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# Physiological Downstream Consequences and Tradeoffs Associated with CO<sub>2</sub> Compensation in Marine Fish

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UNIVERSITY OF MIAMI

PHYSIOLOGICAL DOWNSTREAM CONSEQUENCES AND TRADEOFFS  
ASSOCIATED WITH CO<sub>2</sub> COMPENSATION IN MARINE FISH

By

Rachael M. Heuer

A DISSERTATION

Submitted to the Faculty  
of the University of Miami  
in partial fulfillment of the requirements for  
the degree of Doctor of Philosophy

Coral Gables, Florida

December 2015

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PHYSIOLOGICAL DOWNSTREAM  
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Physiological Downstream Consequences  
and Tradeoffs Associated with CO<sub>2</sub> Compensation  
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Fish are renowned for their ability to defend blood pH following exposure to elevated ambient CO<sub>2</sub>, a trait that has undoubtedly led to their ability to thrive successfully in a wide variety of habitats. While the defense of internal pH is critical for survival, the sustained elevation of HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> that occurs as a part of this compensatory response could potentially lead to sub-lethal impacts. This point is underscored by a growing number of studies demonstrating negative effects of CO<sub>2</sub>, even at relatively low CO<sub>2</sub> tensions. The goal of this dissertation was to assess potential downstream consequences and/or tradeoffs associated with compensation for CO<sub>2</sub> exposure in marine fish. This was achieved by examining the intestinal response of the Gulf toadfish (*Opsanus beta*) and the neurological response of the Spiny damselfish (*Acanthochromis polyacanthus*). Results from this dissertation indicate that ocean acidification relevant CO<sub>2</sub> exposure levels predicted for year 2300 stimulates HCO<sub>3</sub><sup>-</sup> loss through the intestine that is seemingly counterproductive to whole-animal acid-base balance. This base loss incurred an increased energetic demand on isolated intestinal tissue, providing evidence that low level CO<sub>2</sub> exposure could lead to increases in baseline

metabolism. Increasing the range of tested CO<sub>2</sub> levels in the toadfish (up to 20,000 μatm CO<sub>2</sub>; ~2 kPa) led to the first broad characterization of intestinal transport physiology during elevated CO<sub>2</sub>. Despite a consistent increase in intestinal HCO<sub>3</sub><sup>-</sup> secretion with CO<sub>2</sub> exposure, the quantity and composition of intestinally produced carbonates did not change. Finally, the last portion of this dissertation involved a shift in focus to downstream impacts of CO<sub>2</sub> compensation in the brain. To my knowledge, this dissertation provides the first direct measurements of extracellular and intracellular HCO<sub>3</sub><sup>-</sup> in a coral reef species exposed to an ocean acidification relevant CO<sub>2</sub> level (1900 μatm CO<sub>2</sub>; 0.19 kPa) that also induces a behavioral disturbance. These measurements support the hypothesis that CO<sub>2</sub> compensation leads to changes in gradients across neuronal membranes that alter behavior. Overall, results from this dissertation indicate that CO<sub>2</sub> compensation may lead to an unexpected sensitivity for certain tested endpoints. The potential for sub-lethal impacts on fish is particularly topical, since it is unclear how fast fish will adapt in response to oceans that are currently undergoing acidification at a rapid rate.

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## **Chapter 1: Introduction**

As atmospheric CO<sub>2</sub> has increased from preindustrial (280 p.p.m. CO<sub>2</sub>) to present day values (~390 p.p.m. CO<sub>2</sub>) (Feely et al. 2009; Meehl et al. 2007), equilibration with the ocean has led to a corresponding pH decline of 0.1 and is projected to undergo changes over the next two centuries at rates that have not been seen in the last 300 million years, ultimately leading to a pH decline of up to 0.77 by year 2300 (Caldeira and Wickett 2003; Meehl et al. 2007). Global mean oceanic CO<sub>2</sub> values are expected to reach 1,000 μatm CO<sub>2</sub> by year 2100 and 1,900 μatm CO<sub>2</sub> by year 2300 (Caldeira and Wickett 2003; Meehl et al. 2007), levels expected to impact a wide array of organisms (Doney et al. 2009; Kroeker et al. 2013).

### ***Current state of ocean acidification research: a brief meta-analysis***

Using a quantitative approach to enumerate the diversity and threshold levels of particular taxonomic groups can be an effective tool to identify patterns and determine critical knowledge gaps in the realm of ocean acidification research, as seen in previous meta-analyses (Kroeker et al. 2013; Kroeker et al. 2010; Wittmann and Pörtner 2013). The present analysis begins with an examination of ocean acidification studies over time and across taxa to explore the evolution and current status of fish studies in a broader context. In this analysis, an examination of peer-reviewed literature from 2000 to Jan 15, 2014 revealed that research across most taxa began to rapidly expand after 2007 (Fig. 1.1). The calcifying Cnidarians and Molluscs are the most studied taxonomic groups and have seen

the most rapid increase in the number of studies, averaging ~52–57 studies per year since 2012 (Fig. 1.1). The low number of studies on marine worms and sponges stands in stark contrast to the aforementioned groups, with ~5–8 studies per year from 2012–2013, suggesting research on these less prominent but ecologically important groups is needed. Pisces, Echinoderms, and Crustaceans are at an intermediate level (~27–30 studies/year). Arguably, considering the ecological and economical importance of fish, this group has not received the attention it deserves.

### ***Current state of ocean acidification research and fish***

Before 2009, studies examining CO<sub>2</sub> effects on fish were performed at relatively high levels and were largely mechanistic in nature, demonstrating that fish are effective acid-base regulators (Fig. 1.1). This regulatory ability, combined with early studies showing effects on growth and survival only at high CO<sub>2</sub> exposures (Ishimatsu et al. 2008), undoubtedly led to the idea that fish were not particularly sensitive to CO<sub>2</sub>. Broadening the scope of tested endpoints revealed that fish are far more sensitive to low CO<sub>2</sub> levels (less than ~2,000 μatm CO<sub>2</sub>; 0.2 kPa) than predicted. This shift was largely prompted by the olfactory disturbance noted in the orange clownfish at ~1,000 μatm (0.1 kPa), published by Munday and colleagues in 2009 (Munday et al. 2009a). The compensatory response during acid-base regulation (see section below) is now hypothesized to be responsible for a large number of downstream effects, resulting in a variety of physiological and behavioral alterations.

Fig. 1.1: Number of ocean acidification relevant papers published each year from 2000 through 2013 for different phylogenetic animal groups. Literature searches were performed using Web of Science combining the search terms ["Ocean acidification" or hypercap\* or hypercarb\* or CO<sub>2</sub>] and one of the following: [Fish or Fishes or Pisces or teleost\* or elasmobranch\*], [Mollus\* Not Molluscum or gastropod or bivalve\* or abalone\* or oyster\* or squid\* or octop\*], [Cnidaria\* or coral\* or anemone\* or hydra or hydras or jellyfish\*], [Crustacean\* or shrimp\* or crab or crabs or copepod\* or krill\* or barnacle\*], [Echinoderm\* or starfish or urchin\* or sand dollar\* or "sea cucumber"], [Worm\* or annelid\* or polychaet\* or hirudin\* or oligochaet\* or platyhelminth\* or nemertea\* or nematode\* or flatworm\* or turbellar\* or monogen\* or trematoda\* or cestoda\* and roundworm\*] or [Porifera or sponge\*]. Due to large volume of terrestrial studies including worms, the above search terms were combined with AND [Marine or seawater or ocean\*] for this group. Search results were subsequently screened manually to include only peer-reviewed studies examining impacts of elevated ambient CO<sub>2</sub> regardless of PCO<sub>2</sub> levels employed. Papers were included in multiple groups if appropriate and relevant reviews of particular subject areas were also included.

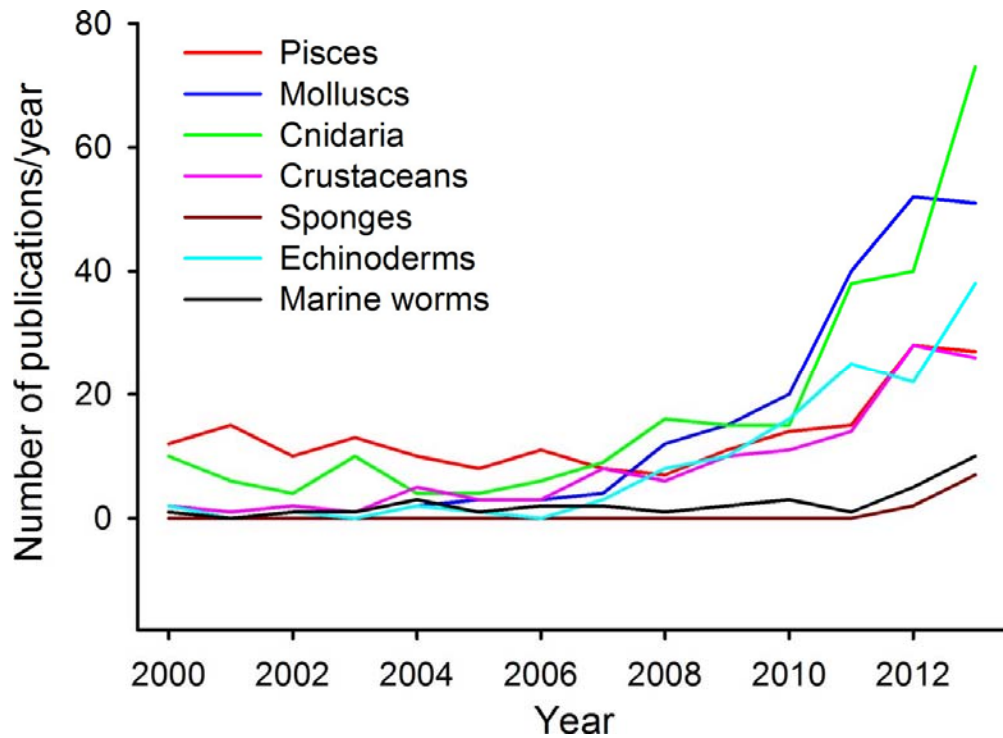
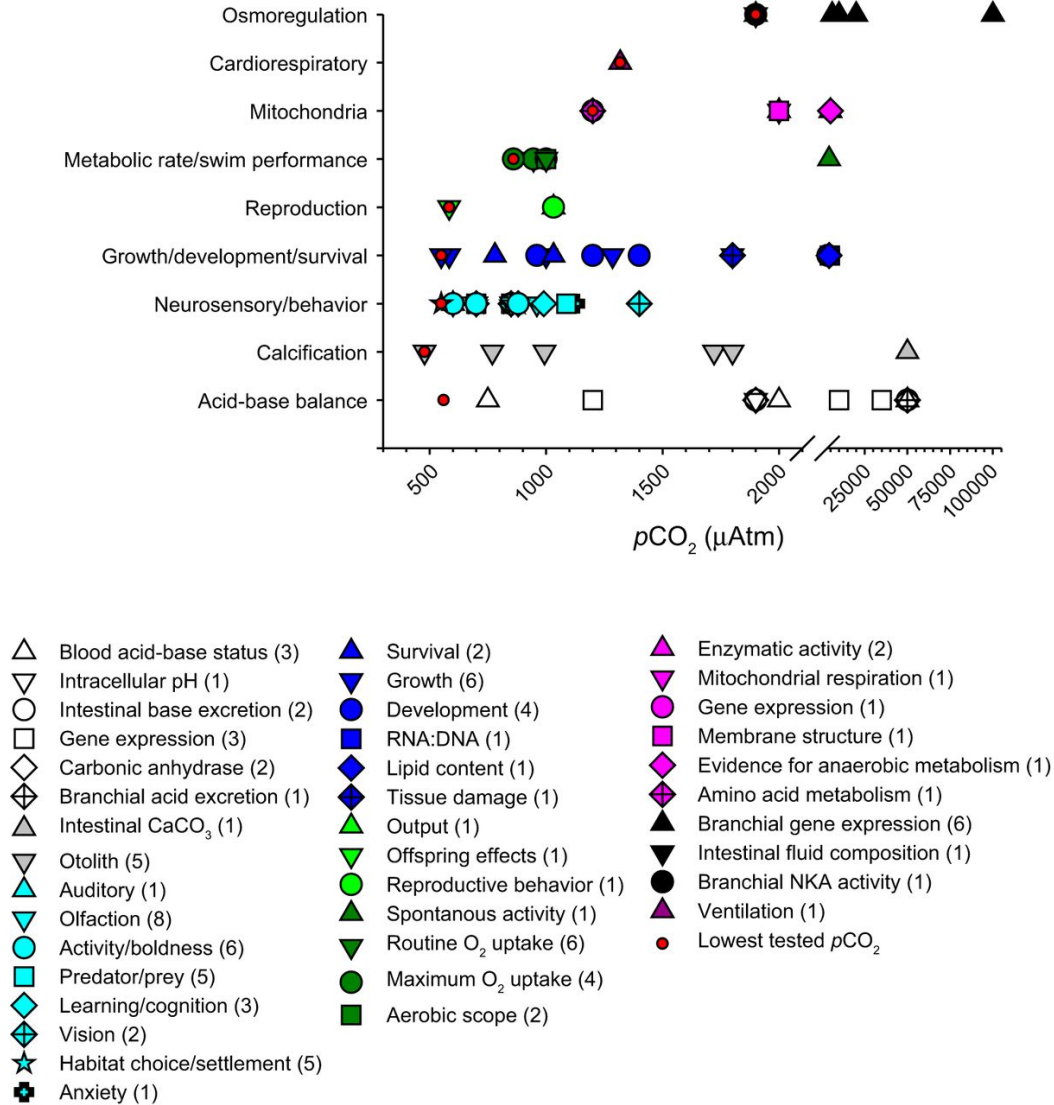


Fig. 1.2: Visual summary of information presented in (Heuer and Grosell 2014). Individual reported CO<sub>2</sub> effects within in each of the 9 effect categories discussed in (Heuer and Grosell 2014) (represented by common color) are presented as a function of PCO<sub>2</sub> levels. Each major category was then further broken into sub-categories shown by individual symbols (see legend). The number of individual reported CO<sub>2</sub> effects within each sub-category are represented in parentheses. In addition, the lowest tested PCO<sub>2</sub> level within each category is included (**red**). Individual publications may be included in more than one effect sub-category; however, each data point represents the lowest tested effect level in one sub-category for a particular species. Accordingly, studies examining multiple species could be counted multiple times across sub-categories. Species examined in multiple studies were only reported once at the lowest effect level in a sub-category. It is important to note that the only sub-category (ventilation) effect reported in the Cardiorespiratory category is from a freshwater species (see text). See (Heuer and Grosell 2014) for more detail and literature references.



In an expanded examination of the fish taxa, the minimum CO<sub>2</sub> level that induced a significant effect and/or the maximum reported level with no significant effect were documented for all tested parameters and species (Heuer and Grosell 2014). From this analysis, parameters showing a significant CO<sub>2</sub> effect were compiled and organized into nine categories according to apparent sensitivity and putative causal relationships (acid-base balance, calcification, neurosensory and behavior, growth, development and survival, reproduction, metabolic rate and swim



performance, mitochondrial function and metabolic pathways, cardiorespiratory, and osmoregulation) and further subdivided within these categories into commonly themed subcategories (Fig. 1.2).

One noticeable trend is that reported effects under 2,000  $\mu\text{atm CO}_2$  in “neurosensory and behavior”, “growth, development, and survival”, and “metabolic rate and swim performance” tend to dominate pertinent literature (total numbers 31, 12, and 11, respectively) and represent measurements on a diverse array of species. “Acid-base balance”, “mitochondrial function, and metabolic pathways”, “osmoregulation,” “reproduction”, and “cardiorespiratory physiology” have generally been understudied (total numbers 6, 5, 3, 3, and 1, respectively) and are reflective of fewer tested species. Interestingly, all categories show observed effects at or close to the lowest tested  $\text{PCO}_2$  (partial pressure of carbon dioxide) values. Certain categories including “osmoregulation”, “mitochondrial function, and metabolic pathways” have not been tested below 1,900 (.19 kPa), and 1,200  $\mu\text{atm CO}_2$  (.12 kPa), respectively, and therefore may be more sensitive than currently recognized. Generally, results from this analysis suggest that fish may be far more sensitive to ocean acidification than previously predicted (Gutowska et al. 2008; Kroeker et al. 2010; Melzner et al. 2009; Portner 2008), at least for certain endpoints. In four of the nine major categories, significant effects of  $\text{CO}_2$  are noted below 600  $\mu\text{atm CO}_2$ , a level expected by the middle of this century. Some degree of adaptation is bound to occur, but it is unknown if ~30 years is enough time to keep pace with the rate of change in the environment, especially for species that reach sexual maturity late. In the majority of studies reporting the most sensitive

endpoints, such as increased calcification in otoliths and broad neuronal disruptions linked to GABA<sub>A</sub> receptors, effects are arguably linked to compensation for acid-base balance (see section below). However, few studies have quantified the time course, threshold, effects of long-term chronic exposure, and magnitude of change in acid-base chemistry that occur in extracellular and intracellular fluids in fish during low level CO<sub>2</sub> exposure. These types of physiological measurements, in addition to molecular and biochemical techniques, are of central importance to assessing the impacts of ocean acidification on fish populations, especially since there is likely substantial variation across species, life stages, and environments. While the idea that changes in acid-base balance to cope with future projected ocean acidification may present fish with a need for physiological tradeoffs and metabolic costs is certainly not new (Ishimatsu et al. 2008; Melzner et al. 2009; Portner et al. 2004), additional mechanistic work in this research area is needed.

Broadly, the unifying goal of my dissertation is to examine the potential downstream consequences and/or tradeoffs associated with compensation for CO<sub>2</sub> exposure in marine fish. Accordingly, specific objectives for the dissertation will focus on 3 of the 9 major categories shown in Fig. 1.2: calcification, osmoregulation, and neurosensory/behavioral impacts. I believe that compensatory acid-base regulation is at the basis for effects observed in these general categories and will therefore briefly introduce this topic before describing my detailed research objectives.

***Acid-base balance in marine fish***

Teleost fish maintain relatively constant, yet temperature-dependent, alkaline pH (7.7-8.1) in extracellular fluids ( $pH_e$ ) (Marshall and Grosell 2006). Owing to the largely  $O_2$ -based ventilatory drive, the relatively high  $CO_2$  solubility in water, and the countercurrent exchange of water and blood flow at the gill,  $CO_2$  is readily excreted into the water resulting in low plasma  $CO_2$  partial pressure ( $PCO_2$ ) in strict water breathers compared with air breathing vertebrates (arterial  $PCO_2$  ~2.6 kPa or ~2600  $\mu$ atm in fish versus ~5.3 kPa or ~53,000  $\mu$ atm in humans) (Claiborne 1998; Evans et al. 2005; Heisler 1988; Perry and Gilmour 2006). Respiratory adjustments to control acid-base balance are thus relatively inefficient strategies in water-breathing compared to air-breathing animals due to the low scope for  $PCO_2$  change (Evans et al. 2005; Perry and Gilmour 2006), although cardio-respiratory responses by fish exposed to elevated  $CO_2$  have been noted (Vulesevic et al. 2006). The primary response of fish to an acid-base balance disturbance is to adjust blood plasma  $HCO_3^-$  levels through the differential regulation of  $H^+$  and  $HCO_3^-$  excretion rates (metabolic adjustment) (Cameron 1978; Evans et al. 2005; Marshall and Grosell 2006; Perry et al. 2010; Perry and Gilmour 2006; Randall et al. 1982).

Historically, elevated  $CO_2$  has been frequently used in studies of acid-base balance to induce a respiratory acidosis in freshwater and marine fish (Cameron and Randall 1972; Haswell et al. 1980; Janssen and Randall 1975; Lloyd and White 1967; Randall et al. 1976). Most studies demonstrate full and efficient pH compensation within hours to days of the initial acidosis despite continued

exposure to hypercarbia at levels exceeding 10,000  $\mu\text{atm}$ , a process that is achieved by retention and/or uptake of  $\text{HCO}_3^-$  and/or by an increased net acid secretion (Cameron 1978; Cameron and Iwama 1989; Claiborne et al. 2002; Evans et al. 2005; Goss et al. 1992; Heisler 1989, 1993; Larsen et al. 1997; Marshall and Grosell 2006; McDonald et al. 1989; Michaelidis et al. 2007; Perry 1982; Perry and Gilmour 2006; Perry et al. 2003; Randall et al. 1982; Toews et al. 1983; Wheatly et al. 1984; Wood et al. 1984). Thus, fish exposed to elevated  $\text{CO}_2$  effectively regulate extracellular pH but display continuously elevated  $\text{PCO}_2$  and  $\text{HCO}_3^-$  in their extracellular fluids. While these observations clearly illustrate fish are highly efficient acid-base regulators, they have undoubtedly led to the general assumption that compensation confers broad  $\text{CO}_2$  tolerance (Gutowska et al. 2008; Kroeker et al. 2010; Melzner et al. 2009; Portner 2008).

The first study to investigate acid-base balance responses of a marine fish to ocean acidification relevant  $\text{PCO}_2$  levels (560-1900  $\mu\text{atm}$ ; 0.06-0.19 kPa) revealed that 750  $\mu\text{atm}$   $\text{CO}_2$  induced an acidosis in the Gulf toadfish (*Opsanus beta*) (Esbaugh et al. 2012). Similar to fish tested at higher levels, compensation was achieved through the sustained elevation of plasma  $\text{HCO}_3^-$  (Esbaugh et al. 2012). This study validated that fish exposed to low levels of  $\text{CO}_2$  also defended pH, but are faced with sustained elevated levels of  $\text{HCO}_3^-$  and  $\text{PCO}_2$ . Although not specifically measured in Esbaugh et al 2012, it is typical for fish exhibiting elevated levels of plasma  $\text{HCO}_3^-$  experience an equimolar reduction in plasma  $\text{Cl}^-$ . This new steady state is now hypothesized by many to be the underlying cause of  $\text{CO}_2$

induced sub-lethal effects noted in fish and serves as a foundation for hypotheses outlined in the rest of this dissertation.

***Chapter 2 Objectives: Downstream impacts of elevated CO<sub>2</sub> on intestinal base secretion in the marine Gulf toadfish (Opsanus beta)***

One particular area of the body that could potentially be impacted by sustained increases in plasma HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> following CO<sub>2</sub> exposure is the marine fish intestine. In this organ, routine osmoregulatory processes involve transport of acid and base equivalents from the plasma (Grosell 2011a), and thus could be sensitive to changes in acid-base parameters that occur as a result of CO<sub>2</sub> compensation.

In order to survive in a dehydrating environment, marine fish must ingest seawater and absorb Na<sup>+</sup> and Cl<sup>-</sup> across the intestinal epithelia, a process that enables water absorption (Grosell et al. 2007; Skadhauge 1974; Smith 1930). Up to 70% of Cl<sup>-</sup> uptake occurs in exchange for HCO<sub>3</sub><sup>-</sup>, ultimately connecting water absorption to intestinal HCO<sub>3</sub><sup>-</sup> secretion (Grosell 2006; Grosell and Genz 2006). Fortunately, a thorough body of work has examined intestinal HCO<sub>3</sub><sup>-</sup> secretion in the context of both osmoregulation and digestion (Taylor and Grosell 2009; Taylor et al. 2010; Wilson et al. 2002), providing a foundation on which to build hypotheses regarding the potential impacts of elevated CO<sub>2</sub>.

There are two sources of HCO<sub>3</sub><sup>-</sup> available for secretion from the cell into the intestinal lumen. For species studied thus far, HCO<sub>3</sub><sup>-</sup> transport from the blood into the cell occurs via basolateral Na<sup>+</sup>:HCO<sub>3</sub><sup>-</sup> co-transporter NBC1 (SLC4a4) (Taylor et al. 2010). The second source of HCO<sub>3</sub><sup>-</sup> is derived from endogenous CO<sub>2</sub>

produced from the high metabolic rates of the intestinal epithelium. This  $\text{CO}_2$  becomes hydrated through cytosolic carbonic anhydrase (CAc; equivalent to mammalian CAII).  $\text{HCO}_3^-$  derived from both sources cross the apical membrane into the intestinal lumen through an electrogenic apical  $\text{Cl}^-:\text{HCO}_3^-$  anion exchanger (SLC26a6) (Grosell et al. 2009b; Kurita et al. 2008). With few exceptions (Grosell et al. 2009a), these two sources of  $\text{HCO}_3^-$  generally contribute equally to overall intestinal  $\text{HCO}_3^-$  secretion (Grosell and Genz 2006; Wilson and Grosell 2003).

Methodical and thorough analysis of  $\text{HCO}_3^-$  transport by Taylor and colleagues in 2010 was instrumental in forming hypotheses for the present chapter (Taylor et al. 2010). After isolating toadfish intestine to examine transport characteristics in a combined Ussing chamber/pH stat system, Taylor found that increasing  $\text{HCO}_3^-$  concentration in a blood-side (serosal) saline led to an increase in  $\text{HCO}_3^-$  secretion into the gut-side (mucosal) saline. In addition, measurements suggested that  $\text{HCO}_3^-$  rather than  $\text{Na}^+$  dictated transport rates through basolateral transporter NBC1. Finally, Taylor's work indicated that basolateral  $\text{HCO}_3^-$  movement through NBC1 rather than apical transport via anion exchanger slc26a6 was likely the rate-limiting step for transepithelial secretion. In combination, these results suggested that the intestine is sensitive to changes in plasma  $\text{HCO}_3^-$ , and could be responsive to changes in blood chemistry induced during low-level  $\text{CO}_2$  exposure. Thus, the first objective of the second chapter was to determine if elevated plasma  $\text{HCO}_3^-$  and  $\text{PCO}_2$  that occur in response to 1900  $\mu\text{atm}$   $\text{CO}_2$  in the Gulf toadfish (Esbaugh et al. 2012) would lead to a stimulation in intestinal  $\text{HCO}_3^-$  secretion *in vivo*.

A second strategy employed during routine osmoregulation that has the potential to be impacted by low level CO<sub>2</sub> exposure is intestinal carbonate formation. As marine fish drink seawater to absorb Na<sup>+</sup>, Cl<sup>-</sup>, and water, divalent ions such as Ca<sup>2+</sup> and Mg<sup>2+</sup> are concentrated since the intestine is relatively impermeable to these ions (Wilson et al. 2002). Elevated levels of these divalent ions, combined with the high concentration of HCO<sub>3</sub><sup>-</sup> secreted into the lumen leads to alkaline conditions (pH in some cases exceeding 9.0) (Wilson et al. 2002), that promote the formation of carbonates with some degree of Mg<sup>2+</sup> incorporation (Perry et al. 2011; Walsh et al. 1991; Wilson et al. 1996). Carbonate formation reduces luminal osmotic pressure as much as 100 mOsm and thereby facilitates osmotic fluid absorption by the intestine (Anderson et al. 2010; Grosell 2011a; Wilson et al. 2002).

Since elevated CO<sub>2</sub> was predicted to increase HCO<sub>3</sub><sup>-</sup> secretion rates into the lumen, it was not unreasonable to suspect that the production of calcium carbonate may scale accordingly. However, assuming an increase in production, it is possible that already low Ca<sup>2+</sup> concentrations in luminal fluids could become limiting, leading to increased Mg<sup>2+</sup> incorporation into carbonates. Thus, the 2<sup>nd</sup> objective of chapter 2 was to determine if elevated plasma HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> that occur in response to 1900 μatm CO<sub>2</sub> in the Gulf toadfish (Esbaugh et al. 2012) would lead to a stimulation in carbonate production or altered carbonate composition (Mg<sup>2+</sup> content).

The relevance of this objective is expanded beyond the whole-animal level, since fish-produced carbonates are estimated to contribute between 3 to 15% to

the global marine inorganic carbon cycle (Wilson et al. 2009). These carbonates are likely to undergo some degree of dissolution at shallower depths compared to carbonates produced by other calcifying organisms due to their high  $Mg^{2+}$  content (Wilson et al. 2009; Woosley et al. 2012). Modeling potential changes to production or solubility under future ocean acidification scenarios could be an important component in estimating shifts to the global oceanic carbon cycle as oceans continue to acidify.

Prior to the onset of this dissertation, only one study examined the base content of rectal fluid and carbonates following short-term exposure to high  $CO_2$  ( $50,000 \mu atm CO_2$ ) in a marine teleost, the Plainfin midshipman (Perry et al. 2010). Thus, the series of experiments performed in Chapter 2 were among the first to provide details on the intestinal response to  $CO_2$  exposure. Accordingly, Chapters 3 and 4 follow up on many of these initial findings, making the dissertation heavily focused on a specific organ. Nonetheless, it is important to note that all chapters, regardless of the target study organ or species, fall under the unifying theme of this dissertation: to examine the potential downstream consequences and/or tradeoffs associated with compensation for  $CO_2$  exposure in marine fish.

***Chapter 3 Objectives: Assessing the regulatory capacity and energetic costs associated with  $CO_2$  compensation in the intestine***

Findings from Chapter 2 revealed that toadfish exposed to  $1900 \mu atm$  for 3 days experienced a ~34% increase in their total rectal base secretion rate. These results indicated that the intestinal tract is impacted by increased  $HCO_3^-$  and/or  $PCO_2$  that occurs following compensation for a  $CO_2$  induced respiratory acidosis.



Interestingly, the loss of  $\text{HCO}_3^-$  through the intestine appeared to be counterproductive to whole-body acid base balance, where the retention and/or uptake of  $\text{HCO}_3^-$  is necessary to defend blood pH. Thus in Chapter 2, a downstream impact was observed, but the mechanism or regulatory responses and/or potential costs or tradeoffs associated with this impact were not addressed.

The first objective of Chapter 3 was to determine if the intestine of the Gulf toadfish would dynamically regulate transporters and enzymes associated with  $\text{HCO}_3^-$  secretion to reduce  $\text{CO}_2$ -induced intestinal base loss in favor of whole-body  $\text{HCO}_3^-$  retention. To achieve this goal, the  $\text{HCO}_3^-$  secretion rate of intestinal tissue from toadfish acclimated for at least two weeks to control or 1900  $\mu\text{atm}$   $\text{CO}_2$  was measured *in vitro*, using a combined Ussing chamber/pH stat experimental system. A decline in  $\text{HCO}_3^-$  secretion in fish acclimated to  $\text{CO}_2$  would suggest an attempt by the fish to reduce base loss, whereas no difference between control and  $\text{CO}_2$  acclimated tissue would indicate toadfish are unable to regulate intestinal base loss. Contrary to both of these hypotheses,  $\text{HCO}_3^-$  secretion rates from tissues obtained from  $\text{CO}_2$ -acclimated fish were higher than tissue from control fish, indicating a stimulation rather than a reduction in  $\text{HCO}_3^-$  transport pathways, potentially incurring a metabolic cost. Thus, the second objective of Chapter 3 was to determine if increased intestinal ion transport that occurs in response to  $\text{CO}_2$  acclimation would be reflected in an increased tissue metabolic demand.

***Chapter 4 Objectives: Further dissecting the response of the intestine to elevated CO<sub>2</sub> over a range of CO<sub>2</sub> levels***

As previously discussed, only one study outside of the present dissertation has examined how intestinal transport pathways may be impacted by CO<sub>2</sub> (Perry et al. 2010). The large difference in exposure levels (1900  $\mu$ atm vs. 50,000  $\mu$ atm CO<sub>2</sub>) in only two species makes it difficult to establish patterns in the intestinal response to hypercarbia (elevated ambient CO<sub>2</sub>). The gill has been rightfully well-studied with respect to acid-base balance, since it is the dominant organ responsible for regulating internal CO<sub>2</sub> levels through metabolic and to a lesser extent, respiratory adjustments (Brauner and Baker 2009; Perry and Gilmour 2006). It is nonetheless surprising that the intestine has received relatively little attention in the context of acid-base balance. The secretion of HCO<sub>3</sub><sup>-</sup> through the intestine appears to be a unifying response in the multifunctional gut of marine fish (Grosell 2006; Grosell and Jensen 1999; Grosell et al. 2001), and its role in osmoregulation and digestion have been more firmly established (Genz et al. 2008; Taylor and Grosell 2009; Taylor et al. 2010). Findings from Chapter 2, showing an unexpected 34% increase in HCO<sub>3</sub><sup>-</sup> secretion at relatively low CO<sub>2</sub> levels that only appears to be further stimulated by longer term acclimation to CO<sub>2</sub> (Chapter 3), clearly demonstrate that more information is needed to provide a complete picture of the intestinal contribution to whole-body acid base balance. Thus, building on the knowledge and techniques applied in the first two chapters, Chapter 4 provided the unique opportunity to contribute to this gap and more carefully examine how exposure to CO<sub>2</sub> alters properties of the intestine.

Accordingly, the approach employed in Chapter 4 was to expose Gulf toadfish to a range of CO<sub>2</sub> levels (control, 5,000, 10,000, and 20,000 μatm CO<sub>2</sub>) to better characterize the intestinal response to CO<sub>2</sub>. Thus, this chapter still focused on the downstream effects of compensation for a CO<sub>2</sub>-induced respiratory acidosis, just at higher CO<sub>2</sub> levels that capitalized on the strengths of using dose-dependent exposures to better elucidate mechanisms. This chapter also allowed for the pursuit and verification of unexpected findings in chapter 2.

Despite the large increase in base secretion observed in Chapter 2 at 1900 μatm CO<sub>2</sub>, carbonate production remained unchanged. It was unclear whether an increase would be evident at higher CO<sub>2</sub> levels, and if so, at what threshold such an increase would occur. Thus, one specific objective of Chapter 4 was to test if higher CO<sub>2</sub> levels would result in the hypothesized increase in carbonate formation and excretion.

In addition to assessing carbonate production across CO<sub>2</sub> levels, another objective of this chapter was to ensure that surgical procedures associated with attaching rectal collection sacs commonly used in Chapter 2 and in other studies, did not affect the ability to detect changes in carbonate production. To evaluate this methodology, carbonate measurements from rectal collection sacs were compared to measurements taken directly from the toadfish intestine through spot sampling. Yet another advantage of this study design was that carbonates excreted from these spot-collected fish could also be collected from the tank bottom over a discrete time interval, allowing for the investigation of dissolution potential.

Overall, the use of high CO<sub>2</sub> levels in this chapter went beyond the scope of ocean acidification relevant levels used in other dissertation chapters. However, this objective was still within the overall goal of this dissertation; to assess the downstream impacts or consequences of compensation for a CO<sub>2</sub> induced acidosis.

***Chapter 5 Objectives: Downstream impacts of elevated CO<sub>2</sub> on neurosensory and behavioral endpoints in the Spiny damselfish (*Acanthochromis polyacanthus*)***

Similar to the apparent deviation using high levels of CO<sub>2</sub> in Chapter 4, Chapter 5 also is unique in that the focus of downstream effects and consequences of CO<sub>2</sub> are shifted away from the intestine and to the brain and associated neurosensory and behavioral impacts.

The most extensive body of research examining the effects of ocean acidification on fish has focused on sensory systems and behavior. Robust and consistent disturbances have been noted across a range of sensory systems including olfaction (Cripps et al. 2011; Devine et al. 2012a; Devine and Munday 2012; Devine et al. 2012b; Dixson et al. 2010; Munday et al. 2009a; Munday et al. 2010; Munday et al. 2012b; Ou et al. 2015), hearing (Simpson et al. 2011), vision (Chung et al. 2014; Ferrari et al. 2012b; Forsgren et al. 2013), and has also been implicated in processes linked to general cognitive function including changes in lateralization (Domenici et al. 2012; Jutfelt et al. 2013; Lai et al. 2015), activity (Cripps et al. 2011; Devine et al. 2012b; Ferrari et al. 2011a; Munday et al. 2010; Munday et al. 2012b), boldness (Ferrari et al. 2011a; Munday et al. 2010; Munday

et al. 2012b), and learning (Chivers et al. 2013; Ferrari et al. 2012a; Jutfelt et al. 2013). With the exception of a few tested species, including the Atlantic cod (Jutfelt and Hedgärde 2015), and the temperate wrasse (Sundin and Jutfelt 2015), these impairments have been noted in species at numerous life stages and in both tropical and temperate species. Most importantly, almost all lowest PCO<sub>2</sub> exposures that induced significant effects in this topic area (Heuer and Grosell 2014) have been reported below IPCC models for the end of this century (<1000  $\mu$ atm CO<sub>2</sub>) (Meehl et al. 2007), providing evidence that fish may be far more sensitive to ocean acidification than previously predicted. In fact, at least three studies to date have linked lab-demonstrated sensory disruptions to increased mortality in the field (Chivers et al. 2013; Ferrari et al. 2011a; Munday et al. 2010). The implications of these disturbances are expected to be substantial if adaptation or acclimation does not keep pace with the rate of environmental change and include changes to dispersal (Munday et al. 2009a), recruitment (Ferrari et al. 2011a), connectivity (Munday et al. 2009a), social interactions (Devine et al. 2012b), predator-prey dynamics (Allan et al. 2013; Cripps et al. 2011; Ferrari et al. 2011a; Ferrari et al. 2012b; Ferrari et al. 2011b; Lönnstedt et al. 2013; Munday et al. 2012b), population replenishment (Dixon et al. 2010; Munday et al. 2010), biodiversity (Munday et al. 2009a; Munday et al. 2010), habitat preference (Devine et al. 2012a; Devine and Munday 2012) and settlement timing (Devine et al. 2012a); all of which could drastically affect population and ecosystem dynamics (see (Briffa et al. 2012; Munday et al. 2012a) for reviews). Common to most of these studies is the hypothesis that the physiological mechanism underlying the

observed effects is alterations to ion gradients across neurons following compensation for a CO<sub>2</sub>-induced acidosis.

