO_2 excretion. In *T. trichopterus*, the elevation in $\dot{M}CO_2$ in hypercapnia was attributed to an increase in metabolic rate from increased air breathing evidenced by an elevated $\dot{M}O_2$ and an overall R not different from control (R = 0.81).

Comparison between forced emersion and hypercapnia

Increases in $\dot{M}CO_{2\ air}$ can be achieved though both exposure to aquatic hypercapnia or emersion though an elevation of blood PCO_2 and F_{AB}. In *P. labradei*, the rate of pulmonary CO_2 exchange achieved though forced emersion was similar to that when exposed to aquatic hypercapnia of 15 - 40 mmHg CO_2 (Fig. 2). If blood PCO_2 was in equilibrium with the aquatic environment, this is within the range found in other air-breathing fishes during emersion (DeLaney et al., 1977; Ishimatsu and Itazawa, 1983; Randall et al., 1978b). Elevated blood PCO_2 is common in air-breathing fishes and is met with an ability to accumulate bicarbonate for blood acid-base compensation up to 15-20 mmHg CO_2 and a preferential regulation of intracellular pH at higher levels of hypercapnia (Shartau and Brauner, 2014). To minimize the need for acid-base compensation, control of CO_2 exchange must be obtained. The ability of *P. labradei* to maintain pH and CO_2 equilibrium both on land or in aquatic hypercapnia therefore depends on both its ability to detect and drive pulmonary ventilation in response to CO_2.

Implications

Both during emersion and aquatic hypercapnic exposure, the normal route of CO_2 aquatic elimination via the water is restricted, resulting in an elevation of arterial PCO_2 (a respiratory acidosis). Blood PCO_2 continues to rise and pH falls until the rate CO_2 exchange can meet the rate of metabolic production by another means. On land this can be though cutaneous or pulmonary respiration, and in aquatic hypercapnia, solely though the lungs. Skin functions as an effective respiratory surface in water and air due to water's high capacity for CO_2 and air's low viscosity. However, these exposed surfaces must remain permeable and moist making animals dependent on cutaneous respiration vulnerable to desiccation and limited from longer terrestrial sojourns. Although cutaneous respiration has been shown to be regulated though capillary recruitment, increases in gas exchange rates during times of elevated activity or temperature are usually met by active forms of ventilation (Reviewed in Feder and Burggren, 1985). Cutaneous respiration is therefore most effective in moist areas when gas exchange demands are low.

Lungs provide a respiratory surface with high diffusive capacity for gas exchange which are protected from the outside environment. However, this protection results in a ventilatory limitation that effects CO_2 and O_2 differently and has an associated energetic cost. Due to the higher tissue conductance of CO_2 relative to O_2, lungs tend to be ventilatory limited for CO_2 while diffusion limited to O_2 exchange (DeJours, 1981). Thus, high rates of ventilation favor CO_2 exchange, yet are energetically costly, while much lower rates are required for effective O_2 exchange. Animals inhabiting moist environments, such as amphibians, utilize cutaneous respiration to passively maintain CO_2 offloading, releasing active forms of ventilation (e.g., pulmonary) to be paced at maintaining O_2 rather than CO_2 homeostasis (Feder and Burggren, 1985). The ultimate outcome is maximizing gas exchange and minimizing energetic cost by exploiting the difference in respiratory gases and bimodal breathing systems.

Although early tetrapods were thought to have heavy scales and an epidermis too thick to allow efficient gas exchange, more recent insights consider cutaneous respiration possible (Clack, 2012). Grooves for blood vessels through dermal bone indicate the presence of an epidermal layer with capillaries superficial enough for O_2 and CO_2 diffusion. Further, *P. labradei* and *E. calabaricus* demonstrate that a large proportion of gas exchange can take place through thick scales. It has been suggested that a gas permeable integument would be disadvantageous in aquatic hypercapnia due to CO_2 loading (Ultsch 1996). However, studies investigating perfusion of cutaneous surfaces in amphibians show that its permeability can be regulated in response to unfavorable respiratory conditions (Feder and Burggren, 1985). It is thus possible that cutaneous exchange provided the first tetrapods on land a low-cost mode of eliminating CO_2 during emersion. Although many air-breathing fishes demonstrate a CO_2 respiratory drive, central CO_2 receptors are only unequivocally present in the Sarcopterygian lineage (Milsom, 2002). Regardless of whether amphibious behavior appeared before the presence of central CO_2 reception, cutaneous respiration may have played a valuable role in staving off respiratory acidosis on land in primitive tetrapods freeing lungs functioned primarily for O_2 acquisition. Cutaneous respiration could then be slowly phased out during the shift from O_2 to CO_2 ventilatory drive, present in all current terrestrial vertebrates, and as higher selective pressures were placed on an animal’s aerobic capacity and desiccation resistance further from the water’s edge.

References

Babiker, M. M. (1984). Development of dependence on aerial respiration in Polypterus senegalus (Cuvier). *Hydrobiologia* **110**, 351–363.

Brauner, C. J. and Baker, D. W. (2009). Patterns of acid–base regulation during exposure to hypercarbia in fishes. In *Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects*, pp. 43–63.

Burggren, W. W. (1979). Biomodal Gas Exchange During Variation in Environmental Oxygen and Carbon Dioxide in the Air Breathing Fish, *Trichogaster trichopterus*. *J. Exp. Biol.* **82**, 197–213.

Burggren, W. and Haswell, S. (1979). Aerial CO2 excretion in the obligate air breathing fish *Trichogaster trichopterus*: A role for carbonic anhydrase. *J. Exp. Biol.* **82**, 215–225.

Clack, J. A. (2012). *Gaining Ground: The Origin and Early Evolution of Tetrapods*. 2nd ed. (ed. Farlow, J. O.) Bloomington, Indiana: Indiana University Press.

Damsgaard, C., Gam, L. T. H., Dang, D. T., Van Thinh, P., Huong, D. T. T., Wang, T. and Bayley, M. (2015). High capacity for extracellular acid-base regulation in the air-breathing fish *Pangasianodon hypophthalmus*. *J. Exp. Biol.* **218**, 1290–1294.

Daxboeck, C., Barnard, D. K. and Randall, D. J. (1981). Funtional morphology of the gill of the bowfin, *Amia calva L.*, with special reference to their significance during air exposure. *Respir. Physiol.* **43**, 349–364.

DeJours, P. (1981). *Principles of Comparative Respiratory Physiology - P. Dejours - 1981.pdf*.

DeLaney, R. G., Lahiri, S., Hamilton, R. and Fishman, P. (1977). Acid-base balance and plasma composition in the aestivating lungfish (*Protopterus*). *Am. J. Physiol.* **232**, R10–R17.

Feder, M. E. and Burggren, W. W. (1985). Cutaneous gas exchange in vertebrates: design, patterns, control and implications. *Biol. Rev. Camb. Philos. Soc.* **60**, 1–45.

Graham, J. B. (1997). *Air-breathing Fishes - Evolution, diversity and adaptation*. San Diego, CA: Academic Press.

Graham, J. B. and Baird, T. A. (1984a). The Transition to Air Breathing in Fishes III. Effects of Body Size and Aquatic Hypoxia on the swamp eel *Synbranchus Marmoratus*. *J. exp. Biol.* **375**, 357–375.

Graham, J. B. and Baird, T. A. (1984b). III. Effects of body size and aquatic hypoxia on the aerial gas exchange of the swamp eel *Synbranchus marmoratus*. *J.Exp.Biol.* **375**, 357–375.

Graham, J., Lai, N., Chiller, D. and Roberts, J. (1995). The transition to air breathing in fishes. V. Comparative aspects of cardiorespiratory regulation in *Synbranchus marmoratus* and *Monopterus albus* (Synbranchidae). *J. Exp. Biol.* **198**, 1455–67.

Graham, J. B., Wegner, N. C., Miller, L. A., Jew, C. J., Lai, N. C., Berquist, R. M., Frank, L. R. and Long, J. A. (2014). Spiracular air breathing in polypterid fishes and its implications for aerial respiration in stem tetrapods. *Nat. Commun.* **5**,.

Hutchinson, V. H., Whitford, W. G. and Kohl, M. (1968). Relation of body size and surface area to gas exchange in anurans. *Physiol. Zool.* 65–82.

Inoue, J. G., Miya, M., Tsukamoto, K. and Nishida, M. (2003). Basal actinopterygian relationships: A mitogenomic perspective on the phylogeny of the "ancient fish." *Mol. Phylogenet. Evol.* **26**, 110–120.

Ishimatsu, A. and Itazawa, Y. (1981). Ventilation of the Air· breathing Organ in the Snake-head *Channa argus*. *Japanese J. Ichthyol.* **28**, 276–283.

Ishimatsu, A. and Itazawa, Y. (1983). Blood oxygen levels and acid-base status following air exposure in an air-breathing fish, *Channa argus*: The role of air ventilation. *Comp. Biochem. Physiol. A. Comp. Physiol.* **74**, 787–793.

Jew, C. J., Wegner, N. C., Yanagitsuru, Y., Tresguerres, M. and Graham, J. B. (2013). Atmospheric oxygen levels affect mudskipper terrestrial performance: Implications for early tetrapods. *Integr. Comp. Biol.* **53**, 248–257.

Lechleuthner, A., Schumacher, U., Negelem, R. D. and Welsch, U. (1989). Lungs of *Polypterus* and *Erpetoicthys*. *J. Morphol.* **201**, 161–178.

Magid, A. M. A. (1966). Breathing and function of the spiracles in *Polypterus senegalus*. *Anim. Behav.*

14, 530–533.

Magid, A. M. A., Vokac, Z. and Ahmed, N. E. D. (1970). Respirtory Function of the Swim-bladders of the Primitive Fish *Polypterus Senegalus*. *J. exp. Biol.* 27–37.

Martin, K. L. M. (1991). Aerial Respiration in an Intertidal Sculpin, *Clinocottus analis*. *Physiol. Zool.* **64**, 1341–1355.

Martin, K. L. M. (1995). Time and tide wait for no fish: Intertidal fishes out of water. *Environ. Biol. Fishes* **44**, 165–181.

Martin, K. L. M., Berra, T. M. and Allen, G. R. (1993). Cutaneous Aerial Respiration during Forced Emergence in the Australian salamanderfish, *Lepidogalaxias salamandroides*. *Copeia* **1993**, 875–879.

Milsom, W. K. (2002). Phylogeny of CO2/H+ chemoreception in vertebrates. *Respir. Physiol. Neurobiol.* **131**, 29–41.

Milsom, W. K. (2012). New insights into gill chemoreception: Receptor distribution and roles in water and air breathing fish. *Respir. Physiol. Neurobiol.* **184**, 326–339.

Pelster, B., Bridges, C. R. and Grieshaber, M. K. (1988). Physiological adaptations of the intertidal rockpool teleost Blennius pholis L., to aerial exposure. *Respir. Physiol.* **71**, 355–373.

Perry, S. F. (2007). Swimbladder-lung homology in basal osteichthyes revisited. In *Fish Respiration and the Environment*, pp. 41–54.

Perry, S. F., Gilmour, K. M., Swenson, E. R., Vulesevic, B., Chew, S. F. and Ip, Y. K. (2005). An investigation of the role of carbonic anhydrase in aquatic and aerial gas transfer in the African lungfish *Protopterus dolloi*. *J. Exp. Biol.* **208**, 3805–3815.

Pettit, M. J. and Beitinger, T. L. (1981). Aerial respiration of the brachiopterygian fish, *Calamoichthys calabaricus*. *Comp. Biochem. Physiol. Part A Physiol.* **68**, 507–509.

Pettit, M. J. and Beitinger, T. L. (1985). Oxygen Acquisition of the Reedfish , Erpetoichthys Calabaricus. *J. Exp. Biol.* **114**, 289–306.

Randall, D. J., Farrell, A. P. and Haswell, M. S. (1978a). Carbon dioxide excretion in the pirarucu

(*Arapaima giga*), an obligate air-breathing fish. *Can. J. Zool.* **56**, 977–982.

Randall, D. J., Farrell, A. P., Haswell, M. S. and Farrell, A. P. (1978b). Carbon dioxide excretion in the jeju, *Hoplerythrinus unitaeniatus*, a facultative air-breathing teleost. *Can. J. Zool.* **56**, 970–973.

Sacca, R. and Burggren, W. (1982). Oxygen uptake in air and water in the air-breathing reedfish *Calamoichthys calabaricus*: role of skin, gills and lungs. *J. Exp. Biol.* **97**, 179–186.

Sanchez, A. P., Giusti, H., Bassi, M. and Glass, M. L. (2005). Acid-base regulation in the South American lungfish *Lepidosiren paradoxa*: Effects of prolonged hypercarbia on blood gases and pulmonary ventilation. *Physiol. Biochem. Zool.* **78**, 908–915.

Sayer, M. D. J. (2005). Adaptations of amphibious fish for surviving life out of water. *Fish Fish.* **6**, 186–211.

Shartau, R. B. and Brauner, C. J. (2014). Acid-base and ion balance in fishes with bimodal respiration. *J. Fish Biol.* **84**, 682–704.

Standen, E. M., Du, T. Y. and Larsson, H. C. E. (2014). Developmental plasticity and the origin of tetrapods. *Nature* **513**, 54–58.

Ultsch, G. R. (1996). Gas exchange, hypercarbia and acid-base balance, paleoecology, and the evolutionary transition from water-breathing to air-breathing among vertebrates. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **123**, 1–27.

Urbina, M. A., Meredith, A. S., Glover, C. N. and Forster, M. E. (2014). The importance of cutaneous gas exchange during aerial and aquatic respiration in galaxiids. *J. Fish Biol.* **84**, 759–773.

West, J. B. (2012). *Respiratory Physiology, The Essentials*. 9th ed. La Jolla, California: Lippincott Williams & Wilkins.

Whitford, W. G. and Hutchison, V. H. (1960). Gas Exchange in Salamanders. *Physiol. Zool.* **37**, 273–279.

Wilhelm, B. C., Du, T. Y., Standen, E. M. and Larsson, H. C. E. (2015). *Polypterus* and the evolution of fish pectoral musculature. *J. Anat.* 511–522.

Willmer, E. N. (1934). Some observations on the respiration of certain tropical fresh-water fishes.

J.Exp.Biol. **11**, 283–306.

Zhang, J., Taniguchi, T., Takita, T. and Ali, A. B. (2003). A study on the epidermal structure of Periophthalmodon and Periophthalmus mudskippers with reference to their terrestrial adaptation. *Ichthyol. Res.* **50**, 310–317.

CHAPTER 2

The Effects of Aquatic Hypercapnia on Respiration in Air-breathing Fishes

Introduction

The role of aquatic hypoxia in the evolution, physiology, and behavior of air-breathing fishes has been well studied (Graham, 1997). In contrast, the influence of aquatic hypercapnia (high CO_2) has received relatively little attention. The focus on aquatic hypoxia stems from the hypothesis that reductions in aquatic O_2 drove the evolution of vertebrate air-breathing and terrestriality. In addition, the relatively low solubility of O_2 compared to CO_2 in water led to the notion that CO_2 was not a significant stimulus in shaping ventilatory and gas exchange patterns. In aquatic environments hypoxia is common, whereas the high solubility of CO_2 allows unhindered offloading of metabolically produced CO_2 (DeJours, 1981). Thus, unloading of CO_2 by water breathers is effective and arterial blood PCO_2 rarely exceeds 5 mmHg. Consequently, studies of respiration in air-breathing fishes typically focus on aquatic hypoxia and normocapnia, stimulating fish to obtain O_2 from the atmosphere via the air-breathing organ and releasing CO_2 into the water via the gills or integument. This uncoupling of O_2 and CO_2 exchange between respiratory organs is reflected in numerous reports of respiratory exchange ratios (R; VCO_2/VO_2) of the air-breathing organ between 0.01 and 0.30 (reviewed in Graham, 1997), while R for aquatic branchial exchange is often greater than 1.0 (Martin, 1995). Although gases exhaled from air-breathing organs have been shown to have an R elevated in some obligate air breathers (*Arapima gigas*) or in aquatic hypoxia (*Channa argus*, *Anabas testudineus*, *Trichogaster trichopterus*), R in submerged air-breathing fishes remains in this low range (Burggren, 1979; Glass et al., 1986; Hughes and Singh, 1970; Randall et al., 1978). This indicates that CO_2 offloading at the gills remains sufficient despite the reductions in gill ventilation and surface area, a characteristic of many air-breathing fishes. Interestingly, when water is removed as a sink for CO_2, and the animals are only in air, such as for intertidal amphibious fishes, R can exceed 0.7 (Martin, 1995).

The notion that aquatic environments do not pose a challenge to CO_2 exchange disregards the extent of which CO_2 can accumulate within various aquatic environments. Hypercapnia and hypoxia can often occur together and are common in tropical freshwater habitats (Ultsch, 1987; Willmer, 1934). These environments generally have heavy organic loads and are turbid and stagnant, creating high rates of bacterial respiration, little photosynthetic activity, and low mixing with the atmosphere. The levels of CO_2 can also be elevated through bacterial anaerobic fermentation resulting in hypercapnic/hypoxic environments. Elevated CO_2 levels above 60 mmHg have been shown to occur naturally alongside aquatic hypoxia (Ultsch, 1996; Willmer, 1934). In these marginal aquatic habitats, the scarcity for O_2 is hypothesized to be the driving force behind the evolution of vertebrate air breathing, though the abundance of CO_2 may have played an additional, important role preparing early respiratory systems for terrestriality (Ultsch, 1987; Ultsch, 1996). Anthropogenic influences can also exacerbate these conditions through numerous avenues such as eutrophication from agricultural runoff, burning of fossil fuels, resequestration and injection of atmospheric CO_2 into deep ocean sites, and aquaculture (Brauner and Baker, 2009; Damsgaard et al., 2015a; Ishimatsu et al., 2005). In densely packed aquaculture ponds hypercapnia is a critical factor restricting growth (summarized in Lefevre et al., 2014). In these situations, the common practice of pure O_2 injection into the water to meet the aerobic demands of growing fish, result in an aeration rate that is insufficient in removing the associated metabolically produced CO_2 (Hu et al., 2011; Lefevre et al., 2014). For example, in Vietnamese aquaculture ponds used to grow *Pangasianodon hypophthalmus*, aquatic PCO_2 values above 34 mmHg are measured (Damsgaard et al., 2015a). In this example, it is possible that aquatic CO_2 levels may have been higher as the measured levels approached the maximum detection range of the measurement devices (personal communication).

Regardless of the source, in aquatic hypercapnia, the gradient by which CO_2 is passively offloaded from the gills to the water becomes reduced and thus branchial exchange can become ineffective at maintaining CO_2 homeostasis. Furthermore, if ambient PCO_2 exceeds that of ventral aortic blood, the diffusion gradient is reversed resulting in branchial loading of CO_2 and elevation of arterial PCO_2. Aquatic hypercapnia thus creates a rising respiratory acidosis until CO_2 offloading can be adjusted to meet metabolic production by allowing blood PCO_2 to increase and maintaining outward diffusion gradient across the gills (pattern 1) or switching gas exchange to the air-breathing organ, increasing reliance of aerial respiration (pattern 2).

Pattern 1: CO_2 exchange via the gills

For purely water breathing fishes offloading CO_2 at the gills by preserving a favorable blood-water diffusion gradient is the only mechanism available for CO_2 homeostasis. It requires an elevation of blood PCO_2 to levels greater than aquatic PCO_2 and adjustment to the accompanying respiratory acidosis. This strategy and the associated acid-base compensation incurs an elevated energetic cost of ion transport through the accumulation of bicarbonate at the gills and depletion of plasma chloride (Brauner and Baker, 2009). This mechanism benefits from a large gill surface area providing for sufficient CO_2 efflux and ion/pH-regulation (Brauner and Baker, 2009). In species of fish adopting pattern 1, aquatic hypoxia would drive the rate of air-breathing (F_{AB}) to match aerial O_2 consumption ($MO_{2\ air}$), rather than CO_2 aerial offloading ($MCO_{2\ air}$).

Pattern 2: CO_2 exchange via the air breathing organ

In air breathing fish exposed to hypercapnic aquatic environments, this alternative mode of CO_2 exchange switches the primary avenue for CO_2 exchange from the gills to the air-breathing organ. Consequently, this strategy requires a respiratory system sensitive to CO_2, similar to that present in terrestrial vertebrates. Species adopting this pattern would reduce blood/water exchange for O_2 and CO_2. For example, such mechanism would include a small gill surface area, large diffusion distances, and restricting water- and/or blood-perfusion of branchial surfaces though reduced branchial ventilation and shunting of blood past the gills. These morphological and physiological features in some air-breathing fishes minimize the potential for transbranchial O_2 loss, as well as CO_2 loading (Graham, 1997; Randall et al., 1981; Ultsch, 1996). Air-breathing in such species would be elevated in aquatic hypercapnia, and F_{AB} would match $MCO_{2\ air}$, rather than $MO_{2\ air}$, in fish adopting pattern 2.