Disruption to endpoints representing broad cognitive impairment such as lateralization and learning suggest that ocean acidification affects central neural processing rather than individual sensory systems in isolation (Devine et al. 2012a; Domenici et al. 2012; Ferrari et al. 2012a; Ferrari et al. 2012b; Nilsson et al. 2012; Wisenden 2012). Disruption of multiple sensory systems in a single damselfish species (*Pomacentrus amboinensis*) (Ferrari et al. 2011a; Ferrari et al. 2012a; Ferrari et al. 2012b; Lönnstedt et al. 2013) and in the orange clownfish (*Amphiprion percula*) (Munday et al. 2010; Simpson et al. 2011) further support this idea. Interestingly, the minimum CO<sub>2</sub> level needed to invoke these responses varies, which could reflect differences in the processing of signals from each sensory system and/or how these signals are integrated into neuronal systems (Ferrari et al. 2012b). Changes in sensory system structures have been investigated but have generally not been linked to behavior, further supporting broader neural disruptions as the cause of behavioral changes (Munday et al. 2009a). For example, olfactory disturbances were not found to be linked with structural changes in the nasal area (Munday et al. 2009a) and increases in otolith size have not been convincingly linked to behavioral endpoints. In clownfish, auditory disturbance occurs at a much lower CO<sub>2</sub> threshold (600  $\mu$ atm CO<sub>2</sub>) (Simpson et al. 2011) than what is observed for increases in otolith growth (1700  $\mu$ atm CO<sub>2</sub>) (Munday et al. 2011b), and cobia displaying increased otolith growth (800  $\mu$ atm CO<sub>2</sub>) show only minor changes in swimming activity (Bignami et al. 2013b). Evidence for centralized processing

impacts is also present in studies where fish prefer an inappropriate scent over control water (Dixson et al. 2010; Munday et al. 2009a; Munday et al. 2010; Munday et al. 2012b), show a lack of preference when one should be present (Devine et al. 2012a; Devine and Munday 2012; Devine et al. 2012b; Munday et al. 2009a; Munday et al. 2009b; Simpson et al. 2011), or a switch from preference to avoidance (Cripps et al. 2011; Devine et al. 2012a). While the input, processing, and integration of sensory signals in the brain occur rapidly, sensory and behavioral responses in CO<sub>2</sub> exposed fish typically take ~4 days to manifest (Munday et al. 2010) and are reversible after ~2 or 12 days once fish are returned to control water (tropical damselfish and temperate Californian rockfish, respectively) (Hamilton et al. 2014; Munday et al. 2010). As previously suggested, these findings indicate that contact of the sensory structure with seawater of altered PCO<sub>2</sub> does not instantly change input but modifies responses on a longer timescale that could involve regulation of neuronal processes related to acid-base balance compensation (Chivers et al. 2013; Cripps et al. 2011; Domenici et al. 2012; Ferrari et al. 2012a; Ferrari et al. 2012b; Jutfelt et al. 2013; Munday et al. 2009a; Munday et al. 2010; Nilsson et al. 2012; Simpson et al. 2011). Few studies have discussed specific neuronal cells/regions (Allan et al. 2013; Allan et al. 2014; Hamilton et al. 2014) or brain hemisphere communication errors (Domenici et al. 2012) that could be linked to specific observed behavioral alterations. Certain neurological disruptions, represented by a set of locomotor and non-locomotor aspects of escape time, may be attenuated in juveniles when parents are also exposed to CO<sub>2</sub> (Allan et al. 2014), however, a recent study examining olfactory

and lateralization responses of offspring from parents exposed to high CO<sub>2</sub> exhibited little scope for transgenerational acclimation (Welch et al. 2014).

An important stride in understanding the basis for neurosensory disruptions in fish was reported by Nilsson and colleagues in 2012, who demonstrated that the primary inhibitory neurotransmitter GABA and its accompanying GABA<sub>A</sub> receptors in the nervous system were associated with disruptions in olfaction and lateralization during CO<sub>2</sub> exposure (Nilsson et al. 2012). In this study, the addition of gabazine, a GABA<sub>A</sub> receptor antagonist, was found to reestablish proper response to olfactory cues and lateralization in CO<sub>2</sub> exposed fish. Since the GABA<sub>A</sub> receptor exhibits conductance for both HCO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup>, it has been suggested that decreases in plasma Cl<sup>-</sup> and increases in HCO<sub>3</sub><sup>-</sup> that occur during compensation for a CO<sub>2</sub> induced acidosis alter normal neuronal cell membrane ion gradients, leading to an attenuation of current observed during GABA binding to GABA<sub>A</sub> receptors. This alteration under elevated CO<sub>2</sub> conditions leads to change in response to GABA, thus providing a possible explanation for the alleviation of CO<sub>2</sub> effects with gabazine addition (Chivers et al. 2013; Nilsson et al. 2012). Recently, two subsequent studies have reinforced GABA<sub>A</sub> involvement in CO<sub>2</sub>-related sensory impairment related to learning (Chivers et al. 2013) and anxiety (Hamilton et al. 2014). In the former study, CO<sub>2</sub> exposed fish taught to recognize a predator cue in the presence of gabazine were not able to respond to this cue one day after the exercise due to lasting CO<sub>2</sub> effects, but were able to successfully respond 5 days later when CO<sub>2</sub> effects would have ceased. This finding demonstrated that fish could receive sensory information during CO<sub>2</sub> exposure but



could not properly process the signals. (Chivers et al. 2013). In the latter study, CO<sub>2</sub> fish treated with muscimol, a GABA<sub>A</sub> receptor agonist, exhibited increased anxiety levels while control fish treated with the same drug exhibited reduced anxiety (Hamilton et al. 2014). These divergent responses using a drug designed to open the GABA<sub>A</sub> receptor effectively demonstrated that changes within the body during CO<sub>2</sub> exposure are capable of inducing a change in current (Hamilton et al. 2014). Several subsequent studies have demonstrated similar alleviation with the use of pharmaceutical compounds in different species using various behavioral assays (Chung et al. 2014; Lai et al. 2015; Ou et al. 2015).

The hypothesis that the GABA<sub>A</sub> receptor is linked to CO<sub>2</sub>-induced behavioral disturbances is well-supported, but few studies have provided simultaneous measurements of extracellular and intracellular HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> at levels relevant to ocean acidification projections (Esbaugh et al. 2012; Strobel et al. 2012). Furthermore, no study to date has matched these gradients to a known induced behavioral disturbance in any fish. Therefore, the overall objective of Chapter 5 was to test the prediction that altered intracellular and extracellular HCO<sub>3</sub><sup>-</sup> due to CO<sub>2</sub> compensation occurs in a species and at ambient CO<sub>2</sub> levels that result in behavioral disturbances. To achieve this objective, intracellular whole-brain HCO<sub>3</sub><sup>-</sup> and pH (pH<sub>i</sub>) as well as and extracellular HCO<sub>3</sub><sup>-</sup> levels in blood plasma were measured in the spiny damselfish (*Acanthochromis polyacanthus*) following 4 days of exposure to 1900 μatm CO<sub>2</sub>. A second goal was to apply these measured values in an assessment of GABA<sub>A</sub> receptor function by calculating the reversal potential for GABA<sub>A</sub> ( $E_{GABA}$ ) in control and CO<sub>2</sub>-exposed fish. Finally, a third goal

was to determine if spiny damselfish exposed to the applied CO<sub>2</sub> level (1900 μatm CO<sub>2</sub>) would display altered behavioral responses to olfactory cues using a two-choice flume system (Munday et al. 2009a; Welch et al. 2014). To my knowledge, this is the first study to report direct measurements of both intracellular and extracellular HCO<sub>3</sub><sup>-</sup> and intracellular pH<sub>i</sub> in a coral reef fish species and the first study to match such measurements with behavioral observations. While plasma HCO<sub>3</sub><sup>-</sup> measurements have served as an anchor for other downstream effects noted in the present dissertation in the toadfish, well-documented and consistent behavioral assays needed to assess CO<sub>2</sub> induced effects would have to be performed. Accordingly, it became clear that performing measurements on a species with a robust behavioral assay would be more fruitful.

Until recently, fish were generally considered to be less sensitive to CO<sub>2</sub> than other, more traditional calcifying organisms. After an expanded analysis of tested endpoints that started near the onset of my dissertation research, this viewpoint has changed considerably. The compensatory response associated with a CO<sub>2</sub>-induced acidosis is now hypothesized to be the underlying cause of a variety of downstream impacts in many topic areas. Thus, my dissertation has allowed me to follow and contribute to this rapidly expanding realm of research in “real time”. While the detailed experiments of this dissertation vary in terms of the study organ or the target level of CO<sub>2</sub> exposure, the underlying goal is to examine the potential consequences associated with compensation for a CO<sub>2</sub> induced acidosis in marine fish.

Flexibility afforded to me by my committee in qualifying exam topics largely broadened by interests, ultimately motivating me to write a first-authored review article on “Physiological impacts of elevated CO<sub>2</sub> and ocean acidification marine fish” (Heuer and Grosell 2014). This article is not a direct part of my dissertation but reflects a synthesis of a wide range of topics and most certainly informed the choices of specific objectives, while placing this dissertation in a broader context.

## Chapter 2: Downstream impacts of elevated CO<sub>2</sub> on intestinal base secretion in the Gulf toadfish (*Opsanus beta*)

### Summary

Oceanic CO<sub>2</sub> has increased from 280 to 380 μatm since preindustrial times and is expected to reach 1,900 μatm by 2300. In addition, regional upwelling zones exhibit levels up to 2,300 μatm, making exploration at future global projected CO<sub>2</sub> levels ecologically relevant today. Recent work has demonstrated that CO<sub>2</sub> exposure as low as 780 μatm induces acidosis in toadfish (*Opsanus beta*), leading to metabolic compensation by retention of blood HCO<sub>3</sub><sup>-</sup> in an effort to defend pH. Since increased serosal HCO<sub>3</sub><sup>-</sup> translates to increased HCO<sub>3</sub><sup>-</sup> secretion rates in isolated intestinal tissue, I predicted that blood elevation of HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> during exposure to 1,900 μatm CO<sub>2</sub> would increase *in vivo* base secretion rates. Rectal fluid and CaCO<sub>3</sub> excretions were collected from toadfish exposed to 380 (control) and 1,900 μatm CO<sub>2</sub> for 72 hours. Fluids were analyzed for pH, osmolality, ionic composition, and total CO<sub>2</sub>. Precipitated CaCO<sub>3</sub> was analyzed for titratable alkalinity, Mg<sup>2+</sup>, and Ca<sup>2+</sup> content. Fish exposed to 1,900 μatm CO<sub>2</sub> exhibited higher rectal base excretion rates, higher rectal fluid HCO<sub>3</sub><sup>-</sup> (mmol l<sup>-1</sup>), and lower fluid Cl<sup>-</sup> (mmol l<sup>-1</sup>) than controls, suggesting increased intestinal anion exchange as a result of the compensated respiratory acidosis. This study verifies that imminent projected CO<sub>2</sub> levels expected by the year 2300 lead to greater intestinal HCO<sub>3</sub><sup>-</sup> loss, a process that acts against compensation for a CO<sub>2</sub>-induced acidosis.

## Background

Anthropogenic input to the global carbon cycle has already increased oceanic CO<sub>2</sub> from preindustrial values (~280 μatm) to current levels of ~380 μatm, and decreased ocean pH by 0.1 (Caldeira and Wickett 2003; Fabry et al. 2008; Meehl et al. 2007). Values of oceanic CO<sub>2</sub> are expected to climb to 1,900 μatm by the year 2300, inducing an oceanic pH decline of up to 0.77 units, an extraordinary rate of change that has not been observed in the past 300 million years (Caldeira and Wickett 2003; Meehl et al. 2007). In addition to these predicted elevations of ambient CO<sub>2</sub>, current regional CO<sub>2</sub> levels can reach values as high as 1,000–2,300 μatm, corresponding to pH 7.75 and 7.49, respectively (Feely et al. 2008; Thomsen et al. 2010). Coastal and estuarine areas close to urban areas may be particularly likely to display elevated CO<sub>2</sub> levels since nutrient and organic matter runoff can lead to increased PCO<sub>2</sub> and hypoxia from respiration, magnifying PCO<sub>2</sub> increases resulting from ocean acidification (Cai et al. 2011; Feely et al. 2010). Ocean acidification has particularly deleterious effects on many calcifying organisms due to the impacts of decreased pH on the calcium carbonate saturation state, resulting in less [CO<sub>3</sub><sup>2-</sup>] available for shell formation and maintenance (Doney et al. 2009; Fabry et al. 2008; Orr et al. 2005). Reduced calcification is expected by the year 2300 across a broad array of species including blue mussels (Thomsen et al. 2010), sea urchins (Byrne et al. 2011), corals (Kleypas and Yates 2009), foraminifera and coccolithophores (Riebesell 2004; Riebesell et al. 2000), pteropods (Lischka et al. 2011), and abalone larvae (Byrne et al. 2011). Troubling findings related to reproduction have also been observed, including reduced

fertilization and settlement success in corals (Albright and Langdon 2011) and decreased egg production in shrimp (Kurihara et al. 2008). Increased calcification rates have been noted in some species following CO<sub>2</sub> exposure, often with an associated cost, such as reduced buoyancy in cephalopods (Gutowska et al. 2010), and decreased muscle mass in a species of brittlestar (Wood et al. 2008).

Recent research on marine teleosts exposed to CO<sub>2</sub> levels <1,900 μatm has focused on sensory systems of younger animals and areas of the body responsible for forming calcium carbonates, including the skeleton and otolith. Fish otoliths were found to increase in size in white sea bass (Checkley et al. 2009) and juvenile clownfish (Munday et al. 2011b) with elevated ambient CO<sub>2</sub> concentrations (~1,000, 1,700 μatm, respectively). However, lower level CO<sub>2</sub> exposures predicted over the next century (600, 725, 850 μatm) did not elicit changes in otolith size, shape, symmetry or skeletal formation in the spiny damselfish (Munday et al. 2011a). Juvenile clownfish exposed to 600 μatm CO<sub>2</sub> also displayed no changes in otolith size but did show a lack of avoidance to predator sounds, suggesting a disturbance of the auditory sensing system at this CO<sub>2</sub> level (Simpson et al. 2011). Juvenile clownfish have also been found to be attracted to predator olfactory cues (~1,000 μatm) (Dixson et al. 2010), unresponsive to appropriate settlement olfactory stimuli at ~1,000 μatm, and attracted to inappropriate settlement sites at ~1,700 μatm, despite no change in body morphology (Munday et al. 2009a). Researchers examining the background mechanism responsible for neurological disruption have proposed that GABA<sub>A</sub> receptor function is altered by changes in Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> during acid-base regulation, affect behavioral lateralization and

olfactory ability in clownfish and could explain other sensory system disruptions described in previous studies (Nilsson et al. 2012).

Marine teleosts are strong acid-base regulators and actively defend blood pH from respiratory acidosis at high levels of CO<sub>2</sub> with a well-documented metabolic compensation mechanism. The events following onset of exposure to elevated ambient CO<sub>2</sub> include an initial increase in plasma PCO<sub>2</sub>, decrease in pH, followed by the retention of HCO<sub>3</sub><sup>-</sup> to bring pH levels back to normal (Baker et al. 2009; Brauner and Baker 2009; Claiborne and Evans 1992; Evans et al. 2005; Ishimatsu et al. 2004; Larsen et al. 1997; Perry and Gilmour 2006). Despite the array of studies explicating this response, few studies have examined the impacts of current regional and near-future hypercarbia on the physiology of adult marine teleosts. Exposure to 1,000 μatm CO<sub>2</sub> induces an acidosis in toadfish, causing an early decrease in blood pH and an increase in partial pressure of CO<sub>2</sub> (PCO<sub>2</sub>) (Esbaugh et al. 2012). To combat this acidosis, elevation of blood HCO<sub>3</sub><sup>-</sup> levels occurs within 4–8 h of exposure, and stable levels are reached within 24 h (Esbaugh et al. 2012). Elevation of plasma HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> may have implications for other areas of the body where acid and base equivalents are exchanged for other solutes, as seen during routine osmoregulation in the marine fish intestine. In order to stay hydrated, marine fish exposed to salinity levels above the isosmotic point (~10 ppt) must drink seawater to obtain and uptake Na<sup>+</sup> and Cl<sup>-</sup>, a process that ultimately drives water absorption. (Genz et al. 2008; Grosell et al. 2011; Grosell et al. 2007; Skadhauge 1974; Smith 1930). Interestingly, substantial Cl<sup>-</sup> absorption occurs in exchange for HCO<sub>3</sub><sup>-</sup>, linking water absorption to base

secretion (Grosell et al. 2011; Grosell and Genz 2006; Grosell et al. 2009b). Bicarbonate from the plasma is transported across the basolateral membrane via  $\text{Na}^+ : \text{HCO}_3^-$  cotransporter NBC1 or comes from intracellular  $\text{CO}_2$  as it becomes hydrated via carbonic anhydrase (Taylor et al. 2010). An examination of  $\text{HCO}_3^-$  movement across the basolateral membrane of isolated anterior toadfish intestine via NBC1, revealed that elevating serosal  $[\text{HCO}_3^-]$  resulted in increased luminal  $\text{HCO}_3^-$  secretion rates. This suggests that the intestine, in addition to the gill, may be important for setting blood  $\text{HCO}_3^-$  concentrations through transepithelial secretion (Taylor et al. 2010). During hypersalinity, increased expression of NBC1 further suggests a link between osmoregulatory machinery and acid-base regulation (Taylor et al. 2010). On this background, I hypothesized that elevation of blood  $\text{HCO}_3^-$  and  $\text{PCO}_2$  during 72-h exposure to 1,900  $\mu\text{atm CO}_2$  in toadfish would activate intestinal transport pathways, increasing intestinal base secretion rates that would act against the compensatory mechanism for  $\text{CO}_2$ -induced acidosis. Waters reaching this  $\text{CO}_2/\text{pH}$  level have already been documented in the Baltic Sea (Thomsen et al. 2010) and coastal areas in the Gulf of Mexico (Cai et al. 2011) and can be expected well before year 2300 (global projected level) in certain estuaries and coastal upwelling zones (Feely et al. 2010; Feely et al. 2008).

Furthermore, recent research has highlighted the formerly understated importance of  $\text{CaCO}_3$  formed in the intestine of marine teleost fish to the oceanic carbon cycle (Wilson et al. 2009). Bicarbonate secreted into the intestinal lumen creates a highly alkaline environment that promotes precipitation with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  to form highly soluble calcite crystallites (Perry et al. 2011; Walsh et al. 1991;



Wilson et al. 1996) and promotes increased water absorption by reducing luminal osmotic pressure (Grosell et al. 2009b; Whittamore 2012; Whittamore et al. 2010). These precipitates are predicted to dissolve at a shallower depth than calcites from many other calcifying organisms and are estimated to contribute between 3% and 15% of carbonate to the marine inorganic carbon cycle (Wilson et al. 2009) and 14%–70% in certain tropical carbonate sediments (Perry et al. 2011). A second hypothesis tested in this study was that toadfish exposed to hypercarbia would secrete more  $\text{CaCO}_3$  precipitates proportional to predicted increases in intestinal base secretion. It was also predicted that these precipitates would be more soluble due to higher  $\text{Mg}^{2+}:\text{Ca}^{2+}$  ratios, since increased precipitation rates may deplete already depressed luminal fluid  $\text{Ca}^{2+}$  levels and increase the proportion of abundant luminal  $\text{Mg}^{2+}$  in precipitates (Grosell et al. 2011). Understanding how a large taxonomic group like the teleosts will compensate or adapt to elevated  $\text{CO}_2$  and possibly alter the inorganic carbon cycle is vital.

## **Materials and Methods**

### ***Experimental Animals***

Gulf toadfish (*Opsanus beta*) were obtained from commercial shrimp fishermen trawling Biscayne Bay, Miami, from August 2010 to January 2011. Gulf toadfish are known to inhabit seagrass beds throughout Biscayne Bay and Florida Bay and experience large diurnal fluctuations in dissolved oxygen levels ( $\sim 1.3\text{--}6.1$  ppm  $\text{O}_2$ )  $\text{O}_2$  (McDonald et al. 2007);  $4.1\text{--}10.8$  ppm  $\text{O}_2$  (Serafy et al. 1997),  $\text{PCO}_2$ ,

and pH (138  $\mu\text{atm}$  and 0.13, respectively) (Yates et al. 2006). On arrival, toadfish were treated for ecto-parasites (McDonald et al. 2003) and sorted by size into 40-L tanks (8–10 per tank) with aerated, sand-filtered, flow-through seawater from Bear Cut (22°–25°C, 30–35 ppt salinity) at the Rosenstiel School of Marine and Atmospheric Science, Miami. Pieces of polyvinylchloride tubing were placed in tanks as shelter to reduce stress and decrease aggressive behavior. Toadfish were fed squid to satiation biweekly but starved for at least 72 h before experimentation to ensure an empty digestive tract. Toadfish were acclimated to laboratory tanks for at least 2 weeks before experimentation, and experimental procedures were performed according to current University of Miami animal care protocol (IACUC 10-293).

### ***Surgical Procedure and Hypercarbia Exposure Protocol***

To determine rectal base excretion rate and  $\text{CaCO}_3$  production by fish under control and elevated  $\text{CO}_2$  conditions, fish were fitted with rectal collection sacs as outlined in Genz et al. (2008). Toadfish ( $n=8$  for each treatment) were placed in 1-L aerated, flow-through acclimation chambers at ambient  $\text{CO}_2$  levels for 24 h before surgery. Similar body masses were used in control ( $65.8 \pm 2.2$  g) and 1,900  $\mu\text{atm}$   $\text{CO}_2$  ( $65.0 \pm 3.6$  g) treatments. Toadfish were then anesthetized using 0.2 g  $\text{l}^{-1}$  MS-222 solution buffered with 0.3 g  $\text{l}^{-1}$   $\text{NaHCO}_3$ , weighed, and affixed with a rectal collection sac made from a nitrile balloon attached to a 1-cm segment of a 1-mL syringe (Becton-Dickinson) that was heat flared at both ends (Genz et al. 2008). Rectal sacs were attached with a running suture stitched around the rectal

opening and pulled taut around the open end of the syringe, permitting rectal contents to drain into the collection sac while preventing seawater intrusion.

Post-surgery, toadfish recovered in experimental chambers where they remained for the next 72 h while being exposed to their respective treatment of either 380  $\mu\text{atm}$  (control) or 1,900  $\mu\text{atm}$   $\text{CO}_2$ . Seawater in both treatments was constantly aerated; however, seawater flow was interrupted during distinct time intervals in order to quantify branchial acid flux for a concurrent study (Esbaugh et al. 2012). Constant carbon dioxide concentrations were achieved using a WTW 3310 pH meter and a pH electrode (SenTix 41, Logilo Systems, Tjele, Denmark) connected to a  $\text{PCO}_2/\text{pH}$  DAQ-M digital relay instrument (Loligo) controlled by CapCTRL software (Loligo) following guidelines provided by the manufacturer. Calibrating this automated negative feedback system involved measuring pH in two known  $\text{CO}_2$  partial pressures and extrapolating a  $\text{pH}/\text{PCO}_2$  standard curve to indirectly measure  $\text{PCO}_2$  through the pH of seawater. Control conditions (380  $\mu\text{atm}$ ) displayed a pH of approximately 8.1 and the 1,900  $\mu\text{atm}$   $\text{CO}_2$  treatment resulted in a decline of  $\sim 0.6$  pH units. Once target pH/  $\text{PCO}_2$  levels were met, total  $\text{CO}_2$  measurements combined with pH measurements were found to corroborate target  $\text{PCO}_2$  levels when placed into CO2SYS software. Values of pH recorded by the stat system were compared with independent pH measurements and were consistently found to be in good agreement. A recent study using a similar setup and  $\text{CO}_2$  set point produced mean  $\text{CO}_2$  exposure values of  $1,911 \pm 13$   $\mu\text{atm}$  when monitored over 15 days. The system can oscillate up to 15% from the desired set-

point but typically stays between 4% and 10% from the 1,900  $\mu\text{atm}$  setting. The pH range associated with the deviations is between  $\sim 7.5$  and 7.6.

### ***Sampling Procedures***

After 72 hours of exposure, toadfish were euthanized by an overdose of 0.2 g l<sup>-1</sup> MS-222 buffered with 0.3 g l<sup>-1</sup> NaHCO<sub>3</sub>. The rectal collection sac was immediately clamped with a hemostat to prevent backflow of contents into the gastrointestinal tract during sampling. Fluid and precipitates contained in the collection sac were immediately transferred to pre-weighed 15-mL BD Falcon tubes. To ensure complete sampling, any precipitates inside the syringe portion of the drainage tube were considered part of the rectal collection and combined with contents of the rectal collection sac.

### ***Analytical Procedures***

Once tubes were re-weighed to determine sample mass, samples were centrifuged (4,000 rpm, 15 min; Eppendorf centrifuge 5810 R), to separate fluid and precipitates. The fluid obtained from the rectal collection sacs was immediately isolated and measured for pH (PHM201, Radiometer, Copenhagen), osmolality (Wescor Vapro vapor pressure osmometer, Logan, UT), and total CO<sub>2</sub> (Corning 965, Medfield, MA), with the remaining fluid stored at  $-20^{\circ}\text{C}$  for later analysis of ionic concentrations. Bicarbonate equivalents in rectal fluid were determined from

total CO<sub>2</sub> and pH measurements using the Henderson-Hasselbach equation as in previous studies (Genz et al. 2008).

Solid precipitate components were resuspended in 10 mL deionized water and sonicated (Kontes Micro Ultrasonic Cell Disruptor) before determining total bicarbonate/carbonate through double-endpoint titrations. Prepared samples were aerated with N<sub>2</sub> gas for 15 min before initial pH determination and continuously gassed with N<sub>2</sub> for the duration of the titration. Samples were first titrated with 0.2 mol l<sup>-1</sup> HCl to a stable pH below 3.80, maintained at this pH for 15 min under continuous gassing with N<sub>2</sub>, and returned to initial pH using 0.02 mol l<sup>-1</sup> NaOH. Acid and base were added using 2.0-mL micrometer syringes (GS-1200, Gilmont Instruments). Sample pH was monitored using Ag/AgCl combination electrodes (PHC3005-8, Radiometer Analytical) attached to a pH meter (PHM 201, Radiometer Analytical MeterLab). Bicarbonate and carbonate equivalents in precipitates were calculated by subtracting the amounts of NaOH added from the amount of HCl added to prepared samples.

Following titration, dissolved solid samples were analyzed for Mg<sup>2+</sup> and Ca<sup>2+</sup>, and fluids obtained from the rectal collection sac were analyzed for ionic composition. The amount of Mg<sup>2+</sup> and Ca<sup>2+</sup> (μmol) in the precipitate samples were used to determine the Mg<sup>2+</sup>: Ca<sup>2+</sup> ratios. Cations were analyzed using flame atomic absorption spectrometry with an air/acetylene flame, (Varian 220, Palo Alto, CA). Concentrations of Cl<sup>-</sup> were measured using a chloride titrator (CMT10 chloride titrator; Radiometer, Copenhagen).

### ***Data Analysis and Statistics***

Total rectal base excretion rate ( $\mu\text{mol kg}^{-1} \text{h}^{-1}$ ) was determined by adding fluid  $\text{HCO}_3^-$  measurements and  $\text{HCO}_3^-$  in solid precipitates determined through double endpoint titrations. Drinking rate ( $\text{mL kg}^{-1} \text{h}^{-1}$ ) was estimated by multiplying the rectal  $\text{Mg}^{2+}$  concentration by the rectal fluid excretion rate ( $\text{mL kg}^{-1} \text{h}^{-1}$ ) and dividing by  $50 \text{ mmol l}^{-1} \text{Mg}^{2+}$ , a value representative of average  $\text{Mg}^{2+}$  concentration in seawater (Genz et al. 2008). This method has been previously verified to reflect values comparable to direct isotopic drinking rate measurements (Genz et al. 2008).

All values are reported as means  $\pm$  SEM. Control ( $380 \mu\text{atm}$ ) and experimental ( $1,900 \mu\text{atm}$ ) values were compared using unpaired Student's t-tests. Two-tailed t-tests were used unless specifically stated otherwise. Data sets lacking normality were compared using Mann-Whitney rank sum tests. Significance for all tests was determined at  $P < 0.05$ .

## **Results**

### ***Rectal Base Excretion Rate***

Total rectal base excretion rate ( $\mu\text{mol kg}^{-1} \text{h}^{-1}$ ), measured from fluid and solid (precipitate) base components in a rectal collection sac over 72 hours, was significantly higher in toadfish exposed to  $1,900 \mu\text{atm CO}_2$  ( $64.2 \pm 6.6 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ) compared with toadfish exposed to ambient control  $\text{CO}_2$  levels ( $380 \mu\text{atm}$ ,  $48 \pm 5.9 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ; one-tailed Student's t-test; Fig. 2.1). The fluid base component

was the major contributor to the observed total difference and showed a significant difference during hypercarbia compared with control exposures, while the solid (precipitate) component exhibited no difference (Fig. 2.1).

### ***Rectal Base Excretion Rate and Predicted Future CO<sub>2</sub> Levels***

Using a previously reported *in vitro* relationship between serosal HCO<sub>3</sub><sup>-</sup> concentration and HCO<sub>3</sub><sup>-</sup> secretion rate in isolated toadfish intestine, expected increases in HCO<sub>3</sub><sup>-</sup> secretion rates were predicted at measured blood plasma levels corresponding to various ambient CO<sub>2</sub> levels (380, 560, 750, 1,000, 1,900 μatm) (Esbaugh et al. 2012). As plasma HCO<sub>3</sub><sup>-</sup> increased from 3.29 ± 0.17 mmol L<sup>-1</sup> (control) to 6.29 ± 0.15 mmol L<sup>-1</sup> (1,900 μatm CO<sub>2</sub>), predicted HCO<sub>3</sub><sup>-</sup> secretion rates increased from 0.56 μmol cm<sup>2</sup> h<sup>-1</sup> to 0.69 μmol cm<sup>2</sup> h<sup>-1</sup> (Fig. 2.2A). The values of HCO<sub>3</sub><sup>-</sup> secretion rate per cm<sup>2</sup> (Fig. 2.2A) at each CO<sub>2</sub> level translated to predicted increases in overall intestinal base secretion relative to control values (Fig. 2.2B). As expected, rectal base excretion increased during exposure to 1,900 μatm; however, the observed increase of 33.6% was even greater than the predicted 22.4% (Fig. 2.2B).

Fig. 2.1: Rectal base excretion rates ( $\mu\text{mol kg}^{-1} \text{h}^{-1}$ ) in toadfish fitted with rectal collection sacs exposed to control ( $380 \mu\text{at CO}_2$ ) and hypercapnia ( $1,900 \mu\text{atm CO}_2$ ) over 72 h. Total rectal base excretion is the sum of solid (precipitate) and fluid base equivalents. An asterisk indicates statistical significance from control (Student's t-test, one-tailed for total rectal base excretion rate,  $P < 0.05$ ). Values are means  $\pm$  SEM ( $n=8$ ).

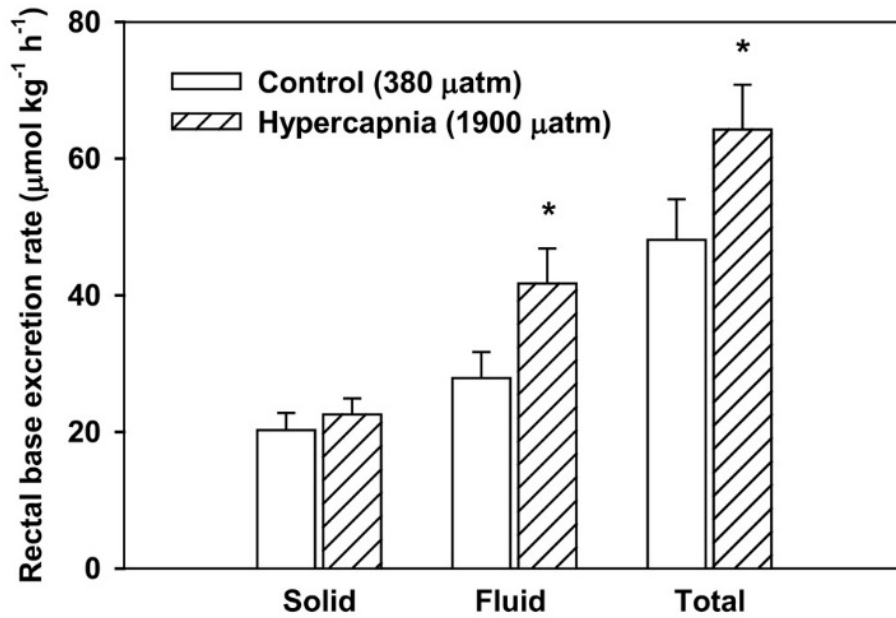
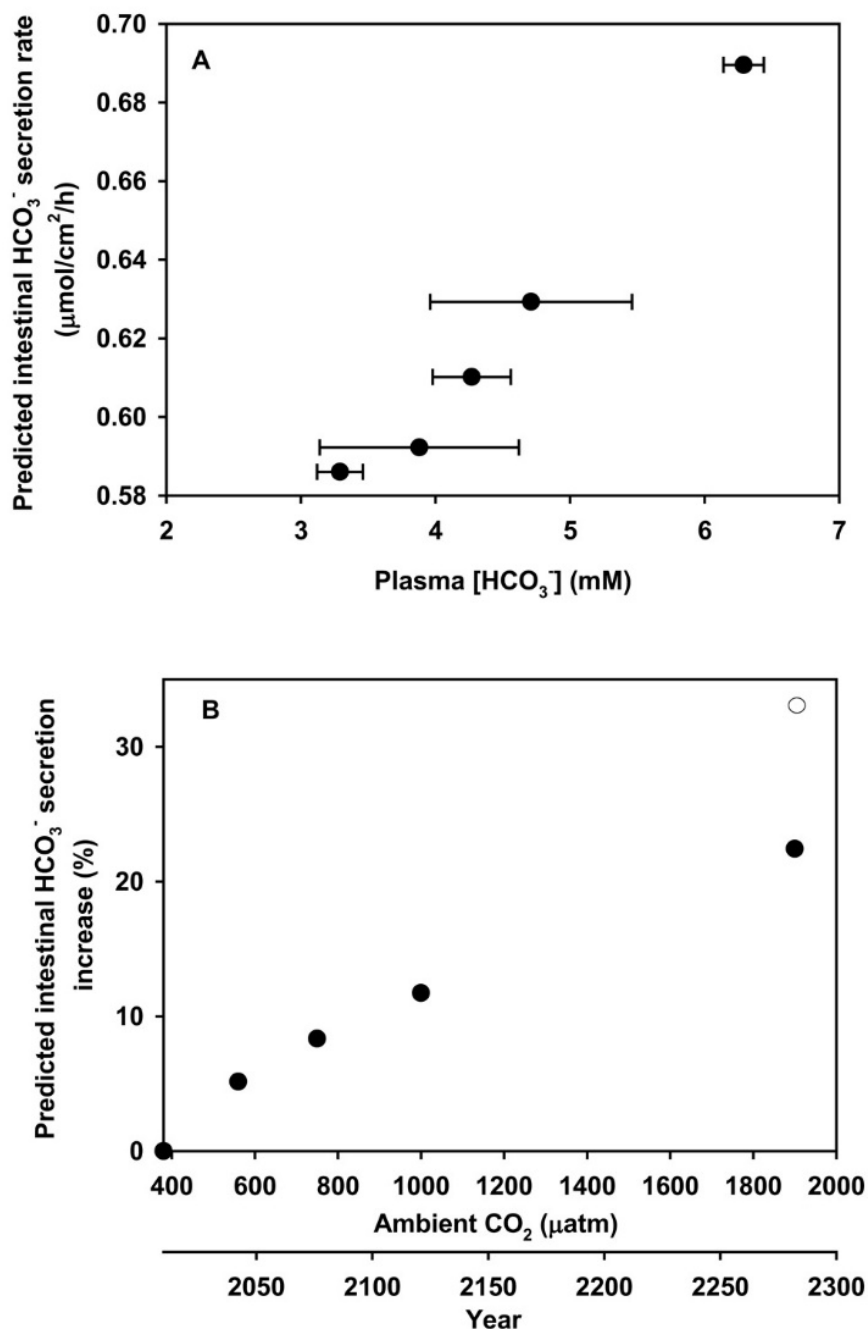




Fig. 2.2: A) Predicted intestinal  $\text{HCO}_3^-$  secretion rate ( $\mu\text{mol cm}^2 \text{h}^{-1}$ ) based on measured blood plasma  $[\text{HCO}_3^-]$  in toadfish after 24 hours of treatment exposures as reported in Esbaugh et al. (2012). Predicted  $\text{HCO}_3^-$  secretion rates were calculated using  $\text{HCO}_3^-$  secretion rates of isolated toadfish anterior intestine as a function of serosal  $\text{HCO}_3^-$  concentration (Taylor et al. 2010). Values are means  $\pm$  SEM. B) Predicted intestinal  $\text{HCO}_3^-$  secretion percent increase compared with controls ( $380 \mu\text{atm CO}_2$ ) corresponding to projected future atmospheric  $\text{CO}_2$  concentrations (Meehl et al. 2007). Open circle indicates actual percent increase in  $\text{HCO}_3^-$  excretion rate after exposure to  $1,900 \mu\text{atm CO}_2$  (33.6%).