These patterns represent the two extreme ends of the spectrum and we anticipate the diversity of air-breathing fishes to demonstrate a gradient of responses based on their capacities for air and water breathing. Using this framework, this study seeks to understand how aquatic conditions influence the distribution of O_2 and CO_2 exchange between the gills and air breathing organs in three species of tropical freshwater fishes indigenous to Southeast Asia, with each species possessing independently evolved air-breathing organs and variation in their capacities for air-breathing, water-breathing, and amphibiousness. The first species, *Monopterus albus*, is an eel-like amphibious burrow dweller which breathes air by inflating paired buccal pouches lined with vascularized epithelia (Hughes and Datta Munshi, 1979). Because the gills of *M. albus* are highly reduced making it an obligate air breather, we also predict a reduced capacity for aquatic CO_2 exchange, favoring respiratory Pattern 2 when exposed to aquatic hypercapnia. The second species, *Clarias batrachus*, is a facultative air-breather and a member of the walking catfishes, a well-known genus of air breathers (Jordan, 1976). Walking catfish are amphibious and known to make voluntary excursion onto land to feed or in search of new habitats (reviewed in Graham, 1997). The air-breathing organ in *C. batrachus* consists of paired superbranchial chambers located dorsal-posterior to the branchial cavity lined with respiratory epithelia, gill fans that separate the air chamber from the branchial cavity, and bony outcroppings derived from the 2nd and 4th gill arch, known as a tree organ, that fill the chamber (Hughes and Datta Munshi, 1979). Although *C. batrachus* lack the extreme gill reductions found in *M. albus*, its amphibious behavior suggests a capacity for adequate aerial CO_2, suggesting a respiratory pattern between 1 and 2. Finally, *Pangasianodon hypophthalmus* is a non-amphibious air-breathing catfish, which utilizes a modified swim bladder for air breathing. It is a facultative air breather with a gill surface area that has been shown to be plastic, varying to a size comparable to water-breathing fishes, and with a very low diffusion distance (Lefevre et al., 2011; Phuong et al., 2017; Phuong et al., 2018). This high capacity for capacity for aquatic gas exchange suggest *P. hypophthalmus* will favor respiratory pattern 2 when exposed to aquatic hypercapnia.

Materials and Methods

Specimens

Specimens of *M. albus* (248 ± 25 g, mass ± S.E.; n=8), were obtained from an aquaculture facility in Can Tho, Vietnam and shipped to Aarhus University, Aarhus, Denmark and housed at 27℃. Specimens of *C. batrachus* (47 ± 7 g; n=6) and *P. hypophthalmus* (28 ± 7 g; n=7) were obtained through the aquarium trade and housed in aquaria at 30℃. Fish were maintained in a 12/12 h light cycle and fasted at least 24 h before experimentation. All experiments were performed at Aarhus University and approved by the Danish Ministry of Food Agriculture and Fisheries (2016-15-0201-00865).

Bimodal respirometry

To measure the simultaneous rates of O_2 uptake from air and water and CO_2 release into air, fish were placed in a bimodal respirometer described in (Lefevre et al., 2016a). This respirometer consisted of a water phase and an air phase in which the animal could freely breathe either media (Fig. 1). The water phase acted as an intermittent closed respirometer consisting of the holding chamber (2.5 l), an O_2-optode (Visiferm DO Arc 120, Hamiltion, Reno, US), a circulation pump, and a flush pump automated by a custom made controller box (Aarhus University, Aarhus, DK). O_2 uptake from the water ($\dot{M}O_{2\ \mathrm{water}}$) was calculated by measuring the decline in PO_2 in the water phase over a 15-minute measurement period. After each measurement period, the water was flushed for 15 minutes with new water. The air phase of the bimodal respirometer was modified for flow-through respirometry in which the air phase was continuously perfused with room air during the measurement period. The air stream was then dried using a ND-2 air sample drier (Sable Systems, Las Vegas, US) and a $CaCO_2$ column, and then measured for O_2 (570A Oxygen Analyzer, Servomex, Crowborough, UK) and CO_2 (CA-10, Sable Systems, Las Vegas, US) concentrations. The flow-through air phase system revealed dips and peaks in O_2 and CO_2 concentrations corresponding to air-breathing events. The integration of these dips and peaks were used to calculate the volume of aerial O_2 consumption ($VO_{2\,\mathrm{air}}$) and aerial CO_2 release ($VCO_{2\,\mathrm{air}}$), similar to methods used in (Jew et al., 2013). Injecting known volumes of O_2 and CO_2 into the chamber verified these measurements. $MO_{2\,\mathrm{air}}$ and $MCO_{2\,\mathrm{air}}$ are the mass specific mass of O_2 and CO_2 exchanged at the air phase. Air-breathing frequency (F_{AB}) was also measured from the continuous flow-through sampling of the air phase by measuring the timing interval between dips and peaks in O_2 and CO_2 content, respectively. O_2 partitioning ($\%MO_{2\,\mathrm{air}}$), the percentage of O_2 consumption from air relative to total O_2 consumption from both air and water ($MO_{2\,\mathrm{total}}$) and R were calculated using the equations:

$$\%M\mathrm{O}_{2\,\mathrm{air}} = \frac{M\mathrm{O}_{2\,\mathrm{air}}}{M\mathrm{O}_{2\,\mathrm{total}}} \times 100$$

$$\mathrm{R} = \frac{\dot{V}\mathrm{CO}_{2\,\mathrm{air}}}{\dot{V}\mathrm{O}_{2\,\mathrm{air}}}$$

Aquatic gas manipulation

To manipulate aquatic PO_2 and PCO_2 levels, respirometers were placed in large plastic tubs filled with water external to the respirometers. PO_2 was regulated in the external water by bubbling room air and N_2 through air stones controlled by a Hamilton optode connected to a controller box. PCO_2 was regulated by bubbling compressed CO_2 though microbubble diffusers controlled by an Oxyguard Pacific Commander System with a pH and a PCO_2 probe (Oxyguard International A/S, Farum, Denmark). Water inside the respirometers was equilibrated with external PO_2 and PCO_2 conditions during flush periods when water was exchanged.

Fishes were allowed acclimate to the respirometers for 10 h in air equilibrated water (normal conditions: PO_2=159 mmHg, PCO_2=0 mmHg), followed by 10 hours of aquatic hypoxic normocapnia (PO_2 =50 mmHg, PCO_2= 0 mmHg), 10 h of aquatic hypoxic hypercapnia (PO_2=50 mmHg, PCO_2=38 mmHg), and 10 h of normoxic hypercapnia (PO_2 =159 mmHg, PCO_2=38 mmHg). The level of hypoxia was chosen based on a compromise between the PO_2 required to elicit a significant increase in air breathing (Thomsen et al., 2017) and the frequency of changing tanks of compressed N_2. All trials were started at approximately 2 p.m. which may have resulted in a time-of-day effect, however this schedule was necessary to ensure trials were completed during a limited time window. The level of hypercapnia was chosen to matched those found in *P. hypophthalmus* aquaculture ponds by (Damsgaard et al., 2015a). Only the last 5 hours of each conditions were used and averaged together to avoid the transition period between treatment in an attempt capture these animals in a steady state. An exception was made for the hypoxic hypercapnia treatment, in which a consistent and contrasting trend was observed between species, and the data was consequently averaged across three 3 h intervals. After each trial, specimens were removed from the respirometers and weighed, and background respiration was measured for at least 1 hour. To correct for diffusion of atmospheric O_2 into the water phase, empty chambers were also run in hypoxia. Because all trials were run in the same treatment order, there exist the possibility of a confounding order effect. However, this was unavoidable as fish required time to adjust to air breathing inside the respirometry chamber under normal conditions to avoid mortalities. It was necessary for the hypercapnia treatment to be last due to the extended period of time required to return PCO_2 to normal levels via aeration. All fish were tested at their respective holding tank temperatures.

Data analysis

Data were recorded in AcqKnowledge, BIOPAC Systems, Inc. and analyzed in R. Statistical significance ($P<0.05$) of variables between conditions was determined by repeated-measures ANOVA with a post-hoc Tukey Test.

Results

Aerial CO_2 Exchange

For all species tested, $MCO_{2\ air}$ remained low in normal and hypoxic normocapnic conditions and was significantly elevated in hypoxic hypercapnia ($P<0.001$) (Table 1). In *M. albus,* however, $MCO_{2\ air}$ was only significantly elevated after 9 h ($P<0.001$), while the other two species remained elevated throughout the exposure period. Subsequently, in normoxic hypercapnia, $MCO_{2\ air}$ in both *C. batrachus* and *M. albus* fell slightly whilst remaining above control levels ($P<0.001$, $P=0.034$).

In all species, aquatic hypoxia alone failed to elicit a rise in aerial R, while exposure to hypoxic hypercapnia or normoxic hypercapnia significantly elevated R ($P<0.001$) (Table 1). With hypoxic hypercapnic exposure, R in *C. batrachus* and *P. hypophthalmus* were elevated and plateaued after 6 and 3 h, respectively, while in *M. albus* R continued to rise over 3, 6, and 9 h.

Aerial and Aquatic O_2 Exchange

In *M. albus* and *C. batrachus*, low aquatic O_2 reduced $MO_{2\ water}$ significantly compared to normal conditions ($P<0.001$, $P<0.001$) and was further reduced in hypoxic hypercapnia (Table 1). Hypercapnia alone also depressed $MO_{2\ water}$ compared to normal conditions in both species ($P<0.001$, $P<0.001$), however less so than when hypoxia was also present. In *M. albus*, $MO_{2\ air}$ did not significantly change across all conditions ($P=0.291$), however reductions in $MO_{2\ water}$ increased $\%MO_{2\ air}$ in response to hypoxia and hypoxic hypercapnia compared to normal ($P<0.001$, $P<0.001$). Changes in $MO_{2\ water}$, however, did not have an effect on $MO_{2\ total}$ across conditions. In contrast, $MO_{2\ air}$ in *C. batrachus* was increased in hypoxia and hypoxic hypercapnia ($P<0.001$, $P<0.001$), however, $MO_{2\ total}$ was still depressed in all treatments compared to normal ($P<0.001$). The resulting $\%MO_{2\ air}$ had an inverse response to MO_2

$_{water}$ in each condition. In *P. hypophthalmus*, no major changes were identified for $MO_{2\ water}$, $MO_{2\ air}$, $MO_{2\ total}$ or $\%MO_{2\ air}$ between hypoxia and hypoxic hypercapnia.

Air-Breathing Frequency

Air-breathing frequency in *C. batrachus*, increased significantly in aquatic hypoxia compared to normal conditions (P<0.001), remained elevated throughout exposure to aquatic hypoxic hypercapnia (P<0.001), and then significantly decreased upon exposures to normoxic hypercapnia (P=0.013) (Table 1). *P. hypophthalmus* show a similar trend with no change in F_{AB} between aquatic hypoxia and hypoxic hypercapnia (P=0.211). *M. albus*, on the other hand, showed a much different air-breathing response than the other two species with no change between normal and hypoxic conditions (P=0.998) and a slow graded increase over the 9 hours of hypoxic hypercapnic exposure. In normoxic hypercapnia, F_{AB} was reduced from hypoxic hypercapnia but remained elevated with respect to normal conditions, although not statistically significant from either.

	Condition	Normal	Hypoxic Normocap	Hypoxic Hypercapn	Hypoxic Hypercapn	Hypoxic Hypercapn	Normoxic Hypercapnia
M. albus	$MO_{2\text{ water}}$	15 ± 1^{a}	5 ± 1^{b}	3 ± 1^{c}	3 ± 1^{c}	3 ± 1^{c}	10 ± 1^{d}
	$MO_{2\text{ air}}$	17 ± 3^{a}	16 ± 2^{a}	19 ± 4^{a}	19 ± 5^{a}	20 ± 3^{a}	11 ± 2^{a}
	$MO_{2\text{ total}}$	32 ± 2^{a}	20 ± 2^{a}	22 ± 4^{a}	22 ± 5^{a}	23 ± 3^{a}	22 ± 2^{a}
	$\%MO_{2\text{ air}}$	52 ± 6^{a}	76 ± 4^{b}	79 ± 7^{b}	78 ± 11^{b}	83 ± 4^{b}	48 ± 7^{a}
	$MCO_{2\text{ air}}$	6 ± 1^{a}	3 ± 1^{a}	8 ± 2^{a}	17 ± 5^{b}	26 ± 3^{b}	18 ± 4^{b}
	R	0.26 ± 0.02^{a}	0.12 ± 0.01^{b}	0.33 ± 0.04^{a}	0.69 ± 0.08^{ac}	0.98 ± 0.06^{d}	1.10 ± 0.10^{d}
	F_{AB}	8 ± 1^{a}	6 ± 1^{a}	10 ± 2^{a}	14 ± 4^{ab}	21 ± 3^{b}	14 ± 3^{ab}
C. batrachus	$MO_{2\text{ water}}$	230 ± 31^{a}	40 ± 6^{b}	21 ± 3^{c}	16 ± 6^{c}	17 ± 4^{c}	81 ± 7^{d}
	$MO_{2\text{ air}}$	30 ± 24^{a}	159 ± 18^{b}	141 ± 9^{b}	145 ± 13^{b}	142 ± 9^{b}	78 ± 10^{a}
	$MO_{2\text{ total}}$	261 ± 34^{a}	211 ± 15^{b}	170 ± 9^{b}	161 ± 10^{b}	165 ± 7^{b}	164 ± 10^{b}
	$\%MO_{2\text{ air}}$	9 ± 6^{a}	79 ± 4^{bc}	87 ± 1^{b}	90 ± 3^{b}	89 ± 3^{b}	48 ± 6^{c}
	$MCO_{2\text{ air}}$	3 ± 3^{a}	11 ± 3^{a}	61 ± 8^{bc}	81 ± 10^{b}	77 ± 9^{b}	40 ± 7^{c}
	R	0.06 ± 0.01^{a}	0.04 ± 0.01^{a}	0.31 ± 0.02^{b}	0.39 ± 0.02^{c}	0.39 ± 0.03^{c}	0.35 ± 0.03^{bc}
	F_{AB}	4 ± 3^{a}	22 ± 4^{b}	23 ± 2^{b}	27 ± 4^{b}	25 ± 3^{b}	13 ± 2^{a}
P. hypoph.	$MO_{2\text{ water}}$	-	191 ± 43^{a}	203 ± 40^{a}	218 ± 45^{a}	174 ± 31^{b}	-
	$MO_{2\text{ air}}$	-	102 ± 16^{a}	136 ± 13^{ab}	82 ± 18^{a}	153 ± 5^{b}	-
	$MO_{2\text{ total}}$	-	287 ± 29^{a}	339 ± 30^{b}	301 ± 43^{ab}	343 ± 39^{ab}	-
	$\%MO_{2\text{ air}}$	-	39 ± 7^{ab}	41 ± 6^{ab}	30 ± 7^{a}	46 ± 5^{b}	-
	$MCO_{2\text{ air}}$	-	3 ± 1^{a}	63 ± 9^{b}	34 ± 7^{c}	67 ± 6^{b}	-
	R	-	0.02 ± 0.00^{a}	0.33 ± 0.03^{b}	0.30 ± 0.02^{b}	0.32 ± 0.02^{b}	-
	F_{AB}	-	17 ± 3^{ab}	23 ± 4^{ab}	15 ± 4^{a}	24 ± 3^{b}	-

Table 2.1. **Bimodal Respirometry Variables for *M. albus, C. batrachus, and P. hypophthalmus.*** Mean respiratory variables under various conditions for *M. albus* (n=8), *C. batrachus* (n=6), and *P. hypophthalmus* (n=7). Superscript letters indicated statistically significant differences between conditions determined by repeated-measures ANOVA and post-hoc Tukey Test.
$MO_{2\text{ water}}$, aquatic O_2 uptake; $MO_{2\text{ air}}$, aerial O_2 uptake; $MO_{2\text{ total}}$, total O_2 uptake; $MCO_{2\text{ air}}$, aerial CO_2 release; $\%MO_{2\text{ air}}$, percent of total O_2 uptake from air, R, respiratory exchange ratio. Units for F_{AB}, MO_2, and MCO_2 are h^{-1}, mg O_2 kg^{-1} hr^{-1}, and mg CO_2 kg^{-1} hr^{-1}, respectively.

Respiratory and Ventilatory Relationship

Figure 1AD shows the relationship between F_{AB} and $MO_{2\ air}$, $MCO_{2\ air}$, and R in each species. In *M. albus*, although F_{AB} and $MCO_{2\ air}$ are strongly matched across all conditions, $MO_{2\ air}$ and R also show increases with $F_{AB.}$ Normoxic (circles) and hypoxic points (squares) largely encompassed the same F_{AB} range in *M. albus*, while instead the normocapnic (blue) and hypercapnic (red) are separated into lower range and the higher range of F_{AB}, respectively.

In *C. batrachus* and *P. hypophthalmus,* F_{AB} closely matched $MO_{2\ air}$ across all conditions (Fig. 1BC), while $MCO_{2\ air}$ and R deviated between the normocapnic (blue) and hypercapnic (red) conditions (Fig. 1EFHI). $MCO_{2\ air}$ and R remained low in normocapnic conditions while showing increases with F_{AB} in hypercapnic conditions. F_{AB} in normocapnic and hypercapnic conditions occupy the full range in *C. batrachus*, while normoxic points (circles) occupy the lower bounds compared to hypoxic points (squares). *P. hypophthalmus* also shows the same overlap of F_{AB} across normocapnic (blue) and hypercapnic (red) conditions.

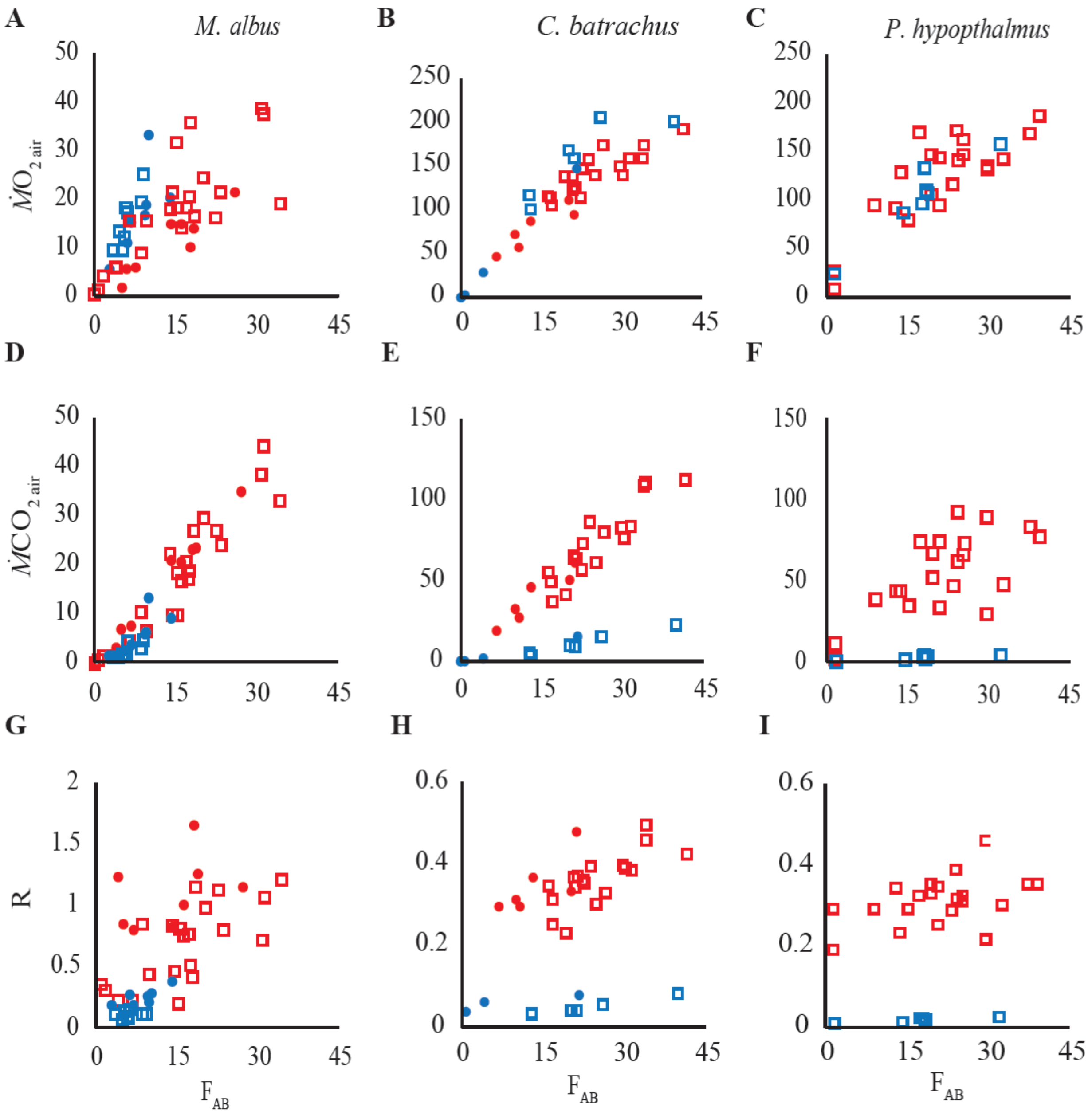

Figure 2.1. Aerial Gas Exchange in Relation to Air-breathing Frequency for *M. albus*, *C. batrachus*, and *P. hypophthalmus*.