### ***Rectal Fluid Ions and Drinking Rate***

All ion concentration measurements ( $\text{mmol L}^{-1}$ ) were taken from fluid in the rectal collection sac after 72 hours of exposure to either control ( $380 \mu\text{atm CO}_2$ ) or hypercarbia ( $1,900 \mu\text{atm CO}_2$ ) conditions. Both the concentration of  $\text{Cl}^-$  and osmolality (mOsm) were significantly lower in the rectal fluid of fish exposed to  $1,900 \mu\text{atm CO}_2$  ( $70.0 \pm 5.4 \text{ mmol L}^{-1}$ ,  $341.8 \pm 7.4 \text{ mOsm}$ ) when compared with concentrations in controls ( $99.1 \pm 6.4 \text{ mmol L}^{-1}$ ,  $373.4 \pm 8.0 \text{ mOsm}$ ; Fig. 2.3). In addition,  $\text{Na}^+$  tended to be lower in fish exposed to  $1,900 \mu\text{atm}$  ( $32.4 \pm 3.5 \text{ mmol L}^{-1}$ ) versus controls ( $42.7 \pm 5.6 \text{ mmol L}^{-1}$ ), although this difference was not statistically significant ( $P < 0.148$ , Fig. 2.3). The concentration of  $\text{HCO}_3^-$  was significantly higher in the rectal fluid of toadfish exposed to hypercarbia ( $96.5 \pm 4.5 \text{ mmol L}^{-1}$ ) compared with the concentration in controls ( $77.2 \pm 3.0 \text{ mmol L}^{-1}$ ; Fig. 2.3). Concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were not significantly different between control and hypercarbia exposures (Table 2.1). No other ions showed significant concentration differences or obvious trends (data not shown).

Both the fluid excretion rate ( $\text{mL kg}^{-1} \text{ h}^{-1}$ ) and the drinking rate ( $\text{mL kg}^{-1} \text{ h}^{-1}$ ) tended to be higher in hypercarbia-exposed toadfish; however, these differences were not statistically significant ( $P < 0.288$ ,  $P < 0.219$ , respectively; Table 2.2)

Fig. 2.3:  $\text{Cl}^-$ ,  $\text{HCO}_3^-$ ,  $\text{Na}^+$  ( $\text{mmol L}^{-1}$ ), and osmolality (mOsm) in rectal fluid in control ( $380 \mu\text{atm CO}_2$ ) and hypercarbia exposed ( $1,900 \mu\text{atm CO}_2$ ) toadfish over 72 hours. An asterisk indicates statistical significance from control at  $P < 0.05$  (Student's  $t$ -test). Values are means  $\pm$  SEM (N=8).

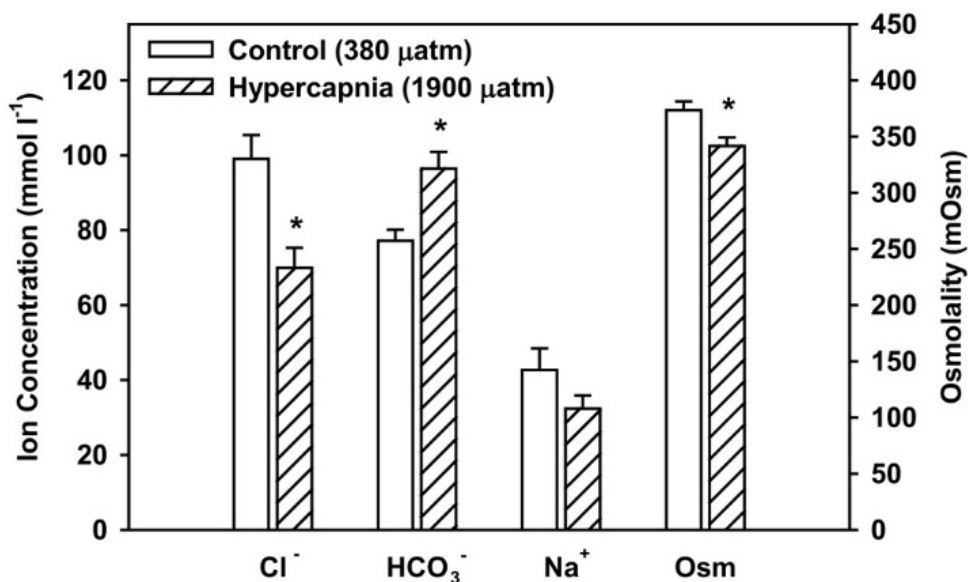


Table 2.1: Rectal fluid ion concentrations of *Opsanus beta* exposed to 72 hours of control ( $380 \mu\text{atm CO}_2$ ) and hypercarbic ( $1,900 \mu\text{atm CO}_2$ ) conditions

	$\text{Ca}^{2+}$ ( $\text{mmol L}^{-1}$ )	$\text{Mg}^{2+}$ ( $\text{mmol L}^{-1}$ )
380	$3.9 \pm 0.4$	$168.5 \pm 5.4$
1,900	$4.1 \pm 0.3$	$170.3 \pm 4.9$

Values are means  $\pm$  SEM (n=8)

Table 2.2: Rectal fluid excretion rate and estimated drinking rate of *Opsanus beta* exposed to control ( $380 \mu\text{atm CO}_2$ ) and hypercarbic ( $1,900 \mu\text{atm CO}_2$ ) conditions

	Control $380 \mu\text{atm CO}_2$	Hypercarbia $1900 \mu\text{atm CO}_2$
Rectal fluid excretion rate	$0.36 \pm 0.05$	$0.43 \pm 0.05$
Estimated drinking rate	$1.20 \pm 0.14$	$1.45 \pm 0.14$

Fluid excretion rate ( $\text{mL kg}^{-1} \text{h}^{-1}$ ) was calculated from accumulated rectal fluid over 72 hours. Drinking rates ( $\text{mL kg}^{-1} \text{h}^{-1}$ ) were estimated using the rectal fluid excretion rate ( $\text{mL kg}^{-1} \text{h}^{-1}$ ), the concentration of  $\text{Mg}^{2+}$  in rectal fluid, and the  $\text{Mg}^{2+}$  in seawater (Genz et al. 2008) Values are means  $\pm$  SEM (n=8)

***Precipitate base and ion analysis***

There was no difference in the ratio of precipitate base to the total base between fish in control and hypercarbia exposures (Fig. 2.4). Contrary to predictions,  $Mg^{2+}$  to  $Ca^{2+}$  ratios in rectal precipitates were also not significantly different, although fish exposed to 1,900  $\mu\text{atm}$   $\text{CO}_2$  tended to have slightly higher ratios (Mann-Whitney rank sum,  $P < 0.234$ , Fig. 2.4). Further evaluation comparing the relative fractions of  $Ca^{2+}$  and  $Mg^{2+}$  in rectal fluid and precipitate also showed no differences (Table 2.3). In both treatments, the fraction of  $Mg^{2+}$  present in the precipitate was low ( $0.10 \pm 0.01$ ) compared with that of the rectal fluid ( $0.90 \pm 0.01$ ; Table 2.3). This trend was opposite with respect to  $Ca^{2+}$ , where the precipitate contained the majority of  $Ca^{2+}$  in the control and hypercarbia exposures ( $0.81 \pm 0.02$  and  $0.84 \pm 0.02$ , respectively), and only a small proportion of the  $Ca^{2+}$  was present in the rectal fluid ( $0.16 \pm 0.02$  and  $0.19 \pm 0.02$ , respectively; Table 2.3).

Fig. 2.4: Fraction of base in solid precipitate compared to total base in fluid and precipitate (Student's t-test,  $P < 0.288$ ). Ratio of  $\mu\text{mol Mg}^{2+}$  to  $\mu\text{mol Ca}^{2+}$  in rectal precipitate in control ( $380 \mu\text{atm CO}_2$ ) and hypercarbia exposed ( $1,900 \mu\text{atm CO}_2$ ) toadfish over 72 hours (Mann-Whitney rank sum test,  $P < 0.219$ ). Values are means  $\pm$  SEM ( $n=8$ ).

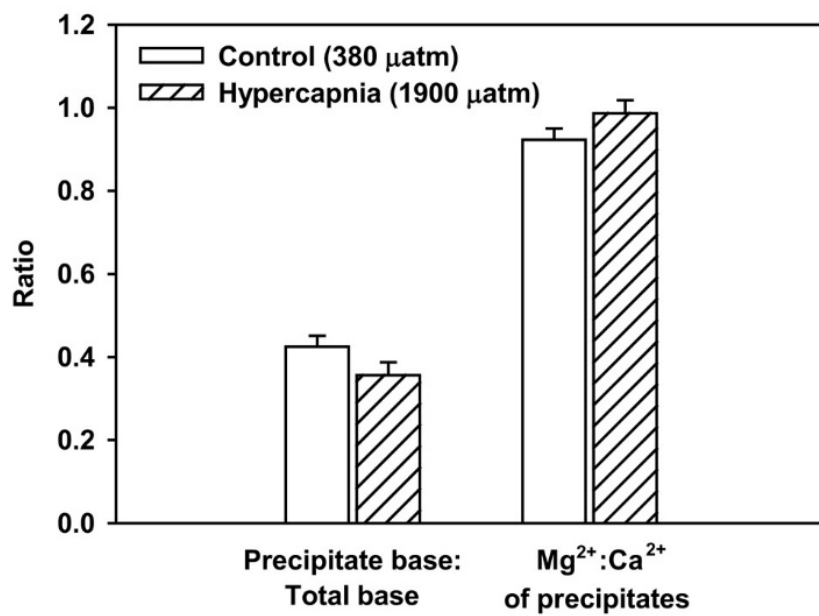


Table 2.3: Fraction of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in rectal fluids and rectal precipitates in *Opsanus beta* exposed to control ( $380 \mu\text{atm CO}_2$ ) and hypercarbic ( $1,900 \mu\text{atm CO}_2$ ) conditions over 72 hours.

	$\text{Ca}^{2+}$		$\text{Mg}^{2+}$	
	Control	Hypercarbia	Control	Hypercarbia
Fraction in fluid	$0.16 \pm 0.02$	$0.19 \pm 0.02$	$0.90 \pm 0.01$	$0.90 \pm 0.01$
Fraction in pellet	$0.84 \pm 0.02$	$0.81 \pm 0.02$	$0.10 \pm 0.01$	$0.10 \pm 0.01$

Values are means  $\pm$  SEM ( $n=8$ )

## Conclusions

### ***Rectal Base Excretion Rates***

Significantly increased rectal base excretion in toadfish exposed to 1,900  $\mu\text{atm}$   $\text{CO}_2$  compared with control exposures (Fig. 2.1) over 72 hours suggests that the intestinal tract is influenced by a compensated respiratory acidosis induced by small increases in  $\text{CO}_2$ . The majority of literature on the physiological response of fish to hypercarbia has utilized high levels of  $\text{CO}_2$  (7–16 mmHg or  $\sim 9,200$ – $21,000$   $\mu\text{atm}$ ) (Brauner and Baker 2009) to elucidate compensation mechanisms and upper tolerance limits. While this response has been well characterized, few studies have used lower levels of exposure relevant to current regional conditions and imminent ocean acidification predictions, since adult fish are known to be especially strong acid-base regulators. Two major findings prompted the investigation of the role of the intestinal tract in acid-base balance during low-level hypercarbia. First, hypercarbia exposure has been found to induce blood elevation of  $\text{HCO}_3^-$  and  $\text{PCO}_2$  in response to  $\text{CO}_2$  levels as low as 1,000  $\mu\text{atm}$  (Esbaugh et al. 2012). Second, serosal elevation of  $\text{HCO}_3^-$  has been shown to stimulate basolateral NBC1 and increase transepithelial  $\text{HCO}_3^-$  transport in isolated intestinal tissue (Taylor et al. 2010), suggesting a similar response *in vivo*. In combination, these results suggest that low-level hypercarbia may affect gastrointestinal  $\text{HCO}_3^-$  secretion.

Exposure to elevated  $\text{CO}_2$  promoted increased intestinal  $\text{HCO}_3^-$  secretion, presumably due to the increased serosal  $\text{HCO}_3^-$  concentrations that have been previously demonstrated (Esbaugh et al. 2012). However, this base loss is

counterproductive during a CO<sub>2</sub>-induced respiratory acidosis since the retention of HCO<sub>3</sub><sup>-</sup> is necessary to defend blood pH. Elevated intestinal base loss during compensation for low-level hypercarbia likely necessitates an increased demand on branchial adjustments, either through acid extrusion or HCO<sub>3</sub><sup>-</sup> retention. For toadfish, exposure to nominally HCO<sub>3</sub><sup>-</sup>-free water (182 μM) prevented pH compensation at 1,900 μatm CO<sub>2</sub> (Esbaugh et al. 2012), suggesting branchial uptake of HCO<sub>3</sub><sup>-</sup> is the dominant mechanism employed to combat the respiratory acidosis. Whether or not increased branchial adjustment in response to intestinal base loss would come at an increased metabolic cost to the animal remains to be investigated.

When examined *in vitro*, base secretion rates of toadfish intestine (anterior) increased in a dose-dependent manner depending on serosal HCO<sub>3</sub><sup>-</sup> concentrations (Taylor et al. 2010). Rectal base excretion rates have been previously estimated to be as high as 135 μmol kg<sup>-1</sup> h<sup>-1</sup> in fish exposed to 5% CO<sub>2</sub> (50,000 μatm CO<sub>2</sub>) (Perry et al. 2010). Together these studies support a dose-dependent, although not linear, response. Thus, although intestinal base secretion was only measured at 1,900 μatm CO<sub>2</sub> in this study, it is likely that base secretion values would mirror the dose-response pattern observed in blood PCO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> (Esbaugh et al. 2012) between 1,000 and 1,900 μatm CO<sub>2</sub>. Using the previously reported relationships between HCO<sub>3</sub><sup>-</sup> secretion rates of isolated toadfish anterior intestine (μmol cm<sup>-2</sup> h<sup>-1</sup>) and serosal HCO<sub>3</sub><sup>-</sup> concentrations (Taylor et al. 2010), along with measured blood plasma HCO<sub>3</sub><sup>-</sup> levels at 1,900 μatm CO<sub>2</sub> (Esbaugh et al. 2012), I predicted a 22.5% increase in intestinal HCO<sub>3</sub><sup>-</sup> secretion as a result of

1,900  $\mu\text{atm CO}_2$  exposure (Fig. 2.2). It is important to note that a percent increase value was used because isolated tissue values were reported in  $\mu\text{mol cm}^{-2} \text{h}^{-1}$ , while  $\mu\text{mol kg}^{-1} \text{h}^{-1}$  was measured in this *in vivo* study. Interestingly, the observed actual increase in rectal base excretion was 33.6% (Fig. 2.2), around 1.5 times higher than predicted. The relationship between serosal  $[\text{HCO}_3^-]$  and intestinal secretion rates was determined in isolated anterior toadfish intestine (Taylor et al. 2010), and it cannot be excluded that more distal segments contributed transepithelial  $\text{HCO}_3^-$  secretion to yield the higher than predicted rectal base secretion rate observed in this study. In addition, integrative, whole animal processes such as neuronal and/or hormonal interactions may have also contributed to higher than predicted  $\text{HCO}_3^-$  secretion rates.

In addition to transepithelial  $\text{HCO}_3^-$  secretion via NBC1, carbonic anhydrase-mediated endogenous  $\text{CO}_2$  hydration also accounts for  $\text{HCO}_3^-$  secreted into the lumen. The share of these two sources of  $\text{HCO}_3^-$  varies among species (Grosell 2006; Grosell et al. 2005; Trischitta et al. 2011). It is therefore possible that elevated  $\text{CO}_2$  hydration also contributed to the higher than expected intestinal base secretion as elevated plasma  $\text{CO}_2$  observed during hypercarbia may result in elevated cytosolic  $\text{CO}_2$  in the intestinal epithelial cells. Future studies should attempt to differentiate endogenous and transepithelial sources of  $\text{HCO}_3^-$  in rectal fluids during hypercarbia exposure.



### ***Cascade Effects on Intestinal Transport***

Chloride concentrations were found to be significantly lower in the rectal fluid of fish exposed to 1,900  $\mu\text{atm CO}_2$  compared with those in controls (Fig. 2.3). This result, in combination with elevated rectal  $\text{HCO}_3^-$  concentrations with 1,900  $\mu\text{atm CO}_2$  exposure versus those of controls likely reflects elevated anion exchange activity via SLC26a6 in the apical membrane following increased activity of basolateral NBC1 by elevated serosal  $\text{HCO}_3^-$  concentration (Fig. 2.5). Interestingly, low luminal  $\text{Cl}^-$  concentration tends to favor continued  $\text{Cl}^-$  transport via SLC26a6 over apical cotransporter NKCC2, which is utilized more for chloride transport during high  $\text{Cl}^-$  concentrations when  $\text{HCO}_3^-$  secretion is inhibited (Grosell 2011a; Taylor et al. 2010).

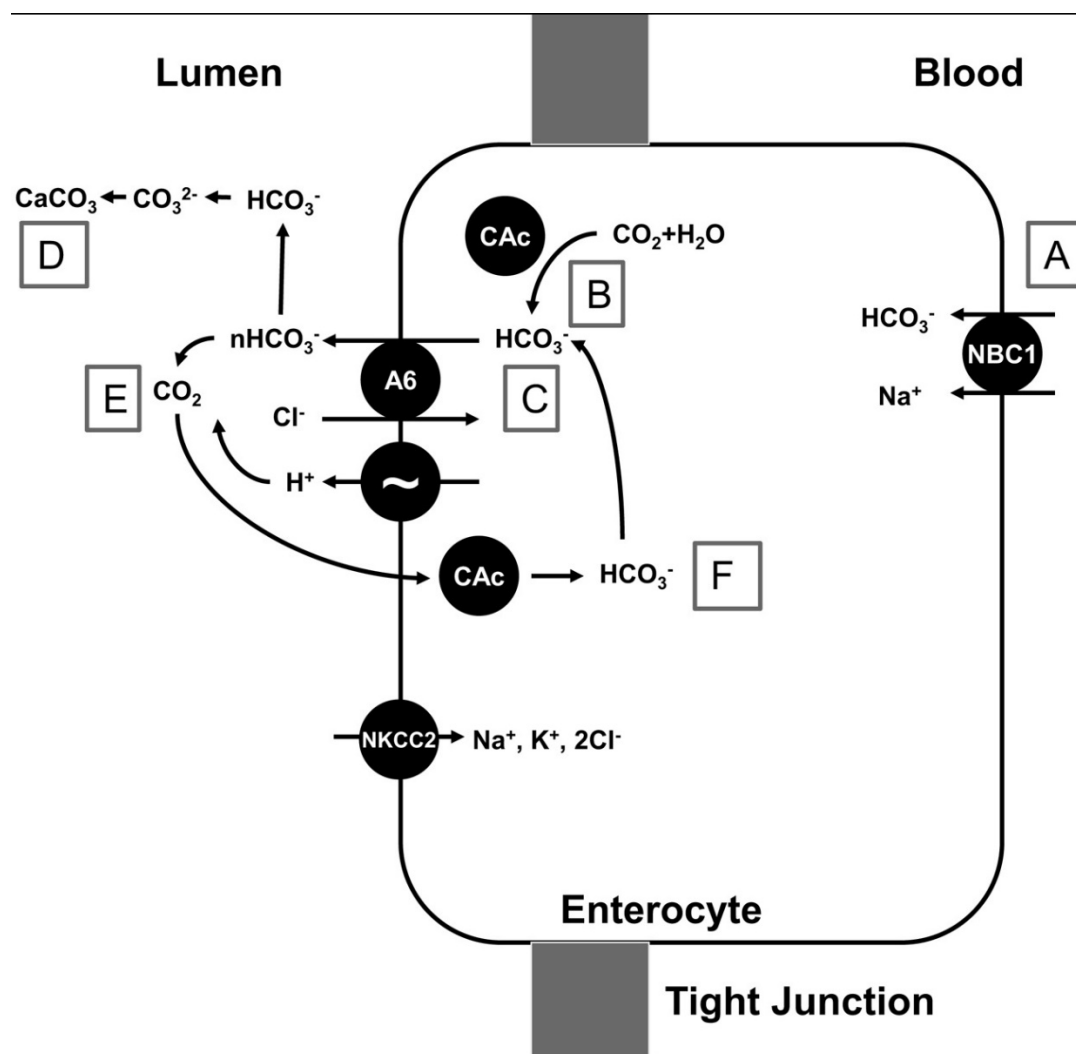
Low luminal  $\text{Cl}^-$  concentrations following stimulated anion exchange in toadfish exposed to 1,900  $\mu\text{atm CO}_2$  were predicted to increase drinking since luminal  $\text{Cl}^-$  levels control drinking rate in eel (Ando and Nagashima 1996). However, while the rectal fluid excretion rate and the estimated drinking rate ( $\text{mL kg}^{-1} \text{h}^{-1}$ ) tended to be higher in toadfish exposed to 1,900  $\mu\text{atm CO}_2$ , these differences were not significant (Table 2.2). To further investigate fluid movement through the intestine, the total fluid absorption rate was calculated by taking the difference between the estimated drinking rate and the rectal fluid excretion rate (Genz et al. 2008). This rate was calculated to be  $0.84 \text{ mL kg}^{-1} \text{h}^{-1}$  for controls and  $1.02 \text{ mL kg}^{-1} \text{h}^{-1}$  for toadfish exposed to 1,900  $\mu\text{atm CO}_2$ . Despite the trend for an increase in estimated drinking rate and fluid absorption, the fractional fluid absorption was relatively similar at 70.0% and 70.3%, for control and for toadfish

exposed to 1,900  $\mu\text{atm CO}_2$ . Thus, it was proposed that although fractional fluid absorption did not change with hypercarbia, blood  $\text{HCO}_3^-$  levels elevated by hypercarbia initiated a series of events that activated osmoregulatory machinery for a non-osmoregulatory process, leading to increased intestinal anion exchange,  $\text{Cl}^-$  uptake, decreased luminal  $\text{Cl}^-$  concentration, and significantly lower osmolality in the lumen of hypercarbic fish.

### ***Intestinal Precipitate Formation***

Contrary to expectations, no difference was observed in solid precipitates produced in control compared with toadfish exposed to 1,900  $\mu\text{atm CO}_2$  (Fig. 2.1). Differences in precipitate titratable alkalinity have been noted at 5%  $\text{CO}_2$  exposure (50,000  $\mu\text{atm CO}_2$ ) (Perry et al. 2010), but since elevated precipitate formation was not observed in this study, the threshold for observing this effect remains to be determined. A recent study of fish carbonate production across the Bahamian Archipelago noted large variation in production rates that varied with body mass, with smaller fish typically producing more carbonates per unit mass (Perry et al. 2011). Although this study did not find evidence for elevated  $\text{CaCO}_3$  formation during exposure to elevated  $\text{CO}_2$ , possible effects of fish size and life history should be investigated.

Fig. 2.5: Proposed effects of 1,900  $\mu\text{atm}$   $\text{CO}_2$  exposure in the intestinal epithelia of Gulf toadfish (*Opsanus beta*). A) Elevated serosal  $\text{HCO}_3^-$  concentration stimulates NBC1 in the basolateral membrane, increasing cytosolic  $\text{HCO}_3^-$ . B) Increased serosal  $\text{PCO}_2$  reduces the concentration gradient between serosal and cytosolic  $\text{CO}_2$ , leading to reduced  $\text{CO}_2$  diffusion and increased cytosolic  $\text{CO}_2$  concentration. This elevated  $\text{CO}_2$  is hydrated via carbonic anhydrase, also contributing to increased availability of cytosolic  $\text{HCO}_3^-$ . C) Increased  $\text{HCO}_3^-$  from (A) and (B) leads to increased SLC26a6 anion exchange activity, increasing luminal  $[\text{HCO}_3^-]$  and decreasing  $[\text{Cl}^-]$ . Low luminal  $[\text{Cl}^-]$  may stimulate drinking and favors continued transport via SLC26a6. D)  $\text{CO}_3^{2-}/\text{HCO}_3^-$  combine with  $\text{Ca}^{2+}$  to form  $\text{CaCO}_3$  precipitates, lowering osmolality, although not at elevated rates with hypercarbia exposure. E) A fraction of elevated luminal  $\text{HCO}_3^-$  combines with  $\text{H}^+$  from the apical  $\text{H}^+$  pump (Grosell et al. 2009b; Guffey et al. 2011), generating  $\text{CO}_2$  that will equilibrate with the enterocytes. F) Endogenous  $\text{CO}_2$  is converted to  $\text{HCO}_3^-$  via carbonic anhydrase. This additional  $\text{HCO}_3^-$  is available for further anion exchange via SLC26a6.



There was also no difference in the amount of base present in the solid precipitate compared to the total combined base in fluid and precipitate from fish in control and hypercarbia exposures (Fig. 2.4). This result was unexpected considering that an increase in total rectal  $\text{HCO}_3^-$  excretion would be predicted to provide more substrate for  $\text{CaCO}_3$  formation and, thus, increased amount of base excretion in the pellet form. It was hypothesized that the predicted increase in carbonate substrate would deplete already depressed levels of  $\text{Ca}^{2+}$  normally present in the intestinal lumen and shift  $\text{CaCO}_3$  composition in favor of higher  $\text{Mg}^{2+}$  content since this ion is abundant in the intestinal fluid and is also a component of fish-produced  $\text{CaCO}_3$  (Wilson et al. 2009). However, while the  $\text{Mg}^{2+}:\text{Ca}^{2+}$  ratio in fish exposed to 1,900  $\mu\text{atm CO}_2$  tended to be slightly higher than the ratio in control exposures, this difference was not significant (Fig. 2.4).

In both treatments,  $\text{Mg}^{2+}$  levels in precipitates were low compared with levels in rectal fluid (Table 2.3). Conversely, the majority of  $\text{Ca}^{2+}$  was present in the precipitate in control and hypercarbia exposures, and only a small proportion of the  $\text{Ca}^{2+}$  was present in the rectal fluid (Table 2.3). A recent study has classified “high” Mg calcites as greater than 4 mole%  $\text{MgCO}_3$  and stated an average range for fish between 18% and 39% mole%  $\text{MgCO}_3$  (Perry et al. 2011), values exceeded in this study at  $47.9\% \pm 0.7\%$   $\text{Mg}^{2+}$  content for control fish (data not shown). In contrast to the procedures applied by Perry et al., precipitates were not rinsed in this study before titrations. This procedural difference may account for the very high values for  $\text{Mg}^{2+}$  content observed in this study, but regardless, it is clear that fish-produced  $\text{CaCO}_3$  is  $\text{Mg}^{2+}$  rich. These observations verify the unique chemical

nature of fish precipitates and may suggest that an upper level of  $Mg^{2+}$  in fish-produced precipitates exists regardless of  $HCO_3^-$  secretion rates and luminal  $Ca^{2+}$  concentrations.

### ***Potential Caveats***

Despite their utility, rectal collection sacs provide a composite of secretions over 72 hours and cannot be used to examine the time course of an acid-base disturbance. We surmise that intestinal base secretion rates likely stabilized after around 4 hours of exposure to 1,900  $\mu\text{atm}$   $CO_2$ , since intestinal conditions would be expected to mirror plasma conditions (Esbaugh et al. 2012; Taylor et al. 2010). However, it is unclear how much later intestinal fluids would depict these changes in intestinal transport function as a volume of such fluids is constantly residing in the lumen. In any case, it is clear that the content of the rectal collection sacs is the sum of fluids residing in the intestine before experimentation and the altered transport rates of the epithelium during the experiment. However, the error resulting from this fact is conservative, as it would act to reduce the difference between the control group and the group exposed to 1,900  $\mu\text{atm}$ . There are two alternatives to the technique used in this study; *in situ* perfusion of the intestine and collection of excreted precipitates from the tanks. Unfortunately, Gulf toadfish do not tolerate the surgery involved in the *in situ* perfusion procedures (M. Grosell, personal observation). Collection of precipitates from the tanks could be done to avoid influence of intestinal fluids residing in the intestine before exposure if a “pre-exposure” period was used. However, this technique would not allow for

quantification of rectal fluid and  $\text{HCO}_3^-$  excretion rates and would not allow for determination of chemical composition of excreted rectal fluids.

### ***Conclusions and Future Directions***

Elevated rectal base excretion rates by toadfish exposed to 1,900  $\mu\text{atm CO}_2$  (~year 2300 and relevant to certain regions at present) over 72 hours suggests that the gastrointestinal tract is influenced by changes in blood chemistry induced during a hypercarbic respiratory acidosis. We propose that blood elevation of  $\text{HCO}_3^-$  and  $\text{PCO}_2$  activated apical and basolateral  $\text{HCO}_3^-$  ion transporters in epithelial cells and increased intestinal  $\text{HCO}_3^-$  secretion rates that work against bicarbonate retention mechanisms needed during metabolic compensation.

Increased base secretion, combined with significantly lower  $\text{Cl}^-$  concentration and luminal osmolality highlights a need for comprehensive investigations into transporters previously identified both in osmoregulation and high-level hypercarbia exposure. In order to elucidate adaptive capacity, we are currently examining intestinal tissue isolated from fish exposed long term to 1,900  $\mu\text{atm CO}_2$  levels to determine whether downregulation of intestinal  $\text{HCO}_3^-$  secretion occurs to retain blood  $\text{HCO}_3^-$  and minimize base loss. Previous *in vitro* isolated intestinal tissue examination of Plainfin midshipman at high levels of  $\text{CO}_2$  (5%; 50,000  $\mu\text{atm}$ ) indicated no downregulation to minimize base loss; however, exposures were for 48 hours (Perry et al. 2010) and possibly too short to reveal acclimation responses.

Understanding the physiological changes induced by elevated CO<sub>2</sub> levels in fish is imperative, especially since regional areas are already experiencing CO<sub>2</sub> levels between 1,000 and 2,300  $\mu$ atm (Fabry et al. 2008; Thomsen et al. 2010). Furthermore, it is worth noting that fish with larger body mass (Portner and Farrell 2008) and/or fish living in areas with little environmental fluctuation (Portner et al. 2004) may have greater difficulty adjusting to elevated CO<sub>2</sub>-induced changes in the environment.

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### **Chapter 3: Assessing the regulatory capacity and energetic costs associated with CO<sub>2</sub> compensation in the intestine**

#### **Summary**

Energetic costs associated with ion and acid-base regulation in response to ocean acidification have been predicted to decrease the energy available to marine fish for basic life processes (Ishimatsu et al. 2008). However, the estimated low cost of ion regulation (6-15% of standard metabolic rate) (Ishimatsu et al. 2008) and inherent variation associated with whole-animal metabolic rate measurements (Roche 2013) have made it difficult to consistently demonstrate a cost (Clark 2013; Heuer and Grosell 2014). In this chapter, I aimed to gain resolution in assessing the energetic demand associated with acid-base regulation by examining ion movement and O<sub>2</sub> consumption rates of isolated intestinal tissue from marine Gulf toadfish acclimated to control or near-future CO<sub>2</sub> levels (1900  $\mu$ atm CO<sub>2</sub>; year 2300). This highly active tissue readily exchanges ions with the environment to maintain water balance. CO<sub>2</sub> exposure caused a 13% increase of intestinal HCO<sub>3</sub><sup>-</sup> secretion that was not dynamically regulated by the animal. Isolated tissue from CO<sub>2</sub>-exposed toadfish also exhibited an 8% higher O<sub>2</sub> consumption rate than tissue from control fish. The present findings show that compensation for ocean acidification leads to a maladaptive persistent base loss that incurs an energetic expense at the tissue level. Sustained increases to baseline metabolic rate could lead to energetic reallocations away from other life processes at the whole-animal level.



## Background

Although fish respond effectively and restore blood pH during CO<sub>2</sub> induced acid-base balance disturbance, recent studies have noted potential impacts of ocean acidification across a range of areas including neurosensory disruptions, altered mitochondrial function, and changes to metabolic rate (Heuer and Grosell 2014; Munday et al. 2012a; Nilsson et al. 2012; Rummer 2013; Strobel et al. 2012). A suggested unifying hypothesis is that the compensatory response induced during CO<sub>2</sub> exposure to correct blood pH may have maladaptive downstream consequences or induce tradeoffs (Heuer and Grosell 2014; Nilsson et al. 2012; Portner et al. 2004). This pH compensation is associated with a sustained increase of extra and intracellular concentration of HCO<sub>3</sub><sup>-</sup> in response to the elevated partial pressure of CO<sub>2</sub> (PCO<sub>2</sub>) (Esbaugh et al. 2012; Heuer and Grosell 2014). Assuming the rate of adaptation does not keep pace with a rapidly acidifying ocean, fish in future oceans will require consistent and elevated levels of ion exchange to sustain elevated HCO<sub>3</sub><sup>-</sup> and normal pH, a process that is anticipated to add to the cost of basic maintenance of homeostasis, most often quantified as standard metabolic rate (the minimum O<sub>2</sub> consumption rate of a resting animal in the post-absorptive state) (Brett and Groves 1979).

The gill, intestine, and kidney are the organs involved with acid-base balance and osmoregulation in marine fish (Perry and Gilmour 2006). Estimates vary widely, but metabolic cost of ion regulation has been proposed to range from 6-15% of whole-animal standard metabolic rate (Ishimatsu et al. 2008). More targeted estimates of ion transport using specific isolated organs suggest that the

gills and intestine account for ~4% (Morgan and Iwama 1999) and 5.6% (Taylor and Grosell 2009) of standard metabolic rate, respectively, which combined would account for about 10% of whole-animal standard metabolic rate. Interestingly, despite the need for  $\text{HCO}_3^-$  retention during a  $\text{CO}_2$ -induced acidosis, toadfish experience ~34% increase in  $\text{HCO}_3^-$  ion loss through the intestine when exposed to near-future  $\text{CO}_2$  scenarios (1900  $\mu\text{atm CO}_2$ ) (Heuer et al. 2012). Although the intestine is a highly metabolically active tissue central to water balance and survival in seawater, the functional consequence of this relatively high  $\text{HCO}_3^-$  loss during  $\text{CO}_2$  acclimation has not been examined. Elevated  $\text{HCO}_3^-$  secretion may also stimulate an increase in fish  $\text{CaCO}_3$  production and release to the marine environment, potentially altering the marine carbon cycle (Wilson et al. 2009). Since teleosts are the largest vertebrate group with a vital role in oceanic food webs, it is important to understand how impacts to the intestine during ocean acidification could affect both the fish and its surrounding environment. The first goal of the present study was to determine if the intestine of a marine teleost, an organ known to show plasticity in other ion regulatory challenges (Genz et al. 2008), would dynamically regulate intestinal function to reduce  $\text{CO}_2$ -induced intestinal  $\text{HCO}_3^-$  loss in favor of whole-body  $\text{HCO}_3^-$  retention following exposure to 1900  $\mu\text{atm CO}_2$ . If dynamic regulation was occurring in tissue from fish exposed to  $\text{CO}_2$  to reduce  $\text{HCO}_3^-$  loss, lower  $\text{HCO}_3^-$  secretion rates would be expected in tissue from  $\text{CO}_2$  acclimated fish. A second goal was to determine if the increase in intestinal ion transport that occurs in response to 1900  $\mu\text{atm CO}_2$  exposure would be associated with an increased tissue metabolic demand.