Aerial O_2 consumption ($MO_{2\ air}$), aerial CO_2 offloading ($MCO_{2\ air}$) and respiratory exchange ratio (R) in relation to air-breathing frequency (F_{AB}) for individuals of *M. albus*, *C. batrachus*, and *P. hypophthalmus* under normal (blue circles), hypoxic normocapnic (blue squares), hypoxic hypercapnic (red squares), and normoxic hypercapnic (red circles) aquatic conditions. Units for F_{AB}, MO_2, and MCO_2 are h^{-1}, mg O_2 kg^{-1} hr^{-1}, and mg CO_2 kg^{-1} hr^{-1}, respectively.

Discussion

Air breathing in vertebrates has independently evolved 65 times and has generated a diversity of systems and methods for gas exchange (Farrell, 2011; Graham, 1997; Ishimatsu, 2012; Martin, 2014; Shartau and Brauner, 2014). Although hypoxia is usually considered the primary driver of air-breathing among fishes, we found a spectrum of respiratory responses in three species exposed to a combination of aquatic hypoxia and hypercapnia.

A large amount of variation exists both between the literature and our data and within the literature itself. In *M. albus*, although $MO_{2\ \mathrm{water}}$ was similar to values reported in Lefevre et al., (2016) using similar methods, our air-breathing variables ($MO_{2\ \mathrm{air}}$, $\%MO_{2\ \mathrm{air}}$, and F_{AB}) under normal conditions were lower, although $\%MO_{2\ \mathrm{air}}$ was similar under hypoxia and hypoxic hypercapnia. A difference in time of day (overnight vs. in the morning when facility staff was present) when measurements were taken, might contribute to this reduction in air-breathing as *M. albus* retracts from quietly ventilating at the surface when disturbed (personal observation). Singh and Hughes (1971) found in active *C. batrachus*, a similar $MO_{2\ \mathrm{total}}$ to fish in this study, but a $\%MO_{2\ \mathrm{air}}$ = 63% as compared to 9% in this study under normal conditions. Jordan (1976) reported a similar $\%MO_2$ (10%) to our study, but a much lower $MO_{2\ \mathrm{total}}$ (104 at 25°C as compared to 261 mg O_2 kg^{-1} hr^{-1} at 30°C in this study). Despite these differences likely caused by contrasting methods (e.g., 6 l vs 2.5 l respirometry chambers, manual vs. automatic sampling, volumetric vs. flow through respirometry, temperature), our R and F_{AB} values were similar to those in both studies. Data for *P. hypophthalmus* also differed from Lefevre et al. (2011) where a slightly less hypoxic treatment (45 mmHg) resulted in higher $MO_{2\ \mathrm{water}}$ and lower $\%MO_{2\ \mathrm{air}}$. Our $\%MO_{2\ \mathrm{air}}$ values do, however, lie within theirs upper reported range for *P. hypophthalmus*. Despite the variation from other studies, much of which can be likely attributed to changes in behavioral state or difference in methods, the conclusions from this study are not changed as they are based on comparisons of repeated measure across conditions and thus serve as their own controls.

The presence of aquatic hypercapnia had a dramatic influence on aerial CO_2 exchange within species while aquatic hypoxia had little effect. In aquatic hypercapnia, blood PCO_2 likely became elevated from an initial influx of exogenous CO_2 and a buildup of metabolically produced CO_2 as the outward blood-water diffusion gradient at the gills and integument became reduced or even reversed. Elevated blood PCO_2 thus increased diffusion into the air-breathing organ and the CO_2 content of each breath, resulting in elevated $MCO_{2\ air}$ and R in all species in this study.

It is not surprising that we observed $MCO_{2\ air}$ and R increases with F_{AB}. Due to the high diffusive constant of CO_2 relative to O_2, during a breath hold the PCO_2 in the air-breathing organ quickly equilibrates with the blood, while PO_2 continues to be extracted over time (West, 2012). Ventilation is thus the limiting step to aerial CO_2 exchange, and the longer a breath is held, the lower R of exhaled gas becomes. This has been shown in *S. marmoratus* by Lomholt and Johansen (1976) in which air sampled via a buccal catheter had an R of 0.7 immediately after inhalation of an air breath and 0.2 after 10 minutes of a breath hold, as well as in other studies with similar findings in *A. gigas*, *C. argus,* and *Polypterus senegalus* (Ishimatsu and Itazawa, 1981; Magid et al., 1970; Randall et al., 1978). Thus regardless of whether air-breathing is used for PCO_2 regulation or is in response to O_2 demand and aerial CO_2 exchange was merely a passive by-product of shorter breath hold duration, we expect elevated $MCO_{2\ air}$ and R with increases in F_{AB}. Our study was not able to determine the contribution of $MCO_{2\ air}$ to internal PCO_2 regulation, however, it is likely to aid in species where F_{AB} is sensitive to aquatic hypercapnia, such as in *M. albus* and *C. batrachus* (Table 1).

In *M. albus*, respiratory patterns for $MCO_{2\ air}$ in Figure 1D match the increases in F_{AB} consistent with pattern 2, and suggesting active offloading of CO_2 via the air-breathing organ. Regulation is the most likely scenario in *M. albus*, as supported by elevated F_{AB} in response to aquatic hypercapnia shown both in Table 1 and Figure 1A, where normocapnic points (blue) remain in the lower F_{AB} range compared to hypercapnic points (red). The lack of a direct effect of aquatic hypoxia on F_{AB} in *M. albus* may be due to its extremely high hemoglobin affinity (P_{50}=4.1 mmHg at 3% CO_2, Damsgaard et al., 2014), far below the PO_2 used in this study. However, $MO_{2\ air}$ in Figure 1A appears similar to that predicted by pattern 1 indicating some air-breathing response but to internal O_2 demands rather than external conditions (e.g., hypoxia vs. hypercapnia, Table 1). Still, hypercapnic points tend to deviate to the right of the normocapnic points, indicating that for the same $MO_{2\ air}$, F_{AB} is further elevated by CO_2 (Fig. 1A). In *M. albus*, air breathing seems to play a primary role in offloading and regulating internal CO_2. Reduced gill surface area and high arterial blood PCO_2 under normal conditions (>20 mmHg; Damsgaard et al., 2014) further suggest that the gills of *M. albus* are ineffective at aquatic CO_2 offloading and aerial exchange of CO_2 a necessity.

The respiratory patterns of *C. batrachus* in Figure 1B and Table 1 fit those characteristic of pattern 1, suggesting an air-breathing system mainly driven by aquatic hypoxia. Increases in F_{AB} are clearly matched with $MO_{2\ air}$, and data points representing conditions with normal PO_2 levels (circles) cluster along the lower F_{AB} range compared to low PO_2 levels (squares). However, aquatic hypercapnia alone also elevates F_{AB} above normal conditions (Table 1) indicating a sensitivity to CO_2. The combined effect of hypoxia and hypercapnia did not elevate F_{AB} above that of hypoxia alone, suggesting maximum respiratory drive can be elicited solely by O_2 stress. $MCO_{2\ air}$ in *C. batrachus* in Figure 1E remains low for normocapnic points (blue) while hypercapnic points (red) increase along with F_{AB} further suggesting a dominant control of respiration by O_2 but also a sensitivity to CO_2 when hypercapnia is present. Despite elevations in $MCO_{2\ air}$, low R values indicated CO_2 offloading is primarily occurring aquatically (Fig. 1H). Taken together, air breathing in *C. batrachus* is driven by both O_2 and CO_2, and CO_2 is exchanged at both the gills and air-breathing organ.

P. hypophthalmus offloads PCO_2 almost completely through the gills (pattern 1) as evidenced by data shown in Table 1 and respiratory patterns in Figure 1CF and uses air breathing solely to maintain O_2

homeostasis. Ventilatory and gas exchange patterns during normal conditions and normoxic hypercapnia were not determined for *P. hypophthalmus* in this study, as pilot data showed very low air-breathing rates, making it difficult to obtain air-phase measurements. This is in agreement with other studies that show negligible air breathing in aquatic normoxia and hypercapnia (Lefevre et al., 2011; Thomsen et al., 2017). Regardless, *P. hypophthalmus* shows a ventilatory response and CO_2 and O_2 exchange consistent with pattern 1, with close overlap of hypoxic and hypoxic hypercapnic $MO_{2\ \mathrm{air}}$ points (Fig. 1C), and no change in F_{AB} with the addition of hypercapnia (Table 1).

R values from this study are difficult to interpret because $MCO_{2\ \mathrm{air}}$ can be both metabolically and environmentally derived. Hence we cannot calculate the actual proportion of CO_2 respired into the air versus water. Without blood PCO_2 measurements, we cannot determine whether these animals were in a steady state, however, the rate of change in R during the course of hypoxic hypercapnia exposure may be an indicator of changes in blood PCO_2. At the onset of hypoxic hypercapnia, *P. hypophthalmus* immediately increased R, while in *C. batrachus* and *M. albus* R slowly increased after 6 and 9 h respectively (Table 1). The elevation in R likely reflects the time course of changes in blood PCO_2. In *P. hypophthalmus* the time course of changes in R matched those of blood gas measurement in Damsgaard et al. 2015 (<3 h to equilibration), however, similar data has not been collected on the other two species. The much slower change in R in *C. batrachus* and *M. albus* may be due to a slower rate of loading of external CO_2 at the gills and integument. The rate of CO_2 loading would be determined by the branchial and cutaneous perfusion and diffusion capacities (i.e., diffusion distance and surface area). *P. hypophthalmus* has both a high surface area and thinner diffusion distance (212 $mm^2\ g^{-1}$, 1.67 µm) and thus a higher diffusion capacity than *C. mossambicus* (17.33 $mm^2\ g^{-1}$, 1.97 µm), a related species to *C. batrachus* (Maina and Maloiy, 1986; Phuong et al., 2017). Although measurements of gill surface area could not be found for *M. albus*, it likely has the lowest diffusive capacity of the three species with a significantly reduced gill surface area (filaments on only the first three gill arches) and a higher diffusion distance (4.09 µm) found in a relative, *M. cuchia* (Hughes and Datta Munshi, 1979; Iversen et al., 2013).

M. albus is further insulated from aquatic hypercapnia as it uses its buccal chamber for air-breathing, such that during a breath hold, gill ventilation ceases (Iversen et al., 2013). Although the integument in another synbranchid (*S. marmoratus*) is known to contribute between 30-60% of $MO_{2\ \mathrm{total}}$ (Graham et al., 1986), its permeability may be overstated due to its relatively low overall metabolic rate. However, $MO_{2\ \mathrm{water}}$ may still be maintained through these surfaces using an extremely high hemoglobin affinity (Damsgaard et al., 2014). In *M. albus*, its mode of breathing as well as the morphological features that insulate the blood from the aquatic environment may be responsible for the putative slow change in internal PCO_2 and R. Blood PCO_2 may even be maintained below aquatic conditions as was found in *Protopterus dolloi* exposed to aquatic hypercapnia (40 mmHg), where blood PCO_2 remained constant, attributed to increased pulmonary CO_2 excretion (Perry et al., 2005). Even if *M. albus* is unable to maintain its blood PCO_2 below the ambient levels when exposed to hypercapnia for prolonged durations, a slower rate of CO_2 uptake will of course also slow the rate at which the acid-base disturbance occurs, giving more time for pH compensation by transepithelieal ion-transfer. This will potentially reduce the magnitude of the pH disturbance relative to the perturbation otherwise observed if air-breathing had not been utilized. This cannot be determined directly from our results, as the difference in the rate and size of acid-base disturbance would have to be compared between fish with and without access to the surface (i.e. only with aquatic gas exchange available) – which is not possible in an obligate air-breather such as *M. albus*.

Along with R, F_{AB} was also elevated slowly in *M. albus* and may indicate the presence of internal facing CO_2/H^+ chemoreceptors. Change in F_{AB} with exposure to hypoxic hypercapnia match a slow graded change in internal PCO_2 rather than the immediate and sustained elevation of the external environment. Similarly, in the spotted gar, which is argued to possess central CO_2/H^+ chemoreceptors (Wilson et al., 2000), exposure to aquatic hypercapnia of 8 mmHg caused a gradual increase in F_{AB} over an 8 hour period (Smatresk and Cameron, 1983). Regardless of internal or external facing chemoreception, the air-breathing response to aquatic hypercapnia alone is present in some fishes (*Ancistru chargresi*, *Hypostomus plecostomus*, *S. marmoratus*, *Lepisosteus osseus*, *A. calva*) but absent or blunted in others

(*Misgurnus anguillicaudatus, Gillicthys mirabilis, Electrophorus electricus, P. hypophthalmus*) (Graham, 1997; Milsom, 2012; Thomsen et al., 2017).

How animals respond to hypercapnia is an important issue from an environmental, anthropogenic, and evolutionary perspective. Air-breathing fishes have evolved to thrive in these marginal aquatic habitats that are both hypoxic and hypercapnic. Presumably, the first terrestrial vertebrates emerged from similar habitats, likely utilizing many earlier adaptations to these harsh aquatic conditions for life on land. One central theme in the terrestrial transition is the shift from water to air breathing and insulation from aquatic conditions. Hypoxia has been thought to be the dominant driver in this transition, and hypothesized to cause the reduction in gill diffusion capacity found in numerous fishes to limit transbranchial O_2 loss (Graham, 1997; Randall et al., 1981). The reduced ability to breath water likely served as a more important preadaptation for life on land rather than the capacity to breath air in terms of CO_2 exchange. However, hypoxia alone does not always guarantee these changes and the transition from water to air breathing. As evidenced by *P. hypophthalmus,* O_2 exchange can be shifted to air breathing, while CO_2 exchange remains at the gills and dependent on the aquatic environment. Rather, how a fish responds to hypercapnia and where they lie along spectrum of responses between pattern 1 or 2 may be a good predictor of amphibiousness and an important factor as a preadaptation for terrestrial life. Our data show a correlation between pattern 2 and amphibiousness where the exchange of O_2 and CO_2 are recoupled. Ultsch (1996), proposed that physiological traits that allowed survival in hypercapnic bodies of water provided a suite of preadaptations for terrestrial life that also include elevated blood PCO_2 and bicarbonate buffering capacity, reduced pH, and an air-breathing drive sensitive to CO_2/H^+.

Findings from this study demonstrate contrasting effects of aquatic hypercapnia on three phylogenetically diverse species of air-breathing fishes. All fishes can increase CO_2 exchange at the air-breathing organ, however, some do so by actively increasing air ventilation and others only as a passive property of elevated PCO_2 in the blood diffusing into the air-breathing organ. In the latter case, CO_2 exchange is mainly maintained through gills. These differences stem from the independent evolutions of air-breathing system among fishes yielding unique respiratory systems, behaviors, and physiological responses. Our data do not evaluate which method is more effective in dealing with aquatic hypercapnia, but rather demonstrate the diversity of possible evolutionary outcomes in response to the same environmental challenge.

Funding

This research was supported by the Journal of Experimental Biologist Travelling Fellowship (JEBTF-150426) to the primary author and the Danish Ministry of Foreign Affairs (DANIDA).

References

Brauner, C. J. and Baker, D. W. (2009). Patterns of acid–base regulation during exposure to hypercarbia in fishes. In *Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects*, pp. 43–63.

Burggren, W. W. (1979). Biomodal Gas Exchange During Variation in Environmental Oxygen and Carbon Dioxide in the Air Breathing Fish, *Trichogaster trichopterus*. *J. Exp. Biol.* **82**, 197–213.

Damsgaard, C., Findorf, I., Helbo, S., Kocagoz, Y., Buchanan, R., Huong, D. T. T., Weber, R. E., Fago, A., Bayley, M. and Wang, T. (2014). High blood oxygen affinity in the air-breathing swamp eel *Monopterus albus*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **178**, 102–108.

Damsgaard, C., Gam, L. T. H., Dang, D. T., Van Thinh, P., Huong, D. T. T., Wang, T. and Bayley, M. (2015a). High capacity for extracellular acid-base regulation in the air-breathing fish *Pangasianodon hypophthalmus*. *J. Exp. Biol.* **218**, 1290–1294.

Damsgaard, C., Phuong, L. M., Huong, D. T. T., Jensen, F. B., Wang, T. and Bayley, M. (2015b). High affinity and temperature sensitivity of blood oxygen binding in *Pangasianodon hypophthalmus* due to lack of chloride-hemoglobin allosteric interaction. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **308**, R907–R915.

DeJours, P. (1981). *Principles of Comparative Respiratory Physiology - P. Dejours - 1981.pdf*.

Farrell, A. P. (2011). *Encyclopedia of Fish Physiology: From Genome to Enviornment*. (ed. Farrell, A. P.), Joseph, C. J.), Richards, J. G.), and Stevens, E. D.) Elsevier Inc.

Glass, M. L., Ishimatsu, A. and Johansen, K. (1986). Responses of aerial ventilation to hypoxia and hypercapnia in *Channa argus*, an air-breathing fish. *J. Comp. Physiol. B* **156**, 425–430.

Graham, J. B. (1997). *Air-breathing Fishes - Evolution, diversity and adaptation*. San Diego, CA: Academic Press.

Graham, J. B., Baird, T. A. and Stockmann, W. (1986). The transition to air breathing in fishes : IV . Impact of branchial specializations for air breathing on the aquatic respiratory mechanisms and ventilatory cost of the swamp eel *Synbranchus Marmoratus*. *J.Exp.Biol.* 83–106.

Hu, Y., Ni, Q., Wu, Y., Zhang, Y. and Guan, C. (2011). Study on CO2 removal method in recirculating aquaculture waters. *Procedia Eng.* **15**, 4780–4789.

Hughes, G. M. and Datta Munshi, J. S. (1979). Fine Structure of the Gills of Some Indian Air-Breathing Fishes. *J. M* **160**, 169–194.

Hughes, G. M. and Singh, B. N. (1970). Respiration in an Air-Breathing Fish , the Climbing Perch *Anabas Testudineus Bloch I* . Oxygen Uptake and Carbon Dioxide Release. 265–280.

Ishimatsu, A. (2012). Evolution of the Cardiorespiratory System in Air-Breathing Fishes. *Aqua-BioScience Monogr.* **5**, 1–28.

Ishimatsu, A. and Itazawa, Y. (1981). Ventilation of the Air· breathing Organ in the Snake-head *Channa argus*. *Japanese J. Ichthyol.* **28**, 276–283.

Ishimatsu, A., Hayashi, M. and Lee, K. (2005). Physiological effects on fishes in a high CO2 world. *J. Geophys. Res.* **110**, 1–8.

Iversen, N. K., Lauridsen, H., Huong, D. T. T., Van Cong, N., Gesser, H., Buchanan, R., Bayley, M., Pedersen, M. and Wang, T. (2013). Cardiovascular anatomy and cardiac function in the air-breathing swamp eel (*Monopterus albus*). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **164**, 171–180.

Jew, C. J., Wegner, N. C., Yanagitsuru, Y., Tresguerres, M. and Graham, J. B. (2013). Atmospheric oxygen levels affect mudskipper terrestrial performance: Implications for early tetrapods. *Integr. Comp. Biol.* **53**, 248–257.

Jordan, J. (1976). The inflence of body weight on gas exchange in the air-breathing fish, *Clarias batrachus*. *Comp. BIoehem. Physiol.* **53A**, 305–310.

Lefevre, S., Huong, D. T. T., Wang, T., Phuong, N. T. and Bayley, M. (2011). Hypoxia tolerance and partitioning of bimodal respiration in the striped catfish (*Pangasianodon hypophthalmus*). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **158**, 207–214.

Lefevre, S., Wang, T., Jensen, A., Cong, N. V., Huong, D. T. T., Phuong, N. T. and Bayley, M. (2014). Air-breathing fishes in aquaculture. What can we learn from physiology? *J. Fish Biol.* **84**, 705–731.

Lefevre, S., Bayley, M. and Mckenzie, D. J. (2016a). Measuring oxygen uptake in fishes with bimodal respiration. *J. Fish Biol.* **88**, 206–231.

Lefevre, S., Findorf, I., Bayley, M., Huong, D. T. T. and Wang, T. (2016b). Increased temperature tolerance of the air-breathing Asian swamp eel *Monopterus albus* after high-temperature acclimation is not explained by improved cardiorespiratory performance. *J. Fish Biol.* **88**, 418–432.

Lomholt, J. P. and Johansen, K. (1976). Gas Exchange in the Amphibious Fish , *Amphipnous cuchia**. *J. Comp. Physiol. B* **157**,.

Magid, A. M. A., Vokac, Z. and Ahmed, N. E. D. (1970). Respirtory Function of the Swim-bladders of the Primitive Fish *Polypterus Senegalus*. *J. exp. Biol.* 27–37.

Maina, J. N. and Maloiy, G. M. O. (1986). The morphology of the respiratory organs of the African air-breathing catfish (*Clarias mossambicus*): A light, electron and scanning microscopic study, with morphometric observations. *J. Zool.* **209**, 421–445.

Martin, K. L. M. (1995). Time and tide wait for no fish: Intertidal fishes out of water. *Environ. Biol. Fishes* **44**, 165–181.

Martin, K. L. (2014). Theme and variations: Amphibious air-breathing intertidal fishes. *J. Fish Biol.* **84**, 577–602.

Milsom, W. K. (2012). New insights into gill chemoreception: Receptor distribution and roles in water and air breathing fish. *Respir. Physiol. Neurobiol.* **184**, 326–339.

Perry, S. F., Gilmour, K. M., Swenson, E. R., Vulesevic, B., Chew, S. F. and Ip, Y. K. (2005). An investigation of the role of carbonic anhydrase in aquatic and aerial gas transfer in the African lungfish *Protopterus dolloi*. *J. Exp. Biol.* **208**, 3805–3815.

Phuong, L. M., Huong, D. T. T., Nyengaard, J. R. and Bayley, M. (2017). Gill remodelling and growth rate of striped catfish *Pangasianodon hypophthalmus* under impacts of hypoxia and temperature. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* **203**, 288–296.

Phuong, L. M., Thi, D., Huong, T., Malte, H., Nyengaard, J. R. and Bayley, M. (2018). Ontogeny and morphometrics of the gills and swim bladder of air-breathing striped catfish *Pangasianodon* *hypophthalmus*. *J.Exp.Biol.* **221**,.

Randall, D. J., Farrell, A. P. and Haswell, M. S. (1978). Carbon dioxide excretion in the pirarucu (*Arapaima giga*), an obligate air-breathing fish. *Can. J. Zool.* **56**, 977–982.

Randall, D. J., Cameron, J. N., Daxboeck, C. and Smatresk, N. (1981). Aspects of bimodal gas exchange in the bowfin,. *Amia calva L.* (Actinopterygii: Amiiformes). *Respir. Physiol.* **43**, 339–348.

Shartau, R. B. and Brauner, C. J. (2014). Acid-base and ion balance in fishes with bimodal respiration. *J. Fish Biol.* **84**, 682–704.

Smatresk, N. J. and Cameron, J. N. (1983). Respiration and Acid-Base Physiology of the Spotted Gar, A Bimodal Breather: I. Normal Values, and the Response to Severe Hypoxia. *J. Exp. Biol.* **96**, 263–280.

Thomsen, M. T., Wang, T., Milsom, W. K. and Bayley, M. (2017). Lactate provides a strong pH-independent ventilatory signal in the facultative air-breathing teleost *Pangasianodon hypophthalmus*. *Sci. Rep.* **7**, 6378.

Ultsch, G. R. (1987). The potential role of hypercarbia in the transition from water-breathing to air-breathing in vertebrates. *Evolution (N. Y).* **41**, 442–445.

Ultsch, G. R. (1996). Gas exchange, hypercarbia and acid-base balance, paleoecology, and the evolutionary transition from water-breathing to air-breathing among vertebrates. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **123**, 1–27.

West, J. B. (2012). *Respiratory Physiology, The Essentials*. 9th ed. La Jolla, California: Lippincott Williams & Wilkins.

Willmer, E. N. (1934). Some observations on the respiration of certain tropical fresh-water fishes. *J.Exp.Biol.* **11**, 283–306.

Wilson, R. J., Harris, M. B., Remmers, J. E. and Perry, S. F. (2000). Evolution of air-breathing and central CO2/H+ respiratory chemosensitivity: new insights from an old fish? *J. Exp. Biol.* **203**, 3505–3512.

CHAPTER 3

Hypoxia and Hypercapnia Avoidance Behaviors in Air-breathing Fishes

Introduction

Fish that live in tropical freshwater habitats must overcome harsh environmental conditions. These environments generally have heavy organic loads and are turbid and stagnant, creating high rates of bacterial respiration, little photosynthetic activity, and low mixing with the atmosphere. The result is animals inhabiting these areas frequently experience hypoxic and hypercapnic aquatic conditions, and such environments pose a significant challenge for gas exchange (Ultsch, 1996; Willmer, 1934). Aquatic hypoxia can limit an animal's capacity for aerobic metabolism, reducing its ability for locomotion, growth, reproduction, and survival. Aquatic hypercapnia is also known to negatively impact growth and survival by causing a respiratory acidosis and, at high enough levels, an anesthetic affect (Bernier and Randall, 1998; Brauner and Baker, 2009; Ishimatsu et al., 2005; Ross et al., 2001).

One strategy of dealing with these conditions is behavioral; simply avoid aquatic hypoxia and hypercapnia and move to another area. Both laboratory and field studies have shown fishes possess hypoxic and hypercapnic avoidance behaviors, actively seek out other areas with more favorable conditions (Beitinger and Pettit, 1984; Black et al., 1954; Kates et al., 2012; Kramer, 1987). However, leaving an area is not without its costs, as it limits an animal's habitat use area, for forging, predator evasion, and mating opportunities (Kramer, 1983; Kramer, 1987). Avoidance behavior is thus an integrated response that involves the tradeoffs between the cost incurred from inhabiting poor aquatic conditions and the opportunity cost of leaving.

One adaptation to inhabiting waters that frequently become hypoxic and hypercapnic is the ability to breath air (Graham, 1997). In fact, hypoxia has been hypothesized to be the primary environmental stressor that drove the 64 independent evolutions of air breathing in fishes. Air-breathing enables these fishes to use the atmosphere as an auxiliary source of O_2, thereby allowing the rate O_2 consumption to continue independently of aquatic O_2 content. These fishes circumvent detrimental effects of aquatic hypoxia and possess a distinct competitive advantage in marginal aquatic habitats through increased aerobic capacity and habitat utilization. However, the cost of swimming to the surface to breathe air has its own tradeoffs as well summarized (Kramer, 1987). These include the energetic and time costs of surfacing and increased predation risk. Thus, the relationship between air-breathing and habitat selection is complex, poorly understood, and likely differs among the diversity of air-breathing fish species and their ecological roles.

Avoidance behavior to both hypoxia and hypercapnia are of increasing interest in both wildlife management and the aquaculture industry. Avoidance behaviors have been proposed for invasive species control utilizing a plum of hypercapnia as non-physical dispersal barrier and in aquaculture to drive self-transferring of fish between tanks (Clingerman et al., 2007; Kates et al., 2012). Little is known about the avoidance behaviors in air-breathing fishes which is of increasing relevance as the use of these fishes in aquaculture has seen significant growth over the last two decades. Understanding the fundamental biology of air-breathing fishes and defining favorable grow out conditions is vital for the healthy and efficient production of these animals (Lefevre et al., 2014).

To test how air-breathing influences hypoxia and hypercapnia avoidance, we observed the behavior of two air-breathing fishes with and without air access. The first species, *Polypterus lapradei* is native to African tropical freshwater habitats and a member of a primitive predatory family of fishes possessing paired ventral lungs. The second species, *Pangasianodon hypophthalmus*, is a catfish native to Southeast Asia rivers and utilizes a modified gas bladder for breathing air. Although air-breathing in both species is driven by O_2, only in *Po. lapradei* is air-breathing also driven by CO_2 (Ch.1, Ch.2)(Thomsen et al., 2017). Therefore, we expect air-breathing to effect hypoxia avoidance in both *Po. lapradei* and *Pa. hypophthalmus*, but hypercapnia avoidance in only *Po. lapradei.*

Materials and Methods

Polypterus lapradei and *Pangasianodon hypophthalmus* were obtained though the aquarium trade and kept in a recirculating aquarium system at the University of California Irvine on a 12/12 h light cycle. Aquarium water was held at 30°C and fish were feed commercial feed (Starmilling Co., Perris, CA) twice daily.

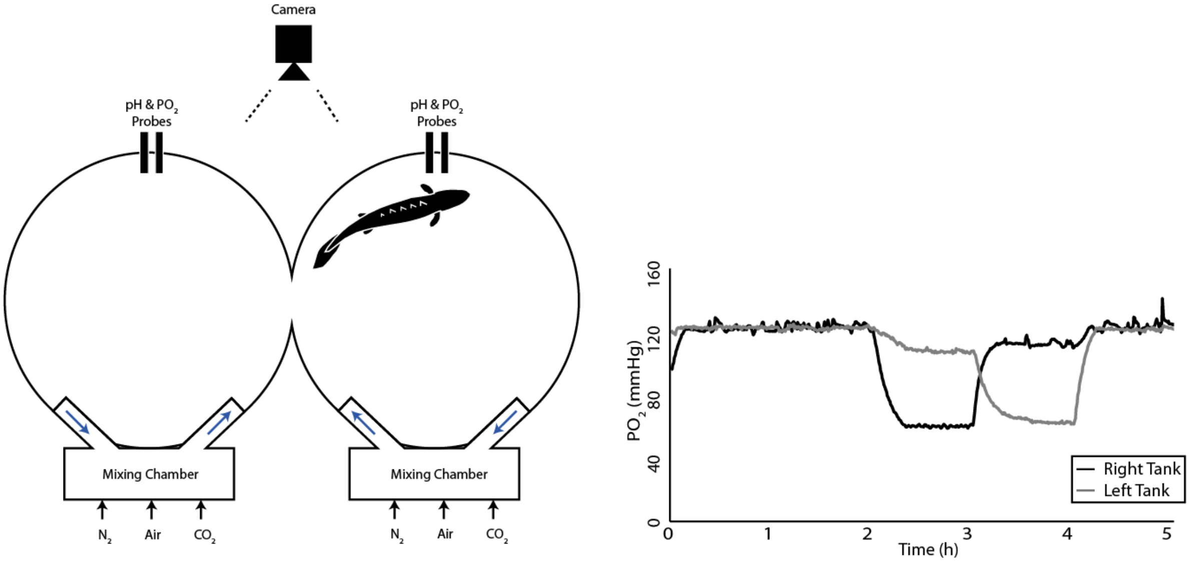

Figure 3.1. Shuttle Box Schematic and PO$_2$ Example Trace.
(A) Shuttle box set and (B) PO$_2$ example during hypoxia trial. (A) Conditions in each arena were monitored separately by PO$_2$ and pH probes and regulated by bubbling compressed gases into mixing chambers. Position was recorded using a camera placed above the shuttle box. (B) Animals were acclimated for 1 hour in air equilibrated water. Position was then monitored for 1 h with left and right side in the same condition, 1 h right side challenge (hypoxia or hypercapnia), 1 h left side challenge, and returned to identical condition again.

To determine if avoidance behavior was present, we used a shuttle box set up consisting of two intersecting circular arenas (diameter=60 cm, depth = 20 cm) allowing a 12 cm wide passageway between either side (Fig. 1). Water on each side was pumped into two elevated 25 l mixing chambers which gravity fed back into their respective arenas creating opposing circular flow.

Shuttle box conditions were monitored and regulated by a custom-built system consisting of O_2 and pH probes (Atlas scientific) connected to an Arduino microcontroller. This system bubbled compressed air, N_2, and CO_2 into the mixing chambers by operating a series of solenoid valves. To convert pH to PCO_2, a calibration curve was made using a pH probe and gas mixer Figure 2. One fish was placed at a time in a random arena and were allowed 1 hour of acclimation, followed by another hour during which conditions in both arenas were identical to serve as a control. A hypoxic or hypercapnic challenge was then imposed on one side, after which conditions were switched between sides, and then brought back to identical levels again, each for an hour. Aquatic PO_2 and PCO_2 levels tested are reported in Table 1. During hypoxia trials PCO_2 was held at ambient (0 mmHg), and during hypercapnic trials PO_2 remained above 130 mmHg. For trials where air access was denied, a plastic netting and with PVC frame was placed below the water's surface. PO_2 and pH were recorded every 30 s, and fish position was monitored at 30 s intervals using a time lapse camera. Videos were digitized in MatLab using DLTdv digitizing tool (Hedrick, 2008). Differences in time spent on the right versus left arena during the phases of each trail were analyzed for statistical significance by repeated measures ANOVA with post-hoc Tukey test or paired T-test. To describe the degree of avoidance behavior, an avoidance score for each trial was calculated using the following equations:

$$Avodience\ score\ (\%) = \frac{Time\ spent\ outside\ hypoxic\ arena - \frac{Total\ Time}{2}}{\frac{Total\ Time}{2}} x100$$

This score represents the percentage of time spend outside of the hypoxic arena above random chance (half of total time). An avoidance score of 0% would mean equal time was spent in each arena, and 100%

would mean the entire time was spent outside the hypoxia arena. This value was averaged for individual across right and left side challenges, and the formula was for hypercapnia as well.

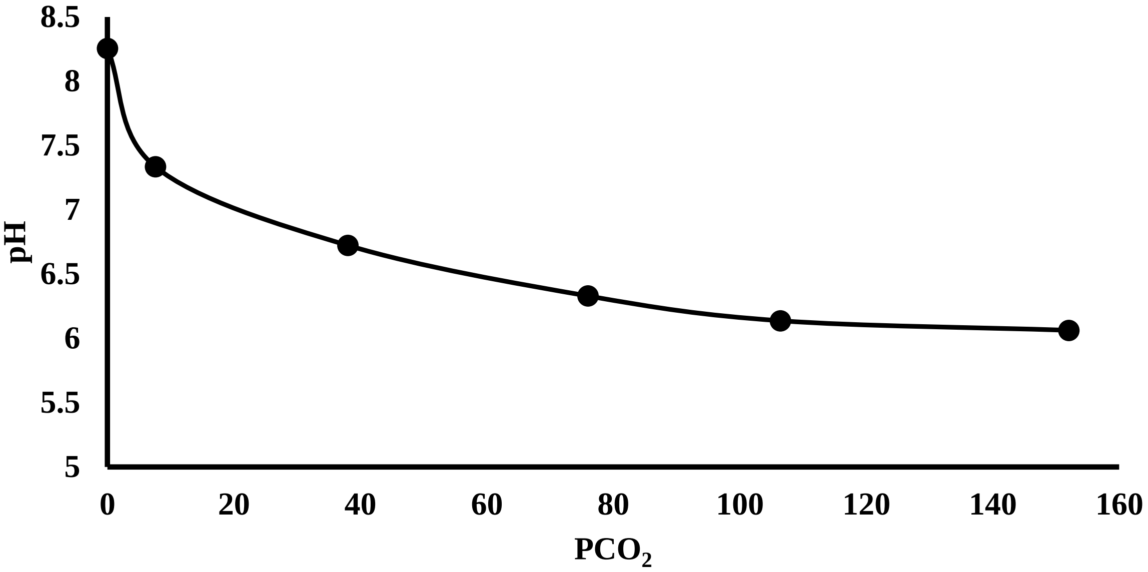

Figure 3.2. Calibration curve for pH/PCO$_2$ conversion.
Calibration curve was created by bubbling known CO$_2$ and air mixtures into a water sample and recording pH at equilibrium. T=30°C, Total Alkalinity = 120 CaCO$_3$ eq.

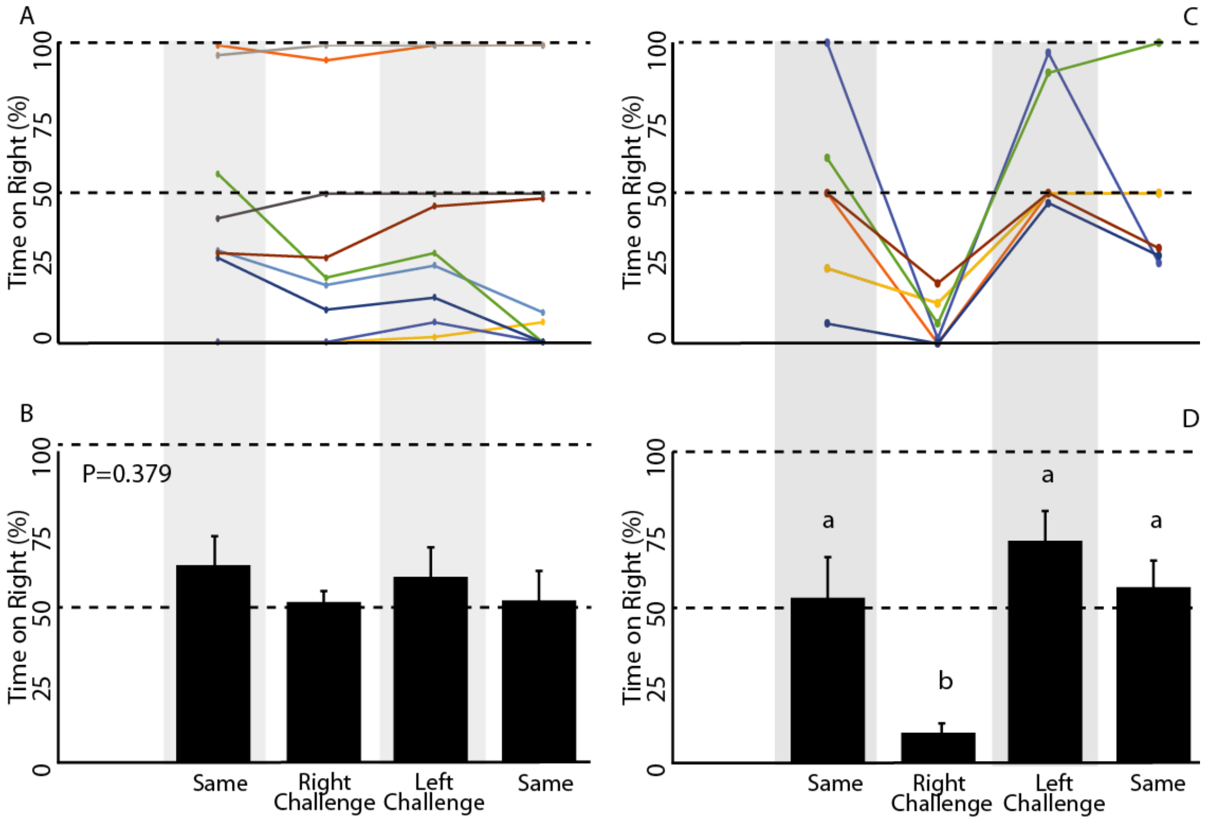

Figure 3.3. Example data from *Po. lapradei* hypoxia trials (AB) with and (CD) without air access. Top panels (AC) show percentage of time spent in right arena for individual fish by colors and bottom panels (BD) show mean ± s.e.m. Letters signify statistical significance ($p<0.05$) determined by ANOVA with post-hoc Tukey Test.

Results

Hypoxia avoidance

At 75 mmHg $O_{2,}$ *Po. lapradei* spent equal time in either arena across treatments when able to air breathe but demonstrated a hypoxia avoidance behavior when air access was denied (Table 1, Fig. 3). This resulted in a contrasting in hypoxia avoidance score with (8±4 %) and without air access (71±14 %) under the identical hypoxic intensities.

Pa. *hypophthalmus* did not demonstrate any hypoxia avoidance behavior at any level of hypoxia tested even when air breathing was denied (Table 1). No difference in time spent in either arena during a hypoxia challenge of 75 mmHg with or without air-access, nor was there a difference at 50 or 37 mmHg O_2. Nevertheless, at 37 mmHg O_2, *Pa*. *hypophthalmus* was observed attempting to breathe air indicating hypoxic stress. Hypoxia avoidance score remained low (1-17%) in all trials, even without air access.

Species	High PO_2 (mmHg)	Low PO_2 (mmHg)	Air access	N (mass)	Avoidance Score (%)	Avoidance Behavior
Po. lapradei	115	75	Yes	8 (196±16 g)	8±4	No
	115	75	No	7 (178±12 g)	71±13	Yes
Pa. hypophthalmus	115	75	Yes	11 (72±10 g)	2±14	No
	115	75	No	9 (75±6 g)	1±5	No
	70	50	No	6 (66±6 g)	17±14	No
	50	37	No	5 (67±5 g)	3±7	No

Table 3.1. Hypoxia Avoidance Trial PO_2 and Results.
Avoidance behavior was determined present if location between right side and left side challenge significantly differed by ANOVA. PCO_2=0 mmHg for all trials. Avoidance score represents the percentage of time spend outside of the hypoxic arena above random chance (half of total time).

Hypercapnia avoidance

Po. Lapradei demonstrated a hypercapnia avoidance behavior with and without the ability to air breathe, yet the threshold was elevated when air breathing was denied (Table 2). No difference in time spent in either arena was observed at 38 mmHg CO_2 without air access. At 57 mmHg CO_2, *Po. Lapradei* spent significantly less time in the high PCO_2 arena when air access was denied (avoidance score= 68±14 %) but not when air access was available (15±15 %). With a hypercapnia challenge of 137 mmHg *Po. Lapradei* avoided hypercapnia even when able to breathe air (avoidance score=52±16%).

Pa. *hypophthalmus* displayed a hypercapnia avoidance behavior, but access to air-breathing did not change the threshold of this behavior (Table 2). No difference in the time spent in either arena was observed in 38 mmHg CO_2 with air access. In 57 mmHg CO_2, no statistical difference was detected with a repeated measures ANOVA with or without air access. However, due to the ANOVAs' low statistical power resulting from low sample sizes, paired t-tests were run between right and left side challenges treatments and revealed statistical significance both with and without air-access. Regardless the small difference in avoidance scores (21±7 % with and 32±13 % without air access) indicate air breathing had little impact behavior. With a hypercapnia challenge of 137 mmHg *Pa*. *hypophthalmus* avoided hypercapnia even when able to breathe (avoidance score =79±9%).