## **Materials and Methods**

### ***Animal collection and care***

Gulf toadfish (*Opsanus beta*) were obtained from local shrimpers as by-catch in Biscayne Bay and acclimated to flow-through, aerated, sand-filtered seawater from Bear Cut, FL (22-25°C, 32-35 p.p.t) in the laboratory at the University of Miami for at least two weeks prior to experimentation. During this period, fish were fed squid twice weekly. Once introduced to experimental tanks, toadfish ( $\text{HCO}_3^-$  secretion mass range: 21.7-30.9 g,  $\text{O}_2$  consumption mass range: 28.1-48.6 g) were fed 5% of their body weight weekly. Food was withheld at least 4 days prior to experimentation, a time period previously demonstrated to ensure that confounding effects of specific dynamic action (SDA) would not be factor for  $\text{HCO}_3^-$  secretion or oxygen consumption measurements (Taylor and Grosell 2009). Fish were sacrificed using a lethal dose of 0.2 g/L MS-222 buffered with 0.3 g/L  $\text{NaHCO}_3$  in accordance with University of Miami animal care protocols (IACUC 13-225).

### ***General experimental procedures***

Toadfish were acclimated to either control or high  $\text{CO}_2$  (~440 and ~1900  $\mu\text{atm}$   $\text{CO}_2$ , respectively; see “seawater acclimation” below) for 2-4 weeks, a time period previously deemed sufficient to elicit a  $\text{CO}_2$  compensatory response in the toadfish (Esbaugh et al. 2012). Following acclimation, anterior intestinal tissue was dissected and immediately placed between two half-chambers where the tissue

was bathed on either side by salines representative of *in vivo* conditions. Gut lumen-side (mucosal) salines were identical throughout all treatments, however, two different blood-side (serosal) salines (“control” or “CO<sub>2</sub>”) were used to mimic measured values at control or 1900  $\mu$ atm CO<sub>2</sub> (Table 3.1) (Esbaugh et al. 2012). These Ussing chambers were combined with pH-stat titration to allow for measurement of HCO<sub>3</sub><sup>-</sup> secretion rates from the blood-side to the gut-side saline (Grosell and Genz 2006). In addition, the O<sub>2</sub> consumption rate of isolated intestinal segments were measured using a custom-built epithelial respirometer (Taylor and Grosell 2009). Each mounted tissue was treated with both the control and the CO<sub>2</sub> serosal saline, allowing for examination of the effect of prior CO<sub>2</sub> acclimation along with the effect of blood-side saline conditions that mimicked *in vivo* measured blood chemistry. Isolated setups are described in further detail below.

### ***Data analysis***

Data from the Ussing chamber/pH stat setup and the intestinal tissue respirometry setup were analyzed using a two-way ANOVA (acclimation and saline as factors), followed by Holm-Sidak tests for multiple comparisons when appropriate. Significance was determined at P<0.05 for all tests and all values are presented as means  $\pm$  s.e.m.

Table 3.1: Serosal saline composition used in isolated tissue experiments for both Ussing/pH stat and oxygen consumption measurements

Constituent	Serosal		Mucosal
	Control serosal	CO <sub>2</sub> serosal	
NaCl (mmol <sup>-1</sup> )	151	151	69
KCl (mmol <sup>-1</sup> )	3	3	5
MgSO <sub>4</sub> (mmol <sup>-1</sup> )	0.88	0.88	77.5
MgCl <sub>2</sub> (mmol <sup>-1</sup> )	-	-	22.5
Na <sub>2</sub> HPO <sub>4</sub> (mmol <sup>-1</sup> )	0.5	0.5	-
KH <sub>2</sub> PO <sub>4</sub> (mmol <sup>-1</sup> )	0.5	0.5	-
CaCl <sub>2</sub>	1	1	5
Hepes, free acid	3	3	-
Hepes, sodium salt	3	3	-
Urea	4.5	4.5	-
Glucose	5	5	-
Osmolality (mOsm l <sup>-1</sup> )	320**	320**	320**
pH	7.8†	7.8†	7.8
Gas††	0.225% CO <sub>2</sub> in O <sub>2</sub> *	0.462% CO <sub>2</sub> in O <sub>2</sub> *	O <sub>2</sub>
HCO <sub>3</sub> <sup>-</sup> (mmol <sup>-1</sup> )	3.30*	6.33*	-

\*Values designed to mimic measured or calculated PCO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> values in Gulf toadfish exposed to control or ~1900 μatm CO<sub>2</sub> (Esbaugh et al. 2012)

\*\*Adjusted with mannitol to ensure transepithelial isomostic conditions in all experiments.

†pH 7.8 was maintained by pH-stat titration

††Salines gassed for at least one hour prior to experimentation. All salines in O<sub>2</sub> consumption setup were gassed with air.

### ***In vitro Ussing chamber/pH stat electrophysiological and bicarbonate secretion measurements***

Simultaneous measurement of electrophysiological parameters and bicarbonate secretion of isolated tissue was achieved using Ussing chamber systems (Physiological Instruments, San Diego, CA, USA) combined with automated pH-stat titration (TIM854/856 Titralab and Titramaster software v.5.1.0, Radiometer, Copenhagen, Denmark) (Grosell and Genz 2006). Following acclimation as described above, anterior intestine segments were mounted on

tissue holders designed to expose 0.71 cm<sup>2</sup> of tissue to two half-chambers (1.6 mL) in the Ussing chamber system (P2400, Physiological Instruments). In this setup, isolated tissue was bathed in pre-gassed serosal or mucosal salines continuously mixed by air-lift gassing and held at 25°C using a recirculating water bath. Mucosal saline composition remained unaltered throughout all experiments (Table 3.1) and was gassed with 100% O<sub>2</sub>. “Control” and “CO<sub>2</sub>” serosal salines were designed to mimic *in vivo* HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> levels previously measured in the plasma of toadfish during exposure to control and 1900 μatm CO<sub>2</sub> (3.3 and 6.3 mM HCO<sub>3</sub><sup>-</sup>, 0.225 and 0.462% CO<sub>2</sub> (Esbaugh et al. 2012), respectively (Table 3.1).

A pH electrode (PHC4000-8, Radiometer, Denmark) and an acid-dispensing microburette were submerged into the mucosal half-chamber. The rate acid titrant addition and the titrant concentration (0.005 N HCl) needed to hold the mucosal saline at a constant pH of 7.8 were used to calculate the epithelial HCO<sub>3</sub><sup>-</sup> secretion rate. Electrophysiological measurements were taken simultaneously with bicarbonate secretion rates (described below). Once tissue preparations were considered stable based upon steady bicarbonate secretion and electrophysiological parameters, a minimum 30-minute measurement interval was recorded prior to a saline change. Each isolated intestinal tissue received both control and CO<sub>2</sub> serosal saline treatments. Although tissues have been demonstrated to be viable for at least 5 hours in prior studies using this species and setup (Grosell and Genz 2006), the order of serosal salines applied were altered. During a saline change, the first serosal saline was carefully removed with a syringe, the half chamber was rinsed, and replaced by the second serosal saline

treatment. Measurements post-saline change were continued until stable levels were recorded for a minimum of 30 minutes.

### ***In vitro oxygen consumption measurements***

Following the same acclimation procedures outlined for bicarbonate secretion experiments, the oxygen consumption rate of isolated anterior intestine was measured using a custom-designed epithelial respirometer (Loligo Systems, Tjele, Denmark). Intestinal tissue was mounted so that 0.87 cm<sup>2</sup> of tissue was exposed to two half-chambers (2.80 mL) with mucosal saline on the gut side and one of two serosal saline treatments (described above) on the blood-side. All salines were pre-gassed with air (100% O<sub>2</sub> saturation) rather than custom O<sub>2</sub> mixes to make measurements of oxygen consumption rates comparable to whole-animal measurements. Saline HCO<sub>3</sub><sup>-</sup> concentrations were kept the same in the control and CO<sub>2</sub> serosal salines (Table 3.1).

Salines in half chambers were continuously mixed by micromagnetic glass-coated Teflon stir bars (Loligo Systems), and a teflon tissue mount ensured that the system was gas-tight (Taylor and Grosell 2009). Oxygen measurements were conducted using a fiber-optic cable secured to the outside wall of either glass half-chamber that illuminated a fiber-optic sensor spot glued to the inside wall of each respective side. Each cable was connected to a separate single-channel oxygen meter (Fibox 3) used in conjunction with Oxy-View software (PST3-V6.02; PreSens, Regensburg, Germany). Prior to daily experiments, calibrations were

performed with salines pre-gassed with air for 100% air saturation and gassed with N<sub>2</sub> conditions representing no oxygen saturation.

Intermittent-flow respirometry was performed to determine oxygen consumption rates of isolated tissue by flushing and replacing salines using a manual gravity-fed system. Flush cycles guaranteed complete saline replacement during open cycles and time intervals during closed measurements were limited to ~20 minutes, to ensure that air saturation of tissue did not drop below 80%, since values below 75% were previously shown to restrict this tissue (Taylor and Grosell 2009). Previous use of this respirometry system has shown negligible rates of gas back-flux with the atmosphere or across chambers and a constant O<sub>2</sub> consumption rate at air saturation above 75% (Taylor and Grosell 2009). Tissue O<sub>2</sub> consumption was estimated from the mucosal and serosal O<sub>2</sub> depletion rates.

### ***Electrophysiological measurements***

In the Ussing chamber systems (Physiological Instruments, San Diego, CA, USA), current and voltage electrodes attached to an amplifier measured transepithelial potential (TEP, mV) differences between a baseline 0  $\mu$ A current clamp and a 3 second pulse of 50  $\mu$ A that was applied every 60s. Measurements were logged using logged using Acknowledge software (v. 3.8.1, BIOPAC Systems). TEP values are reported with a luminal reference of 0 mV and conductance were calculated using Ohm's law.



### **Seawater manipulation**

Stable CO<sub>2</sub> levels were achieved using a Loligo pH-based negative feedback system (Loligo Systems, Tjele, Denmark). In this system, a standard curve was generated based on the relationship between known gas standards and seawater pH. Once a pH setpoint corresponding to 1900 μatm CO<sub>2</sub> was calculated following calibration and measurement of ambient seawater, 100% CO<sub>2</sub> was gassed directly into flow-through tanks via solenoid valves controlled by pH electrodes (Sentix H, wtw Germany) and a PCO<sub>2</sub>/pH DAQ-M digital relay instrument connected to CapCTRL software (Loligo Systems, Tjele, Denmark) to achieve the desired PCO<sub>2</sub> setpoint. PCO<sub>2</sub> levels typically stayed within 4-10 percent of setpoint values. Independent pH measurements were taken multiple times a week using a separate pH electrode (pH<sub>NBS</sub>, PHC3005, Radiometer, France). Target CO<sub>2</sub> levels were confirmed with measurements of seawater TCO<sub>2</sub> using a Corning 965 CO<sub>2</sub> Analyzer (Corning 965, Corning Diagnostics, UK). TA (titratable alkalinity) and PCO<sub>2</sub> levels were calculated from measured pH<sub>NBS</sub> and TCO<sub>2</sub> measurements in CO2SYS software (Pierrot et al. 2006). These calculations confirmed target PCO<sub>2</sub> values for control (ambient-~439 μatm CO<sub>2</sub>) and 1900 (1878 μatm CO<sub>2</sub>) were reached. Water chemistry parameters including temperature and salinity are reported in Table 3.2.

Table 3.2: Water chemistry parameters. Values are presented as means ± standard deviation. C and H stand for control (ambient) and high CO<sub>2</sub>, respectively (1900 μatm CO<sub>2</sub> nominal value).

	pH	PCO <sub>2</sub> (μatm)	Alkalinity (μmol kg <sup>-1</sup> )	TCO <sub>2</sub> (μmol kg <sup>-1</sup> )	Salinity (p.p.t.)	Temp.(°C)
C	8.15 ± .03	439 ± 24	2294 ± 42	2037 ± 31	34.1 ± 0.5	22.5 ± 1.1
H	7.58 ± .02	1878 ± 54	2334 ± 41	2303 ± 40	34.2 ± 0.5	22.5 ± 1.2

## Results

### ***Bicarbonate secretion rates of isolated tissue***

Contrary to expectations, anterior intestinal tissue from CO<sub>2</sub> acclimated toadfish exhibited significantly increased HCO<sub>3</sub><sup>-</sup> secretion rates (μmol cm<sup>-2</sup> h<sup>-1</sup>) when compared to control tissue under identical conditions (Fig. 3.1). This result indicated prior acclimation to CO<sub>2</sub> does not suppress but stimulates intestinal HCO<sub>3</sub><sup>-</sup> transport by around 13%. In addition, within both the control and CO<sub>2</sub> acclimated fish, HCO<sub>3</sub><sup>-</sup> secretion rates using serosal salines mimicking plasma conditions at 1900 μatm CO<sub>2</sub> were significantly higher than HCO<sub>3</sub><sup>-</sup> secretion rates under control serosal salines (Two-way ANOVA, Fish treatment P<0.009, Saline P<0.001, Fish treatment x saline P<0.490, Fig. 3.1). TEP and conductance remained stable during experiments and were in agreement with earlier reports (Table 3.3) (Grosell and Genz 2006; Taylor and Grosell 2009).

### ***Oxygen consumption rates of isolated tissue***

Under both saline compositions, tissue from 1900 μatm CO<sub>2</sub> acclimated toadfish showed a significant 8% increase in oxygen consumption rate compared to tissue from control acclimated fish. In contrast to HCO<sub>3</sub><sup>-</sup> secretion rates, oxygen consumption rates of isolated tissue from both control and 1900 μatm CO<sub>2</sub> exposed fish showed no effect of saline composition (Two-way ANOVA, Fish treatment P<0.033, Saline P<0.769, Fish treatment x saline P<0.509, Fig. 3.2). A similar relationship was observed when data was corrected for body mass (Fig. 3.3).

Fig. 3.1: Bicarbonate secretion rates of isolated anterior intestine from control and CO<sub>2</sub> acclimated toadfish: Effect of blood-side saline composition on HCO<sub>3</sub><sup>-</sup> secretion rates (means ± s.e.m.) of isolated anterior intestinal tissue obtained from Gulf toadfish acclimated to control (~440 μatm CO<sub>2</sub>, n=9) or ~1900 μatm CO<sub>2</sub> (n=10) for 2-4 weeks. Tissues from either control or 1900 μatm CO<sub>2</sub> acclimated fish mounted in this dual chambered Ussing/pH-stat system were bathed on either side by salines that mimicked *in vivo* ionic composition. Each tissue received two blood-side serosal saline treatments, control and CO<sub>2</sub> saline, representative of HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> previously measured in toadfish blood following control and 1900 μatm CO<sub>2</sub> exposure (Table 3.1). \*significant acclimation effect, †significant saline effect.

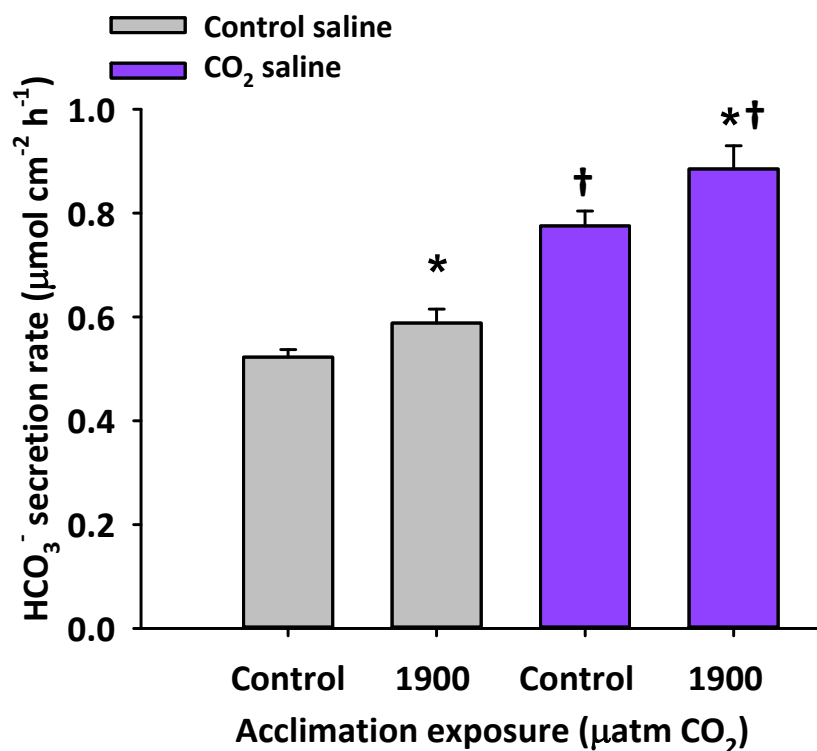


Fig. 3.2: Oxygen consumption of isolated anterior tissue from control and CO<sub>2</sub> acclimated toadfish: Effect of blood-side saline composition on oxygen consumption rates (means  $\pm$  s.e.m.) of isolated anterior intestinal tissue taken from toadfish acclimated to control ( $\sim 440 \mu\text{atm CO}_2$ ; n=12) or  $\sim 1900 \mu\text{atm CO}_2$  (n=8) for 2-4 weeks. Tissues from either control or 1900  $\mu\text{atm CO}_2$  acclimated fish mounted in this dual-chambered epithelial respirometer were bathed on either side by salines designed to mimic *in vivo* ionic composition. Each tissue received two blood-side serosal saline treatments, control saline and CO<sub>2</sub> saline, that were representative of HCO<sub>3</sub><sup>-</sup> and previously measured in toadfish blood following acclimation at control and 1900  $\mu\text{atm CO}_2$  (Table 3.1). \*significant acclimation effect.

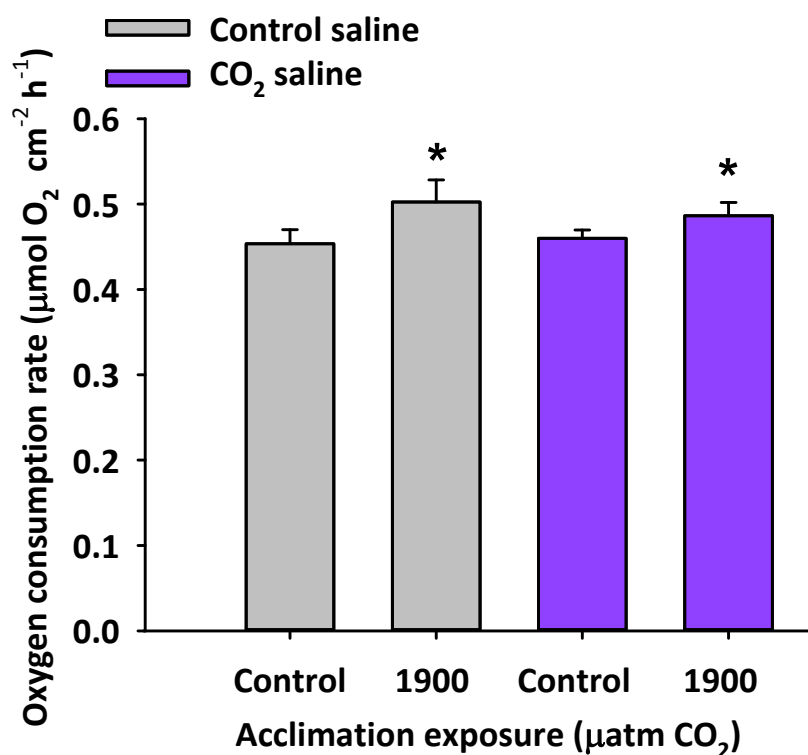


Fig. 3.3: Mass normalized oxygen consumption of isolated anterior tissue from control and CO<sub>2</sub> acclimated toadfish: Effect of blood-side saline composition on oxygen consumption rates (means  $\pm$  s.e.m.) of isolated anterior intestinal tissue taken from toadfish acclimated to control ( $\sim$ 440  $\mu$ atm CO<sub>2</sub>; n=12) or  $\sim$ 1900  $\mu$ atm CO<sub>2</sub> (n=8) for 2-4 weeks. Tissues from either control or 1900  $\mu$ atm CO<sub>2</sub> acclimated fish mounted in this dual-chambered epithelial respirometer were bathed on either side by salines designed to mimic *in vivo* ionic composition. Each tissue received two blood-side serosal saline treatments, control saline and CO<sub>2</sub> saline, that were representative of HCO<sub>3</sub><sup>-</sup> and previously measured in toadfish blood following acclimation at control and 1900  $\mu$ atm CO<sub>2</sub> (Table 3.1). \*significant acclimation effect.

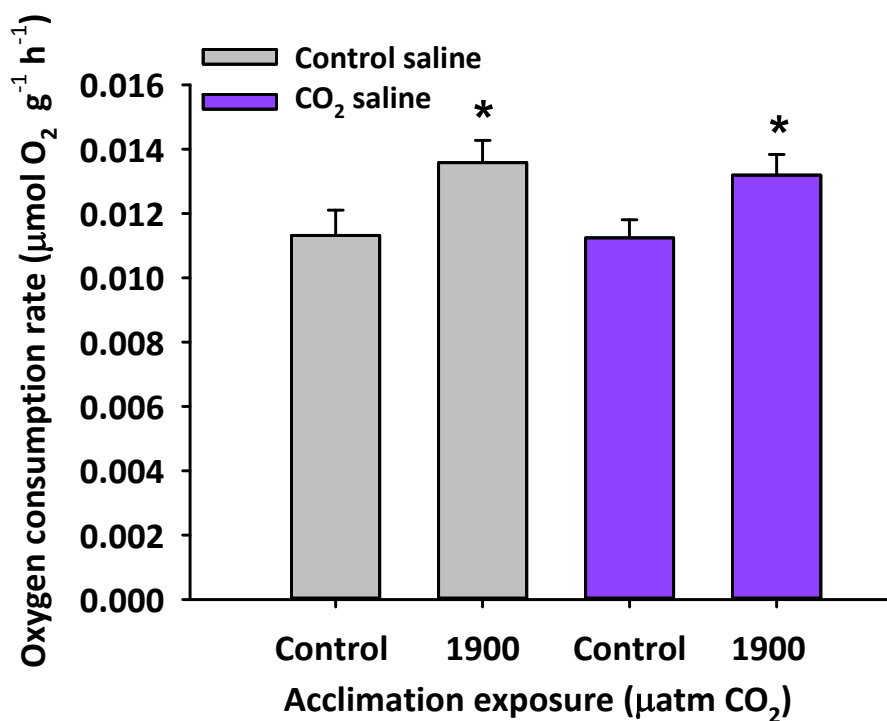


Table 3.3 Electrophysiological measurements from isolated toadfish intestinal tissue

Fish acclimation and serosal saline type	Transepithelial potential (TEP) (mV)	Conductance ( $\mu$ Si)
Anterior control tissue		
Control serosal saline	$-17.71 \pm 1.38$	$9.78 \pm 0.24$
CO <sub>2</sub> serosal saline	$-18.06 \pm 0.71$	$9.97 \pm 0.21$
Anterior CO <sub>2</sub> tissue		
Control serosal saline	$-17.63 \pm 0.98$	$10.34 \pm 0.27$
CO <sub>2</sub> serosal saline	$-16.73 \pm 1.35$	$10.16 \pm 0.31$

Values are means  $\pm$  s.e.m. See Table 3.1 for control and CO<sub>2</sub> saline compositions

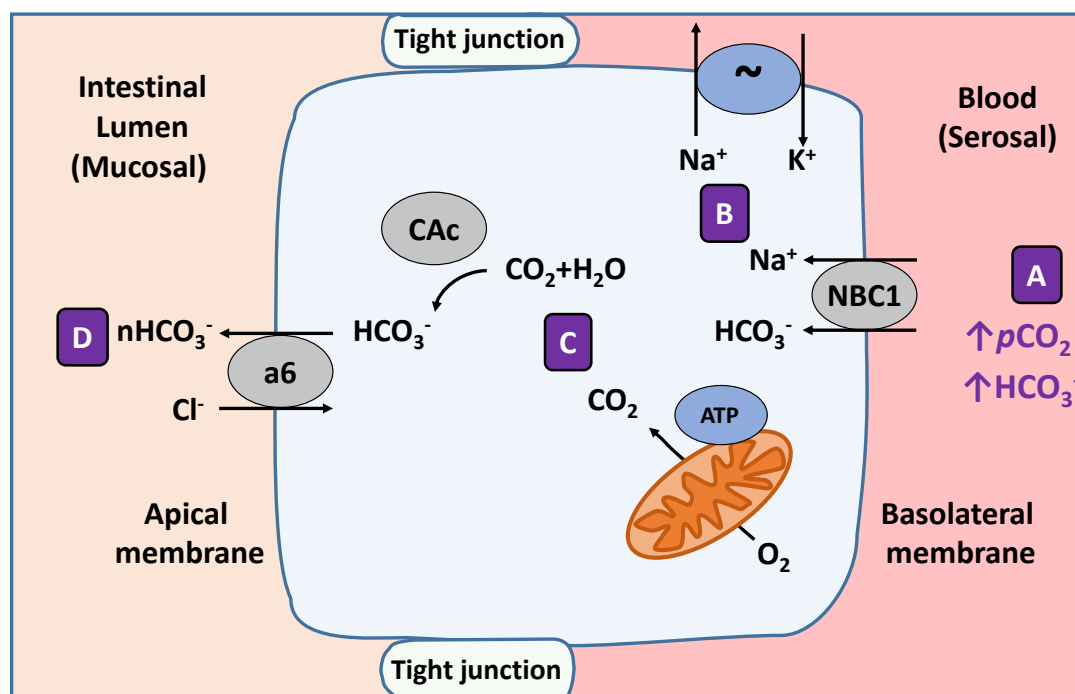
### **Conclusions**

These results indicate that the marine fish intestine has a higher metabolic demand at 1900  $\mu$ atm CO<sub>2</sub>, a level predicted for year 2300 and currently seen in upwelling coastal areas (Feely et al. 2008). This demand is likely attributed to an increase in intestinal HCO<sub>3</sub><sup>-</sup> loss from the body. Similar to other fish experiencing elevated CO<sub>2</sub> in a marine environment, Gulf toadfish have been shown to defend blood pH following exposure to 1900  $\mu$ atm CO<sub>2</sub> by sustaining elevated levels of HCO<sub>3</sub><sup>-</sup> in the face of higher PCO<sub>2</sub> in the blood (Esbaugh et al. 2012). However, this compensation was associated with an increased HCO<sub>3</sub><sup>-</sup> loss that was presumed to be linked to an activation of existing ionoregulatory transport pathways (Esbaugh et al. 2012; Heuer et al. 2012). These pathways involve the movement of plasma HCO<sub>3</sub><sup>-</sup> into the intestinal lumen in exchange for Cl<sup>-</sup> and are critical for maintaining water balance (Grosell 2011a). However, from a whole-animal acid-base balance perspective, HCO<sub>3</sub><sup>-</sup> loss during CO<sub>2</sub> compensation counteracts the need to retain HCO<sub>3</sub><sup>-</sup> and defend pH, suggesting that fish faced with longer term acclimation to

CO<sub>2</sub> must dynamically downregulate pathways involved in intestinal HCO<sub>3</sub><sup>-</sup> secretion. Comparison of HCO<sub>3</sub><sup>-</sup> secretion rates of intestinal tissue from CO<sub>2</sub> and control acclimated fish under identical saline conditions revealed that CO<sub>2</sub> exposure leads to a stimulation, rather than a downregulation of HCO<sub>3</sub><sup>-</sup> transport pathways.

It was proposed that the 13% increase in HCO<sub>3</sub><sup>-</sup> loss from CO<sub>2</sub> acclimated fish reflects an increased energetic demand, and thus increased CO<sub>2</sub> production, in intestinal tissue. This suggestion is supported by an 8% increase in O<sub>2</sub> consumption in tissue from CO<sub>2</sub> acclimated fish. A detailed mechanistic explanation of this proposed response can be seen in Fig. 3.4. Sustained elevated plasma HCO<sub>3</sub><sup>-</sup> following CO<sub>2</sub> compensation leads to an increase in HCO<sub>3</sub><sup>-</sup> movement from the blood into the intestinal cell that is paired with the movement of Na<sup>+</sup> through basolateral NBC1, a Na<sup>+</sup>-HCO<sub>3</sub><sup>-</sup> co-transporter (Taylor et al. 2010). The sustained Na<sup>+</sup> influx must be compensated for by Na<sup>+</sup> extrusion through the Na<sup>+</sup>-K<sup>+</sup> ATPase (NKA). Increased NKA activity leads to an increased ATP and thus O<sub>2</sub> demand. Meeting this demand results in increased endogenous CO<sub>2</sub> production available for hydration via intracellular carbonic anhydrase (CAc) to form additional HCO<sub>3</sub><sup>-</sup>. Secretion of this excess HCO<sub>3</sub><sup>-</sup> through apical SLC26a6 anion exchange likely accounts for the stimulation of HCO<sub>3</sub><sup>-</sup> loss during CO<sub>2</sub> exposure. Hydration of endogenous CO<sub>2</sub> also results in formation of protons that must be eliminated from the intestinal cell. This proton extrusion may occur via Na<sup>+</sup>-dependent or independent pathways (Grosell 2011a), both of which are energy demanding and could contribute to the observed elevation of O<sub>2</sub> consumption.

Fig. 3.4: Proposed impacts of 1900  $\mu\text{atm}$   $\text{CO}_2$  on intestinal transport physiology in a marine teleost: Compensation for a  $\text{CO}_2$ -induced acidosis increases  $\text{HCO}_3^-$  and  $\text{PCO}_2$  in extracellular fluids (Esbaugh et al. 2012). A) Elevated serosal  $\text{HCO}_3^-$  during  $\text{CO}_2$  exposure stimulates transport via  $\text{Na}^+:\text{HCO}_3^-$  co-transporter, NBC1, leading to both an increase influx of  $\text{HCO}_3^-$  and  $\text{Na}^+$  across the basolateral membrane. B) Sustained influx of  $\text{Na}^+$  via NBC1 likely leads to a demand for increased  $\text{Na}^+$  extrusion via the energy-demanding  $\text{Na}^+ \text{K}^+ \text{ATPase}$  ( $\sim$ ). C) The metabolic demand and increased  $\text{O}_2$  consumption associated with handling an increased  $\text{Na}^+$  influx would generate additional endogenous  $\text{CO}_2$ , increasing substrate for intracellular CAc hydration. D) Intracellular  $\text{HCO}_3^-$  generated via this process likely accounts for the observed stimulation of bicarbonate secretion via SLC26a6 (a6) in isolated tissue from fish acclimated to 1900  $\mu\text{atm}$   $\text{CO}_2$  compared to control fish under serosal salines with identical bicarbonate concentrations. For a more detailed overview of marine fish intestinal transport processes see review (Grosell 2011a).



It is likely that the 8% increase in intestinal tissue  $\text{O}_2$  consumption would not be observable by measurements of whole-animal metabolic rate. Difficulties in picking up small differences in organismal metabolic rate may explain some of the



varying results from studies examining the effects of ocean acidification on fish standard metabolic rate (Heuer and Grosell 2014). In addition, there appears to be considerable intra- and inter-species variation in the response to elevated CO<sub>2</sub> and there is inherent difficulty in comparing measurements using different methodologies (Clark 2013; Roche 2013). As demonstrated in the present study and by others (Kreiss et al. 2015; Stapp et al. 2015; Strobel et al. 2012; Tseng et al. 2013), increased resolution and mechanistic insight into energetic tradeoffs and/or apparent consequences of ocean acidification may be obtained by integrating techniques and methods that examine multiple levels of organization. Altered mitochondrial capacity (Strobel et al. 2012), shifts in energetic budgets (Pan et al. 2015; Strobel et al. 2013b; Tseng et al. 2013), and increased protein turnover (Pan et al. 2015) have all been noted with little impact to whole-animal measurements. While the present study of isolated intestinal tissue precluded normal hormonal cascades or feedback mechanisms, it offered the advantage of careful control of blood-side (serosal) saline conditions and made it possible to identify mechanistic differences in tissue function that were impossible to observe in previous *in vivo* work in Chapter 2 (Heuer et al. 2012).

The marine fish intestine is fine-tuned to changes in plasma HCO<sub>3</sub><sup>-</sup> to aid in water uptake and to handle the alkaline tide associated with digesting a meal (Taylor and Grosell 2009; Taylor et al. 2010). Although the functional consequence of stimulated intestinal HCO<sub>3</sub><sup>-</sup> loss remains to be fully elucidated, the increase in O<sub>2</sub> consumption leads to the conclusion that this CO<sub>2</sub>-induced response is maladaptive but persists for protection of the aforementioned functions. Support

for this conclusion comes from a recent study demonstrating that high levels of CO<sub>2</sub> (9200 μatm) lengthen the amount of time needed to digest a meal (Tirsgaard et al. 2015). An increased metabolic demand from ion transport processes, as seen in the present study, would detract from energy available for digestive functions in the intestine, slowing the process of digestion. Calculations using estimates of toadfish standard metabolic rate (Gilmour et al. 1998), suggest that the energetic cost of CO<sub>2</sub> acclimation in the intestine would account ~0.5% of whole animal O<sub>2</sub> consumption (Taylor and Grosell 2009). Albeit small, any factor that promotes an energy reallocation or increases standard metabolic rate could exacerbate already narrow metabolic constraints (Deutsch et al. 2015) or possibly interact with temperature elevation to increase overall impact (Enzor et al. 2013; Kreiss et al. 2015; Strobel et al. 2013b).

The demand to secrete HCO<sub>3</sub><sup>-</sup> in the intestine to maintain water balance is well-conserved across marine teleosts (Grosell 2011a). Increased intestinal HCO<sub>3</sub><sup>-</sup> secretion with CO<sub>2</sub> exposure as reported here has also been demonstrated in the Plainfin midshipmen (~50,000 μatm CO<sub>2</sub>) (Perry et al. 2010) and suggested from gene expression work in the Japanese ricefish (7000 μatm CO<sub>2</sub>) (Tseng et al. 2013). These three studies suggest that the increased intestinal HCO<sub>3</sub><sup>-</sup> secretion and metabolic demand during CO<sub>2</sub> exposure could be a ubiquitous response to elevated CO<sub>2</sub> throughout marine bony fishes. Finally, intestinal HCO<sub>3</sub><sup>-</sup> secretion results in formation and excretion of CaCO<sub>3</sub> by marine fish which amounts to at least 3-15% of the marine inorganic CaCO<sub>3</sub> production (Wilson et al. 2009). Although a study on the toadfish reported unaltered CaCO<sub>3</sub> excretion rates (Heuer

et al. 2012), increased intestinal  $\text{HCO}_3^-$  loss at elevated  $\text{CO}_2$  in other species may impact the magnitude of this globally important calcification process, a possibility worthy of further study.

Although compensation for elevated  $\text{CO}_2$  in marine fish typically occurs within days (Esbaugh et al. 2012), it cannot be ruled out that longer acclimation periods, transgenerational effects, and/or adaptation may affect the dynamics of acid-base balance. More research on how  $\text{CO}_2$  exposure affects these endpoints is clearly needed.

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## Chapter 4: Further dissecting the response of the intestine to elevated CO<sub>2</sub> over a range of CO<sub>2</sub> levels

### Summary

Most marine teleosts defend blood pH during high CO<sub>2</sub> exposure by sustaining elevated levels of HCO<sub>3</sub><sup>-</sup> in body fluids. In contrast to the gill, where measures are taken to achieve net base retention, elevated CO<sub>2</sub> leads to base loss in the intestine of marine teleosts studied to date. This loss is thought to occur through transport pathways previously demonstrated to be involved with routine osmoregulation. The main objective of this study was to better characterize the intestinal transport physiology of the Gulf toadfish (*Opsanus beta*) when exposed to varied levels of CO<sub>2</sub> (control, 5,000, 10,000 and 20,000 μatm CO<sub>2</sub>). Results of this study suggest that intestinal apical anion exchange is highly responsive to hypercarbia, evidenced by a dose-dependent increase in intestinal luminal HCO<sub>3</sub><sup>-</sup> (mmol l<sup>-1</sup>) that was mirrored by a reduction in Cl<sup>-</sup> (mmol l<sup>-1</sup>). Net Na<sup>+</sup> (mmol l<sup>-1</sup>) transport, drinking rates, and fluid excretion rates were not influenced by hypercarbia, but fractional fluid absorption decreased at the highest level of CO<sub>2</sub>. All measured divalent ion concentrations (Ca<sup>2+</sup>, Mg<sup>2+</sup>, and SO<sub>4</sub><sup>2-</sup>) declined with hypercarbia in intestinal fluid, indicating some degree of alteration to fluid transport. Measures of carbonate composition (Mg<sup>2+</sup> and Ca<sup>2+</sup> content) and production showed no significant changes, indicating that these endpoints are not affected by hypercarbia. This study is among the first to thoroughly characterize how compensation for elevated CO<sub>2</sub> affects transport physiology of the marine fish intestine.