Species	High PCO_2 Low pH	Low PCO_2 High pH	Air access	N (mass)	Avoidance Score (%)	Avoidance Behavior
Po. lapradei	38 mmHg 6.8	19 mmHg 7.0	No	6 (199±19 g)	2±9	No
	57 mmHg 6.5	38 mmHg 6.8	Yes	7 (182±21 g)	15±15	No
	57 mmHg 6.5	38 mmHg 6.8	No	6 (164±23 g)	68±14	Yes
	137 mmHg 6.1	57 mmHg 6.5	Yes	10 (173±19 g)	52±16	Yes
Pa. hypophthalmus	38 mmHg 6.8	19 mmHg 7.0	Yes	15 (82±4 g)	9±8	No
	57 mmHg 6.5	38 mmHg 6.8	Yes	11 (80±9 g)	21±7	No – ANOVA Yes – t-test
	57 mmHg 6.5	38 mmHg 6.8	No	8 (101±5 g)	32±13	No - ANOVA Yes – T-test
	137 mmHg 6.1	57 mmHg 6.5	Yes	10 (99±10 g)	79±9	Yes

Table 3.2: Hypercapnia Avoidance Trial PCO_2 and Results.
Avoidance behavior was determined present if location between right side and left side challenge significantly differed by ANOVA (or T-test if noted). PO_2 > 130 mmHg for all trials. Avoidance score represents the percentage of time spend outside of the hypercapnic arena above random chance (half of total time).

Discussion

Hypoxia avoidance

Air breathing allows fish to access the atmosphere as an auxiliary source of O_2 and enables them to maintain rates of O_2 consumption independent of aquatic supply. Air-breathing fishes therefore elude the high cost of inhabiting hypoxic waters associated with the reduction in metabolism and resulting in a distinct advantage over solely water breathing fishes in similar marginal aquatic habitats. *Po. lapradei* is an air breather and behaved as expected, inhabiting water independent of aquatic PO_2. Thus air-breathing lowers the hypoxic threshold for habitat selection, enabling *Po. lapradei* to increase its viable habitat range. However, *Po. lapradei* does retain a hypoxia avoidance behavior that can be observed when air access is denied. The lack of a hypoxia avoidance behavior was also found in another air-breather from the polypterid family, *Erpetoicthys calabaracus*, with air access, yet without air access was not tested (Beitinger et al., 1985).

In contrast, *Pa. hypophthalmus* did not demonstrate a hypoxia avoidance behavior at any aquatic PO_2 tested, even when air-access was denied. We are confident the severity of hypoxia used in this study (37mmHg) was a significant challenge for *Pa. hypophthalmus* as the air-breathing threshold was reported to be 40-59 mmHg O_2 (Lefevre et al., 2011a; Thomsen et al., 2017) with a P_{crit} without air access of 55 mmHg O_2 (Lefevre et al., 2011a) (Fig. 4). This is further supported by the observation of frequent attempts in the 37 mmHg O_2 trial to reach the surface through the netting and air breathe. *Pa. hypophthalmus* is not tolerant to the levels of hypoxia used in this study, yet a hypoxia avoidance behavior was not observable.

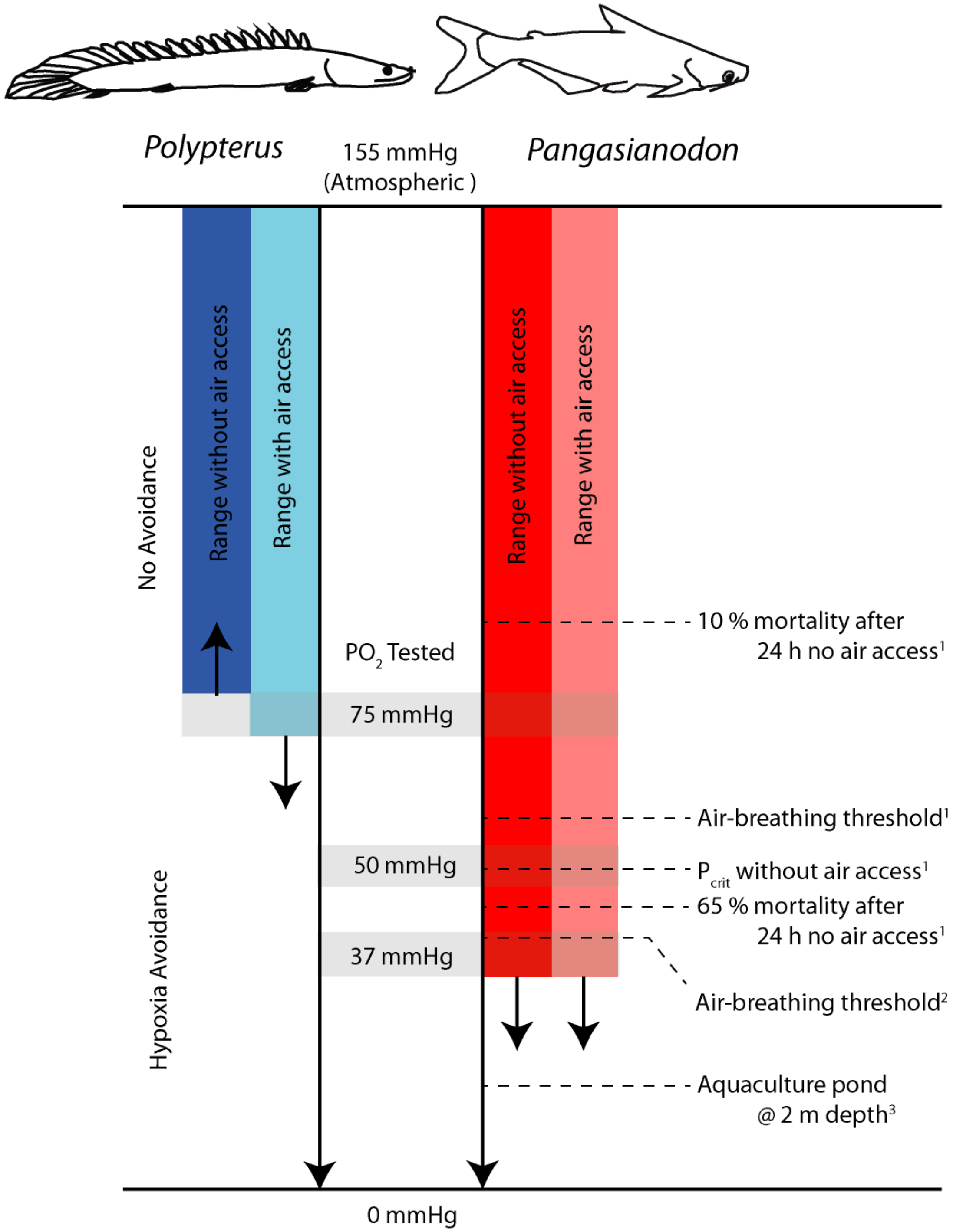

Figure 3.4. Thresholds for hypoxia avoidance behaviors in *Po. lapradei* and *Pa. hypophthalmus*.
[1](Lefevre et al., 2011a) [2](Thomsen et al., 2017) [3](Damsgaard et al., 2015)

In the wild, physical restriction from the water's surface is extremely rare, but there are many behavioral or physiological barriers to air-breathing, including risk of predation and energetic and opportunity costs (Kramer, 1987). Under the framework of optimal use theory where O_2 is the critical resource, Kramer, 1987 models a number of scenarios in which fish in hypoxia would choose a number of alternative behaviors to air-breathing. Under optimal use theory, the chosen behavior will be the one that maximizing the amount of O_2 obtained while minimizing the cost. For example, if occupying hypoxic water at depth and the energetic requirement to traversing the water column is significant, an animal may choose to leave this area rather than stay and breathe air. Depth has been shown to be a large factor determining the cost of air-breathing, particularly in benthic fishes, increasing not only energetic, but time cost of surfacing. In *Coreydoras aeneus,* Kramer and McClure, 1981 showed an inverse relationship between depth (10-120 cm) and air breathing frequency and estimated that the cost of air-breathing can exceed its resting metabolic rate and 25% of its time budget. Elevated energetic demands of air breathing in deeper water has been shown to increase food consumption, as well as reduce the food conversion ratio and growth rate in the striped snakehead (*Channa striata*) (Arunachalam et al., 1976; Pandian and Vivekanandan, 1976; Vivekanandan and Pandian, 1977). Other activities are sometimes prioritized over air breathing as it can represent a significant opportunity cost. For example, *C. aeneus* spends less time air breathing when feeding opportunities are available (Kramer and McClure, 1980) and newts have also been observed to spend less time air breathing during courtship (Halliday, 1977). Air-breathing is also reduced when the predation risk high, as demonstrated by (Kramer et al., 1983) showing reduced breathing rates in six species of air-breathing fishes when an avian predator is present. Under the circumstances above, if the cost of air-breathing is substantial, hypoxia avoidance may be the optimal behavioral response over air-breathing.

Differences in the behavioral ecology of *Po. lapradei* and *Pa. hypophthalmus* may explain the presences and absence of a hypoxia avoidance behavior in these two species. *Po. lapradei* is a sedentary benthic predator only rising on rare occasions to air breathe. The cost of air breathing for *Po. lapradei* would be significantly elevated in deeper water and while stalking prey. In contrast, *Pa. hypophthalmus* is a highly active migratory river fish, inhabiting the upper water column. A telemetry study reported that *Pa. hypophthalmus* rarely descends below 1 m depth in aquaculture ponds (Lefevre et al., 2011b). Its constant presence near the water's surface suggest that air-breathing is always available, and of low time and energetic cost. Further, the metabolic rate of *Pa. hypophthalmus* is 5 times that of *Po. lapradei* (during forced emersion) and in a related species, *Po. senegalus*, (submerged) (Jew in Prep Ch1.) (Babiker, 1984; Lefevre et al., 2011a). Air breathing likely represents a much lower proportion of total energy budget of *Pa. hypophthalmus* than *Po. lapradei*. The selective pressure to maintain a hypoxia avoidance behavior may therefore also be reduced in *Pa. hypophthalmus* if constantly inhabiting hypoxic environments and the cost of air breathing is always low.

One way possibly mechanism by which a hypoxia avoidance behavior may be absent in *Pa. hypophthalmus* is a stronger dependence on internal than external facing O_2 chemoreceptors. External facing O_2 chemoreceptors detect aquatic PO_2, as opposed to internal facing receptors that detect blood PO_2. The orientation of receptors involved in different components of the cardiorespiratory response to hypoxia (e.g., tachycardia, vascular resistance, branchial ventilation and amplitude, air-breathing) have been shown to be variable between water and air-breathing species and within air-breathing species (reviewed in (Milsom, 2012). For example, the air-breathing (and air surface respiration) reflex occurs in response to stimulation of only external facing receptors by NaCN injection into the bucco cavity in tambaqui (*Colosso mesopotamicus*) and bowfin (*Amia calva*), only to internal facing receptors by NaCN injection into the blood in the jeju (*Hoplerythinus unitaeniatus*), gar (*Lepisosteus osseus*), and African lungfish (*Protopterus*), and to both orientations in the mullet (*Mugil cephalu*) (Florindo, 2006; Lahiri et al., 1970; Lopes et al., 2010; Mckenzie et al., 1991a; Mckenzie et al., 1991b; Shingles et al., 2005; Smatresk, 1986; Smatresk and Cameron, 1983; Smatresk et al., 1986). While the hypoxia avoidance behavior has not been linked to chemoreceptor orientation and is an integrated response of multiple physiological and behavioral factors (Kramer, 1987), the ability to detect external O_2 would be an essential part of locating habitats with differing O_2 content. However, detecting external O_2 is likely not important for *Pa. hypophthalmus*, frequently inhabiting hypoxic environments and with a low cost of air-breathing. Rather monitoring internal O_2 would be the more important O_2 homeostasis, resulting in a blunted response to input from external facing O_2 receptors and a low selective pressure for maintaining this system.

Hypercapnia avoidance

Both *Po. lapradei* and *Pa. hypophthalmus* were found to possess a hypercapnia avoidance behavior, however only in *Po. lapradei* was the threshold and degree of avoidance affected by the ability to air-breath. This was expected as *Po. lapradei* is known to have an air-breathing response to aquatic hypercapnia (Ch. 1) while *Pa. hypophthalmus* does not (Thomsen et al., 2017) (Ch. 2).

Air-breathing hyperventilation for the purpose of blood PCO_2 regulation in aquatic hypercapnia has yet to be demonstrated in fishes. The mostly likely reason for the presence of a hypercapnia air-breathing reflex in *Po. lapradei* is in response to changes in internal pH, important to acid-base regulation during amphibious activity. However, *Po. lapradei*, also shows a reduction in branchial ventilation in hypercapnia (Ch.1) when submerged, possibly reducing trans-branchial exchange of CO_2 and transitioning to pulmonary exchange. The current study shows air breathing also elevates the threshold of hypercapnia avoidance behavior and thus an increase in CO_2 tolerance. Although this suggest *Po. lapradei* utilizes air breathing to rectify the respiratory acidosis and regulate blood PCO_2 in hypercapnia, this requires verification by blood PCO_2 measurements.

In contrast, *Pa. hypophthalmus* solely uses branchial ion exchange for acid-base compensation (Damsgaard et al., 2015). Thus, air access had little impact on *Pa. hypophthalmus* during aquatic hypercapnic exposure, offering little change in avoidance behavior (Fig. 5). As with the diversity of air-breathing systems among fishes, dissimilar strategies of CO_2 exchange and coping with hypercapnia in *Pa. hypophthalmus* and *Po. lapradei* are the result of independent colonizations of marginal aquatic habitats. These independent evolutionary histories ultimately determine the role of air-breathing in hypercapnic avoidance behaviors between these two species.

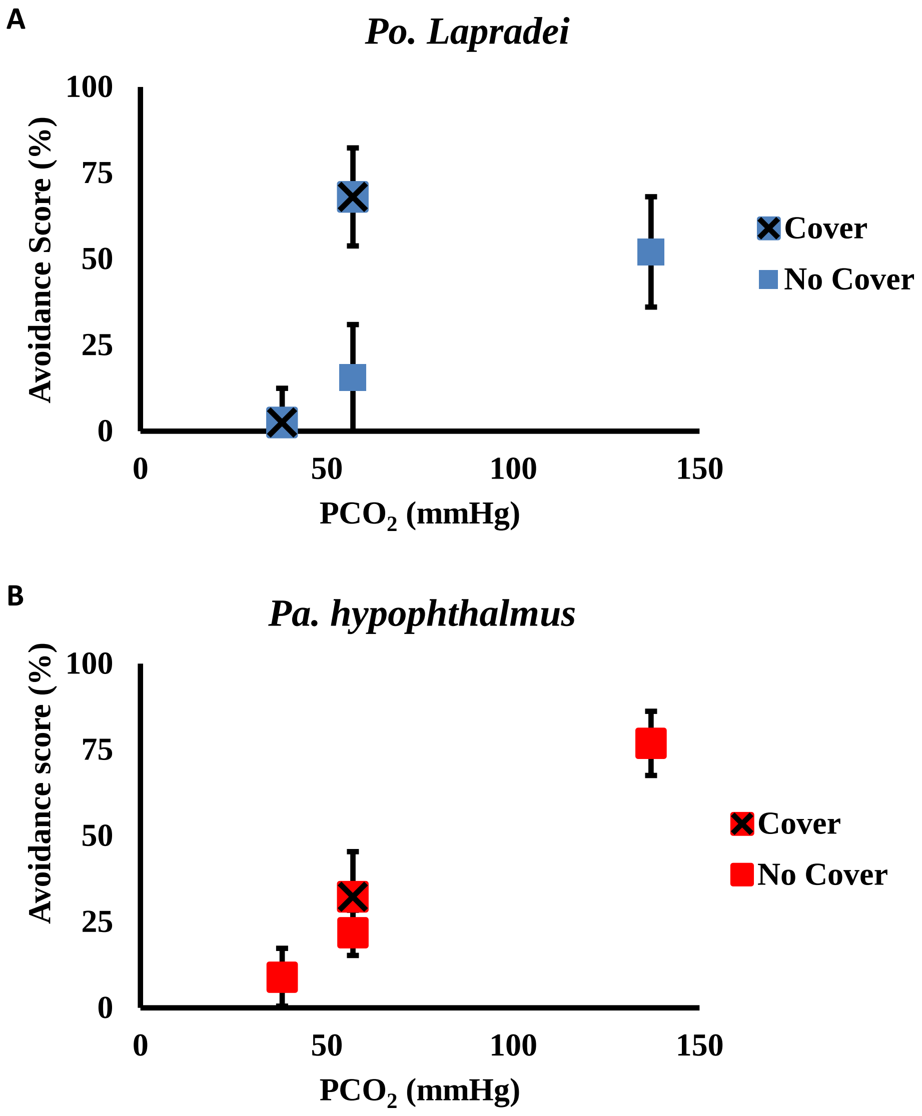

Figure 3.5. Avoidance scores for (A) *Po. lapradei* and (B) *Pa. hypophthalmus* trials.

Values shown are mean ±s.e.m. Avoidance score represents the percentage of time spend outside of the hypoxic or hypercapnic arena above random chance (half of total time). Air access had a large effect on avoidance score under identical PCO_2s in *Po. Lapradei*, while very little impact in *Pa. hypophthalmus.*

Conclusion

Avoidance behavior to hypoxia and hypercapnia in *Po. lapradei* are both influenced by the ability to breath air. *Po. lapradei* possess an O_2 and CO_2 air-breathing reflex and use air-breathing to increase hypoxia and hypercapnia tolerance expanding their viable habitat use area. When unable to air breathe, avoidance behavior becomes more common.

In contrast, in *Pa. hypophthalmus* does not possess a hypoxia avoidance behavior, and hypercapnia avoidance is not influenced by the ability to breath air. Although an O_2 air-breathing drive is present in *Pa. hypophthalmus,* the loss of an avoidance response may be explained by its natural behavior to inhabit the upper water column. It is likely that air-breathing is always accessible to *Pa. hypophthalmus* and thus environmental hypoxia and the need to avoid such conditions is ever an issue. In *Pa. hypophthalmus*, air-breathing plays almost no role in coping with hypercapnia and thus access to air-breathing also has no influence avoidance behavior.

Air breathing clearly enables the inhabitance of hypoxic environments however its role in hypercapnia tolerance is more complex. Avoidance behavior is an integrated response of sensory systems, physiological requirements, and ecological strategies resulting in differences demonstrated here in how this behavior is used, altered, or lost. Many of these influencing factors are likely a result of the diverse natural and evolutionary histories of air breathing systems and ecological roles found among air-breathing fishes. Application of avoidance behaviors for air-breathing fishes both in the use of denying air access and intensity of hypoxia or hypercapnia reflect this diversity and must be characterized on a species by species basis.

References

Arunachalam, S., Vivekanandan, E. and Pandian, T. J. (1976). Food intake, conversion and swimming activity in the air-breathing catfish *Heteropneustes fossilis*. *Hydrobiologia* **51**, 213–217.

Babiker, M. M. (1984). Development of dependence on aerial respiration in *Polypterus senegalus* (Cuvier). *Hydrobiologia* **110**, 351–363.

Beitinger, T. L. and Pettit, M. J. (1984). Comparison of low oxygen avoidance in a bimodal breather, *Erpetoichthys calabaricus* and an obligate water breather, *Percina caprodes*. *Enviornmental Biol. Fishes* **11**, 235–240.

Beitinger, T. L., Pettit, M. J. and Hutchison, V. H. (1985). Oxygen transfer characteristics of the blood of reedfish, *Erpetoichthys calabaricus*. *Comp. Biochem. Physiol. A. Comp. Physiol.* **82**, 553–558.

Bernier, N. J. and Randall, D. J. (1998). Carbon dioxide anaesthesia in rainbow trout: Effects of hypercapnic level and stress on induction and recovery from anaesthetic treatment. *J.Fish Biol.* 621–637.

Black, E. C., Fry, F. E. J. and Black, V. S. (1954). The influence of carbon dioxide on the utilization of oxygen by dome fresh-water fish. *Can. J. Zool.* **32**, 408–420.

Brauner, C. J. and Baker, D. W. (2009). Patterns of acid–base regulation during exposure to hypercarbia in fishes. In *Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects*, pp. 43–63.

Clingerman, J., Bebak, J., Mazik, P. M. and Summerfelt, S. T. (2007). Use of avoidance response by rainbow trout to carbon dioxide for fish self-transfer between tanks. *Aquac. Eng.* **37**, 234–251.

Damsgaard, C., Gam, L. T. H., Dang, D. T., Van Thinh, P., Huong, D. T. T., Wang, T. and Bayley, M. (2015). High capacity for extracellular acid-base regulation in the air-breathing fish

Pangasianodon hypophthalmus. *J. Exp. Biol.* **218**, 1290–1294.

Florindo, L. H. (2006). The role of branchial and orobranchial O2 chemoreceptors in the control of aquatic surface respiration in the neotropical fish tambaqui (Colossoma macropomum): progressive responses to prolonged hypoxia. *J. Exp. Biol.* **209**, 1709–1715.

Graham, J. B. (1997). *Air-breathing Fishes - Evolution, diversity and adaptation*. San Diego, CA: Academic Press.

Halliday, T. R. (1977). The Courtship of European Newts: An Evolutionary Perspective. In *The Reproductive Biology of Amphibians* (ed. Taylor, D.) and Guttman, S.), pp. 185–232. Boston, MA: Springer.

Hedrick, T. L. (2008). Software techniques for two- and three-dimensional kinematic measurements of biological and biomimetic systems. *Bioinspiration and Biomimetics* **3**,.

Ishimatsu, A., Hayashi, M. and Lee, K. (2005). Physiological effects on fishes in a high CO2 world. *J. Geophys. Res.* **110**, 1–8.