## Background

The typical acid-base regulatory response exhibited by fish exposed to elevated ambient CO<sub>2</sub> (hypercarbia) has been well-characterized across a wide array of species and exposure levels (Brauner and Baker 2009; Claiborne et al. 2002; Evans et al. 2005; Heisler 1984; Larsen and Jensen 1997; Perry and Gilmour 2006). After experiencing an initial drop in pH and an increase in PCO<sub>2</sub> at the onset of CO<sub>2</sub> exposure, most fish respond by elevating and maintaining high levels of HCO<sub>3</sub><sup>-</sup>. This compensation leads to an altered steady state, where pH is maintained at pre-exposure levels, but both HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> remain elevated (Esbaugh et al. 2012; Ishimatsu et al. 2004; Larsen and Jensen 1997; Michaelidis et al. 2007; Perry et al. 2010; Toews et al. 1983). The gill is the primary organ used to cope with elevated CO<sub>2</sub> and is estimated to account for ~90% of acid-base regulation in fishes (Claiborne et al 2002; Perry and Gilmour 2006). In most instances, the accumulation of HCO<sub>3</sub><sup>-</sup> in the plasma is usually associated with a corresponding and equimolar decrease in plasma Cl<sup>-</sup> (Brauner and Baker 2009; Claiborne et al. 2002; Larsen and Jensen 1997).

In addition to the gill, the intestine may also contribute to whole body acid-base balance (Perry and Gilmour 2006; Wood et al. 1999). Most evidence to date does not suggest that the intestine actively regulates acid-base disturbances (Perry et al. 2010). However, the intestine is known to be responsive to changes in plasma HCO<sub>3</sub><sup>-</sup> (Heuer et al. 2012; Taylor et al. 2010), and appears to counteract measures employed at the gill to retain HCO<sub>3</sub><sup>-</sup> during elevated CO<sub>2</sub> at least during short-term exposures (Heuer et al. 2012; Perry et al. 2010). In addition,

hypercarbia has recently been demonstrated to prolong specific dynamic action (Tirsgaard et al. 2015). Most patterns of acid and base flux in the intestine have been studied in the context of osmoregulation by manipulating environmental salinity (Genz et al. 2008; Gilmour et al. 2012; Guffey et al. 2011; Sattin et al. 2010; Taylor et al. 2010), since this organ is critical for osmotic homeostasis.

The link between the exchange of acid-base equivalents and osmoregulation lies in processes related to solute-coupled water absorption in the intestine. To stay hydrated, marine fish drink seawater and absorb monovalent ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ), which ultimately drives water from the lumen into the body (Skadhauge 1974; Smith 1930; Usher et al. 1991). In part,  $\text{Cl}^-$  absorption occurs in exchange for  $\text{HCO}_3^-$  on the apical membrane, connecting base secretion to water absorption (Grosell and Genz 2006; Grosell et al. 2009b). One source of  $\text{HCO}_3^-$  for this exchange results from  $\text{Na}^+$ -coupled  $\text{HCO}_3^-$  movement from the blood to the intracellular space via basolateral  $\text{Na}^+$ :  $\text{HCO}_3^-$  co-transporter NBC1 (Taylor et al. 2010). Elevated  $\text{PCO}_2$  also provides a source of  $\text{HCO}_3^-$  for apical anion exchange, since it is substrate for hydration via intracellular carbonic anhydrase (CAc) (Grosell and Genz 2006; Sattin et al. 2010; Wilson et al. 1996). Thus, elevated  $\text{CO}_2$  may increase  $\text{HCO}_3^-$  secretion through increased transepithelial  $\text{HCO}_3^-$  movement via NBC1 or through increased hydration of intracellular  $\text{PCO}_2$ . Furthermore, changes to cellular  $\text{Na}^+$  gradients that occur as a result of an increase in transepithelial  $\text{HCO}_3^-$  movement during elevated  $\text{CO}_2$  could potentially alter the  $\text{Na}^+\text{K}^+\text{ATPase}$  (NKA), leading to increased  $\text{O}_2$  consumption and therefore

metabolic CO<sub>2</sub> production, providing yet another substrate for anion exchange following intracellular hydration.

A short-term study (3 days) exposing toadfish to elevated CO<sub>2</sub> levels relevant for global climate change scenarios predicted for year 2300 (1900 μatm CO<sub>2</sub>) (Meehl et al. 2007), showed a significant increase in rectal base secretion that corresponded to a significant decrease intestinal Cl<sup>-</sup> (mmol l<sup>-1</sup>), suggesting that movement through apical anion exchanger slc26a6 was increased (Heuer et al. 2012). Elevated total CO<sub>2</sub> in the intestinal fluid of Plainfin midshipman (*Porichthys notatus*) exposed to a much higher level of CO<sub>2</sub> (50,000 μatm CO<sub>2</sub>) demonstrated similar results, generally indicating that compensation for CO<sub>2</sub> induces base loss in the intestine that would act against measures taken at the gill to retain HCO<sub>3</sub><sup>-</sup> (Perry et al. 2010). In adult medaka (*Oryzias latipes*) exposed to 7,000 μatm CO<sub>2</sub> for 48 hours, changes in mRNA levels of transporters involved in acid secretion (NHE3), HCO<sub>3</sub><sup>-</sup> regulation (NBCa, NBCb, AE1a, CA15), and various NKA subunits were noted in the intestine (Tseng et al. 2013). Together, these studies indicate that compensation for elevated CO<sub>2</sub> elicits the use of transporters and proteins that are integral in maintaining water balance.

In addition to aiding solute coupled water uptake via anion exchange, HCO<sub>3</sub><sup>-</sup> secreted into the lumen reacts with Ca<sup>2+</sup> to form CaCO<sub>3</sub> to lower luminal osmotic pressure and favor continued osmotic water absorption (Grosell et al. 2009b; Whittamore et al. 2010). Thus, hypercarbia has been previously hypothesized to increase the production of carbonates. This hypothesis was not supported in a study on toadfish (1900 μatm CO<sub>2</sub>) (Heuer et al. 2012); however, in the

midshipmen, exposure to 50,000  $\mu\text{atm CO}_2$  for 48 hours increased carbonate titratable alkalinity (TA) (Perry et al. 2010). It is unclear whether or not differences among these studies was due to exposure level, species-specificity, or a combination of both factors.

Hypercarbia may also affect carbonate composition. As fish drink seawater and monovalent ions and water are absorbed, divalent ions such as  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{SO}_4^{2-}$  are concentrated since they are relatively impermeable to the intestinal epithelia (Genz et al. 2008; Marshall and Grosell 2006). In addition to  $\text{Ca}^{2+}$ , carbonates are also comprised of  $\text{Mg}^{2+}$ , so alterations to fluid absorption could potentially change conditions for  $\text{CaCO}_3$  precipitation in the intestinal lumen. While precipitate  $\text{Mg}^{2+}$  content and thus solubility in seawater have been assessed for a wide-variety of species (Perry et al. 2011; Salter et al. 2012), only one study on the toadfish has examined changes to carbonate composition with  $\text{CO}_2$  exposure (Heuer et al. 2012). This endpoint was not significantly different at 1900  $\mu\text{atm CO}_2$ , however, it is unclear whether higher  $\text{CO}_2$  levels would alter the composition.

While some information is available on how the marine fish intestine responds to hypercarbia (Heuer et al. 2012; Perry et al. 2010; Tirsgaard et al. 2015; Tseng et al. 2013), most *in vivo* work on transport mechanisms is restricted to two species that were investigated at two  $\text{CO}_2$  tensions that differ by 25 fold. The overarching goals of this study were to expose Gulf toadfish (*Opsanus beta*) to a range of  $\text{CO}_2$  levels (control, 5,000, 10,000 and 20,000  $\mu\text{atm CO}_2$ ) to standardize for any species differences note above, and to better characterize intestinal transport mechanisms in response to elevated  $\text{CO}_2$ . Rectal collection sacs were



utilized to examine the effect of hypercarbia on (*Series 1*) the relationship between blood acid-base chemistry and intestinal base secretion, (*Series 2*) ion concentrations in plasma and intestinal fluid, and (*Series 3*) intestinal fluid movement, base secretion rates, and carbonate composition. An additional objective was to validate the use of rectal collection sacs by examining production of carbonates collected directly from the intestine and to examine the potential for dissolution in released carbonates during elevated CO<sub>2</sub> exposure (*Series 4*).

## **Materials and Methods**

### ***Animal collection and care***

Gulf toadfish (*Opsanus beta*) were obtained as by-catch from shrimp fisherman in Biscayne Bay, FL and transported to the University of Miami Rosenstiel School of Marine and Atmospheric Science. On arrival, toadfish were treated for ectoparasites (McDonald et al. 2007) and placed into aerated tanks with flow-through seawater from Bear Cut, Florida (22-27°C, 30-35 p.p.t.). Polyvinylchloride tubing was provided as shelter and all fish were acclimated to the lab for at least two weeks prior to experimentation. Fish were fed squid bi-weekly, but food was withheld for at least 2-3 days prior to experimentation to prevent variation associated with specific dynamic action (SDA) (Taylor and Grosell 2009). All protocols adhered to University of Miami animal care protocols (IACUC 13-3225).

### ***Seawater CO<sub>2</sub> manipulation***

Desired PCO<sub>2</sub> levels were attained using a pH-stat CO<sub>2</sub> dosing system by Loligo Systems (Tjele, Denmark) as described in previous studies (Esbaugh et al. 2012; Heuer et al. 2012). This system utilized pH as a proxy for PCO<sub>2</sub> levels after a standard curve was generated by measuring pH at known CO<sub>2</sub>-air gas mixes and ambient seawater pH. For experiments, a pH setpoint corresponding to each desired PCO<sub>2</sub> level was calculated, and 100% CO<sub>2</sub> was dosed directly into flow-through, aerated tanks to achieve the chosen PCO<sub>2</sub> level. The pH electrode and meter (Sentix H electrode, 3310 pH meter, wtw, Germany) corresponding to each experimental tank were connected to CapCTRL software that dosed CO<sub>2</sub> using solenoid valves controlled by a PCO<sub>2</sub>/pH DAQ-M digital relay instrument (Loligo Systems, Tjele, Denmark). Desired PCO<sub>2</sub> levels remained stable using this system and typically stayed within 4-10 percent of setpoint values. Independent pH<sub>NBS</sub> of experimental tanks was validated on most days during each experimental run using an independent electrode (PHC3005, Radiometer, France). A Corning 965 CO<sub>2</sub> Analyzer (Corning Diagnostics, UK) was used to further validate that desired CO<sub>2</sub> levels were being achieved. TCO<sub>2</sub> readings and pH<sub>NBS</sub> measurements were put into CO2SYS software to define all the other parameters of the carbonate system (PCO<sub>2</sub> and titratable alkalinity (TA) (Pierrot et al. 2006). These results, along with measurements of temperature and salinity, are presented in Table 4.1 and Table 4.2.

Table 4.1: Water chemistry parameters for CO<sub>2</sub> exposure levels

CO <sub>2</sub> level ( $\mu\text{atm CO}_2$ )	pH	PCO <sub>2</sub> ( $\mu\text{atm}$ )	Titrateable Alkalinity ( $\mu\text{mol kg}^{-1}$ )	TCO <sub>2</sub> ( $\mu\text{mol kg}^{-1}$ )
Control	8.14 $\pm$ 0.01	446 $\pm$ 14	2363 $\pm$ 28	2115 $\pm$ 41
5000	7.21 $\pm$ 0.003	4903 $\pm$ 66	2385 $\pm$ 33	2484 $\pm$ 35
10000	6.92 $\pm$ 0.01	9511 $\pm$ 176	2385 $\pm$ 28	2646 $\pm$ 35
20000	6.62 $\pm$ 0.01	19954 $\pm$ 408	2368 $\pm$ 29	2933 $\pm$ 48

Water chemistry parameters for exposures during acclimation to various CO<sub>2</sub> levels. Values are presented as means  $\pm$  s.e.m.

Table 4.2: Temperature and salinity during experimental treatments

CO <sub>2</sub> level ( $\mu\text{atm CO}_2$ )	Salinity (p.p.t.)	Temp. ( $^{\circ}\text{C}$ )
Control	34.9 $\pm$ 0.1	25.2 $\pm$ 0.3
5000	34.8 $\pm$ 0.1	24.8 $\pm$ 0.4
10000	34.7 $\pm$ 0.1	24.7 $\pm$ 0.4
20000	34.9 $\pm$ 0.1	24.7 $\pm$ 0.4

Water chemistry parameters for exposures during acclimation to various CO<sub>2</sub> levels. Values are presented as means  $\pm$  s.e.m.

***Rectal collection sac and blood sampling protocols (Series 1-3)***

Rectal collection sacs were utilized to examine the effect of hypercarbia on (*Series 1*) the relationship between blood acid-base chemistry and intestinal base secretion, (*Series 2*) ion concentrations in plasma and intestinal fluid, and (*Series 3*) intestinal fluid movement, base secretion rates, and carbonate composition. Prior to surgery, individual toadfish were placed into plastic containers with multiple openings to allow for water movement, and submerged directly into experimental tank bottoms already at their respective CO<sub>2</sub> level (control ~400, 5,000, 10,000, and 20,000  $\mu\text{atm}$  CO<sub>2</sub>) for a 3 day acclimation period. The addition of submerged water pumps ensured even mixing in the tank and also facilitated water movement through the plastic containers in which the fish were kept. Fish mass was generally close to 60 g for rectal collection sac experiments (control:  $66.9 \pm 4.2$ , 5,000  $\mu\text{atm}$ :  $60.8 \pm 7.6$ , 10,000  $\mu\text{atm}$ :  $57.2 \pm 4.1$ , 20,000  $\mu\text{atm}$ :  $63.3 \pm 4.9$ ). Following this acclimation period, toadfish were anesthetized (0.2 g l<sup>-1</sup> MS-222 buffered with 0.3 g l<sup>-1</sup> NaHCO<sub>3</sub>) and surgery was performed to attach a rectal collection sac. This sac was made from the tip of a nitrile glove attached to a 1 cm segment of a 1 mL disposable plastic syringe that was heat flared at both ends (Genz et al. 2008). Rectal collection sacs were held in place by suture stitched around the rectal opening. Thus, intestinal secretions over a time period could be collected and kept isolated from seawater. After surgery, toadfish were submerged and recovered in their respective treatment tanks, where they remained for another 3 days.

Following a total 6 day exposure (3 day acclimation, 3 day post-surgery), toadfish were anesthetized with MS-222 (0.2 g l<sup>-1</sup> MS-222 buffered with 0.3 g l<sup>-1</sup>

NaHCO<sub>3</sub>). A blood sample was drawn from the caudal vein into a heparinized syringe and immediately centrifuged to separate red blood cells and the plasma. A hemostat was used to isolate rectal collection sac contents, after which the spinal cord was severed. Plasma was measured for pH (PHC3005, Radiometer, France), total CO<sub>2</sub> (Corning 965, Corning Diagnostics, UK) and osmolality (Wescor Vapro 5520, Logan, UT), and an aliquot of the plasma sample was frozen (-20°C) for later ion analyses. Fish were then dissected and the intestinal tract was removed after clamping the pyloric and rectal sphincter with hemostats. All excretions (fluid and carbonate) from rectal collection sac and samples from the intestinal tract were combined into one sample (pre-weighed 15 mL conical tube) for subsequent analysis.

After the final mass of conical tubes was weighed to determine total intestinal output, tubes were centrifuged (3,000 rpm for 10 minutes) to separate fluid and solid (carbonate) components. Fluid from the collection was isolated and weighed to determine total fluid volume and measured for pH, total CO<sub>2</sub>, and osmolality. An aliquot of the sample was frozen (-20°C) for later ion analyses. For both plasma and rectal fluid, bicarbonate equivalents were calculated from measurements of total CO<sub>2</sub> and pH using the Henderson-Hasselbalch, equation as outlined in a previous study (Genz et al. 2008).

Double endpoint titrations were used to analyze the total bicarbonate/carbonate equivalents in the solid carbonate portion of the rectal sample. The sample was first suspended in 10 mL of nanopure water, sonicated (Kontes Micro Ultrasonic Cell Disruptor), and re-centrifuged (see above). This

procedure was repeated with new nanopure water in order to rinse the precipitates of any residual intestinal fluid that could potentially contaminate the sample. Following the second rinse, nanopure water was removed and replaced by 10 mL of 150 mmol l<sup>-1</sup> NaCl and sonicated a final time. This 150 mmol l<sup>-1</sup> NaCl solution was used to stabilize pH electrode readings. Prior to titration, the sample was gassed with N<sub>2</sub> for a period of 15 minutes and an initial pH reading was determined (PHC 3005-8, Radiometer Analytical). N<sub>2</sub> gassing continued throughout the analysis. Samples were titrated with 0.2 mol l<sup>-1</sup> HCl until a stable pH reading was obtained below 3.8, and then returned to the initial pH using 0.02 mol l<sup>-1</sup> NaOH (BDH Standard for both acid and base, Anachemia Canada, LLC). Acid and base additions were performed using 2 mL microburettes (GS-1200, Gilmont Instruments). Total bicarbonate and carbonate equivalents were determined by subtracting the amount of NaOH from the amount of HCl used during the titration. After titration, samples were frozen (-20°C) for further ion analysis.

Ion concentrations of cations in plasma (Na<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>), rectal fluid (Na<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>), and rectal solid carbonates (Mg<sup>2+</sup>, Ca<sup>2+</sup>) were measured using flame atomic absorption spectrometry with an air/acetylene flame (Varian, Palo Alto, CA). For anions, HCO<sub>3</sub><sup>-</sup> in plasma and rectal fluid was estimated using methods described above, while Cl<sup>-</sup> (plasma and rectal fluid) and SO<sub>4</sub><sup>2-</sup> (rectal fluid) were analyzed using anion chromatography (Dionex 120, Sunnyvale, CA).

Total rectal base secretion rate (μmol kg<sup>-1</sup> h<sup>-1</sup>; *Series 3*) was determined by adding molar amounts of HCO<sub>3</sub><sup>-</sup> equivalents in fluid and solid carbonates. Since temperature affects carbonate precipitation rates in the intestine (Wilson et al.

2009), carbonate production rates were normalized to 25°C by acclimating toadfish to various temperature levels and determining a correction factor (see *Series 5* below). Finally, drinking rate was estimated using an average of  $Mg^{2+}$  and  $SO_4^{2-}$  based estimates of drinking rates as previously described (Genz et al. 2008). Drinking rate was calculated by multiplying the rectal fluid excretion rate ( $ml^{-1} kg^{-1} h^{-1}$ ) by the  $Mg^{2+}$  content ( $mmol l^{-1}$ ) in rectal fluid and dividing this value by  $Mg^{2+}$  content ( $50 mmol l^{-1}$ ) in seawater. The same procedure was performed for  $SO_4^{2-}$ , using a value of  $30 mmol l^{-1}$  in seawater. The fraction of fluid absorbed was calculated by subtracting the rectal fluid excretion rate from the drinking rate and dividing this value by the drinking rate.

***Series 4: Effect of hypercarbia on  $CaCO_3$  collected directly from intestinal tract and from tank bottom***

Direct collection of carbonates from the intestinal tract of undisturbed toadfish was performed to substantiate findings from carbonate production rates in rectal collection sacs in toadfish that underwent surgery. In addition, concurrent collection of carbonates from the tank bottom in this series of the experiment allowed for direct comparison of collection methodologies and, as it turns out, dissolution of excreted  $CaCO_3$ . Toadfish were exposed to control (ambient), 10,000, or 20,000  $\mu atm CO_2$  for 6 days. On day 3, all carbonates and other debris were removed from the tank bottom, so that carbonates could be collected on day 6 to determine a carbonate production rate. On day 6, toadfish were sacrificed, and intestinal carbonates were collected and placed into a conical tube. To increase resolution, the carbonates from 3 fish from each 20 L tank were pooled

for intestinal and tank bottom measurements. The average mass (g) per fish was  $54.0 \pm 2.0$ ,  $54.4 \pm 2.0$ , and  $53.7 \pm 1.4$ , for control, 10,000, and 20,000  $\mu\text{atm CO}_2$  treatments, respectively. Immediately following dissections, carbonates on the bottom of each tank were collected. Double endpoint titrations were performed on samples as outlined above.

### ***Series 5: Effects of temperature on carbonate production***

During the experimental period, ambient temperatures varied more than expected ( $22\text{-}27^\circ\text{C}$ ). To test the effects of temperature on toadfish carbonate production rates, toadfish were exposed to target temperatures of 21, 25, 28, and  $31^\circ\text{C}$  for a total of 4-5 days. The first portion of the exposure (2.5-3.5 days) served as an acclimation period. All carbonates were cleared from the tank bottom following acclimation and the next 1.5 days ( $42.9 \pm 0.3$  hours) served as the collection period for carbonate production rate measurements. All fish were removed at the end of this period and carbonates were collected from the tank bottom. Toadfish were pooled at a density of 3-5 per tank in this portion of the experiment when appropriate. The average mass (g) in each tank for each treatment were  $43.4 \pm 2.5$ ,  $46.7 \pm 5.1$ ,  $41.7 \pm 4.6$ , and  $79.1 \pm 20.3$ , for 21, 25, 28, and  $31^\circ\text{C}$ , respectively. All other analytical procedures were conducted as outlined in *Series 4*. Results from this series were used to normalize all carbonate excretion rates from series 1-3.



### **Data analysis**

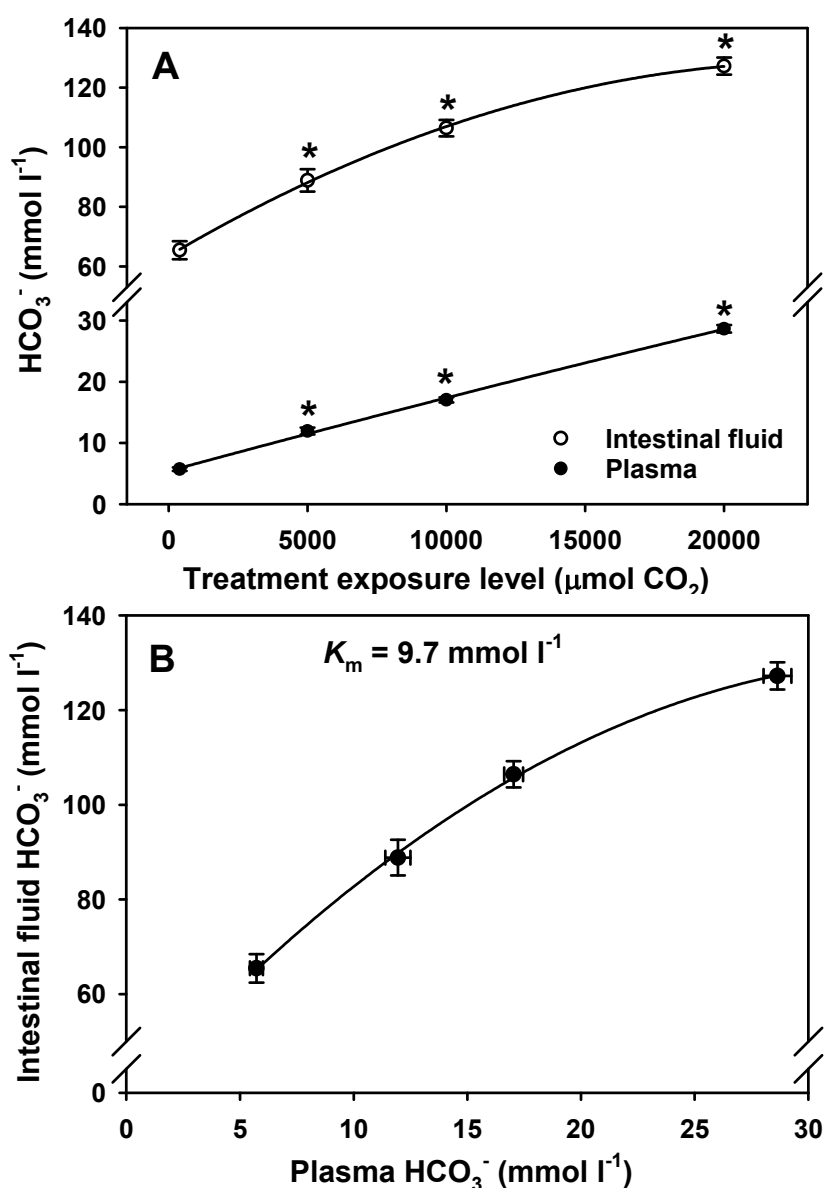
All values are reported as the mean  $\pm$  s.e.m. Since all comparisons involved at least 2 treatment groups in addition to a control group, one-way ANOVA's were used for all statistical comparisons, followed by Holm-Sidak test for multiple comparisons when appropriate. Data that were not normally distributed were tested using the non-parametric Kruskal-Wallis ANOVA on ranks test followed by Dunn's method for multiple comparisons. For temperature data, any follow-up tests were conducted using pairwise comparisons. Significant difference was determined from controls for all tests at  $P < 0.05$ .

### **Results**

#### ***Series 1: Relationship between blood acid-base chemistry and intestinal base secretion***

Plasma  $\text{HCO}_3^-$  exhibited a steady increase as a function of increased  $\text{CO}_2$  levels (quadratic polynomial fit,  $f = 5.41 + 0.0012x + -3.69e-9 x^2$ ,  $r^2 = 0.99$ ), while intestinal fluid  $\text{HCO}_3^-$  ( $\text{mmol l}^{-1}$ ) started to show signs of saturation at higher levels of  $\text{CO}_2$  (quadratic polynomial fit,  $f = 63.55 + 0.0055x + -1.16 e-7 x^2$ ,  $r^2 = 0.99$ ) (Fig. 4.1A). A five-fold increase in plasma  $\text{HCO}_3^-$  from 400  $\mu\text{atm CO}_2$  to 20,000  $\mu\text{atm CO}_2$  translated to around a two-fold increase in  $\text{HCO}_3^-$  ( $\text{mmol l}^{-1}$ ) in intestinal fluids (Fig. 4.1A). When intestinal fluid  $\text{HCO}_3^-$  was expressed as a function of plasma  $\text{HCO}_3^-$  ( $\text{mmol l}^{-1}$ ) (Fig. 4.1B), a quadratic polynomial fit best described the relationship ( $f = 37.34 + 5.29x + -0.075x^2$ ,  $r^2 = 0.99$ ). Analysis of an apparent  $K_m$  value for this relationship was calculated to be 9.7  $\text{mmol l}^{-1}$ .

Fig. 4.1: Relationship between CO<sub>2</sub> exposure level, plasma HCO<sub>3</sub><sup>-</sup> (mmol l<sup>-1</sup>) and intestinal fluid HCO<sub>3</sub><sup>-</sup> (mmol l<sup>-1</sup>): A) Plasma HCO<sub>3</sub><sup>-</sup> and intestinal fluid HCO<sub>3</sub><sup>-</sup> (mmol l<sup>-1</sup>) of Gulf toadfish following 6 days of exposure to control (~400 μatm CO<sub>2</sub>), 5,000, 10,000, and 20,000 μatm CO<sub>2</sub> (n= 17,13, 13, 14 for plasma, and n= 13, 8, 9, 11 for intestinal fluid, respectively at each CO<sub>2</sub> level). The HCO<sub>3</sub><sup>-</sup> concentration (mmol l<sup>-1</sup>) of intestinal fluid was determined from excretions collected in rectal sacs affixed on day 3 of acclimation period and sampled on day 6. B) Intestinal HCO<sub>3</sub><sup>-</sup> (mmol l<sup>-1</sup>) as a function of plasma HCO<sub>3</sub><sup>-</sup> (mmol l<sup>-1</sup>). See text for more details on curves fit to data. Values are means ± s.e.m. \*denotes statistically significant difference from control values (P<0.05).



***Series 2: Effects of hypercarbia on ion concentrations in plasma and intestinal fluid***

Similar to previous studies, the decrease in plasma  $\text{Cl}^-$  (27.9  $\text{mmol l}^{-1}$ ) appeared to be nearly equimolar to the increase in plasma  $\text{HCO}_3^-$  (22.9  $\text{mmol l}^{-1}$ ) when comparing control values to the highest  $\text{CO}_2$  exposure level (20,000  $\mu\text{atm CO}_2$ ). Hypercarbia did not significantly alter  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ , or  $\text{Ca}^{2+}$  plasma concentration ( $\text{mmol l}^{-1}$ ) (Fig. 4.2). Plasma osmolality generally declined with increasing levels of hypercarbia (One-way ANOVA;  $P > 0.032$ ; Table 4.3); however, post-hoc tests only revealed a significant decrease at the 10,000  $\mu\text{atm CO}_2$  exposure level.

A significant increase in intestinal fluid  $\text{HCO}_3^-$  ( $\text{mmol l}^{-1}$ ) at all  $\text{CO}_2$  levels occurred with a corresponding decline in  $\text{Cl}^-$  ( $\text{mmol l}^{-1}$ ) (significant at 10,000 and 20,000  $\mu\text{atm CO}_2$ ; Fig. 4.3A). This change was not equimolar as seen for the plasma, and the ratio of  $\text{HCO}_3^-$  increase to  $\text{Cl}^-$  decrease dropped as  $\text{PCO}_2$  levels increased.  $\text{Na}^+$  ( $\text{mmol l}^{-1}$ ) exhibited a slight increase with increasing exposure level that was not statistically significant (Fig. 4.3A). Outside of a transient non-significant increase in  $\text{SO}_4^{2-}$  at 5000  $\mu\text{atm CO}_2$ , all three divalent ions showed a significant decrease with increasing levels of  $\text{CO}_2$  exposure (Fig. 4.3B). A trend for a decrease in intestinal fluid osmolality was also evident, although this change was not statistically significant (Table 4.4).

Fig. 4.2: Plasma ion concentrations ( $\text{mmol l}^{-1}$ ) of toadfish exposed to various  $\text{CO}_2$  levels: Ion concentrations ( $\text{mmol l}^{-1}$ ) in plasma from Gulf toadfish following 6 days of exposure to control ( $\sim 400 \mu\text{atm CO}_2$ ), 5,000, 10,000, and 20,000  $\mu\text{atm CO}_2$  ( $n=18, 12, 12, 15$  for  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ,  $n= 17, 13, 13, 14$  for  $\text{HCO}_3^-$  and  $n= 18, 9, 12, 14$  for  $\text{Cl}^-$ ). Samples were taken from fish that had a rectal collection sac affixed on day 3 of the exposure period. Note that  $\text{HCO}_3^-$  ( $\text{mmol l}^{-1}$ ) is reported in both Fig 4.1 and 4.2. Values are means  $\pm$  s.e.m. \*denotes statistically significant difference from control values ( $P<0.05$ ).

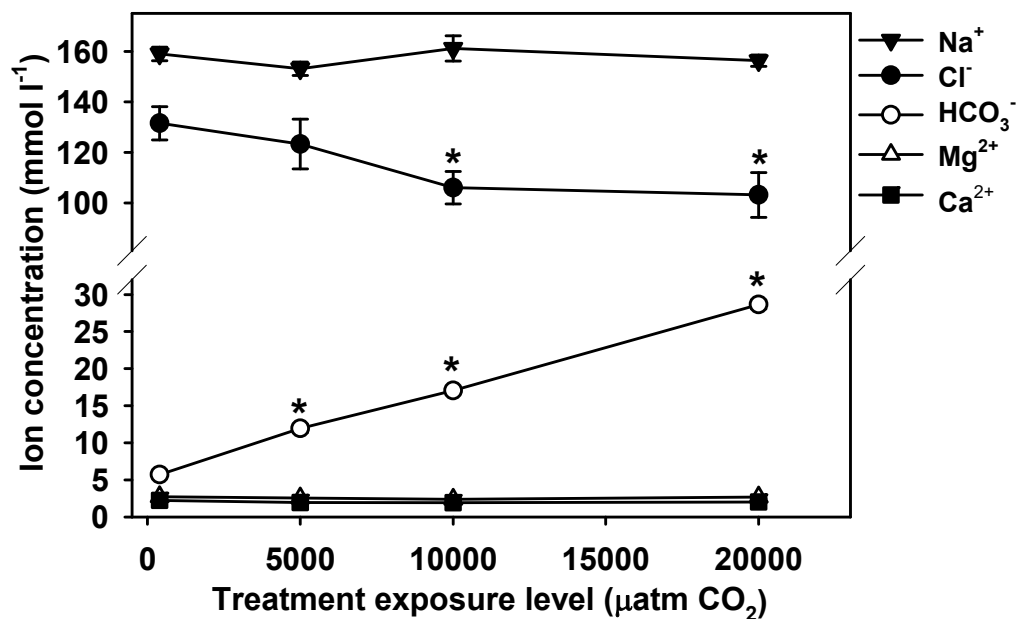


Table 4.3: Osmolality, and pH from plasma of Gulf toadfish (*Opsanus beta*) exposed to various CO<sub>2</sub> levels (μatm CO<sub>2</sub>)

	Plasma	
	mOsm	pH
Control	314.2 ± 2.5 (18)	7.60 ± 0.03 (17)
5000	312.1 ± 2.1 (12)	7.66 ± 0.02 (13)
10000	304.3 ± 2.8* (13)	7.73 ± 0.01 (13)*
20000	307.5 ± 2.3 (15)	7.74 ± 0.01 (14)*

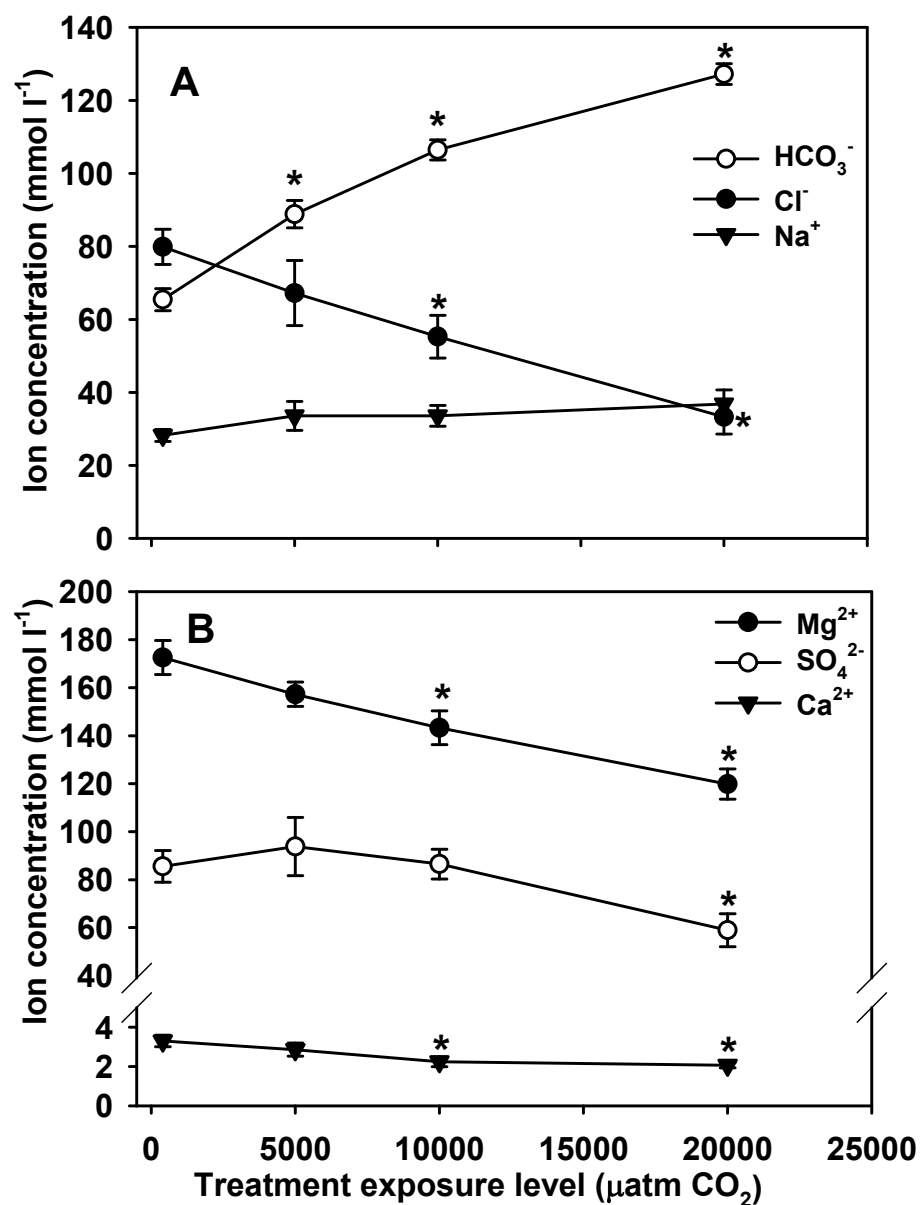
Osmolality (mOsm), and pH in blood plasma of Gulf toadfish exposed to control (~400 μatm CO<sub>2</sub>), 5,000 μatm CO<sub>2</sub>, 10,000 μatm CO<sub>2</sub>, and 20,000 μatm CO<sub>2</sub> for 6 days. Values are means ± s.e.m and n-numbers are in parentheses. \*denotes statistical significance from control values (P<0.05)

Table 4.4: Osmolality, and pH from intestinal fluid of Gulf toadfish (*Opsanus beta*) exposed to various CO<sub>2</sub> levels (μatm CO<sub>2</sub>)

	Intestinal fluid	
	mOsm	pH
Control	330.7 ± 5.8 (13)	8.50 ± 0.02 (13)
5000	327.2 ± 8.0 (8)	8.44 ± 0.03 (8)
10000	321.3 ± 10.0 (9)	8.48 ± .02 (9)
20000	316.0 ± 4.4 (11)	8.31 ± .02 (11)*

Osmolality (mOsm), and pH in intestinal fluid of Gulf toadfish exposed to control (~400 μatm CO<sub>2</sub>), 5,000 μatm CO<sub>2</sub>, 10,000 μatm CO<sub>2</sub>, and 20,000 μatm CO<sub>2</sub> for 6 days. Measurements of intestinal fluid were determined from the fluid component of excretions collected in rectal sacs affixed on day 3 of acclimation period and collected on day 6. Values are means ± s.e.m and n-numbers are in parentheses. \*denotes statistical significance from control values (P<0.05)

Fig. 4.3: Intestinal fluid ion concentrations ( $\text{mmol l}^{-1}$ ) of toadfish exposed to various  $\text{CO}_2$  levels: Concentrations ( $\text{mmol l}^{-1}$ ) of A) monovalent and B) divalent ions from intestinal fluid of Gulf toadfish following 6 days of exposure to control ( $\sim 400 \mu\text{atm CO}_2$ ), 5,000, 10,000, and 20,000  $\mu\text{atm CO}_2$  ( $n= 13, 8, 9, 11$ , respectively for all ions at each  $\text{CO}_2$  level). Ion concentrations of intestinal fluid were determined from excretions collected in rectal sacs affixed on day 3 of acclimation period and sampled on day 6. Note that  $\text{HCO}_3^-$  ( $\text{mmol l}^{-1}$ ) are presented in both Fig 4.1 and Fig 4.3. Values are means  $\pm$  s.e.m. \*denotes statistically significant difference from control values ( $P < 0.05$ ).



***Series 3: Effects of hypercarbia on intestinal fluid movement, base secretion rate, and carbonate composition***

Although  $\text{SO}_4^{2-}$  was lower at the highest  $\text{CO}_2$  tension, both the drinking rate and the rectal fluid excretion rate did not change with  $\text{CO}_2$  exposure (Fig. 4.4). In contrast, the fraction of fluid absorbed from imbibed seawater was significantly lower at 20,000  $\mu\text{atm CO}_2$  (Fig. 4.4).

Similar to findings at lower  $\text{CO}_2$  levels, the fluid base secretion rate and the total (fluid combined with solid  $\text{CaCO}_3$ ) base secretion rate were significantly increased with  $\text{CO}_2$  exposure (Fig. 4.5). The solid, or  $\text{CaCO}_3$ , base secretion did not change with  $\text{CO}_2$  exposure. Consequently, the fluid component of the base secretion became an increasing proportion of the total base secretion at increasing levels of  $\text{CO}_2$  exposure.

It is important to note that there was some variation in ambient water temperatures, generally ranging between 22-27 °C, throughout the rectal collection sac experiments. Since temperature has been previously demonstrated to affect  $\text{CaCO}_3$  precipitation rate in the sheepshead minnow (Wilson et al. 2009), the relationship between temperature and  $\text{CaCO}_3$  production was estimated for the toadfish in the present study (see “*Series 5*” below for more details). Accordingly, solid  $\text{CaCO}_3$  base secretion rates were normalized to 25°C to account for effects of inadvertent temperature variation in rectal collection sac experiments.

Fig. 4.4 Drinking rate, rectal fluid excretion rate, and fraction fluid absorption of toadfish exposed to various CO<sub>2</sub> levels: Estimated drinking rate (ml kg<sup>-1</sup> h<sup>-1</sup>), rectal fluid excretion rate (ml kg<sup>-1</sup> h<sup>-1</sup>), and fractional fluid absorption of Gulf toadfish following 6 days of exposure to control (~400 μatm CO<sub>2</sub>), 5,000, 10,000, and 20,000 μatm CO<sub>2</sub> (n=11, 8, 9, 10, respectively). Drinking rates were estimated using intestinal fluid Mg<sup>2+</sup> and SO<sub>4</sub><sup>2-</sup> concentrations (mmol<sup>-1</sup>) from rectal collection sacs affixed on day 3 and sampled on day 6 of the acclimation period and also from concentrations of these ions in seawater (Genz et al. 2008). The fractional fluid absorption was calculated by dividing the fluid absorption rate by the drinking rate ((drinking rate-fluid absorption rate)/drinking rate). Values are means ± s.e.m. \*denotes statistically significant difference from control values (P<0.05).

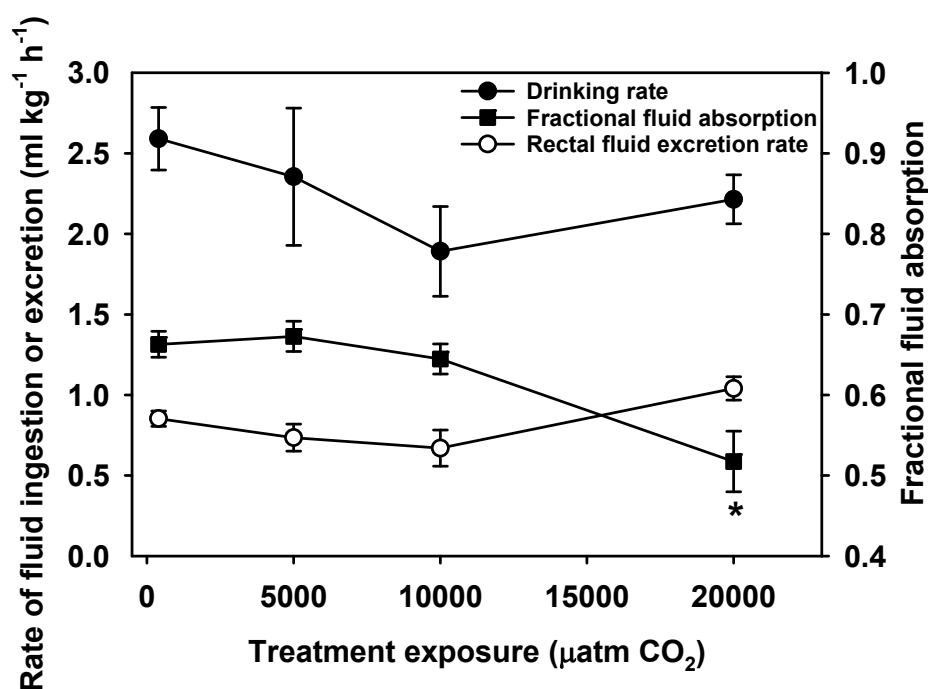
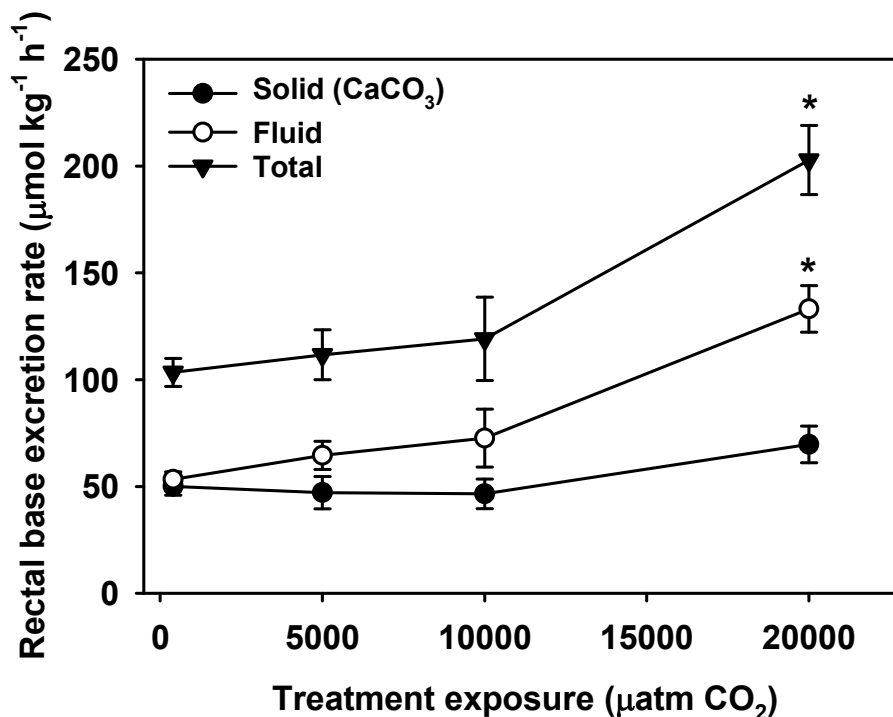




Fig. 4.5: Rectal base secretion rates of toadfish exposed to various CO<sub>2</sub> levels: Rectal base excretion rates ( $\mu\text{mol kg}^{-1} \text{h}^{-1}$ ) of Gulf toadfish following 6 days of exposure to control ( $\sim 400 \mu\text{atm CO}_2$ ), 5,000, 10,000 and 20,000  $\mu\text{atm CO}_2$  (n=11, 8, 9, 10, respectively). Ion concentrations of intestinal fluid was determined from the fluid component of excretions collected in rectal sacs affixed on day 3 of acclimation period and collected on day 6. Total rectal base excretion is the sum of solid (carbonate) and fluid base equivalents. Values are means  $\pm$  s.e.m. \*denotes statistically significant difference from control values ( $P < 0.05$ ).



Ion analysis of carbonates from rectal collection sacs revealed no difference in the proportion of  $\text{CaCO}_3$  as  $\text{Mg}^{2+}$  relative to total  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  ions (Table 4.5), suggesting there were no changes in carbonate composition. The proportion of total  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  in the rectal sac excretion present as solid  $\text{CaCO}_3$  or the intestinal fluid did not change with  $\text{CO}_2$  exposure (Table 4.6). These results also support the finding that the ionic composition of carbonates was not altered.

Table 4.5: Percentage of carbonates as Mg<sup>2+</sup>

CO <sub>2</sub> level ( $\mu$ atm CO <sub>2</sub> )	%Mg
Control	50.7 $\pm$ 1.3
5000	52.6 $\pm$ 2.8
10000	50.5 $\pm$ 1.5
20000	46.8 $\pm$ 1.6

Percentage of whole carbonate (Ca<sup>2+</sup> and Mg<sup>2+</sup>) present as Mg<sup>2+</sup>. Values are means  $\pm$  s.e.m. (P<0.05)

Table 4.6: Relative percent of Mg<sup>2+</sup> and Ca<sup>2+</sup> in intestinal fluid and CaCO<sub>3</sub> (solid) portion of excretions from rectal collection sacs

	Ca <sup>2+</sup>		Mg <sup>2+</sup>	
	% in fluid	% in carbonate	% in fluid	% in carbonate
Control	18.0 $\pm$ 1.8	82.0 $\pm$ 1.8	91.5 $\pm$ 0.4	8.5 $\pm$ 0.4
5,000	19.4 $\pm$ 5.0	80.6 $\pm$ 5.0	90.6 $\pm$ 1.3	9.4 $\pm$ 1.3
10,000	12.5 $\pm$ 1.5	87.5 $\pm$ 1.5	89.4 $\pm$ 0.9	10.6 $\pm$ 0.9
20,000	13.0 $\pm$ 2.1	87.0 $\pm$ 2.1	89.3 $\pm$ 1.1	10.7 $\pm$ 1.1

Percent of Ca<sup>2+</sup> and Mg<sup>2+</sup>, respectively, as fluid and solid (CaCO<sub>3</sub>) component of total excretion collected from Gulf toadfish exposed to control ( $\sim$ 400  $\mu$ atm CO<sub>2</sub>), 5,000, 10,000, and 20,000  $\mu$ atm CO<sub>2</sub> for 6 days (n=11, 8, 9, 10, respectively). Measurements of intestinal fluid were determined from the fluid component of excretions collected in rectal sacs affixed on day 3 of acclimation period and collected on day 6. Values are means  $\pm$  s.e.m. \*denotes statistical significance from control values (P<0.05)

#### ***Series 4: Effect of hypercarbia on CaCO<sub>3</sub> collected directly from intestinal tract and from tank bottom***

To confirm that surgical procedures and the use of rectal sacs outlined above did not affect the ability to detect a change in carbonate formation in the intestine, carbonates were collected directly from undisturbed toadfish exposed to control, 10,000, or 20,000  $\mu$ atm CO<sub>2</sub> for 6 days. Similar to findings from rectal collection sacs, these spot collections from toadfish showed a trend for an increase

in carbonate production with increasing CO<sub>2</sub> levels, however it was not statistically significant (Fig. 4.6A). In contrast to intestinal carbonate production, precipitates collected from the tank bottom were significantly reduced at 20,000  $\mu\text{atm}$  CO<sub>2</sub> (Fig. 4.6B).

### ***Series 5: Effects of temperature on carbonate production***

To account for the effects of temperature on carbonate production, rectal carbonates produced by toadfish over  $\sim 1.5$  days ( $42.9 \pm 0.3$  hours) were collected from the tank bottom. Carbonate production rate ( $\mu\text{mol kg}^{-1} \text{ h}^{-1}$ ) increased significantly with increasing temperature (Fig. 4.7; Linear fit,  $y=1.6455x -26.986$ ,  $r^2=0.96$ ).

Fig. 4.6: Carbonates found in the toadfish intestine compared to carbonate dissolution rate in tank bottom with varied CO<sub>2</sub> exposure: Intestinal carbonate content in A) was determined from carbonates residing in the intestine of fish sacrificed after 6 days of exposure to control (~400 μatm CO<sub>2</sub>), 10,000, and 20,000 μatm CO<sub>2</sub> (n=5 groups of 3 fish). B) Net intestinal carbonate appearance in tanks of Gulf toadfish acclimated to control (~400 μatm CO<sub>2</sub>), 10,000, and 20,000 μatm CO<sub>2</sub> (n=5 groups of 3 fish) for 3 days. All carbonates were cleared from the tank bottom after the first three days, and carbonates were collected from day 3 to day 6 to determine the carbonate production rate. Values are means ± s.e.m. \*denotes statistically significant difference from control values (P<0.05).

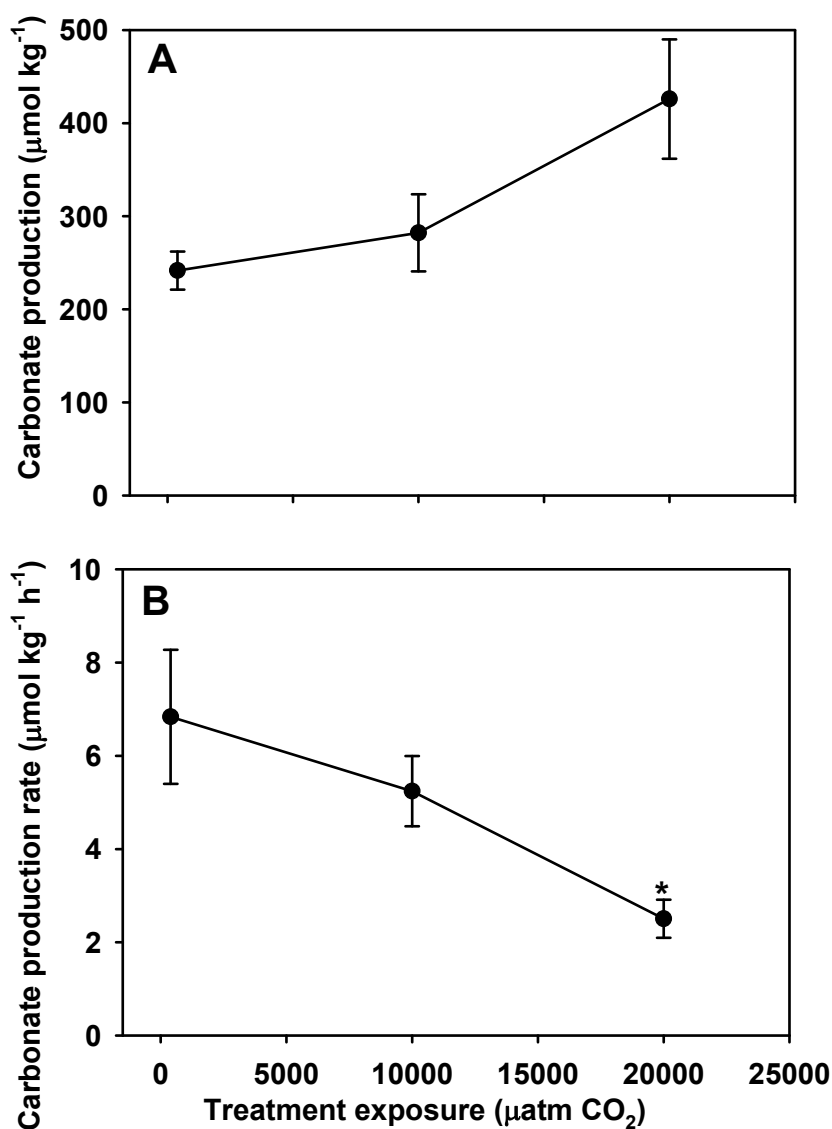
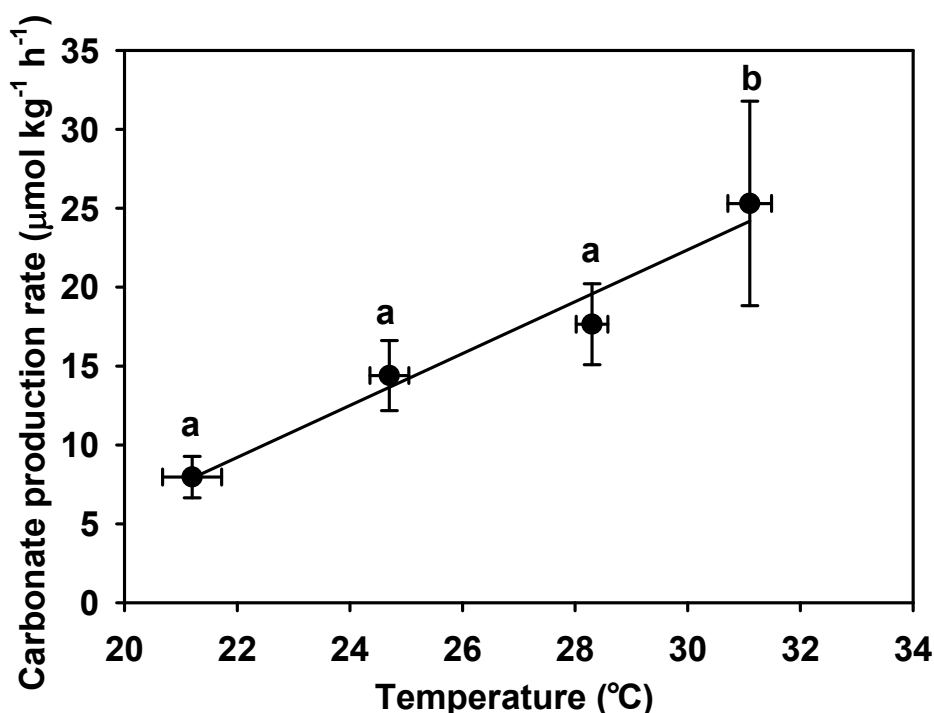


Fig. 4.7: Carbonate production rate ( $\mu\text{mol kg}^{-1} \text{h}^{-1}$ ) of Gulf toadfish as a function of temperature ( $^{\circ}\text{C}$ ) at ambient control  $\text{CO}_2$  ( $\sim 400 \mu\text{atm CO}_2$ ). Carbonate production rates were determined by performing double endpoint titrations (Genz et al 2008) on carbonates collected from the tank bottom following  $\sim 1.5$  days ( $42.9 \pm 0.3$  hours) of exposure. Temperature acclimation n-numbers were 6, 12, 12, and 4, at  $21.2^{\circ}\text{C}$ ,  $24.7^{\circ}\text{C}$ ,  $24.8^{\circ}\text{C}$ , and  $31.1^{\circ}\text{C}$ , respectively. 4). Due to the small size of some fish, carbonates from more than one fish per tank were pooled for analysis (see text for further detail). Values are means  $\pm$  s.e.m. \*letters represent statistically significant differences using a one-way ANOVA, followed by a pairwise multiple comparison (Holm-Sidak;  $P < 0.05$ ). Linear fit ( $y = 1.6455x - 26.986$ ,  $r^2 = 0.96$ )



## Conclusions

### ***Series 1: Relationship between blood acid-base chemistry and intestinal base secretion***

Like many other species, toadfish accumulate  $\text{HCO}_3^-$  to correct pH during a  $\text{CO}_2$ -induced acidosis. This nearly linear rise in plasma  $\text{HCO}_3^-$  was dose-dependent and tightly regulated, suggesting that toadfish have the capacity to cope

with even higher CO<sub>2</sub> levels (Fig. 4.1A). In contrast to the other species, toadfish appear to compensate for a CO<sub>2</sub>-induced acidosis through HCO<sub>3</sub><sup>-</sup> uptake rather than acid excretion, as fish held in nominally HCO<sub>3</sub><sup>-</sup>-free seawater (182 μmol l<sup>-1</sup>) were unable to effectively correct pH (Esbaugh et al. 2012). While this mechanism was used by toadfish exposed to 1900 μatm CO<sub>2</sub>, it is unknown whether or not this strategy would shift to H<sup>+</sup> excretion with increasing CO<sub>2</sub> exposure. Plasma pH was significantly increased in the 10,000 and 20,000 μatm CO<sub>2</sub> exposures compared to controls (Table 4.). This trend would be opposite if compensation were incomplete, as has been noted at high levels of CO<sub>2</sub> in certain species, where pHe is partially compensated and pH<sub>i</sub> is preferentially regulated (Brauner and Baker 2009). Overall, the plasma pH values were low, as would be expected for samples obtained by caudal puncture due to PCO<sub>2</sub> increases in anesthetized and air exposed fish. It is possible that samples from fish exposed to higher CO<sub>2</sub> levels, where blood PCO<sub>2</sub> was higher relative to ambient air, could have experienced greater CO<sub>2</sub> off-gassing, raising pH. Indeed, calculated PCO<sub>2</sub> values for plasma samples approach PCO<sub>2</sub> levels of the water as PCO<sub>2</sub> increased, and was lower than ambient in fish exposed to 20,000 μatm CO<sub>2</sub> (data not shown). Accordingly, caution should be applied when interpreting the apparent overshoot of pH compensation. Though HCO<sub>3</sub><sup>-</sup> accumulation in the plasma does not appear to be limited, HCO<sub>3</sub><sup>-</sup> (mmol l<sup>-1</sup>) in intestinal fluid does appear to plateau (Fig. 4.1A), suggesting that there may be an upper limit to HCO<sub>3</sub><sup>-</sup> secretion into the intestine. Interestingly, when intestinal HCO<sub>3</sub><sup>-</sup> was plotted as a function of plasma HCO<sub>3</sub><sup>-</sup> (Fig. 4.1B), the calculated apparent *K<sub>m</sub>* (9.7 mmol l<sup>-1</sup>) was similar to that noted

during *in vitro* isolated tissue experiments performed in a combined Ussing/pH stat system ( $10.2 \text{ mmol l}^{-1}$ ), and was also similar to the  $K_m$  for  $\text{HCO}_3^-$  uptake in isolated tfNBCe1 expressed in *Xenopus oocytes* ( $8.5 \text{ mmol l}^{-1}$ ) (Taylor et al. 2010). This finding suggests that *in vitro* and *in vivo* transport characteristics are likely similar and lends support to the use of isolated tissue analyses to study intestinal transport mechanisms.

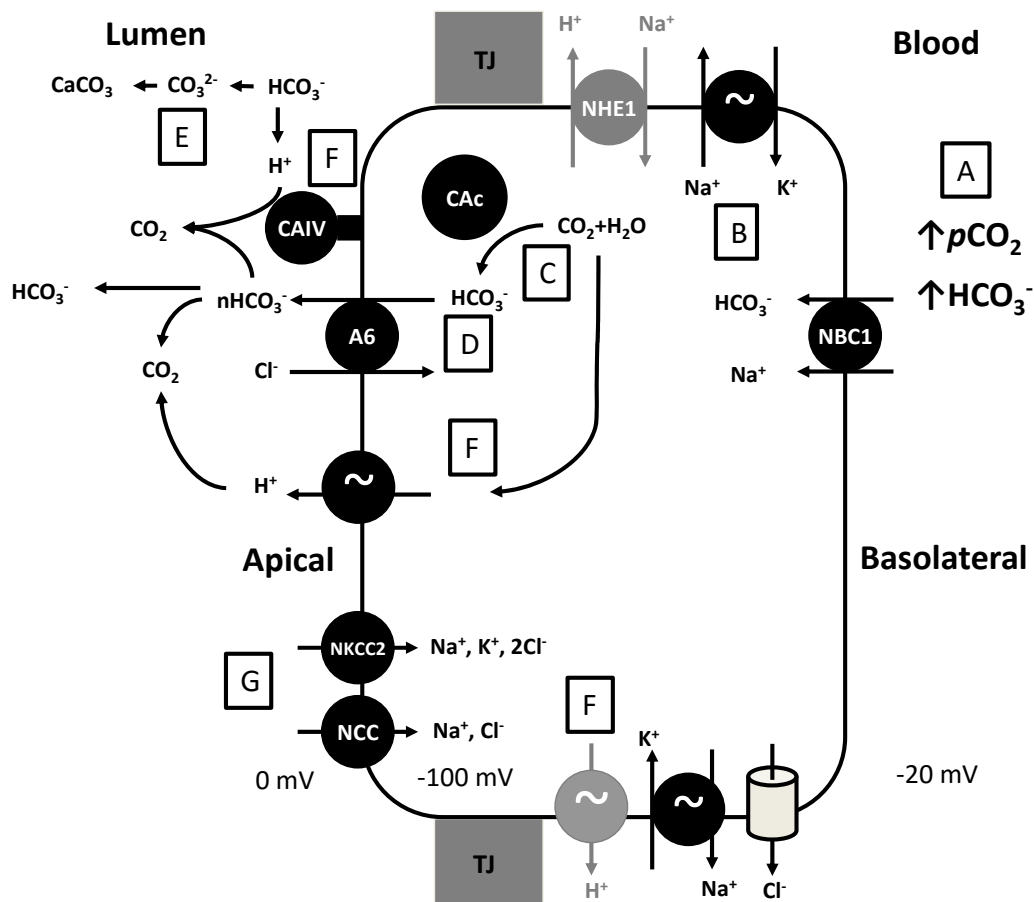
### ***Series 2: Effects of hypercarbia on ion concentrations in plasma and intestinal fluid***

Similar to previous studies (Brauner and Baker 2009; Claiborne et al. 2002; Hayashi et al. 2004; Larsen and Jensen 1997; Toews et al. 1983), the increase in plasma  $\text{HCO}_3^-$  was paired with a nearly equimolar decline in plasma  $\text{Cl}^-$ . In the intestinal fluid, increased  $\text{HCO}_3^-$  ( $\text{mmol l}^{-1}$ ) was mirrored by a reduction in  $\text{Cl}^-$  ( $\text{mmol l}^{-1}$ ), providing robust support to previous findings at lower  $\text{CO}_2$  levels that exposure to  $\text{CO}_2$  impacts apical anion exchange in the intestine (Heuer et al. 2012). This increase in  $\text{HCO}_3^-$  loss from the body counteracts whole-body acid-base balance, since the retention and/or uptake of  $\text{HCO}_3^-$  is necessary for compensation. Anion exchanger *slc26a6* has been cloned in both the seawater acclimated pufferfish (Kurita et al. 2008) and the toadfish (Grosell et al. 2009b), and is an electrogenic exchanger ( $\text{nHCO}_3^-/\text{Cl}^-$ ). Results of the present study comparing the concentrations of  $\text{HCO}_3^-$  and  $\text{Cl}^-$  support this assertion since exchange was not equimolar in control fish. Although it is clear that anion exchange increases drastically with  $\text{CO}_2$  exposure in the intestine, it appears that the ratio of the  $\Delta\text{HCO}_3^-/\Delta\text{Cl}^-$  at each  $\text{CO}_2$  level relative to control values declines as  $\text{CO}_2$  exposure

increased ( $\Delta\text{HCO}_3^-/\Delta\text{Cl}^-$  1.85, 1.66, and 1.33 at 5,000, 10,000, and 20,000  $\mu\text{atm}$   $\text{CO}_2$ , respectively). This change in ratios is not likely due to a shift in  $\text{Cl}^-$  uptake from *slc26a6* to apical NCC or NKCC1 co-transport, since  $\text{Na}^+$  concentrations remained stable across  $\text{CO}_2$  levels (Fig. 4.3 and Fig. 4.8). Alternatively, this reduction could theoretically reflect an increase in the incorporation of  $\text{HCO}_3^-$  into  $\text{CaCO}_3$  precipitates, but there was no significant change in carbonate precipitation. A third viable explanation for this observation is an increase in the activity of the recently recognized apical  $\text{H}^+$  pump. The  $\text{H}^+$  pump secretes  $\text{H}^+$  into the intestinal lumen and titrates some of the secreted  $\text{HCO}_3^-$ , reducing  $\text{HCO}_3^-$  accumulation in the boundary layer, and decreasing osmotic pressure, both of which are thought to facilitate continued anion exchange and water absorption during salinity stress (Grosell 2011a; Guffey et al. 2011) (Fig. 4.8). Significantly lower pH in the intestinal fluid of fish exposed to the highest  $\text{CO}_2$  level (20,000  $\mu\text{atm}$   $\text{CO}_2$ ) also supports the idea of an increase in apical  $\text{H}^+$  pump activity. Suggested impacts of hypercarbia on an existing cellular intestinal transport model are illustrated in Figure 4.8. This cellular model incorporates new findings from this study, such as the lack of difference in  $\text{Na}^+$  transport and potential alterations in  $\text{H}^+$  pump activity.



Fig. 4.8: Proposed effects of elevated  $\text{CO}_2$  on epithelial transport in the Gulf toadfish (*Opsanus beta*): Toadfish exposed to elevated  $\text{CO}_2$  in seawater show a tightly regulated increase of  $\text{HCO}_3^-$  in the blood plasma to defend pH. A) Elevation of plasma  $\text{HCO}_3^-$  likely stimulates  $\text{Na}^+:\text{HCO}_3^-$  co-transporter NBC1 (Taylor et al. 2010), increasing cytosolic levels of  $\text{HCO}_3^-$  and  $\text{Na}^+$ . B) Elevated cellular  $\text{Na}^+$  influx could potentially lead to an increase in  $\text{Na}^+:\text{K}^+$  ATPase mRNA expression and/or activity. Such an elevation would increase energetic demand, leading to an increase in  $\text{O}_2$  consumption and metabolic intracellular  $\text{CO}_2$  production. C) Resulting increased intracellular  $\text{CO}_2$  would provide more substrate for carbonic anhydrase (CAc). Equilibration of plasma  $\text{PCO}_2$  (A) with the intestinal cell provides a second avenue for increased intracellular  $\text{PCO}_2$  available for hydration via CAc. D)  $\text{HCO}_3^-$  resulting from increased basolateral NBC1 transport and/or increased hydration of intracellular  $\text{PCO}_2$  likely leaves the cell on the apical side via SLC26a6 in exchange for  $\text{Cl}^-$ . Impacts to anion exchange are supported by a large increase in  $\text{HCO}_3^-$  ( $\text{mmol l}^{-1}$ ) in intestinal fluid that corresponds to a large decrease in  $\text{Cl}^-$  ( $\text{mmol l}^{-1}$ ) with increasing  $\text{CO}_2$  exposure (Fig. 4.3).  $\text{Cl}^-$  likely exits the cell through chloride channels on the basolateral membrane. E) Some  $\text{CO}_3^{2-}/\text{HCO}_3^-$  combines with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  to form high  $\text{Mg}^{2+}$   $\text{CaCO}_3$ , however, despite an increase in  $\text{HCO}_3^-$  secretion into the intestinal lumen, the production and composition of carbonates are not impacted by hypercarbia in the Gulf toadfish. F) Protons ( $\text{H}^+$ ) generated from intracellular  $\text{CO}_2$  hydration exit the cell, in part, through apical  $\text{H}^+$  pump (vacuolar-ATPase) (Guffey et al. 2011) or potentially through  $\text{Na}^+$ -dependent extrusion. Protons secreted into the lumen may titrate some  $\text{HCO}_3^-$  through dehydration via membrane-bound carbonic anhydrase (CAIV) to form  $\text{CO}_2$  that presumably titrates  $\text{HCO}_3^-$  and reduces luminal osmotic pressure. It is not clear whether or not processes in (F) would be impacted by hypercarbia. G) Despite increased  $\text{Na}^+$  movement into the cell through NBC1 described in (B), there was no change to plasma or luminal  $\text{Na}^+$  ( $\text{mmol l}^{-1}$ ), suggesting that  $\text{Na}^+$  uptake from the lumen into the cell via existing  $\text{Na}^+$  transport pathways (NKCC2 and NCC) are likely not stimulated with hypercarbia. See (Grosell 2011a, b) for further detail on marine fish intestinal cellular transport mechanisms.



All three divalent ions ( $\text{Mg}^{2+}$ ,  $\text{SO}_4^{2-}$ ,  $\text{Ca}^{2+}$ ) were significantly lower at higher  $\text{CO}_2$  levels (Fig. 4.3B). Although these ions are concentrated in the lumen as fish drink seawater since they are relatively impermeable to absorption in the intestine, a relatively large proportion of  $\text{Ca}^{2+}$  in the rectal excretion is incorporated into the precipitation of  $\text{CaCO}_3$ . In isolation, a lower concentration of  $\text{Ca}^{2+}$  ( $\text{mmol l}^{-1}$ ) at higher  $\text{CO}_2$  levels would suggest an increase in precipitation. However, the lack of a significant difference in carbonate rectal output and similar patterns of decline in other divalent ions ( $\text{Mg}^{2+}$  and  $\text{SO}_4^{2-}$ ) suggests that fluid transport is impacted by hypercarbia, at least at high levels of  $\text{CO}_2$ . Decreased fractional fluid absorption at 20,000  $\mu\text{atm CO}_2$  supports this assertion and is discussed below.

***Series 3: Effects of hypercarbia on intestinal fluid movement, base secretion rate, and carbonate composition***

Neither the calculated rate of seawater ingestion (drinking rate) nor the rectal fluid excretion rate were significantly impacted by hypercarbia (Fig. 4.4). The control of drinking is under hormonal control and is responsive to changes in plasma osmolality and external salinity (see (Takei and Loretz 2011) for a detailed review). Luminal levels of  $\text{Na}^+$  and  $\text{Cl}^-$  have also been hypothesized to control drinking rate in the Japanese eel in a feedback system where  $\text{Cl}^-$  inhibits drinking at higher luminal levels but allows for  $\text{Na}^+$  to stimulate drinking at lower levels (Ando and Nagashima 1996). If this is the case for marine teleosts in general, the lack of change in  $\text{Na}^+$  could explain why drinking rate was not influenced by hypercarbia despite dramatic reductions in luminal  $\text{Cl}^-$  ( $\text{mmol l}^{-1}$ ). Considering both the drinking rate and the rectal fluid excretion rate, an estimate of imbibed fluid that

is absorbed can be assumed. At the highest CO<sub>2</sub> level, fish did not change their drinking rate, but absorbed significantly less of the imbibed fluid than control fish (Fig. 4.4). The ultimate cause of this lowered fractional fluid absorption is unclear, especially considering there may have been a slightly more favorable osmotic gradient for water movement from the intestine to the blood. The absolute reduction in osmolality was smaller in the plasma ( $\sim\Delta 6$  mOsm) than what was observed in the intestinal fluid ( $\sim\Delta 15$  mOsm) at 20,000  $\mu\text{atm}$  CO<sub>2</sub>.