Kates, D., Dennis, C., Noatch, M. R. and Suski, C. D. (2012). Responses of native and invasive fishes to carbon dioxide : Potential for a nonphysical barrier to fish dispersal. *Can. J. Fish. Aquac. Sci.* **69**, 1748–1759.

Kramer, D. L. (1983). The evolutionary ecology of respiratory mode in fishes: an analysis based on the costs of breathing. *Environ. Biol. Fishes* **9**, 145–158.

Kramer, D. L. (1987). Dissolved oxygen and fish behavior. *Environ. Biol. Fishes* **18**, 81–92.

Kramer, D. L. and McClure, M. (1980). Aerial respiration in the catfish, *Corydoras aeneus* (Callichthyidae). *Can. J. Zool.* **58**, 1984–1991.

Kramer, D. L. and McClure, M. (1981). The transit cost of aerial respiration in the catfish *Corydoras* *aeneus* (Callichthyidae). *Div. Comp. Physiol. Biochem. , Soc. Integr. Comp. Biol.* **54**, 189–194.

Kramer, D. L., Manley, D. and Bourgeois, R. (1983). The effect of respiratory mode and oxygen concentration on the risk of aerial predation in fishes. *Can. J. Zool.* **61**, 653–665.

Lahiri, S., Szidon, J. P. and Fishman, A. P. (1970). Potential respiratory and circulatory adjustments to hypoxia in the African lungfish. *Fed. Proc.* 1171–8.

Lefevre, S., Huong, D. T. T., Wang, T., Phuong, N. T. and Bayley, M. (2011a). Hypoxia tolerance and partitioning of bimodal respiration in the striped catfish (*Pangasianodon hypophthalmus*). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **158**, 207–214.

Lefevre, S., Huong, D. T. T., Ha, N. T. K., Wang, T., Phuong, N. T. and Bayley, M. (2011b). A telemetry study of swimming depth and oxygen level in a *Pangasius* pond in the Mekong Delta. *Aquaculture* **315**, 410–413.

Lefevre, S., Wang, T., Jensen, A., Cong, N. V., Huong, D. T. T., Phuong, N. T. and Bayley, M. (2014). Air-breathing fishes in aquaculture. What can we learn from physiology? *J. Fish Biol.* **84**, 705–731.

Lopes, J. M., de Boijink, C. L., Florindo, L. H., Leite, C. A. C., Kalinin, A. L., Milsom, W. K. and Rantin, F. T. (2010). Hypoxic cardiorespiratory reflexes in the facultative air-breathing fish jeju (*Hoplerythrinus unitaeniatus*): Role of branchial O2 chemoreceptors. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **180**, 797–811.

Mckenzie, D. J., Aota, S. and Randall, D. J. (1991a). Ventilatory and Cardiovascular Responses to Blood pH , Plasma PCO2 , Blood O2 content, and catecholamines in an air-breathing fish, the bowfin (Amia calva). *1Physiological Zool.* **64**, 432–450.

Mckenzie, D. J., Burleson, M. L. and Randall, D. J. (1991b). The effectos of branchial denervation and pseudobranch ablation on cardioventilatory control in an air-breathing fish. *J. Exp. Biol.* **365**, 347–

365.

Milsom, W. K. (2012). New insights into gill chemoreception: Receptor distribution and roles in water and air breathing fish. *Respir. Physiol. Neurobiol.* **184**, 326–339.

Pandian, T. J. and Vivekanandan, E. (1976). Effects of feeding and starvation on growth and swimming activity in an obligatory air-breathing fish. *Hydrobiologia* **49**, 33–39.

Ross, R. M., Krise, W. F., Redell, L. A. and Bennett, R. M. (2001). Effects of dissolved carbon dioxide on the physiology and behavior of fish in artificial streams. *Environ. Toxicol.* **16**, 84–95.

Shingles, A., McKenzie, D. J., Claireaux, G. and Domenici, P. (2005). Reflex cardioventilatory responses to hypoxia in the flathead gray mullet (*Mugil cephalus*) and their behavioral modulation by perceived threat of predation and water turbidity. *Physiol. Biochem. Zool.* **78**, 744–755.

Smatresk, N. J. (1986). Ventilatory and cardiac reflex responses to hypoxia and NaCN in *Lepisosteus osseus*, an air-breathing fish. *Physiol. Zool.* **59**, 385–397.

Smatresk, N. J. and Cameron, J. N. (1983). Respiration and acid-base physiology of the spotted gar, a bimodal breather: I. Normal values, and the response to severe hypoxia. *J. Exp. Biol.* **96**, 263–280.

Smatresk, N. J., Burleson, M. L. and Azizi, S. Q. (1986). Chemoreflexive responses to hypoxia and NaCN in longnose gar: Evidence for two chemoreceptor loci. *Am. J. Physiol.* **251**, 116–125.

Thomsen, M. T., Wang, T., Milsom, W. K. and Bayley, M. (2017). Lactate provides a strong pH-independent ventilatory signal in the facultative air-breathing teleost *Pangasianodon hypophthalmus*. *Sci. Rep.* **7**, 6378.

Ultsch, G. R. (1996). Gas exchange, hypercarbia and acid-base balance, paleoecology, and the evolutionary transition from water-breathing to air-breathing among vertebrates. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **123**, 1–27.

Vivekanandan, E. and Pandian, T. (1977). Surfacing activity and food utilization in a tropical air-breathing fish exposed to different temperatures. *Hydrobiologia* **54**, 145–160.

Willmer, E. N. (1934). Some observations on the respiration of certain tropical fresh-water fishes. *J.Exp.Biol.* **11**, 283–306.

CHAPTER 4

The Effects of Aquatic Hypercapnia on Stress Blood Biomarkers in *Pangasianodon hypophthalmus*

Introduction

Over the past decade the use of air-breathing fishes in aquaculture has grown dramatically (FAO, 2018; Lefevre et al., 2014). This is partially due to the ability of air-breathing fishes to survive in harsh aquatic environments (low in O_2 and pH, high in CO_2 and nitrogenous waste) reducing operational cost in water turnover, aeration, and mortality. However, survival refers to the ability to tolerate such conditions and does not necessarily confer thriving or maximizing their growth potential. For example, the air-breathing catfish, *Pangasianodon hypophthalmus*, survives in hypoxic aquaculture ponds as low as 8-12 mmHg O_2 (Damsgaard et al., 2015a; Lefevre et al., 2011). Yet providing further oxygenation of these ponds (above P_{crit}) enables higher rates of survival and reduced food conversion ratios (less feed required for growth) (Lefevre et al., 2014). Thus, additional gains in aquaculture production and efficiency can be created by maintaining water quality parameters above what is merely required for survival, and characterizing conditions under which these animals thrive are important for sustaining healthy and productive stocks. The first step in creating an industry framework of data driven standards and practices to optimize production is to identify environmental conditions that are suboptimal, and stressful to such fish species.

Stress in animals is the response to a physical, chemical, or perceived disturbance that threatens homeostasis. Stress is not inherently harmful as these adaptive mechanisms allow an animal to cope with a stressor and reestablish its homeostatic state. However, if a stressor is severe or long-term, and compensation represents a significant energetic cost, these physiological mechanisms can become maladaptive and negatively impact health and survival. Using aquatic hypoxia as an example, swimming to the surface to air-breathe is a stress response that is adaptive as it reestablishes tissue oxygenation. However, if hypoxia is severe and chronic, requiring frequent and energetically expensive trips to the surface, stress can become harmful, reducing feed conversion ratios and growth rates. In the latter case, immediate needs are prioritized to the detriment of long-term investments, and stress becomes maladaptive. The stress response, and the cascade of its effects, are broken down into three general categories: Primary, secondary, and tertiary (Barton, 2002; Wendelaar Bonga, 1997). The primary response is the neuroendocrine response releasing catecholamines from the adrenal medulla, stimulating the hypothalamic-pituitary-innerrenal (HPI) axis to release corticoids into circulation. Next, the secondary response features adjustments at the tissue and system level, including changes in metabolism, respiration, acid-base status, immune function, and changes to plasma and cellular ion and metabolite concentrations. This includes changes in hematological factors such as blood glucose and lactate and hematocrit. The tertiary response involves changes to whole-animal performance resulting from primary and secondary stress, that include growth, disease resistance, metabolic scope, behavior, and survival. Understanding stress physiology and characterizing stressful condition in an artificial environment is essential in aquaculture for ensuring heathy and productive stocks. Growth rates are influenced by food acquisition, consumption, assimilation, and metabolic rate; all factors in stress. Consequently, environmental stressors should be limited to maximize growth and survival.

Stressed caused by aquatic hypercapnia (high CO_2) can limit production in aquaculture (Crocker and Cech Jr., 1996; Danley et al., 2005; Foss et al., 2003; Hu et al., 2011; Lefevre et al., 2014; Petochi et al., 2011). Hypercapnia is caused by lack of aeration and biological respiration, whether it be from high stocking densities and feeding rates or microbial activity. Hypercapnia is exasperated by practices such a pure O_2 injection, where 100% O_2 gas is injected into ponds to meet aerobic demands, yet these aeration rates are insufficient to remove the associated production of CO_2 (Hu et al., 2011).

Exposure to hypercapnia causes lowering (and if high enough, reversal) of the diffusive gradient by which CO_2 is offloaded at the gills. This results in a rise in arterial CO_2 and a respiratory acidosis. Acid-base compensation subsequently occurs through the accumulation of bicarbonate in exchange for chloride ions

(Brauner and Baker, 2009). Although preferential regulation of intracellular pH is likely to occur in air-breathing fishes, a change in extracellular pH would result in elevated transport of ion between intracellular and extracellular spaces (Shartau and Brauner, 2014). Stress can be caused if acid-base homeostasis is disrupted either before compensation can occur, or if hypercapnia intensity is beyond the compensation capacity (Brauner and Baker, 2009). Both intracellular and extracellular pH homeostasis is central to survival in vertebrates as changes in pH are known to alter protein shape and disrupt cell function. All vertebrates are capable of homeostatic compensation to changes in environmental CO_2 and physiological acid-base perturbations. However, in environments that are chronically hypercapnic, the energetic cost of additional ion transport and maintenance of new electrochemical gradients associated with pH compensation can be an added cause of chronic stress. This has been shown in marine fish, when exposed to an elevated PCO_2 of only 1460 µatm (1.2 mmHg), where O_2 consumption of gut tissue increased by 8% due to additional ion transport after 2-4 weeks (Heuer and Grosell, 2016). Finally, blood acidification alters hemoglobin binding affinity though the Root and Bohr effect and can lead to reductions in arterial oxygenation and systemic O_2 transport (Perry et al., 1989).

The acute effects of hypercapnic stress are known to be agitation, behavioral avoidance, increased ventilation and hematocrit, and accumulation of blood catecholamines, cortisol, glucose, and lactate, and an anesthetic effects (Dennis et al., 2016; Iwama et al., 1989; Kates et al., 2012; Petochi et al., 2011; Ross et al., 2001). Chronic (days-months) hypercapnic exposure has been shown to limit food conversion ratio, growth, and survival (Crocker and Cech Jr., 1996; Danley et al., 2005; Hu et al., 2011; Lefevre et al., 2014; Petochi et al., 2011; Ross et al., 2001). For some aquaculture species, CO_2 thresholds for ensuring health and growth have been established. According to Ross et al. 2001, the aquatic PCO_2 should remain at 2 mmHg for maintaining health populations of rainbow trout (*Oncorhynchus mykiss*), while above 8 mmHg becomes harmful for survival. The same aquatic CO_2 standards have yet to been investigated for raising air-breathing fishes. Recent studies indicate that aquaculture ponds for *P. hypophthalmus* exceed 34 mmHg (Damsgaard et al., 2015a). While the productivity of these commercial aquaculture ponds illustrates the extreme hypercapnic tolerance of *P. hypophthalmus*, whether these fish are merely tolerating or thriving under these conditions remains unknown. This study seeks to understand if this level of hypercapnia elicits a stress response in *P. hypophthalmus* for the purpose of indicate if further reducing aquaculture pond CO_2 has the potential to provide additional gains in growth and productivity.

During hypercapnic exposure we anticipate different patterns of stress depending on whether *P. hypophthalmus* is responding to the acid-base disturbance, maintenance of new ionic gradients, or not responding at all. Figure 1 depicts three response patterns that describe changes in a hypothetical stress indicator as well as changes in blood acid-base parameters during exposure to aquatic hypercapnia. If hypercapnia is not found to be stressful, we predict a stress response pattern to reflect model A, where no change occurs between normocapnic and hypercapnic exposure. Model B would correlate pH disturbance with stress, where the initial respiratory acidosis elicits an increase in the stress indicator level yet returns to pre-exposure level as acid-base compensation occurs. Model C indicates hypercapnia is stressful beyond the initial acid-base disturbance due to the elevated cost of transporting ions and maintaining high plasma bicarbonate and low chloride levels.

In this study we measured blood glucose and lactate and hematocrit as indicators of stress in *P. hypophthalmus* when exposed to a level of aquatic hypercapnia mimicking aquaculture conditions.

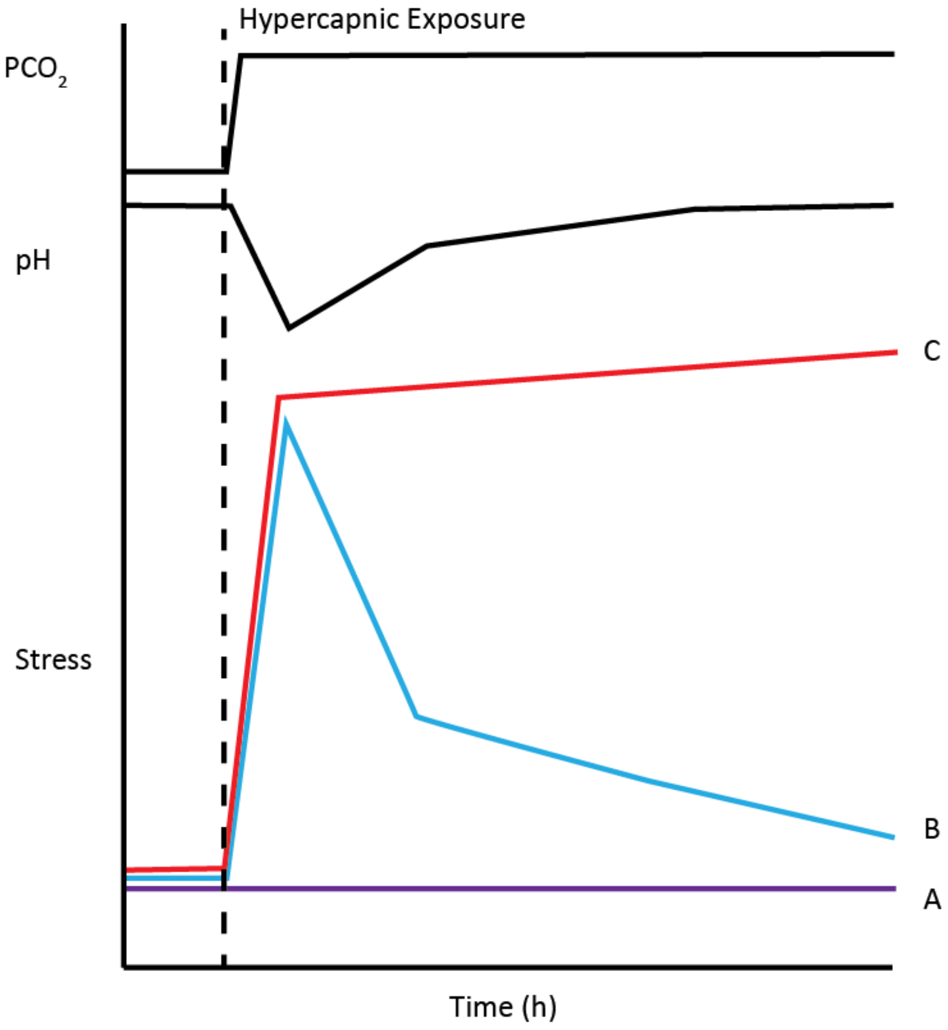

Figure 4.1. Response of Acid-base Parameters and a Hypothetical Stress Indicator During Exposure to Aquatic Hypercapnia.
Model (A) suggests this level of hypercapnia is not stressful. In model (B), stress indicator concentration correlates with acid-base disturbance suggesting the stress response is to the disruption of pH homeostasis. Model (C) stress indicator concentration matches PCO_2. This suggest stress is present beyond pH compensation, possibly due to cost of additional ion transport and maintenance of a new electrochemical gradient.

Materials and Methods

Experimental animals

Pangasianodon hypophthalmus were obtained though the aquarium trade and raised in a recirculating system at the University of California, Irvine at 30°C on a 12:12 h light-dark cycle. Animals were fed commercial pellets (Starmilling Co., Perris, CA) twice daily. Husbandry and experimental procedures in this study were approved under UC Irvine IACUC Protocol # 2013-3105-1.

Animal preparation

Animals were anaesthetized in an aqueous solution of clove oil (0.16 mL L^{-1} clove oil, 1.45 mL L^{-1} ethanol) until cessation of opercular ventilation and transferred to a surgical set up with the gills irrigated with an aerated dilute solution of clove oil (0.11 mL L^{-1} clove oil, 1.00 mL L^{-1} ethanol). A PE50 catheter was implanted into the dorsal aorta through the roof of the mouth using the Seldinger technique with a sharped guitar string as a guide wire. The catheter exited the mouth through a whole drilled in the rostrum and was secured with suture to the upper jaw and dorsal spine. Catheters were filled with heparinized Ringer's (10 UI) and burn sealed. To avoid tangling, catheters were kept at the surface using fly fishing foam strike indicators (Maxcatch Fishing, Qingdao, China) and orthodontic elastic rubber bands. Animals were allowed to recover overnight in the experimental tank.

Experimental Tank

Exposure to hypercapnic conditions occurred in an experimental tank constructed of PVC (180 cm L, 90 cm W, 24 cm H) and divided into thirds to allow fish to be run in triplicate simultaneously, using plastic netting secured with aquarium grade silicon. Experiment tanks were fill with dechlorinated tap water (total alkalinity =120 CaCO3 eq L^{-1}). Water was pumped from one end of the tank into a mixing chamber (60 cm L, 38 cm W, 36 cm H) which gravity fed back into the experimental tank on the opposite end. The mixing chamber contained air stones for aeration and microbubble gas diffusers (PentairAES, Cary, NC) for injecting CO_2. Experimental tank conditions were monitored and regulated by a custom-built system consisting of O_2 and pH probes (Atlas scientific) connected to an Arduino microcontroller. This system bubbled air and CO_2 into the mixing chamber by operating a series of solenoid valves connected to compressed gasses. To convert pH to PCO_2, a calibration curve was made by bubbling various CO_2 mixtures into a water sample and measuring the resulting pH when in equilibrium (Fig. 2). PO_2 was maintained above 136 mmHg O_2 and at 30°C during the entire experiment.

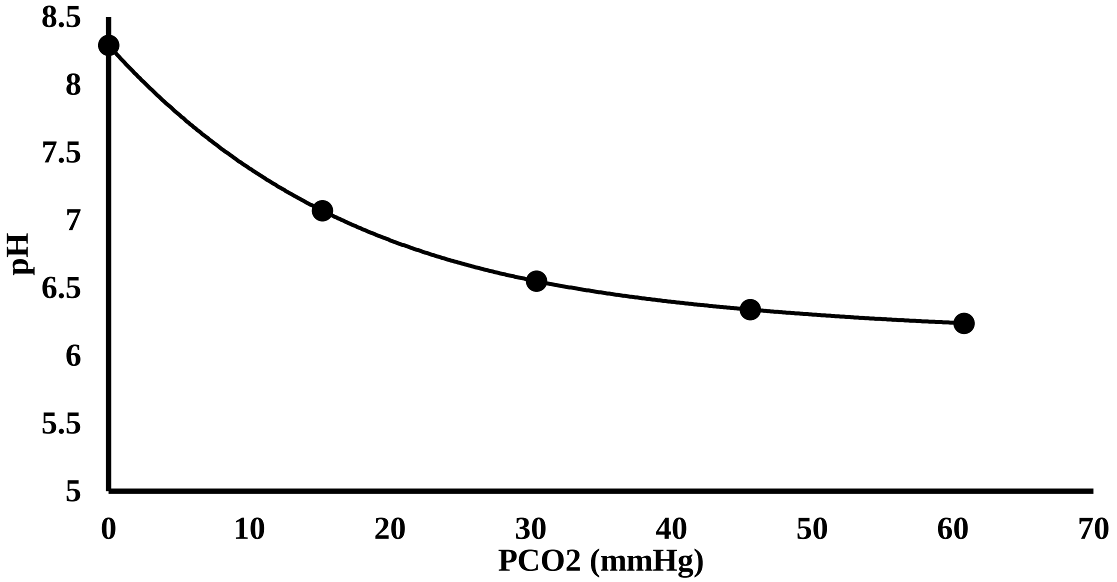

Figure 4.2. PCO_2/pH Calibration Curve. Values generated by bubbling known concentrations of CO_2 into water samples and measuring pH at equilibrium. Total Alkalinity = 120 $CaCO_3$ eq.