Similar to previous findings (Heuer et al 2012), the total and fluid rate of rectal base secretion increased with CO<sub>2</sub> exposure, but there was no change in the solid CaCO<sub>3</sub> base secretion rate (Fig. 4.5), suggesting that carbonate production in the toadfish does not change even at high levels of hypercarbia. Midshipman show an increased titratable alkalinity of carbonates when exposed to 50,000  $\mu\text{atm}$  CO<sub>2</sub> for 48 hours (Perry et al. 2010), so it is unclear whether or not the lack of difference in toadfish reflects a species-specific difference, is related to the level of CO<sub>2</sub> exposure, or ambient temperature (the Midshipmen study was conducted at 13°C). In addition to production rates, the composition of carbonates was unchanged with hypercarbia. It was previously hypothesized that an increase in Mg<sup>2+</sup> incorporation could occur with hypercarbia if already low levels of Ca<sup>2+</sup> became limiting for precipitation with an increase in carbonate production since Mg<sup>2+</sup> is typically high in luminal fluids (Heuer et al. 2012). This was clearly not the case since carbonate production did not change, and divalent ions declined in a similar manner as described above. In the species studied to date, Mg<sup>2+</sup> content and structure of carbonates vary considerably (Perry et al. 2011; Salter et al.

2012). Accordingly, examining both production and composition in other species during hypercarbia may be valuable.

***Series 4: Effect of hypercarbia on CaCO<sub>3</sub> collected directly from intestinal tract and from tank bottom***

To confirm that surgical procedures and the use of rectal sacs did not affect the ability to detect a change in carbonate formation in the intestine, carbonates were spot-collected from undisturbed toadfish exposed to control, 10,000, or 20,000  $\mu\text{atm CO}_2$  using the same exposure time as above (6 days). Carbonates collected using this alternative method also showed no significant increase in production (Fig. 4.6A), confirming that this endpoint does not change in response to hypercarbia in the toadfish. In contrast, collections of carbonates from the tank bottom indicated a significant dose-dependent decrease in carbonate production that was significant at 20,000  $\mu\text{atm CO}_2$ . This decrease is not due to a lack of production, since the intestinal lumen showed constant carbonate levels regardless of ambient  $\text{PCO}_2$ , but rather a product of dissolution that occurred in the tank bottom due to reduced seawater pH. The low value of carbonate production (Fig. 4.6B) during both control and high  $\text{CO}_2$  exposure, as assessed by collection from the tank bottom compared to that found in the rectal collection sac, may suggest that some degree of dissolution in seawater occurs, even at normal seawater pH. Indeed, toadfish carbonates have been found to be 1.95 times more soluble than aragonite (Woosley et al. 2012), pointing to a likelihood of dissolution at normal pH that would be enhanced by reduced seawater pH. The pH in luminal fluids is typically well above seawater pH (8.50 in this particular study), indicating

that carbonates are bathed in highly alkaline conditions prior to excretion. Since the solubility and structure of carbonates varies across species, the stability of carbonates post-excretion may also vary. For example, highly soluble amorphous excretions tend to undergo rapid dissolution in seawater (Foran 2013), and would likely not be a good candidate for collection from the tank bottom as a proxy for excretion rates.

### ***Series 5: Effects of temperature on carbonate production***

As seen in another study on the sheepshead minnow (*Cyprinodon variegatus*) (Wilson et al. 2009), temperature significantly increased carbonate production from the toadfish. The calculated temperature quotient ( $Q_{10}$ ) for this increase was 3.21, a value similar to the previously reported  $Q_{10}$  value (2.33) for carbonate production as a function of temperature in the sheepshead minnow (Wilson et al. 2009). While the mechanism underlying this relationship was not specifically addressed in this study, increased temperature is thought to elevate both baseline metabolism and drinking rate (Wilson et al. 2009).  $\text{CaCO}_3$  excretion rates in rectal collection sac experiments were corrected to a single temperature (25°C) using the relationship between temperature and carbonate production. This adjustment did not change the outcome of the presented experiments significantly but likely provides a better representation of the solid carbonate production rate.

### ***Conclusions and future directions***

It is clear from intestinal fluid measurements that hypercarbia leads to an increase in apical anion exchange in at least two fish species and across a wide

range of CO<sub>2</sub> levels (1,900 to 20,000 μatm CO<sub>2</sub> in the Gulf toadfish, 50,000 μatm CO<sub>2</sub> in the Plainfin midshipmen) (Heuer et al. 2012; Perry et al. 2010). Increased gene expression of AE1a in the intestine of seawater-acclimated medaka following hypercarbia exposure (7,000 μatm CO<sub>2</sub>) also supports these findings (Tseng et al. 2013), suggesting that this response may be ubiquitous among marine teleosts. Continuous base loss through the intestine counteracts measures at the gill to attain net HCO<sub>3</sub><sup>-</sup> retention and defend blood pH. The functional consequence of this sustained base loss is unclear, but could result in a reallocation or an increase in energetic demands on the intestine, since many transporters involved in HCO<sub>3</sub><sup>-</sup> transport are secondarily active and depend on the gradients established by the Na<sup>+</sup>K<sup>+</sup>ATPase (Grosell 2011a).

While impacts to anion exchange with respect to CO<sub>2</sub> are evident across all examined CO<sub>2</sub> levels, the intracellular source of this extra HCO<sub>3</sub><sup>-</sup> could come from transepithelial HCO<sub>3</sub><sup>-</sup> transport and/or increased intracellular CO<sub>2</sub> hydration (Fig. 4.8). Interestingly, the transepithelial movement of HCO<sub>3</sub><sup>-</sup> does not generate a proton. However, the hydration of intracellular CO<sub>2</sub> creates HCO<sub>3</sub><sup>-</sup> available for apical anion exchange while concurrently producing a proton that must be eliminated from the cell to avoid intracellular acidification. Since these sources could differ in terms of the cascade effects on intestinal transport, understanding this balance may be important, especially since species already vary in their modes of Cl<sup>-</sup> absorption. For example, the seawater acclimated trout obtains all of its HCO<sub>3</sub><sup>-</sup> for anion exchange from intracellular hydration (Grosell et al. 2009a), whereas the European flounder (*Platichthys flesus*) and Gulf toadfish can obtain

up to 50% through transepithelial  $\text{HCO}_3^-$  transport (Grosell and Genz 2006; Grosell et al. 2005).

Unlike anion exchange, carbonate production measured using two different methods (rectal collection sacs and spot sampling) does not appear to change under high  $\text{CO}_2$ , at least in the toadfish. A third method, utilizing collections from the tank bottom, illustrated that under high  $\text{CO}_2$  levels, carbonates excreted by fish tend to undergo some degree of dissolution. In addition to production, all measures of carbonate composition, including proportionate amounts of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  exhibited no difference. Since  $\text{Mg}^{2+}$  content is often proportional with solubility (Wilson et al. 2009; Woosley et al. 2012), it is likely that carbonates do not become more soluble with  $\text{CO}_2$  exposure, although ocean acidification could increase dissolution of carbonates. Unlike  $\text{CO}_2$ , temperature does affect carbonate production, which is not unexpected given that elevated temperature elevates metabolic rate. While fluid absorption was lower at the highest  $\text{CO}_2$  level, drinking rate and the rectal fluid excretion rate were not altered with  $\text{CO}_2$ , indicating that hypercarbia does not stimulate an increase in fluid ingestion.

Furthermore, it is interesting that both salinity and hypercarbia result in increased intestinal  $\text{HCO}_3^-$  secretion. Hypersalinity appears to increase the production of carbonates (Genz et al. 2008), while hypercarbia does not. During hypersalinity, the concentrations of both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  are significantly increased in luminal fluids. Since  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were lower although not completely depleted during exposure to  $\text{CO}_2$ , the lack of increased  $\text{CaCO}_3$  precipitation during hypercarbia may reflect substrate availability. The difference in responses to



hypersalinity and hypercarbia is likely due to a difference in drinking rate, which is known to be increased during hypersalinity (Genz et al. 2008) but is not impacted by hypercarbia. Little is known about calcification processes in the marine fish intestine, so additional work in this area would be valuable.

Many studies have examined the basic mechanistic response of fish to CO<sub>2</sub> in the plasma but less is known about intestinal responses. Carbon dioxide levels utilized in this study were admittedly high compared to what would be seen in the environment, even under the most drastic climate change scenarios (Meehl et al. 2007). However, in certain coastal and upwelling areas, CO<sub>2</sub> levels occasionally meet or exceed these predictions (Feely et al. 2008; Melzner et al. 2013; Thomsen et al. 2010) and fish in high density aquaculture settings regularly experience high levels of CO<sub>2</sub> (Tirsgaard et al. 2015). Finally, only a few studies have examined impacts of hypercarbia on osmoregulatory processes (Brauner et al. 2000; Shaughnessy et al. 2015), despite the fact that the maintenance of homeostasis involves the exchange of Na<sup>+</sup> and Cl<sup>-</sup> and/or HCO<sub>3</sub><sup>-</sup> and H<sup>+</sup>. The impact of hypercarbia on osmoregulation is underrepresented in the recently burgeoning area of research looking at the effects of low-level hypercarbia on fish at near-future predicted CO<sub>2</sub> levels (Heuer and Grosell 2014), and further work in this area may aid interpretation of other downstream impacts.

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## **Chapter 5: Downstream impacts of elevated CO<sub>2</sub> on neurosensory and behavioral endpoints in the Spiny damselfish (*Acanthochromis polyacanthus*)**

### **Summary**

Neurosensory and behavioral disruptions have been one of the well-documented responses to ocean acidification-relevant CO<sub>2</sub> exposures, especially in coral reef fish species. The underlying cause of these disruptions is thought to be an alteration of current across the GABA<sub>A</sub> receptor in neuronal cells due to changes in ion gradients (HCO<sub>3</sub><sup>-</sup> and/or Cl<sup>-</sup>) that occur in the body following compensation for elevated ambient CO<sub>2</sub> exposure. Despite this widely documented behavioral response, no studies have measured intracellular and extracellular acid-base parameters in a marine fish that exhibits behavioral alterations at ocean acidification-relevant CO<sub>2</sub> levels. The present study is the first to report intracellular brain HCO<sub>3</sub><sup>-</sup> (mM), brain pH, and extracellular plasma HCO<sub>3</sub><sup>-</sup> in a coral reef fish species exposed to an ocean acidification relevant CO<sub>2</sub> level (1900 μatm CO<sub>2</sub>). Spiny damselfish (*Acanthochromis polyacanthus*) exposed to 1900 μatm CO<sub>2</sub> for 4 days exhibited significantly increased intracellular and extracellular HCO<sub>3</sub><sup>-</sup> concentrations, providing evidence of CO<sub>2</sub> compensation. Damselfish exposed at this CO<sub>2</sub> level were also shown to spend significantly more time in a chemical alarm cue (CAC) compared to control fish, indicating a behavioral alteration. Measurements from this study were used to calculate the reversal potential for GABA<sub>A</sub> ( $E_{GABA}$ ), which supported the hypothesis that altered

ion gradients during compensation for CO<sub>2</sub> could alter GABA<sub>A</sub> receptor function and account for behavioral disturbances noted during low-level CO<sub>2</sub> exposure.

## **Background**

High levels of CO<sub>2</sub> exposure have been frequently employed by physiologists to study acid-base regulatory mechanisms in fish (Brauner and Baker 2009; Claiborne et al. 2002; Evans et al. 2005; Heisler 1984; Larsen and Jensen 1997). In recent years, attention to climate change and ocean acidification has led to a growing number of studies examining the effects of lower level CO<sub>2</sub> on fish (<1900  $\mu$ atm CO<sub>2</sub>). Several investigated endpoints appear to be insensitive to lower levels of CO<sub>2</sub> or may have consequences that are not overtly evident during short-term experiments. However, significant effects have been noted in many areas (Heuer and Grosell 2014), including CO<sub>2</sub>-induced alterations to mitochondrial function (Strobel et al. 2012; Strobel et al. 2013a), metabolic rate (Rummer 2013), otolith growth (Bignami et al. 2013a; Checkley et al. 2009), and reproduction (Miller et al. 2013). The most robust and consistent adverse response to low levels of CO<sub>2</sub> exposure has been noted in behavioral assays designed to assess sensory or cognitive ability. Disruptions to olfaction (Munday et al. 2009a; Munday et al. 2010; Welch et al. 2014), hearing (Simpson et al. 2011), vision (Chung et al. 2014; Ferrari et al. 2012b), lateralization (Domenici et al. 2012; Jutfelt et al. 2013), and learning (Chivers et al. 2013; Ferrari et al. 2012a; Jutfelt et al. 2013) in fish at ocean acidification relevant CO<sub>2</sub> levels demonstrate that CO<sub>2</sub> broadly affects central neuronal processing. Compared to other endpoints,

neurosensory impacts are particularly concerning since these traits appear to show little capacity for transgenerational acclimation (Welch et al. 2014). Furthermore, fish living near highly acidic natural CO<sub>2</sub> vent systems that presumably experience high CO<sub>2</sub> on a more regular basis also exhibit abnormal behavioral responses (Munday et al. 2014). Considering the rapid rate of acidification (Caldeira and Wickett 2003) and the low CO<sub>2</sub> threshold level needed to induce sensory and neurological responses (~600-800  $\mu$ atm CO<sub>2</sub>) (Heuer and Grosell 2014), understanding the physiological mechanism underlying these responses is crucial for assessing risk to fish populations and could aid in predicting adaptive capacity.

Most studies to date suggest that significant CO<sub>2</sub> effects, including behavioral disturbances, result from compensation that fish perform in response to a CO<sub>2</sub>-induced respiratory acidosis. Following exposure to CO<sub>2</sub>, fish correct pH by sustaining elevated levels of HCO<sub>3</sub><sup>-</sup> in intracellular and extracellular fluids (Claiborne et al. 2002; Esbaugh et al. 2012; Ishimatsu et al. 2004; Toews et al. 1983). Consequently, although pH is maintained at pre-exposure levels, HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> remain elevated. Increased plasma HCO<sub>3</sub><sup>-</sup> concentrations are often paired with a corresponding decline in Cl<sup>-</sup>, since equimolar exchange of these ions occurs at the gill as a part of the compensatory process (Brauner and Baker 2009; Claiborne et al. 2002; Larsen and Jensen 1997). Surprisingly, examination of CO<sub>2</sub> acid-base balance disturbances and associated compensatory mechanisms have only been performed at ocean acidification relevant scenarios in a limited number of studies (Esbaugh et al. 2012; Green and Jutfelt 2014; Strobel et al. 2012).

In 2012, Nilsson and colleagues reported a series of seminal experiments on fish, suggesting compensation for CO<sub>2</sub> affects olfaction and lateralization through alterations of ion gradients across neuronal cell membranes, affecting function of the GABA<sub>A</sub> receptor (Nilsson et al. 2012). Under most circumstances, the GABA<sub>A</sub> receptor and its associated neurotransmitter (GABA) are thought to be largely responsible for inhibitory responses throughout the vertebrate nervous system. Nilsson et al 2012 proposed a specific model to illustrate how CO<sub>2</sub> could potentially impact GABA<sub>A</sub> receptor function in fish. Under control conditions HCO<sub>3</sub><sup>-</sup> and/or Cl<sup>-</sup> ions enter the cell through the GABA<sub>A</sub> receptor, leading to cellular hyperpolarization and inhibition which are associated with a normal behavioral phenotype in fish. In contrast, changes in extracellular and/or intracellular HCO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup> that occur during CO<sub>2</sub> compensation are thought to reverse ion movement through the GABA<sub>A</sub> receptor, leading to a depolarizing excitatory response and a disrupted behavioral phenotype (Nilsson et al. 2012). The alleviation of olfactory and lateralization disturbances in CO<sub>2</sub> exposed fish with the treatment of gabazine, a GABA<sub>A</sub> receptor antagonist, effectively implicated GABA<sub>A</sub> receptor involvement in disrupted behavioral responses induced by CO<sub>2</sub>. Since this initial study, the apparent link between CO<sub>2</sub> induced behavioral disturbances and the GABA<sub>A</sub> receptor has been supported by other studies examining a variety of species (tropical and temperate), utilizing many sensory and behavioral assays, and using different GABA<sub>A</sub> receptor antagonists and agonists (Chivers et al. 2013; Chung et al. 2014; Hamilton et al. 2014; Lai et al. 2015; Ou et al. 2015). Further support for the role of the GABA<sub>A</sub> receptor in abnormal behavior during CO<sub>2</sub> exposure has

been provided by theoretical calculations of the GABA<sub>A</sub> receptor equilibrium potential ( $E_{\text{GABA}}$ ) using  $\text{HCO}_3^-$  values estimated from the Gulf toadfish (Heuer and Grosell 2014). Throughout this chapter, it is important to keep in mind that altered ion gradients due to  $\text{CO}_2$  exposure would not necessarily have to cause a current reversal to invoke a behavioral change. Even an attenuation of the normal inhibitory response of the GABA<sub>A</sub> receptor due to changes in ion gradients could alter the function of neurons and account for noted behavioral disruptions.

The hypothesis that the GABA<sub>A</sub> receptor is linked to  $\text{CO}_2$  induced behavioral disturbances is well-supported. However, alterations to acid-base parameters that would lead to an alteration of the current through the GABA<sub>A</sub> receptor have yet to be measured in a marine fish showing a behavioral disruption at ocean acidification relevant levels. Accordingly, the objective of this study was to test the prediction that altered intracellular and extracellular  $\text{HCO}_3^-$  due to  $\text{CO}_2$  compensation occurs in a species and at an ambient  $\text{CO}_2$  level that results in behavioral disturbances. To achieve this objective, one goal of this study was to measure intracellular whole-brain  $\text{HCO}_3^-$  and pH ( $\text{pH}_i$ ) as well as extracellular  $\text{HCO}_3^-$  levels in blood plasma of the spiny damselfish (*Acanthochromis polyacanthus*) exposed to control or 1900  $\mu\text{atm}$   $\text{CO}_2$ . A second goal was to apply the measured values in an assessment of GABA<sub>A</sub> receptor function by calculating  $E_{\text{GABA}}$  in control and  $\text{CO}_2$  exposed fish. A third and final goal was to confirm that spiny damselfish exposed to the applied  $\text{CO}_2$  level displayed altered behavioral responses to olfactory cues using a two-choice flume system (Munday et al. 2009a; Welch et al. 2014). To our

knowledge, this is the first study to report direct measurements of both intracellular and extracellular  $\text{HCO}_3^-$  and intracellular  $\text{pH}_i$  in a coral reef fish species.

## **Materials and Methods**

### ***Fish collection and acclimation***

Adult spiny chromis damselfish (*Acanthochromis polyacanthus*) were collected from inshore reefs at Lizard Island on the Great Barrier Reef, Australia (14° 40' S, 145° 28' E) in April 2015. Fish were caught by barrier netting using SCUBA, and immediately brought back to the Lizard Island Research Station where they were acclimated to flow through seawater tanks for 24 hours prior to the onset of experiments. For brain measurements, fish were  $19.1 \pm 2.3$  and  $17.0 \pm 1.6$  g for control and 1900  $\mu\text{atm}$   $\text{CO}_2$  exposures, respectively. Fish sampled for plasma were  $16.1 \pm 1.4$  and  $15.0 \pm 1.3$ g for control and 1900  $\mu\text{atm}$   $\text{CO}_2$  exposures, respectively.

Damselfish were then transferred to indoor 35L tanks at either control (ambient,  $\sim 450$ ) or 1900  $\mu\text{atm}$   $\text{CO}_2$  for 4 days, a time period previously demonstrated to induce olfactory behavioral abnormalities in other reef species (Cripps et al. 2011; Munday et al. 2010). Fish were kept on a 12: 12 light: dark cycle and at a consistent temperature ( $\sim 27^\circ\text{C}$ ). Fish were held in groups of  $\sim 20$ /tank, provided with PVC pipe segments for shelters, and fed daily until the day prior to sampling. Individual fish were gently netted from the exposure tanks and sacrificed using 0.02 g  $\text{l}^{-1}$  MS-222 0.2 buffered with 0.3 g  $\text{l}^{-1}$   $\text{NaHCO}_3$ . Minimal chase periods ( $< 20$  sec) were necessary to obtain individual fish.



### ***Seawater manipulation***

Lizard Island Research Station flow-through seawater was pumped into two 60 L header tanks at the Lizard Island Research Station. One tank was bubbled with air and served as the control tank, while the second was gassed with CO<sub>2</sub> to achieve ~1900 μatm CO<sub>2</sub>. A feedback system (Aqua Medic AT Control System) was used to dose CO<sub>2</sub> in to the header tank to maintain pH levels at the setpoint necessary to achieve 1900 μatm CO<sub>2</sub>. Seawater from these tanks was gravity fed to experimental tanks, where temperature, salinity, and pH<sub>NBS</sub> were recorded twice daily. Water samples were collected for total alkalinity (TA) three times through the experimental period to verify carbonate chemistry. TA was measured using Gran-titrations (Metrohm 888 Titrando Titrator Metrohm, AG, Switzerland), and referenced with certified material from Dr. A.G. Dickson (Scripps Institute of Oceanography, La Jolla, CA). Values of pH<sub>NBS</sub> and TA were put into CO2SYS using the constants K1 from Merzbach et al (1973) refit by Dickson and Miller (1987), and Dickson for KHSO<sub>4</sub> (Pierrot et al. 2006). All averages of salinity, temperature, pH, and carbonate system parameters are reported in Table 5.1 and Table 5.2.

Table 5.1: Water carbonate chemistry parameters.

	pH	PCO <sub>2</sub> ( $\mu$ atm)	Alkalinity ( $\mu$ mol kg <sup>-1</sup> )	TCO <sub>2</sub> ( $\mu$ mol kg <sup>-1</sup> )
Control (ambient)	8.14 $\pm$ .01	452 $\pm$ 9	2283 $\pm$ 6	1996 $\pm$ 8
1900 $\mu$ atm CO <sub>2</sub>	7.57 $\pm$ .02	1936 $\pm$ 95	2284 $\pm$ 4	2238 $\pm$ 4

Values are presented as means  $\pm$  standard deviation.

Table 5.2: Water chemistry parameters.

	Salinity (p.p.t.)	Temp. ( $^{\circ}$ C)
Control (ambient)	35.1 $\pm$ 0.03	27.1 $\pm$ 0.3
1900 $\mu$ atm CO <sub>2</sub>	35.1 $\pm$ 0.03	27.0 $\pm$ 0.3

Temperature was measured using a Comark meter, and salinity was measured by the Australian Institute of Marine Science (AIMS). Values are presented as means  $\pm$  standard deviation.

***Plasma and tissue analysis (brain  $\text{HCO}_3^-$  and  $\text{pH}_i$ )***

Immediately after being euthanized, the brain was quickly dissected and flash frozen in a mini mortar stored in liquid nitrogen. The tissue was powdered in the mortar using a pestle stored in liquid nitrogen attached to a cordless power tool (Cryogrinder, OPS Diagnostics, New Jersey, USA). The tissue powder was then transferred to a pre-weighed cryotube, sealed, and a final weight was taken to determine tissue mass (g). Tissue homogenization and transfer to cryotubes took place in a glove box containing a  $\text{CO}_2$ -free atmosphere. A buffer containing two metabolic inhibitors, potassium fluoride (0.16 mM) and nitrilotriacetic acid (2.9 M) and adjusted to pH 7.4 with NaOH was added to the sample (250  $\mu\text{l}$ /sample) as outlined in (Pörtner 1990). This mixture was briefly vortexed, centrifuged and immediately placed on ice. This supernatant was used for measurements of both intracellular brain pH and intracellular  $\text{HCO}_3^-$  (mM/kg).

A custom built gas-tight chamber fitted with an electrode (PHC4000-8, Radiometer, France) and surrounded by an acrylic thermostated sleeve was used to measure  $\text{pH}_i$ . Buffer was used to flush out the electrode chamber twice prior to processing each sample. The chamber was then flushed once with, and then injected with the supernatant from the homogenized tissue and pH was recorded. To determine brain  $\text{HCO}_3^-$  (mM/kg), an aliquot of the supernatant (corresponding to 200-1200 nmol) was added to 10 mL of 50 mM NaCl for double endpoint titrations (see below).

Due to the inherent difficulty in sampling smaller fish and the speed required to complete brain homogenization procedures, plasma and brain samples

were not taken from the same individual. Blood was drawn from the caudal vein into a heparinized syringe. Blood was briefly centrifuged for 30 sec and plasma was obtained for analyses.

### ***Double endpoint titrations***

Total bicarbonate and carbonate equivalents were determined in the brain supernatant and the plasma using double endpoint titrations (Brix et al. 2013; Genz et al. 2008). For all samples, an aliquot was pipetted into 10 mL of a 50 mM solution of NaCl in deionized water. Using a NaCl solution allows for stabilization of the electrode but does not affect titrations (Grosell et al. 1999). Following a 15 minute period where samples were bubbled with CO<sub>2</sub>-free gas (either nitrogen or argon) and an initial pH was recorded (PHC 3005-8, Radiometer Analytical). Gassing continued throughout the analysis. Samples were titrated using 0.01 N HCl until a stable reading at or slightly below 3.8 was determined, then titrated back up to the initial pH using 0.01 N NaOH. For quality control, the measured concentration of NaOH was determined to be 0.009482 N following back titrations with the certified acid used in this series of titrations. The same HCl and NaOH titrants were used throughout the course of experiments. Additions of acid and base titrants were dispensed manually using 2 mL microburettes with graduations every 2 uL (GS-1200, Gilmont Instruments). Total HCO<sub>3</sub><sup>-</sup> equivalents in the sample were determined by subtracting the moles of NaOH from the moles of HCl required to bring the sample back to the initial pH (Grosell et al. 1999).

### ***Olfaction behavioral assays***

As outlined in a previous study (Welch et al. 2014), the response to olfactory cues was tested by giving a fish the choice between untreated seawater and seawater containing a chemical alarm cue (CAC). In a two-channel choice flume system, control or CAC treated water was gravity fed into either side at a constant flow rate of 100 mL/min. Validation of equal flow rates was achieved using both a flow meter and a dye test following each water change.

CAC-treated seawater was made by euthanizing a donor fish of the same species with a quick blow to the head and making shallow cuts along the side of the body to mimic an injured conspecific. The fish was then rinsed with 15 mL of seawater that was collected and added to 10 L of seawater that would serve as CAC seawater in the choice system. One donor fish was used per test fish in an experimental run. Previous work has indicated that the behavioral response to a chemical cue is the same in control and elevated CO<sub>2</sub> water in at least two species (Munday et al. 2010). Accordingly, fish in the choice system were tested using seawater from their respective acclimation condition (control or 1900  $\mu$ atm CO<sub>2</sub>).

During choice tests, fish were introduced to the center of the downstream end of the flow chamber and allowed to acclimate for 2 minutes. Following acclimation, the location of the fish was recorded every five seconds over a 2 minute recording period. During a three minute “rest” period, the water sources were switched to eliminate any side biases, and the acclimation and recording period were repeated. To avoid confounding issues of handling stress, fish tested in flume trials were not used for physiological measurements outlined above.

### **Statistical analysis**

Since only two groups were compared in most studies, Student t-tests were used to compare measurements from control and CO<sub>2</sub>-treated fish. Data with *a priori* directional predictions were assessed using a one-tailed t-test and are specifically noted in the text and figures. Data that were non-parametric were analyzed using a Mann-Whitney rank sum test. Significance was determined at P<0.05 for all tests and all values are presented as means ± s.e.m.

### **Results**

Brain HCO<sub>3</sub><sup>-</sup> (mM/kg) and brain pH<sub>i</sub> (Fig. 5.1) were both significantly higher in damselfish exposed to 1900 μatm CO<sub>2</sub> for 4 days when compared to controls (Fig. 5.1; One-tailed significance for brain HCO<sub>3</sub><sup>-</sup>, P<0.03, brain pH<sub>i</sub>, P<0.001). Using these values and pK' and solubility constants from Boutilier et al 1984 (Boutilier et al. 1984), the brain PCO<sub>2</sub> was calculated using the Henderson-Hasselbalch equation and displayed no significant difference between control and CO<sub>2</sub>-exposed fish (Fig 5.1). Plasma HCO<sub>3</sub><sup>-</sup> (mM) was also significantly higher in CO<sub>2</sub> exposed fish compared to controls (P<0.008, Fig. 5.2). Due to low blood volumes and fish size, there was not enough plasma to measure pH in addition to HCO<sub>3</sub><sup>-</sup>, preventing PCO<sub>2</sub> calculations for extracellular fluids. In order to verify that the high levels of HCO<sub>3</sub><sup>-</sup> found for both brain and plasma readings were not an experimental artifact, a series of validation procedures were performed (Fig. 5.3). Double endpoint titrations on a series of blanks (NaCl solution only) and standards in the range of values measured in the brain and plasma samples indicated near perfect agreement between expected and measured values, but also indicated that

there was a small level of background  $\text{HCO}_3^-$  present, represented by the constant offset from a predicted 1:1 slope presented in Figure 5.3. This offset was small (60  $\mu\text{mol}$ ) and was subtracted from all reported values.

Fish acclimated to 1900  $\mu\text{atm CO}_2$  for 4 days spent more than half (53%) of their time in conspecific chemical alarm cue rather than untreated water when tested in two-choice flume chamber. In contrast, control fish spent only ~15% in the CAC (Fig. 5.4,  $P < 0.001$ ).

Fig. 5.1: Brain  $\text{HCO}_3^-$  (mmol/kg tissue), B) intracellular pH and C)  $\text{PCO}_2$  (Means  $\pm$  s.e.m.) in Spiny damselfish (*Acanthochromis polyacanthus*) exposed to either control (value) or 1900  $\mu\text{atm CO}_2$  for 4 days. \*denotes statistical significance from respective control value at  $P < 0.05$ .

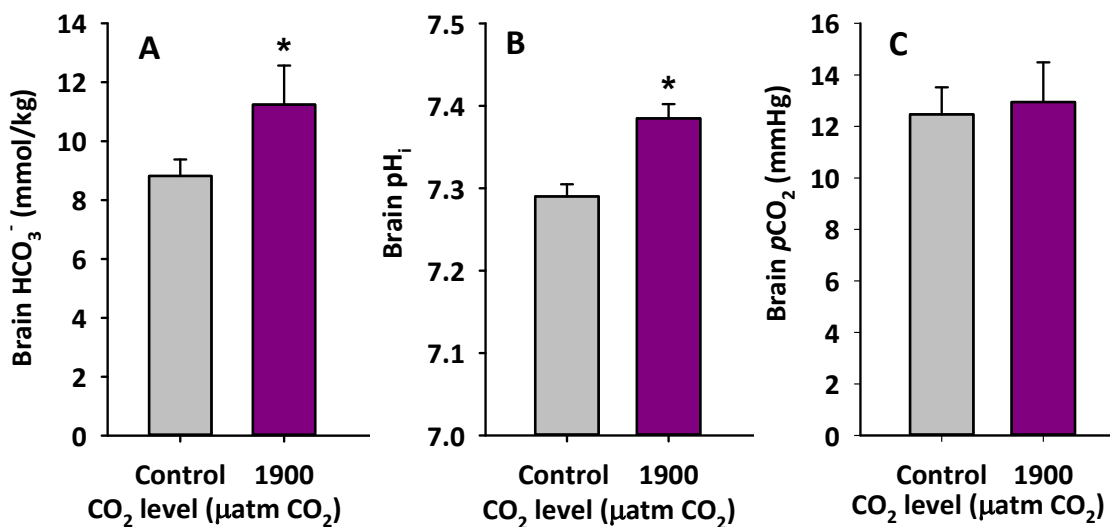


Fig. 5.2: Plasma  $\text{HCO}_3^-$  (mM) (Means  $\pm$  s.e.m.) of Spiny damselfish (*Acanthochromis polyacanthus*) exposed to either control (value) or 1900  $\mu\text{atm}$   $\text{CO}_2$  for 4 days. \*denotes statistical significance from respective control value at  $P < 0.05$ .

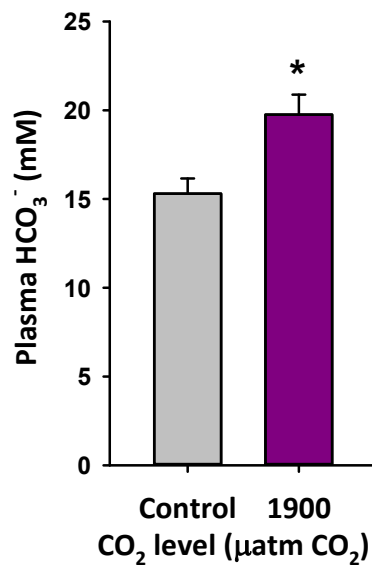




Fig. 5.3 Verification of double endpoint titration methodology using known  $\text{HCO}_3^-$  standards: Double endpoint titrations performed on titration solution (50 mM NaCl) with no addition of  $\text{HCO}_3^-$  (0),  $0.5 \mu\text{mol HCO}_3^-$ , and  $1.0 \mu\text{mol HCO}_3^-$ . The dashed line represents perfect agreement between added and measured values. The solid line represents the linear regression performed on actual measured data (slope=0.98,  $r^2=0.9991$ ). The difference between to the two lines represents background error that was corrected for in reported values.

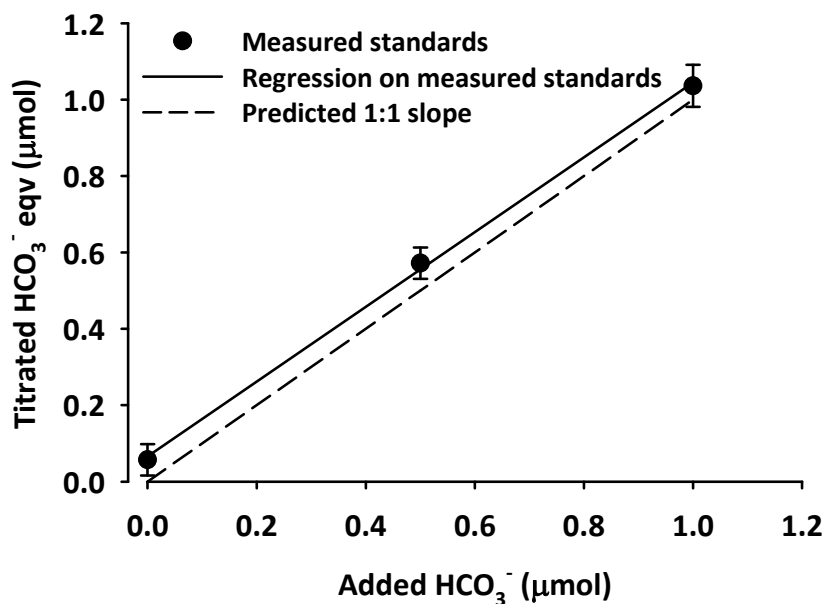
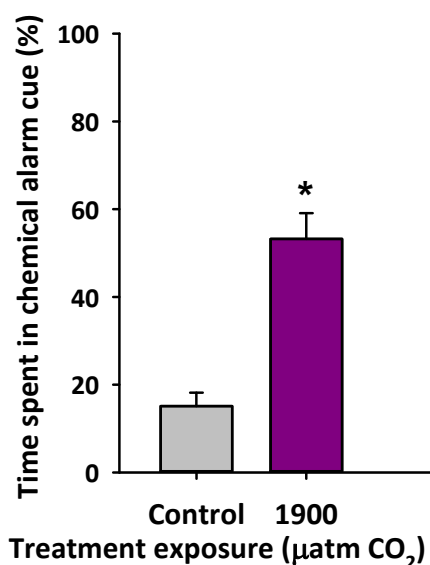


Fig. 5.4 Time spent in chemical alarm cue (CAC) using a two-flume choice chamber in fish exposed to control or 1900  $\mu\text{atm CO}_2$ : Spiny damselfish exposed to either control (value) or 1900  $\mu\text{atm CO}_2$  for 4 days were placed in a two-flume choice chamber. They were offered either control water or water containing a chemical alarm cue (CAC). Percent time (means  $\pm$  s.e.m.) spent in CAC water is shown in figure. \*denotes statistical significance from respective control value at  $P < 0.05$ .



## Conclusions

These results show that spiny damselfish, as expected, compensate for a  $\text{CO}_2$  induced acidosis by elevating plasma  $\text{HCO}_3^-$  following exposure to 1900  $\mu\text{atm CO}_2$  for 4 days and that a corresponding elevation of  $\text{HCO}_3^-$  is observed in the brain (Fig. 5.1 and 5.2). Also as predicted, this compensatory response appears to be associated with a reduced avoidance of a chemical alarm cue (Fig. 5.4), indicating an impairment to olfaction and/or central neuronal processing. Control measurements of brain  $\text{HCO}_3^-$  (8.8 mM) were high in the damselfish compared to other published values but were still within ranges calculated or measured for tissues in other species (0.5-9.2 mM) (Baker et al. 2009; Strobel et al. 2012; Wood

et al. 1990; Wood and Lemoigne 1991). Furthermore, the magnitude of  $\text{HCO}_3^-$  change (2.4 mM) compares to that found in white muscle in both the rockcod (2.7 mM) (Strobel et al. 2012) and the toadfish (3.2 mM) (Esbaugh et al. 2012; Heuer and Grosell 2014) at similar  $\text{CO}_2$  levels (1900-2000  $\mu\text{atm CO}_2$ ). It is important to note that the relative change in ions both inside and outside the cell is most relevant in assessing whether or not  $\text{CO}_2$  compensation alters function of the  $\text{GABA}_A$  receptor.