Sampling and blood analysis

To measure change in blood acid-base status and stress indicator concentrations, 0.15 mL of blood was drawn from the implanted catheters at 0, 3, 24, 48, and 72 h after hypercapnic exposure. Catheters were subsequently flushed with heparinized Ringer's and resealed. Control trials remained in normocapnic conditions and were sampled at 0, 24, 48, and 72 h. Blood pH and PCO_2 was measured using a handheld I-stat blood analyzer with G3+ cartridges (Abbott Point of Care Inc., Princeton, NJ). Hematocrit was measured as the fractional volume of red blood cells after centrifugation at 12,000 rpm for 3 minutes. Blood glucose was measured with a Max Plus Glucose monitor (Nova Biomedical, Waltham, MA) and lactate was measured using a Lactate Plus Meter (Nova Biomedical, Waltham, MA).

To validate lactate and glucose meter measurements, calibration curves (Fig. 3a, 4a) were made by creating predictable mixtures of *P. hypophthalmus* donor blood and concentrated or diluted Ringer's solutions with known concentrations. Calibration curves for known values versus of error of measured values (Δ = Measured – Calculated) were also made (Figure 3b, 4b). Use of the I-stat for blood pH measurements in fish was previously verified by (Harter et al., 2011). (Harter et al., 2011) also reported a relative correlation between PCO_2 and I-stats measurements, albeit a consistent overestimation only when reading below 19 mmHg. Therefore, lower values from the current study should be taken as estimates only, however the relative values are useful.

To verify the range and responsiveness of stress indicators, physically activity was induced by chasing specimen (n=5) by hand in experimental tank to exhaustion (no longer responding to tail pinch) and sampled 30 min post-exercise.

Data analysis

Statistical significance of blood parameters from normocapnia (0 h) was tested by repeated-measures ANOVA with post-hoc Tukey Test. A linear model was used to compare calculated versus measured blood glucose and lactate values, as well as Δ glucose and Δ lactate values. Pre and post-exercise values were compared by paired t-test.

Results

In experimental fish, arterial PCO_2 was significantly increased at 3 h and remained elevated for the following 72 h (Fig. 5). At 3 h, arterial pH was reduced, however only significantly lower than 0 h at 3 h and 24 h. Blood glucose and lactate concentrations and hematocrit were not significantly different from normocapnic values at any time. Lactate measurements were frequently below the detection limit of the instrument. Control fish experienced no change in arterial pH or PCO_2. Blood glucose and lactate values and hematocrit also showed no significant change.

Glucose and lactate validation

Both glucose and lactate measurements were closely correlated with calculated values yielding a highly significant relationship (Table 1, Fig. 3 and 4). Both Δglucose and Δlactate did not show a significant relationship in either slope or y-intercept indicating measured values were not skewed nor did the relationship differ across concentrations.

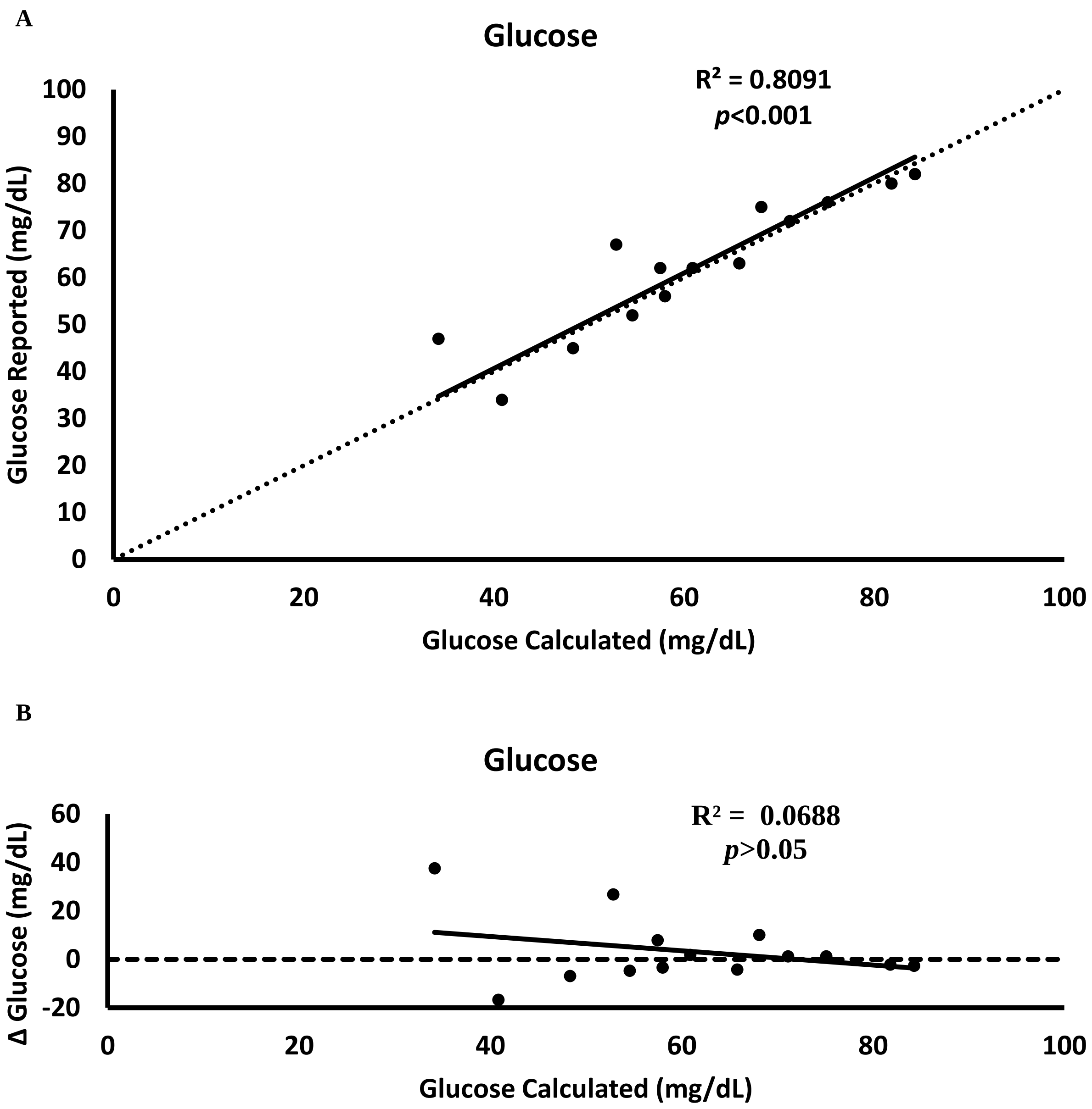

Figure 4.3. Verification of Blood Glucose Measurements. Blood samples from *P. hypophthalmus* were mixed with known concentrations of glucose dissolved in Ringer's solutions (n=12). Known concentrations are compared with analyzer measured values (A) and error (Δ= measured – calculated) (B). Solid line represents fitted linear model (*p*-value shown), dotted line indicates line of identity, dashed line indicates zero.

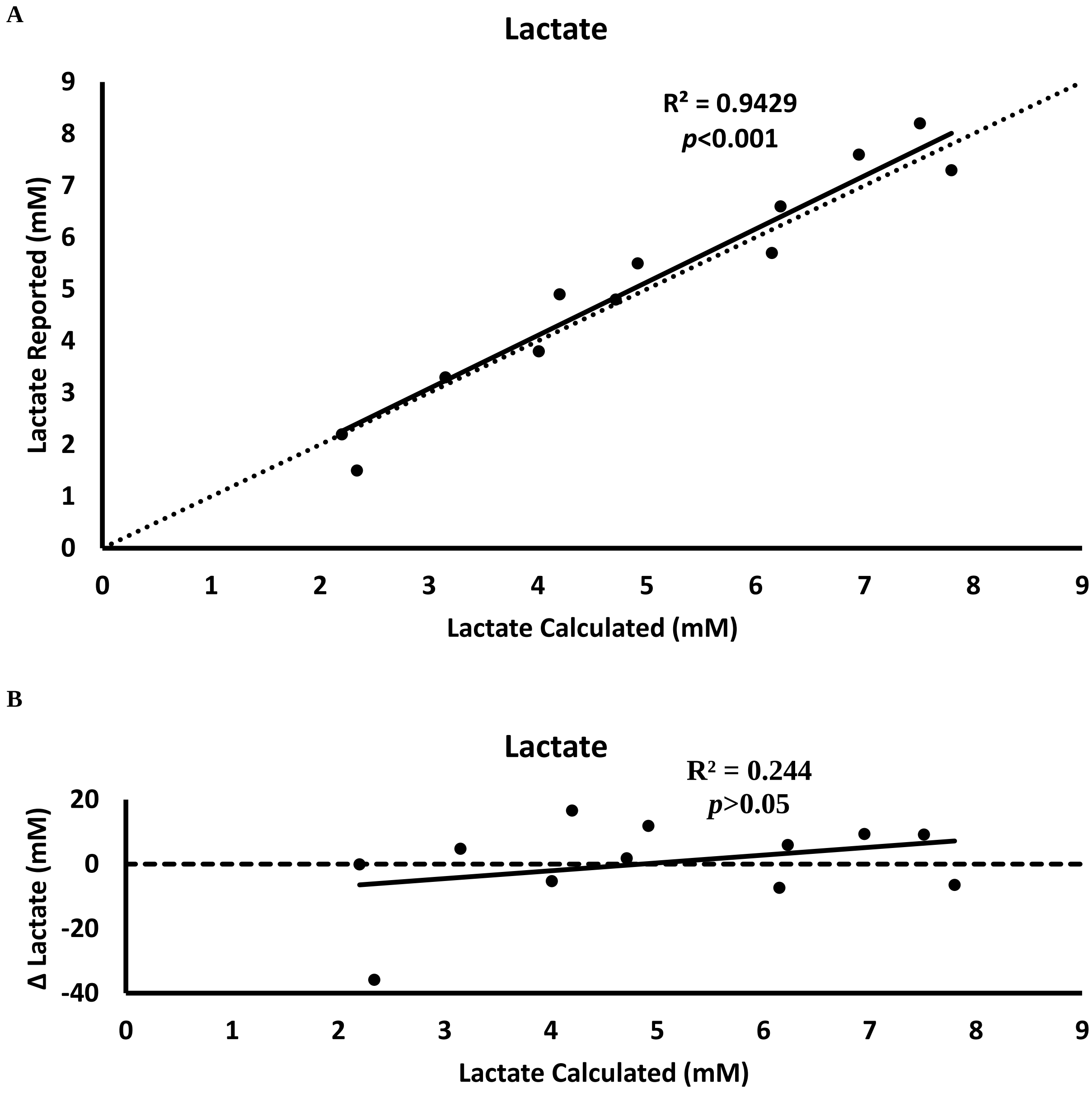

Figure 4.4. Verification of Blood Lactate Measurements.

Blood samples from *P. hypophthalmus* were mixed with known concentrations of lactate dissolved in Ringer's solutions (n=12). Known concentrations are compared with analyzer measured values (A) and error (Δ= measured – calculated) (B). Solid line represents fitted linear model (*p*-value shown), dotted line indicates line of identity, dashed line indicates zero.

Measurement	***A***	***b***	**R^2**	**p-value**
Glucose	8.143 ± 7.351	0.889 ± 0.118*	0.827	<0.001
Δ Glucose	-8.143 ± 7.351	0.111 ± 0.118	0.069	>0.05
Lactate	-0.272 ± 0.438	1.075 ± 0.0819*	0.945	<0.001
Δ Lactate	0.514 ± 0.369	-0.121 ± 0.0671	0.244	>0.05

Table 4.1. Parameter Estimates, R^2, and *p*-values for Glucose and Lactate Meter Validation
Parameter estimates (mean ± s.e.m.), R^2, and *p*-values for the relationships between calculated blood glucose (n=14) and lactate (n=12) versus analyzer measurements and versus error (Δ).
* indicates parameter statistical significance from 0, determined by linear model.
Linear relationship according to: Measured (x) = $a + b \times$ (calculated) and Δ (x)=$a + b \times$ (measured)
Error (Δ) = measured – calculated

Activity Stress

Blood glucose, lactate, and hematocrit values showed a significant increase at 30 min post-exercise Fig. 6). Glucose showed a 60 % increase from 55±6 dL mL^{-1} to 88±4 dL mL^{-1}. Blood lactated was initially not detected (<0.2 mmol L^{-1}) yet rose to 8±3 mmol L^{-1}, and hematocrit was elevated from 22±4 % to 32±4 % post-exercise.

Discussion

No changes in stress indicators were observed in this study suggesting aquatic hypercapnia of 38 mmHg CO_2 does not represent a significant physiological challenge for *P. hypophthalmus* or an environmentally stressful condition.

Although measuring cortisol is useful for studying acute stressors, it is less informative in chronic exposure studies as concentrations in blood are known the decrease over time due to endocrine exhaustion and innerrenal desensitization (Martinez-Porchas et al., 2009). In contrast to cortisol, glucose is a more reliable indicator for chronic studies with a response time of minutes to days after introduction of a stressor. Stress related hyperglycemia during suboptimal or stressful conditions is mediated by cortisol activated glycolytic and gluconeogenic processes, as well as directly by catecholamines in an effort to poise energy stores for a fight-or-flight response (Martinez-Porchas et al., 2009; Wendelaar Bonga, 1997). Blood glucose levels are also positively correlated with metabolic rate during stress exposure (Wendelaar Bonga, 1997). No change in glucose was observed in this study, suggesting no change in activity and a lack of a hypercapnic stress response. Resting blood glucose in *P. hypophthalmus* (40-60 mg dL^{-1}) in aquatic normo- and hypercapnia were similar to levels found in other unstressed fishes (Dennis et al., 2016; Kates et al., 2012; Petochi et al., 2011; Santos et al., 2013). This study verified the use of homecare blood glucose monitor, Max Plus Glucose, for use with fish. Previous studies have found other glucose meters are also useful with fish, albeit recommending the use of relative values, rather than absolute, due to consistent underestimations (Stoot et al., 2014; Wells and Pankhurst, 1999). Our study found that Max Plus Glucose is a valid substituted for these devices, yet with the ability to use absolute values.

Lactate is produced by anaerobic metabolism during activity and is considered an indicator of stress as it is accumulated under suboptimal conditions (Wendelaar Bonga, 1997). Changes in lactate can indicate a change in behavior and an increase in activity in the form of agitation or attempted escape of a stressor by locomotion. No elevation in both blood lactate and glucose, suggesting there is no change in activity associated with seeking new environments and is in agreement with the lack of a hypercapnic avoidance behavior at 38 mmHg reported in Ch.3. Further, branchial ventilation, also an indicator of stress signifying an increase on O_2 consumption due to activity or agitation (Wendelaar Bonga, 1997), was found not to be elevated at this level of hypercapnia (Thomsen et al., 2017). Resting blood lactate both in normo- and hypercapnia were frequently below the detection limit of the Lactate Plus Meter (<0.2 mM) which is not uncommon for other fishes at rest (Bernier and Randall, 1998; Dennis et al., 2016; Kates et al., 2012; Stoot et al., 2014). The use of the Lactate Plus Meter for use in fish was validated in this study. Blood lactate measurements have also been verified for other lactate meters (Brown et al., 2008; Stoot et al., 2014; Wells and Pankhurst, 1999), still, resting blood lactate levels in fishes approach 1 mmol L^{-1} making any handheld lactated meter difficult for use in unstressed animals (Brown et al., 2008).

During stress, interrenal CA release elevates hematocrit through splenic contraction increasing the number of cells in circulation (Wendelaar Bonga, 1997). The resulting increase in hematocrit and hemoglobin concentration improves the O_2 transport capacity to meet the elevated activity demands of a stressful environment. Although changes to the O_2 transport system in response to hypercapnic exposure are sometimes associated with blood acidification and impairment of blood binding due to the Root and Bohr effects (Perry et al., 1989), it is now largely accepted that fish possess CO_2 reflexes independent of blood O_2 status (Gilmour, 2001; Milsom, 2002). Regardless, blood O_2 status in *P. hypophthalmus* is unlikely to be effected by hypercapnia due to the lack of a Root effect in this animals and its extremely high hemoglobin binding affinity (P_{50}=4.6 mmHg O_2) (Damsgaard et al., 2015b). Damsgaard et al., 2015a) reported a hematocrit of 22% in *P. hypophthalmus* which is within the range of our observed values. Our finding that hematocrit did not change suggest circulating levels of CA were also not elevated. Taken together, with no change in other secondary stress response indicators (blood glucose and lactate and hematocrit), we are unlikely to observe a tertiary stress response and a negative impact on health on *P. hypophthalmus* at this level of hypercapnia.

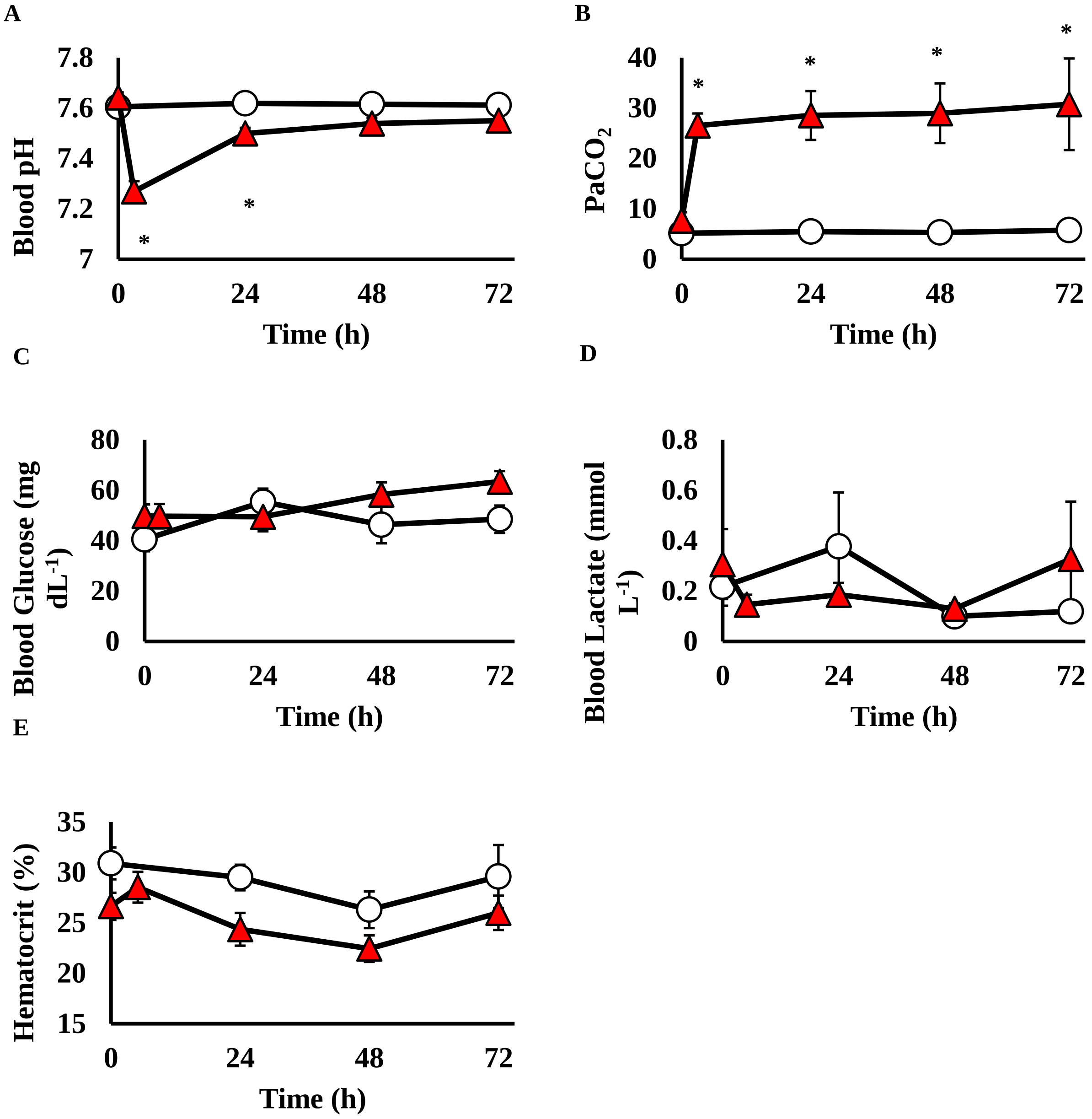

Figure 4.5. Blood Acid-base Parameter and Stress Indicator Concentrations During Exposure to Aquatic Hypercapnia

Mean±s.e.m displayed for blood acid-base parameter and stress indicator concentrations during exposure to aquatic hypercapnia. *P. hypophthalmus* arterial (A) pH, (B) PCO_2, (C) glucose, (D) lactate, and (E) hematocrit were measured in samples drawn via dorsal aortic cannulation. * indicates significant difference from normocapnia (0 h).

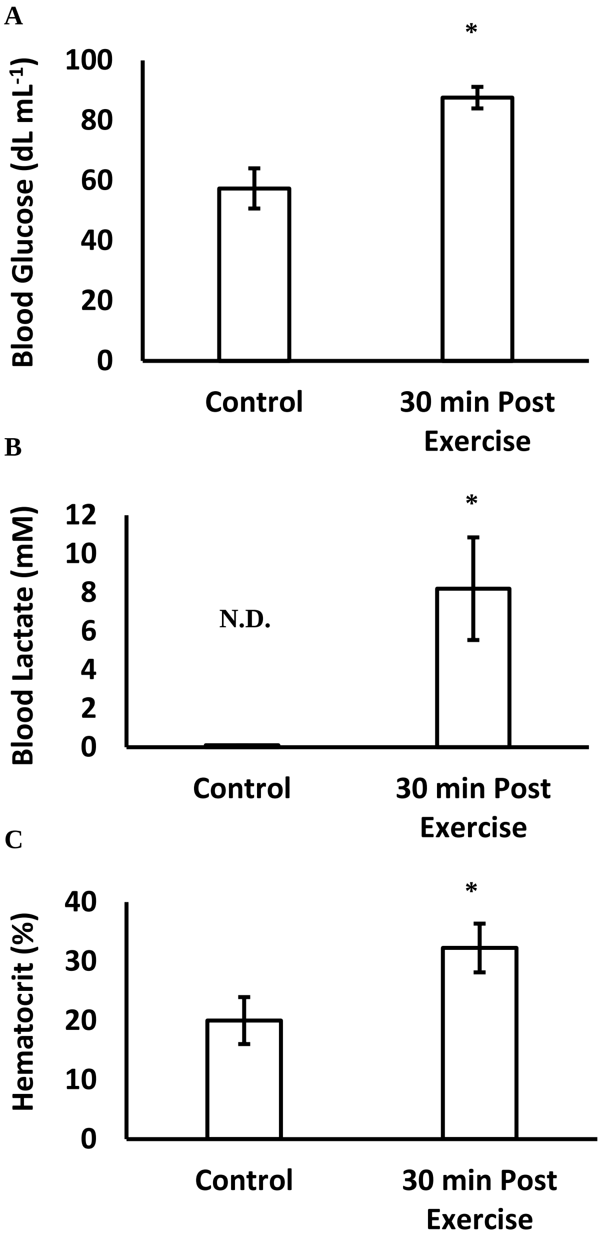

Figure 4.6. The Effect of Exercise on Stress Indicator Concentrations.
Mean±s.e.m. blood glucose (A) and lactate (B) and hematocrit (C) before and 30 min post-exercise in *P. hypophthalmus* (n=5). Control lactate values were not detected (N.D.) due to the minimum detection limit of instruments. * indicates statistical significance by paired t-test.

Effects of hypercapnia

The acute effects of hypercapnia on respiration, blood acid-base status, and behavior in *P. hypophthalmus* have been recently investigated (Damsgaard et al., 2015a; Thomsen et al., 2017), (Jew ch.2 and ch.3). Although exposure to aquatic hypercapnia does increase CO_2 released into the air, air breathing does not aid in blood PCO_2 regulation. Rather, *P. hypophthalmus* responds to hypercapnia as would a solely water breathing fish, blood PCO_2 quickly equilibrates with the aquatic environment and undergoes a rapid drop in pH. At 34 mmHg CO_2, the respiratory acidosis is slowly compensated over 48 hours through branchial ion exchange. In our study exposure to 38 mmHg had a similar compensation time and did not to elicit a change in the stress indicators measured in this study, blood glucose and lactate and hematocrit. This suggest the respiratory acidosis associated with an acute exposure to 38 mmHg is not stressful for *P. hypophthalmus*, nor is the longer term acid-base compensation. In contrast, stress from physical activity elevated all three stress indicators, confirming the responsiveness of these parameters in *P. hypophthalmus*.

Other studies

Studies in other species found CO_2 thresholds occurred at lower levels of hypercapnia than in *P. hypophthalmus.* European sea bass (*Dicentrarchus labrax)* exposed to aquatic hypercapnia of 25 mmHg CO_2 elevated glucose and hematocrit over 96 h (Petochi et al., 2011). The largemouth bass (*Micropterus salmoides*) was reported to have elevated glucose and hematocrit in response to 16 mmHg CO_2 but with no change in lactate (Kates et al., 2012). In this same study, blood glucose and hematocrit increased in the bluegill (*Lepomis macrochirus*) after 1 hour, while in the silver carp (*Hypophthalmichthys molitrix*) and big head carp (*Hypophthalmichthys nobilis*), glucose and lactate increased, and hematocrit instead decreased. Rainbow trout exposed to 37 mmHg CO_2 for the purpose of anesthetization elevated plasma lactate (Bernier and Randall, 1998). Ross et al., 2001 reported exposure to 7.6 mmHg CO_2 resulted an increase in hematocrit in the brook trout (*Salvelinus fontinalis*), black dace (*Rhinichthys atratulus*), and slimy sculpin (*Cottus cognatus*) after 1 hour, as well as an elevation in blood glucose in the book trout.

The above mentioned species all occur in temperate freshwater or saltwater habitats. Little information is available regarding CO_2 thresholds for stress in tropical freshwater fishes or air-breathing fishes except for (Damsgaard et al., 2015a), who also found no change in hematocrit in *P. hypophthalmus* at 34 mmHg CO_2. Air-breathing fishes are known to occur in tropical freshwater habitats which frequently experience hypoxic and hypercapnic conditions (Graham, 1997; Ultsch, 1996; Willmer, 1934). Consequently, hypercapnic thresholds for stress and tolerance are expected differ between air-breathing fishes and those found in temperate or salt water habitats. While hypoxia has been attributed to being the driving force behind the approximately 65 independent evolutions of air breathing in fishes, adaptations to hypercapnia are known to exist as well, such as elevated blood PCO_2, bicarbonate, and capacity for branchial ion exchange (Damsgaard et al., 2015a; Ultsch, 1987). Thus, the lack of a stress response at this comparatively higher PCO_2 is not surprising and likely reflects the evolutionary history of *P. hypophthalmus* inhabiting these marginal aquatic habitats.

Conclusion

This study provides new physiological data on the stress response of *P. hypophthalmus* to hypercapnia and contributes to the estimation of CO_2 threshold levels required to ensure healthy and productive populations. Stress indicators were not changed in response to elevated CO_2, suggesting that the level of hypercapnia found in aquaculture ponds (38 mmHg) is not a significant environmental challenge for *P. hypophthalmus*. It is unlikely therefore that additional aeration for the purpose of controlling PCO_2 below this level will result in additional benefits for production. This agrees with preliminary data on juvenile fish by Bayley et al. (unpublished) showing no change in growth rate when raised in normocapnic versus hypercapnic environments over 3 months. The ability to thrive in hypercapnic environments likely originated as part of a suite of adaptations allowing *P. hypophthalmus* to inhabit the marginal aquatic environments they naturally occur in, making this fish a rational candidate for aquaculture production.

References

Barton, B. A. (2002). Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integr. Comp. Biol.* **42**, 517–525.

Bernier, N. J. and Randall, D. J. (1998). Carbon dioxide anaesthesia in rainbow trout: Effects of hypercapnic level and stress on induction and recovery from anaesthetic treatment. *J.Fish Biol.* 621–637.

Brauner, C. J. and Baker, D. W. (2009). Patterns of acid–base regulation during exposure to hypercarbia in fishes. In *Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects*, pp. 43–63.

Brown, J. A., Watson, J., Bourhill, A. and Wall, T. (2008). Evaluation and use of the Lactate Pro, a portable lactate meter, in monitoring the physiological well-being of farmed Atlantic cod (*Gadus morhua*). *Aquaculture* **285**, 135–140.

Crocker, C. E. and Cech Jr., J. J. (1996). The effects of hypercapnia on the growth of juvenile white sturgeon, *Acipenser transmontanus*. *Aquaculture* **147**, 293–299.

Damsgaard, C., Gam, L. T. H., Dang, D. T., Van Thinh, P., Huong, D. T. T., Wang, T. and Bayley, M. (2015a). High capacity for extracellular acid-base regulation in the air-breathing fish *Pangasianodon hypophthalmus*. *J. Exp. Biol.* **218**, 1290–1294.

Damsgaard, C., Phuong, L. M., Huong, D. T. T., Jensen, F. B., Wang, T. and Bayley, M. (2015b). High affinity and temperature sensitivity of blood oxygen binding in *Pangasianodon hypophthalmus* due to lack of chloride-hemoglobin allosteric interaction. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **308**, R907–R915.

Danley, L., Kenney, P. B. and Hankins, A. (2005). Effects of carbon dioxide exposure on intensively cultured rainbow trout *Oncorhynchus mykiss*: Physiological responses and fillet attributes. *J. World Aquac. Soc.* **36**, 249–261.

Dennis, C. E., Adhikari, S., Wright, A. W. and Suski, C. D. (2016). Molecular, behavioral, and performance responses of juvenile largemouth bass acclimated to an elevated carbon dioxide environment. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **186**, 297–311.

FAO (2018). The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. *THE STATE OF THE WORLD* **Rome**,.

Foss, A., Røsnes, B. A. and Øiestad, V. (2003). Graded environmental hypercapnia in juvenile spotted wolffish (*Anarhichas minor Olafsen*): Effects on growth, food conversion efficiency and nephrocalcinosis. *Aquaculture* **220**, 607–617.

Gilmour, K. M. (2001). The CO2/pH ventilatory drive in fish. *Glass* 219–240.

Graham, J. B. (1997). *Air-breathing Fishes - Evolution, diversity and adaptation*. San Diego, CA: Academic Press.

Harter, T., Shartau, R., Farrell, A. and Brauner, Colin, J. (2011). Portable, fast and inaccurate: validation of the i-STAT system for the analysis of blood parameters in fish. *Conserv. Physiol.* **2**, 26–39.

Heuer, R. M. and Grosell, M. (2016). Elevated CO2 increases energetic cost and ion movement in the marine fish intestine. *Sci. Rep.* **6**, 1–8.

Hu, Y., Ni, Q., Wu, Y., Zhang, Y. and Guan, C. (2011). Study on CO2 removal method in recirculating aquaculture waters. *Procedia Eng.* **15**, 4780–4789.

Iwama, G. K., McGeer, J. C. and Pawluk, M. P. (1989). The effects of five fish anaesthetics on acid–base balance, hematocrit, blood gases, cortisol, and adrenaline in rainbow trout. *Can. J. Zool.* **67**, 2065–2073.

Kates, D., Dennis, C., Noatch, M. R. and Suski, C. D. (2012). Responses of native and invasive fishes to carbon dioxide : Potential for a nonphysical barrier to fish dispersal. *Can. J. Fish. Aquac. Sci.* **69**, 1748–1759.

Lefevre, S., Huong, D. T. T., Ha, N. T. K., Wang, T., Phuong, N. T. and Bayley, M. (2011). A telemetry study of swimming depth and oxygen level in a *Pangasius* pond in the Mekong Delta. *Aquaculture* **315**, 410–413.

Lefevre, S., Wang, T., Jensen, A., Cong, N. V., Huong, D. T. T., Phuong, N. T. and Bayley, M. (2014). Air-breathing fishes in aquaculture. What can we learn from physiology? *J. Fish Biol.* **84**, 705–731.

Martinez-Porchas, M., Martinez-Cordova, L. T. and Ramos-Enriquez, R. (2009). Cortisol and glucose: Reliable indicators of fish stress? *J. Aquat. Sci.* **4**, 158–178.

Milsom, W. K. (2002). Phylogeny of CO2/H+ chemoreception in vertebrates. *Respir. Physiol. Neurobiol.* **131**, 29–41.

Perry, S. F., Kinkead, R., Gallaugher, P. and Randall, D. J. (1989). Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout (Salmo gairneri). *Respir. Physiol.* **77**, 351–363.

Petochi, T., Di Marco, P., Priori, A., Finoia, M. G., Mercatali, I. and Marino, G. (2011). Coping strategy and stress response of European sea bass *Dicentrarchus labrax* to acute and chronic environmental hypercapnia under hyperoxic conditions. *Aquaculture* **315**, 312–320.

Ross, R. M., Krise, W. F., Redell, L. A. and Bennett, R. M. (2001). Effects of dissolved carbon dioxide on the physiology and behavior of fish in artificial streams. *Environ. Toxicol.* **16**, 84–95.

Santos, G. A., Schrama, J. W., Capelle, J., Rombout, J. H. W. M. and Verreth, J. A. J. (2013). Effects of dissolved carbon dioxide on energy metabolism and stress responses in European seabass

(*Dicentrarchus labrax*). *Aquac. Res.* **44**, 1370–1382.

Shartau, R. B. and Brauner, C. J. (2014). Acid-base and ion balance in fishes with bimodal respiration. *J. Fish Biol.* **84**, 682–704.

Stoot, L. J., Cairns, N. A., Cull, F., Taylor, J. J., Jeffrey, J. D., Morin, F., Mandelman, J. W., Clark, T. D. and Cooke, S. J. (2014). Use of portable blood physiology point-of-care devices for basic and applied research on vertebrates: A review. *Conserv. Physiol.* **2**, 1–21.

Thomsen, M. T., Wang, T., Milsom, W. K. and Bayley, M. (2017). Lactate provides a strong pH-independent ventilatory signal in the facultative air-breathing teleost *Pangasianodon hypophthalmus*. *Sci. Rep.* **7**, 6378.

Ultsch, G. R. (1987). The potential role of hypercarbia in the transition from water-breathing to air-breathing in vertebrates. *Evolution (N. Y).* **41**, 442–445.

Ultsch, G. R. (1996). Gas exchange, hypercarbia and acid-base balance, paleoecology, and the evolutionary transition from water-breathing to air-breathing among vertebrates. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **123**, 1–27.

Wells, R. M. G. and Pankhurst, N. W. (1999). Evaluation of simple instruments for the measurement of blood glucose and lactate, abd plasma protein as stress indicators in fish. *J. World Aquac. Soc.* **30**, 2.

Wendelaar Bonga, E. S. (1997). The Stress Response in Fish. *Physiol. Rev.* **77**, 592–616.

Willmer, E. N. (1934). Some observations on the respiration of certain tropical fresh-water fishes. *J.Exp.Biol.* **11**, 283–306.

SUMMARY AND CONCLUSION

Comparative aspects across species

Chapters 1-3 contained comparative studies in which the response of multiple species to environmental challenges were observed. Chapter 1 compared two members of the Polyperid family which showed little difference in their responses to forced emersion likely due to the similarity and homology of their respiratory systems (Graham, 1997). Chapter 2 and 3 investigated a phylogenetically diverse set of species with distinct air-breathing systems, and a spectrum of air and water breathing capacities and amphibious behaviors. The widely differing physiological and behavioral responses observed are a result of the unique integration of both form and function and ecophysiologies. These findings demonstrate the how evolutionary and natural history can shape a diversity of solutions to the same environmental challenge.

Hypercapnia and *P. hypophthalmus*

Our findings in Chapter 2, 3, and 4 show that exposure to hypercapnia at a level of 38 mmHg CO_2 does not create a significant physiological challenge for *P. hypophthalamus*, from a respiratory, behavioral, or stress biomarker assessment, respectively. Although a respiratory acidosis was shown to occur (Ch 4., Damsgaard et al., 2015), this level of hypercapnia did not show any further signs of respiratory distress. No change in air breathing was observed in Chapter 2, in agreement with Thomsen et al., 2017, who also reported branchial ventilation was not altered. No change in respiration further suggesting metabolic rate and behavior was also not altered as a response to aquatic hypercapnia. This observation is in agreement with our blood stress biomarkers in Chapter 4 showing no indication of physiological stress. Chapter 3 reported only a slight hypercapnia avoidance behavior during acute exposure to hypercapnia. It is possible that this would not be observable after 48 h of acclimation once acid-base compensation has occurred.

Taken together, regulation of ponds below 38 mmHg CO_2 appears to have little potential benefit for the aquaculture of *P. hypophthalamus*. This level of CO_2 also would also not be effective for self-tank transfer as shown in *O. mykiss* (Clingerman et al., 2007).

Implications: Aquaculture and Fish that Breathe Air

The potential benefits of, or lack thereof in this case, regulating PCO_2 below 38 mmHg, still must be verified by long-term growth and food conversion studies as well as with experiments assessing the combined effects of multiple environmental stressor. For example, hypercapnia has been shown to enhance nitrite toxicity in *P. hypophthalamus,* although the mechanism is not yet understood (Hvas et al., 2016). Size and development are also an important factor in the ability for *P. hypophthalamus* to cope with environmental challenge. In the same study nitrite susceptibility was found to be size dependent and Phuong et al., 2018 showed gill surface area, potentially available for gas and ion exchange, changes throughout development. It would be important to understand the effects on hypercapnia on different size classes of *P. hypophthalamus* both in its effect on growth, development, and tolerance plasticity. Whether juveniles of *P. hypophthalamus* are more susceptible and early protection from hypercapnia is require, or if early exposure results in adaptive developmental plasticity of tolerance in adult life, as show in zebrafish (*Danio rerio*) for hypoxia tolerance (Shingles et al., 2005), would be valuable knowledge for the aquaculture industry.

Findings from this research provide a starting point for the establishment of CO_2 thresholds for regulation in the cultivation of *P. hypophthalamus* as well as other air-breathing fishes incorporated in this dissertation. I believe the most important implication from the comparative aspect of this work is that air-breathing fishes are different and respond differently to environmental challenges. The diversity of responses shown here stem from unique natural and evolutionary histories. These comparative aspects on gas exchange, acid-base balance, metabolism, and response to environmental challenges in air-breathing fishes have received a recent surge in attention over the past decade, as appreciation of what were once thought to be nuance difference between species may have important economic consequences (Lefevre et al., 2014). Yet what has been uncovered thus far represents only the tip of the iceberg and many future studies are essential for the optimization of aquaculture conditions tailored to the diversity among this group of fishes. Further basic and applied research is necessary to gain a fuller comprehension of these fascinating animals' fundamental biology, not only for the sake of gaining insight into life on our planet, but also necessary for the establishment of evidence-based policies that can improve aquaculture practices and contribute to the ongoing success of the Blue Revolution.

As the growth of the human population continues, we will be faced with the ongoing challenge of feeding a hungry planet and building a sustainable future. Aquaculture remains a fertile opportunity for providing ongoing food security, being the most efficient means of producing animal protein, (1.6 compared to 1.9 for chickens and 8.0 for cattle), while at the same time creating the lowest carbon footprint (one seventh that of cattle) (FAO, 2018; Fry et al., 2018). Despite the projected 37% increase in worldwide aquaculture production by 2030, this growth will only cover 40% of the hike in global fish demand, driven by population and income growth (FAO, 2018). An assessment of Vietnam's aquaculture industry by The World Bank identifies investments in R&D as the primary factor limiting this sectors further expansion and ability to cope with climate change (The World Bank, 2016). My hope is that work done here can to support these efforts, aiding in economic development, food security, reduced industry environmental impact, and the conservation of wild fish stocks and our natural ocean resources.

References

Clingerman, J., Bebak, J., Mazik, P. M. and Summerfelt, S. T. (2007). Use of avoidance response by rainbow trout to carbon dioxide for fish self-transfer between tanks. *Aquac. Eng.* **37**, 234–251.

Damsgaard, C., Gam, L. T. H., Dang, D. T., Van Thinh, P., Huong, D. T. T., Wang, T. and Bayley, M. (2015). High capacity for extracellular acid-base regulation in the air-breathing fish *Pangasianodon hypophthalmus*. *J. Exp. Biol.* **218**, 1290–1294.

FAO (2018). The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. *THE STATE OF THE WORLD* **Rome**,.

Fry, J. P., Mailloux, N. A., Love, D. C., Milli, M. C. and Cao, L. (2018). Feed conversion efficiency in aquaculture: Do we measure it correctly? *Environ. Res. Lett* **13**, 024017.

Graham, J. B. (1997). *Air-breathing Fishes - Evolution, diversity and adaptation*. San Diego, CA: Academic Press.

Hvas, M., Damsgaard, C., Gam, L. T. H., Huong, D. T. T., Jensen, F. B. and Bayley, M. (2016). The effect of environmental hypercapnia and size on nitrite toxicity in the striped catfish (*Pangasianodon hypophthalmus*). *Aquat. Toxicol.* **176**, 151–160.

Lefevre, S., Wang, T., Jensen, A., Cong, N. V., Huong, D. T. T., Phuong, N. T. and Bayley, M. (2014). Air-breathing fishes in aquaculture. What can we learn from physiology? *J. Fish Biol.* **84**, 705–731.

Phuong, L. M., Thi, D., Huong, T., Malte, H., Nyengaard, J. R. and Bayley, M. (2018). Ontogeny and morphometrics of the gills and swim bladder of air-breathing striped catfish *Pangasianodon hypophthalmus*. *J.Exp.Biol.* **221**,.

Shingles, A., McKenzie, D. J., Claireaux, G. and Domenici, P. (2005). Reflex cardioventilatory responses to hypoxia in the flathead gray mullet (*Mugil cephalus*) and their behavioral modulation by perceived threat of predation and water turbidity. *Physiol. Biochem. Zool.* **78**, 744–755.

The World Bank ed. (2016). *Transforming Vietnamese Agriculture: Gaining More from Less*. Hanoi, Vietnam.

Thomsen, M. T., Wang, T., Milsom, W. K. and Bayley, M. (2017). Lactate provides a strong pH-independent ventilatory signal in the facultative air-breathing teleost *Pangasianodon hypophthalmus*. *Sci. Rep.* **7**, 6378.