Interestingly,  $\text{pH}_i$  in the brain of  $\text{CO}_2$ -exposed fish was significantly higher than in control fish ( $\Delta 0.095$ ), demonstrating a  $\text{pH}_i$  overshoot. In the limited number of studies measuring intracellular pH at similar  $\text{CO}_2$  levels, white muscle and liver of the marbled rockcod show compensation with no overshoot (2000  $\mu\text{atm CO}_2$ ) (Strobel et al. 2012), while the white muscle of the Gulf toadfish exhibit a  $\text{pH}_i$  overshoot of a similar magnitude ( $\sim \Delta 0.07$ ; 1900  $\mu\text{atm CO}_2$ ) (Esbaugh et al. 2012) as seen in the damselfish brain. At more extreme levels of  $\text{CO}_2$  (15,000  $\mu\text{atm CO}_2$ ), brain  $\text{pH}_i$  in the white sturgeon also shows an overshoot ( $\Delta 0.3$ ) (Baker et al. 2009). As suggested in (Esbaugh et al. 2012), such an overshoot could result from active intracellular regulation to take up  $\text{HCO}_3^-$  from extracellular fluids or could merely reflect passive uptake due to higher  $\text{HCO}_3^-$  levels in extracellular fluids. Given the relatively high levels of plasma  $\text{HCO}_3^-$  reported in this study, either explanation seems possible. Regardless of the underlying cause, it is clear that  $\text{HCO}_3^-$  availability is not a limiting factor for intracellular compensation for elevated  $\text{CO}_2$ . Interestingly, the overshoot in  $\text{pH}_i$  in damselfish brains means that estimated  $\text{PCO}_2$

levels were not significantly elevated despite elevated ambient CO<sub>2</sub> and elevated intracellular HCO<sub>3</sub><sup>-</sup> concentrations (Fig. 5.1).

While the primary objective of this study was to measure and characterize acid-base parameters in the brain, measurement of plasma HCO<sub>3</sub><sup>-</sup> were also obtained, allowing for better assessment of gradients across neuronal cell membranes. The relative difference in plasma HCO<sub>3</sub><sup>-</sup> concentrations between control and CO<sub>2</sub> exposed fish was similar to that seen in toadfish ( $\Delta$ 3.3 mM; 1900  $\mu$ atm CO<sub>2</sub>), marbled rockcod ( $\Delta$ 3.2 mM; 2000  $\mu$ atm CO<sub>2</sub>), and the spotted catshark ( $\Delta$ 3.0 mM; 1000  $\mu$ atm CO<sub>2</sub>) (Green and Jutfelt 2014). However, the absolute HCO<sub>3</sub><sup>-</sup> levels for both control (15.3 mM) and 1900  $\mu$ atm CO<sub>2</sub> (19.8 mM) exposed damselfish were high compared to values reported in other species. However, a series of double endpoint titrations performed on HCO<sub>3</sub><sup>-</sup> standards and blanks alongside samples validated the obtained values did not reflect analytical errors. Ideally, blood samples should be taken from cannulated resting and unstressed fish using gastight conditions. However, due to the small size of fish in the present study, blood was collected through caudal puncture. Errors associated with this technique can result from fish being anesthetized and briefly air-exposed during sampling, preventing CO<sub>2</sub> excretion. The associated accumulation of CO<sub>2</sub> could result in an overestimation of plasma HCO<sub>3</sub><sup>-</sup> concentrations. Fish blood was sampled quickly (<1 min) to minimize error associated with these measurements, and any error would apply to control as well as CO<sub>2</sub> treated fish. Furthermore, reported values for HCO<sub>3</sub><sup>-</sup> (mM) in fish is relatively broad, ranging from ~3 mM up to ~9 mM in control animals (Baker et al. 2009; Brauner and Baker 2009; Busk et

al. 1997; Esbaugh et al. 2012; Perry et al. 2010; Strobel et al. 2012; Toews et al. 1983; Wood et al. 1990; Wood and Lemoigne 1991). Values greater than 10 have in the rockcod (11.28 mM) (Strobel et al. 2012). Together, the existing range of control values and the potential for high values in some species, lends credence to the values reported here for damselfish. It is important to keep in mind that brain  $\text{HCO}_3^-$  readings in this study were not associated with the same potential errors inherent with plasma measurements and were also at the high end of reported ranges for tissue  $\text{HCO}_3^-$  in other species. To our knowledge, these are the first reported measurements of  $\text{HCO}_3^-$  for any coral reef teleost species, and little is known about their mechanisms of gas exchange and acid-base regulation.

Measurements in the present study showing altered intracellular and extracellular  $\text{HCO}_3^-$  in a species that also shows a  $\text{CO}_2$ -induced olfactory disturbance lends strong support to Nilsson's original hypothesis. For the first time, the measurements reported herein allow for direct calculation of the reversal potential for the  $\text{GABA}_A$  ( $E_{\text{GABA}}$ ) under control conditions and  $\text{CO}_2$  conditions in the damselfish. To calculate  $E_{\text{GABA}}$  the following equation was used (Farrant and Kaila 2007)

$$E_{\text{GABA}} = \frac{RT}{F} \ln \frac{P_{\text{Cl}^-} [\text{Cl}^-]_i + P_{\text{HCO}_3^-} [\text{HCO}_3^-]_i}{P_{\text{Cl}^-} [\text{Cl}^-]_o + P_{\text{HCO}_3^-} [\text{HCO}_3^-]_o} \quad \text{Equation (1)}$$

$R$  is the ideal gas constant,  $T$  is the absolute temperature,  $F$  is Faraday's constant, and  $P$  represents the relative permeability of the  $\text{GABA}_A$  receptor for  $\text{HCO}_3^-:\text{Cl}^-$ .

Intracellular and extracellular values for  $\text{HCO}_3^-$  (Fig 5.1. and Fig. 5.2) were used to calculate  $E_{\text{GABA}}$  for damselfish. Extracellular  $\text{Cl}^-$  was assumed to be 150 mM, a typical value for marine teleosts. Under high  $\text{CO}_2$  conditions,  $\text{HCO}_3^-$  is

generally assumed to increase in extracellular fluids with a corresponding equimolar decrease in  $\text{Cl}^-$  (Brauner and Baker 2009; Claiborne et al. 2002; Larsen and Jensen 1997). Thus, the increase in  $\text{HCO}_3^-$  in control and high  $\text{CO}_2$  was used to adjust extracellular  $\text{Cl}^-$ . Intracellular  $\text{Cl}^-$  was chosen to be 8mM, within the range of values reported from a recent review (6-14 mM) (Delpire and Staley 2014). In the physiological range of both ions,  $\text{GABA}_A$  exhibits conductance for both  $\text{HCO}_3^-$  and  $\text{Cl}^-$ , but tends to be more permeable to  $\text{Cl}^-$  (Lambert and Grover 1995). Different permeability ratios ( $P$ ) have been measured in neurons in invertebrates and mammals ranging from ~0.18-0.6 (Farrant and Kaila 2007). Since values have not been reported for fish,  $E_{\text{GABA}}$  was calculated over a representative range of permeability ratios (0.2-0.5). All input variables are summarized in Table 5.3.

Calculated  $E_{\text{GABA}}$  values for damselfish exposed to control and 1900  $\mu\text{atm}$   $\text{CO}_2$  conditions show a divergent deviation from the commonly assumed resting neuronal membrane potential (-70 mV) under a range of physiologically relevant permeability ratios (0.2-0.25; Fig. 5.5). In these instances,  $E_{\text{GABA}}$  for control damselfish shows a negative deviation from resting, likely conferring a normal hyperpolarizing and inhibitory response. In contrast,  $E_{\text{GABA}}$  for  $\text{CO}_2$ -exposed damselfish show a positive deviation from resting, illustrating the potential for an abnormal depolarizing and excitatory response. In addition to damselfish, the potential for divergent responses from resting potential using calculations of  $E_{\text{GABA}}$  using acid-base parameters has also been estimated for the Gulf toadfish (Heuer and Grosell 2014). Using calculated or measured  $\text{HCO}_3^-$  levels from a previous study (Esbaugh et al. 2012; Heuer and Grosell 2014) and assuming the same  $[\text{Cl}]_i$

level (8mM) as the damselfish, toadfish also show a divergent deviation from the resting membrane potential, however, over a higher range of permeability ratios (0.38-0.5, Fig. 5.5). Finally, intracellular and extracellular values of  $\text{HCO}_3^-$  have also been calculated for white muscle in a polar fish, the marbled rockcod (*Notothenia rossii*) exposed to 2000  $\mu\text{atm CO}_2$ . Using these measurements as a proxy for the brain, it appears that at 8mM  $[\text{Cl}]_i$ , no divergent response would be noted between control and  $\text{CO}_2$  exposed fish. However, if  $\text{Cl}^-$  is assumed to at a lower value in the physiological range (6mM), divergent responses are noted over a wide range of permeability ratios (0.26-0.48; Table 5.3; calculated data not shown). Thus, under a given set of physiologically relevant scenarios in both tropical, subtropical, and polar fish, divergent responses of currents through  $\text{GABA}_A$  are noted with ocean acidification relevant  $\text{CO}_2$  exposure levels. Again, it is important to reiterate that although these calculations can illustrate a divergent deviation from resting, even an attenuation of inhibition could affect behavior.

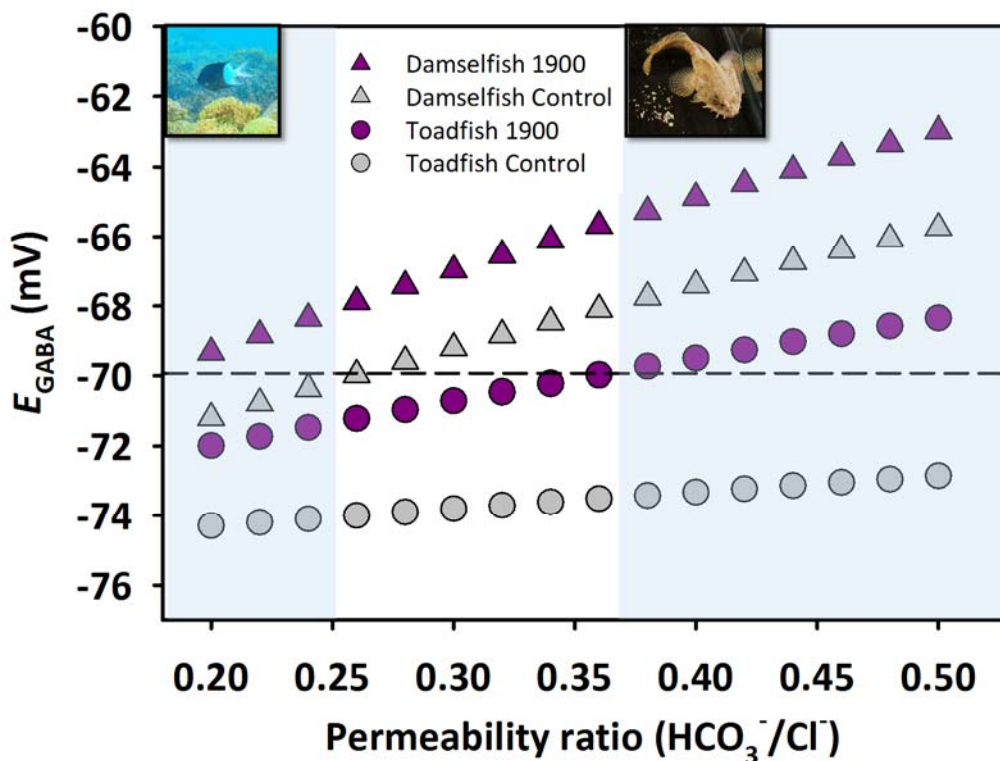
Table 5.3 Values used to calculate  $E_{GABA}$  in Figure 5.5

	Temp. (°C)	$[HCO_3^-]_o$	$[HCO_3^-]_i$	$[Cl^-]_o$	$[Cl^-]_i$
Damselfish					
Control	27	15.3	8.8	150	8
1900 $\mu$ atm $CO_2$	27	19.8	11.2	145.6	8
Toadfish					
Control	25	3.3	1.8	150	8
1900 $\mu$ atm $CO_2$	25	6.3	5.0	147	8
Rockcod					
Control	1	8.05	3.99	150	6
2000 $\mu$ atm $CO_2$	1	11.28	6.72	146.8	6
Rockcod					
Control	7	6.31	5.29	150	6
2000 $\mu$ atm $CO_2$	7	10.08	6.85	146.2	6

Values used for  $E_{GABA}$  calculations (equation 1) in Figure 5.5. Values for toadfish were taken from (Esbaugh et al. 2012) and  $HCO_3^-$  values were calculated in (Heuer and Grosell 2014) from this data. Values for the marbled rockcod were taken from (Strobel et al. 2012).  $E_{GABA}$  calculations for the rockcod are not shown in Fig. 5.5.



Fig. 5.5: Calculated  $E_{GABA}$  values across a range of permeability ratios ( $P_{HCO_3^-}/P_{Cl^-}$ ) for damselfish and toadfish: Calculated  $E_{GABA}$  values based on equation (1) over a range of physiologically relevant  $HCO_3^-/Cl^-$  permeability ratios for the  $GABA_A$  receptor in the Spiny damselfish and the Gulf toadfish. For damselfish, brain and plasma  $HCO_3^-$  concentrations from Fig. 5.1 and 5.2 were used for  $[HCO_3^-]_i$  (intracellular) and  $[HCO_3^-]_o$  (extracellular), respectively. Toadfish values were taken from (Esbaugh et al. 2012) and modeled after calculations in (Heuer and Grosell 2014) using  $[Cl^-]_i$  of 8 mM (Delpire and Staley 2014). Permeability ratios were chosen to represent a range of values previously reported to be physiologically relevant; see review (Farrant and Kaila 2007). For both species, values for extracellular  $Cl^-$  were assumed to be 150 mM (Grosell 2011a), a standard value for marine teleosts and was adjusted assuming equimolar exchange of  $HCO_3^-$  and  $Cl^-$  between extracellular fluids and the environment that has been demonstrated to occur during  $CO_2$  exposure in other teleosts (Brauner and Baker 2009; Larsen and Jensen 1997). Intracellular  $[Cl^-]$  was assumed to be 8mM (Delpire and Staley 2014). Study temperatures of 27°C and 25°C were used for the calculations for damselfish and toadfish, respectively. A standard resting neuronal membrane potential of -70 mV was used to assess divergence from resting. Shaded areas represent the range of permeability ratios in which calculated  $E_{GABA}$  diverges in opposite directions from the resting membrane potential for the species pictured. Theoretically, in these divergent zones, control fish that exhibit a calculated  $E_{GABA}$  below the dashed line (-70mV) would show a normal hyperpolarizing or inhibitory response to GABA corresponding to a normal behavioral phenotype.  $CO_2$ -exposed fish with a calculated  $E_{GABA}$  above the dashed line would show an abnormal depolarizing or excitatory response to GABA corresponding to an abnormal behavioral phenotype. Values used for calculations are summarized in Table 5.3.



Although using the  $E_{\text{GABA}}$  model in the present study is likely an oversimplification of a complex response, it may provide a useful tool for making hypotheses about patterns of behavioral disturbance. For example,  $E_{\text{GABA}}$  calculated using  $\text{HCO}_3^-$  measurements from the polar marbled rockcod experiencing combined temperature and  $\text{CO}_2$  stressors ( $7^\circ\text{C}$ ), also showed divergence from resting membrane potential at  $6\text{mM } [\text{Cl}]_i$ , but over a more narrow range of permeability ratios (0.34-0.44) than with  $\text{CO}_2$  alone (see previous paragraph). Since ocean warming and acidification are expected to occur concomitantly, it is interesting to consider how these co-stressors may influence fish behavior, since  $E_{\text{GABA}}$  is temperature dependent. The above discussion is focused on scenarios of hyperpolarizing and depolarizing effects of  $\text{GABA}_A$  in

control and CO<sub>2</sub> exposed fish, respectively, to account for behavioral impacts of CO<sub>2</sub> exposure. However, it should be noted that even shifts in the degree of current attenuation in response to GABA<sub>A</sub> could potentially alter behavior. Future work on GABA<sub>A</sub> receptors in isolated cells from CO<sub>2</sub> exposed fish would aid in interpreting the mechanism underlying CO<sub>2</sub> induced behavioral alternations. Furthermore, measurements of extracellular and intracellular chloride would be useful in future assessments of the model, since chloride levels may also be altered in a species with higher brain and plasma HCO<sub>3</sub><sup>-</sup> levels.

In conjunction with physiological measurements demonstrating altered ion gradients, there are several other factors that would aid in fully elucidating the mechanism underlying neurological disruption in fish. The GABA<sub>A</sub> receptor can vary in subunit composition which has already been predicted to confer ion permeability differences (Farrant and Kaila 2007). At least in mammals, subunit composition can vary among brain regions (Farrant and Kaila 2007; Henderson 2007; Lambert et al. 2003), with age (Henderson 2007), and developmental stage (Henderson 2007). It would be useful to know the distribution and subunit composition of GABA<sub>A</sub> receptors in fish species with noted behavioral impacts. In addition, ocean acidification could also lead to regulation of neuronal transporters and enzymes involved in HCO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup> transport and is another area of fruitful research.

In summary, this study provides the first direct measurements of extracellular and intracellular HCO<sub>3</sub><sup>-</sup> values in a coral reef species known to exhibit a behavioral disturbance. Using these measurements in calculations of  $E_{\text{GABA}}$

demonstrate that an alteration of ion movement through the GABA<sub>A</sub> receptor under high CO<sub>2</sub> conditions is likely, supporting the hypothesis proposed by Nilsson and colleagues in 2012 (Nilsson et al. 2012). However, more work on other aspects of the GABA<sub>A</sub> receptor distribution and function would greatly aid in detailing the underlying mechanisms associated with behavioral disturbances in CO<sub>2</sub> exposed fish. Neurosensory impacts induced by CO<sub>2</sub> are of particular interest in fish, since evidence to date suggests that the scope for long term acclimation (Munday et al. 2014) and/or transgenerational acclimation (Welch et al. 2014) in these traits may be limited.

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## Chapter 6: Discussion

The body of work comprising this dissertation demonstrates that compensation for CO<sub>2</sub> does not necessarily confer tolerance. Changes in the body that must occur to defend pH (Esbaugh et al. 2012), do in fact, lead to measurable downstream consequences. These impacts have been demonstrated using various whole-animal, isolated tissue, and biochemical techniques across CO<sub>2</sub> levels, species, and body systems in the present dissertation. In addition to contributing primary data to the field of fish physiology, this dissertation also motivated a thorough review of these downstream consequences across disciplines (discussed in the introduction, below and in Heuer and Grosell, 2014).

The onset of this dissertation was timed almost directly with a rapid increase in the number of reported effects of CO<sub>2</sub> on fish, largely prompted by the discovery of severe olfactory alterations in the clownfish at 1000  $\mu$ atm CO<sub>2</sub> (Munday et al. 2009a). Nearly all explanations for noted significant effects are attributed to early research demonstrating the typical compensatory response to elevated ambient CO<sub>2</sub> discussed in earlier chapters (Brauner and Baker 2009; Claiborne et al. 2002; Evans et al. 2005; Larsen and Jensen 1997; Toews et al. 1983). Following this field of study lead to the publication of a 1<sup>st</sup> authored review article in 2014 (Heuer and Grosell 2014). This article focused on identifying areas of research that would benefit from more study of mechanistic physiology. Based on the citations so far, it has been well-received in the international scientific community, thus representing a considerable contribution to the field of research examining the impacts of ocean acidification on fish. This review

article also prompted collaborations with a highly regarded research group that culminated in the data presented in Chapter 5.

Initial collaborative work I co-authored served as an important linchpin for at least two Chapters (2 and 3) of this dissertation and included measurements of plasma  $\text{HCO}_3^-$  and  $\text{PCO}_2$  in the Gulf toadfish exposed to a range of ocean acidification relevant  $\text{CO}_2$  levels (560, 780, 1000, and 1900  $\mu\text{atm CO}_2$ ) (Esbaugh et al. 2012). The relevance of this study to my dissertation was two-fold. One, this study was the first to report measurements showing compensation in a marine fish at ocean acidification relevant levels (780  $\mu\text{atm CO}_2$  and up), giving credence to hypotheses that compensation could account for noted negative effects in fish at low  $\text{CO}_2$  levels. This article is now one of the more widely cited papers in the field (30 citations according to Web of Science). The second reason this paper was relevant to my dissertation was that it provided blood chemistry measurements in study species (Gulf toadfish) at the  $\text{CO}_2$  level used for Chapter 2 and 3, anchoring any downstream noted effects to a discrete plasma measurement.

Prior to the onset of Chapter 2, little was known about the intestinal response to elevated  $\text{CO}_2$ . Laid on the groundwork of previous studies indicating the intestine could be sensitive to plasma  $\text{HCO}_3^-$  levels (Taylor and Grosell 2009; Taylor et al. 2010), this study was the first to characterize intestinal transport physiology at low levels of  $\text{CO}_2$  and laid the foundation for dissertation Chapters 3 and 4. The primary and most important finding in Chapter 2 was that Gulf toadfish exposed to 1900  $\mu\text{atm CO}_2$  experienced a substantial increase in intestinal  $\text{HCO}_3^-$  secretion. This base loss was seemingly counterproductive to whole-animal acid-

base balance, where fish employ measures to retain or uptake  $\text{HCO}_3^-$  to maintain pH at pre-exposure levels. In addition, a reduction in intestinal  $[\text{Cl}^-]$  associated with this  $\text{HCO}_3^-$  loss indicated that base loss occurs through transport pathways previously demonstrated to be significant in marine fish osmoregulation. Notably, these transport pathways in the intestine show considerable plasticity during salinity manipulations (Genz et al. 2008; Guffey et al. 2011; Sattin et al. 2010). A clear need for follow up work to examine whether or not the toadfish regulated  $\text{HCO}_3^-$  loss during  $\text{CO}_2$  exposure was addressed in Chapter 3.

In Chapter 3, an integrative approach was used to link findings from Chapter 2 showing increased  $\text{HCO}_3^-$  loss at the whole animal level, to controlled examination of isolated tissue. It was assumed that toadfish exposed to  $1900 \mu\text{atm}$   $\text{CO}_2$  over a longer acclimation period (>2 weeks), would likely regulate  $\text{HCO}_3^-$  loss noted in the intestine in Chapter 2, in favor of retaining base for whole body acid-base homeostasis. Contrary to expectations, isolated tissue analyses comparing control and  $\text{CO}_2$  acclimated fish indicated that  $\text{HCO}_3^-$  secretion rate was not suppressed, but rather stimulated with  $\text{CO}_2$  exposure. This finding suggested that increased ion movement associated with a stimulation of  $\text{HCO}_3^-$  transport pathways would likely incur an increased energetic demand at the tissue level. This suggestion was confirmed by directly measuring  $\text{O}_2$  consumption rates of isolated intestinal tissue from  $\text{CO}_2$  acclimated fish. Thus, Chapter 3 represented a unique situation where an identified downstream impact of  $\text{CO}_2$ , stimulated  $\text{HCO}_3^-$  loss through the intestine, could be directly associated with an energetic cost. This contribution to the field is significant, since metabolic costs associated with ion

regulation are notoriously variable and difficult to measure because they represent a relatively small proportion of whole-animal resting metabolic rate (Brett and Groves 1979; Ishimatsu et al. 2008; Morgan and Iwama 1999). In addition, this chapter employed the use of a novel methodology (Taylor and Grosell 2009), a custom respirometer, to measure fine-tuned differences in oxygen consumption in intestinal tissue that has only been previously used in studies examining the energetic cost of digestion (Secor et al. 2012; Taylor and Grosell 2009). Interestingly, a recent study on the Atlantic cod has demonstrated that higher levels of CO<sub>2</sub> exposure (9,200  $\mu$ atm CO<sub>2</sub>) prolong specific dynamic action (SDA) (Tirsgaard et al. 2015), a finding that would align with an elevated baseline metabolic cost associated with increased ion regulation at low levels of CO<sub>2</sub> exposure. These findings potentially put the results of Chapter 3 in a broader context.

Although strategies at the cellular level may differ, the compensatory response in the blood that occurs in response to a CO<sub>2</sub> induced acidosis appears to be consistent across species and exposure levels (Brauner and Baker 2009; Claiborne et al. 2002; Esbaugh et al. 2012; Evans et al. 2005; Heisler 1984; Larsen and Jensen 1997; Perry and Gilmour 2006). Furthermore, intestinal HCO<sub>3</sub><sup>-</sup> secretion has been noted in all marine teleosts examined to date (Grosell 2006; Grosell and Jensen 1999; Grosell et al. 2001). Together, these observations suggest that the increase in HCO<sub>3</sub><sup>-</sup> secretion and sustained energetic cost associated with increased ion transport could potentially be a ubiquitous response to elevated CO<sub>2</sub>.



Chapter 2 and 3 provided evidence that altered blood chemistry following compensation for 1900  $\mu\text{atm}$   $\text{CO}_2$  stimulates  $\text{HCO}_3^-$  secretion in the intestine that is associated with an energetic cost at the tissue level. The use of a consistent  $\text{CO}_2$  level in these studies added congruity and strength and placed the findings in an environmentally relevant context for ocean acidification (Caldeira and Wickett 2003) and current conditions in some coastal and upwelling areas (Feely et al. 2008; Melzner et al. 2013; Thomsen et al. 2010). However, using one  $\text{CO}_2$  level did not incorporate the advantage conferred by dose-dependent responses that may provide a more in-depth view of relationships between a stressor and a particular variable. Thus, Chapter 4 capitalized on the strength of dose-dependence to provide a more thorough characterization of the potential downstream consequences of  $\text{CO}_2$  compensation in the intestine using higher  $\text{CO}_2$  levels (control, 5,000, 10,000, and 20,000  $\mu\text{atm}$   $\text{CO}_2$ ). In addition, using a range of  $\text{CO}_2$  exposures also allowed for further investigation into responses that were predicted but not observed in the toadfish at lower levels of  $\text{CO}_2$  in Chapter 2.

For example, despite an expectation for an increase in intestinal carbonate production that would theoretically scale to the observed increase in  $\text{HCO}_3^-$  secretion, there was no difference in the quantity or composition of carbonates in toadfish exposed to 1900  $\mu\text{atm}$   $\text{CO}_2$  in Chapter 2. Predictions of elevated carbonate release by fish exposed to elevated  $\text{CO}_2$  seem reasonable and are interesting in the context of ocean acidification as it would represent an unusual scenario of elevated calcification and carbonate production in response to reduced ambient aragonite saturation. Notably, fish produced carbonates contribute

significantly to the oceanic inorganic carbon cycle and sedimentation of  $\text{CaCO}_3$  (Perry et al. 2011; Salter et al. 2012; Wilson et al. 2009). Such elevated carbonate excretion was observed for midshipmen exposed to 50,000  $\mu\text{atm CO}_2$  (Perry et al. 2010) (4-5 fold). The large disparity in test levels (x25 fold) and measurement in only two species, clearly show that carbonate formation is understudied with respect to hypercarbia. Research conducted in Chapter 4 revealed no change to carbonate production by toadfish, even at the highest  $\text{CO}_2$  exposure level (20,000  $\mu\text{atm CO}_2$ ), meaning either this increase does not occur, or alternatively, that the techniques employed did not possess the resolution to detect such a response.

Carbonate collection methods vary across studies. The three primary methodologies include the use of a surgically attached rectal collection sac, spot collections taken directly from the intestine of undisturbed fish, or collection of excreted carbonates from the bottom of the tank. In Chapter 2 and Chapter 4, rectal collection sacs were used, since they confer the advantage of assessing a rate of rectal base output, however, they do involve some degree of disturbance to the fish. In Chapter 4, spot collections from the intestine at matching high  $\text{CO}_2$  levels also showed no difference in carbonate production by toadfish, confirming that surgical procedures did not alter this response. In contrast, carbonates collected from the tank bottom over a 3 day period showed signs of dissolution with increasing  $\text{CO}_2$ . Results from this chapter advise caution in using tank bottom collections for studies involving  $\text{CO}_2$  manipulation, especially at high levels of  $\text{CO}_2$  (and thus low seawater pH).

Regardless of pH, the fate of fish produced carbonate upon excretion to the environment is currently being debated in the literature. Evidence suggests that high  $Mg^{2+}$  content makes fish produced carbonates much more soluble than carbonates produced by many other oceanic organisms, and could explain the increase in total alkalinity seen at shallow depths in the ocean (Wilson et al. 2009; Woosley et al. 2012). These precipitates are also hypothesized to be important components of carbonate sediments in certain areas like the Bahamas, comprising about 14-70% of samples (Perry et al. 2011). Interestingly, carbonate crystalline morphology and  $Mg^{2+}$  content can vary widely depending on the species, but most carbonates range between 18-39%  $Mg^{2+}$  (Perry et al. 2011). Recent reports from the gilt-head seabream, *Sparus aurata*, have shown the highest  $Mg^{2+}$  content to date at 54 mol%  $Mg^{2+}$  (Foran 2013). Results from Chapter 2 and Chapter 4 indicate that toadfish carbonates tend to be on the high end of the average range between 45-50%  $Mg^{2+}$ , but this composition does not change with ambient  $CO_2$  exposure.

As mentioned previously, fish produced carbonates contribute significantly to global oceanic  $CO_3^{2-}$  production (Perry et al. 2011; Salter et al. 2012; Wilson et al. 2009). This dissertation is the first to report measurements of carbonate composition and one of two to report carbonate production rates during  $CO_2$  exposure (Perry et al. 2010). All marine teleosts studied to date produce intestinal carbonates (Perry et al. 2011; Salter et al. 2012; Wilson et al. 2009), and thus measurements reported here are important in the broader context of the field. Other research groups are currently examining these questions and this dissertation will allow for comparisons among species. Even assuming all

carbonate characteristics remain the same prior to excretion, it is probable that the potential for dissolution prior to sedimentation could increase as oceans continue to acidify. Such dissolution could potentially buffer or protect alkalinity of surface waters in areas of high fish density (such as for example coral reefs), but could also reduce the flux of carbonate to deeper sediments.

In combination, Chapters 2-4 of the dissertation outline a set of effects that occur in response to a CO<sub>2</sub>-induced acidosis. Building on previous intestinal cellular models (Grosell 2011a), it is clear that CO<sub>2</sub> affects apical anion exchange. Elevated O<sub>2</sub> consumption of isolated tissue supports the assertion that there is an increase in ion transport. This increase is mostly likely rooted in an increase in Na<sup>+</sup>-coupled HCO<sub>3</sub><sup>-</sup> transport across the basolateral membrane via NBC1. This increased activity of basolateral NBC1 appears to alter demands to the Na<sup>+</sup>-K<sup>+</sup> ATPase (NKA). This dissertation lays the groundwork for a number of future studies to more fully elucidate these mechanisms. For example, future studies examining gene expression and protein activity of these transporters would be useful in conjunction with isolated intestinal tissue work using pharmaceutical reagents.

Similar to Chapters 2-4, Chapter 5 investigates the downstream effects of CO<sub>2</sub> compensation, however, this final research chapter deviates from the previous chapters by focusing on the marine fish brain and neurosensory systems. Prior to this dissertation, more than 30 studies suggested that CO<sub>2</sub> levels expected before year 2100 would induce drastic and disorienting neurosensory disruptions in fish if adaptation did not keep pace with the rate of environmental change. The

pervasiveness of these issues across sensory systems and endpoints like learning and lateralization, provided strong evidence for broader neurological malfunction during CO<sub>2</sub> exposure.

Most of these studies have speculated that altered ion gradients due to CO<sub>2</sub> compensation underlie these effects. Chapter 5 is the first, to my knowledge, to perform measurements of extracellular plasma as well as intracellular brain HCO<sub>3</sub><sup>-</sup> in an attempt to address this hypothesis. Measurements from this chapter showed that exposing a coral reef fish (the spiny damselfish) to 1900 μatm CO<sub>2</sub> led to a retention of HCO<sub>3</sub><sup>-</sup> in both the brain and the plasma, and overshoot of pHi in the brain. As expected, this response appeared to be associated with a behavioral disturbance. The contribution of this Chapter 5 to the field of ocean acidification research is considerable, since it substantiates the hypothesis that CO<sub>2</sub>-induced changes in ion gradients have the potential to alter the functionality of the GABA<sub>A</sub> receptor (Nilsson et al. 2012); a hypothesis that has been supported in many follow up experiments since its initial publication in 2012 (Chivers et al. 2013; Chung et al. 2014; Hamilton et al. 2014; Lai et al. 2015; Ou et al. 2015). Since behavioral disturbances have been noted across a wide array of exposure levels and species, and do not appear to be curtailed by transgenerational acclimation (Welch et al. 2014), the interest and impact of this work could be substantial following its dissemination. Furthermore, data for this chapter was forged from an international collaboration with a highly regarded research group responsible for the first publication and many subsequent reports demonstrating neurological disruptions in fish following CO<sub>2</sub> exposure. Research linked to this Chapter 5 is still ongoing

and includes analysis of mRNA levels in the brain and measures of whole-animal metabolic rate.

Integrating knowledge that originated in my qualifying exams examining the family of GABA receptors, I was able to take Chapter 5 one step further and integrate measured values of  $\text{HCO}_3^-$  from damselfish into calculations of the reversal potential for GABA ( $E_{\text{GABA}}$ ) under control and 1900  $\mu\text{atm}$   $\text{CO}_2$  conditions. Using this approach, I was able to demonstrate that an alteration of  $\text{GABA}_A$  current could occur with  $\text{CO}_2$  exposure under a given set of physiologically relevant parameters. This approach was also employed with success using calculated and measured values for both the sub-tropical toadfish (Esbaugh et al. 2012; Heuer and Grosell 2014), and the polar marbled rockcod (Strobel et al. 2012) exposed at the same  $\text{CO}_2$  level, demonstrating the potential of this model as a useful tool on which to base and test future hypotheses.

For example, one could examine if the presence or absence of a behavioral impact could be matched to whether or not an attenuation of current through the  $\text{GABA}_A$  receptor under a given set of measured conditions. The temperate wrasse (Sundin and Jutfelt 2015) and the Atlantic cod (Jutfelt and Hedgärde 2015) are both species that do not exhibit certain behavioral alterations and would be useful to examine in this context. A testable prediction is that these species do not exhibit drastic alterations of  $\text{HCO}_3^-$  gradients during  $\text{CO}_2$  exposures. On the same note,  $E_{\text{GABA}}$  may be useful to further investigate species that show large amounts of variation in response at a particular  $\text{CO}_2$  level. For example, olfactory responses in the damselfish (*Pomacentrus wardii*) exposed to 700  $\mu\text{atm}$   $\text{CO}_2$  (Munday et al.

2010) show a large degree of variation in response among individuals. Here, individuals displaying behavioral abnormality would be predicted to have more pronounced alterations of  $\text{HCO}_3^-$  gradients than those displaying normal behavior during  $\text{CO}_2$  exposure. Admittedly, use of this model would be strengthened with measured values for chloride and  $\text{GABA}_A$  receptor permeability in fish. Since the  $E_{\text{GABA}}$  equation is temperature-dependent, it also invites hypotheses related to temperature under future climate change scenarios.

In summary, this dissertation demonstrates that marine fish exposed to elevated  $\text{CO}_2$  are effective at compensating for an acidosis by correcting pH, but that this compensation does not confer tolerance. This concept is demonstrated by alternations to intestinal transport that lead to an increased tissue energy consumption and through noted behavioral disturbances that occur alongside altered brain neurophysiology. This dissertation is arguably of particular relevance, because of its value to both physiologists interested in mechanisms underlying intestinal transport and a broader group of scientists that are currently more focused on quantifying the effects of low level  $\text{CO}_2$  exposure on fish at ocean acidification relevant levels. One particular strength of this dissertation is the choice of species for which a set of background knowledge was already available and that were well-suited for the measurements in question.

Though noted effects in this dissertation do not result in direct mortality (and rarely do in other species), sub-lethal impacts at climate change relevant  $\text{CO}_2$  levels can affect the fitness of individuals that extends across populations and communities. Now that many sensitive (and less sensitive) endpoints have been

identified and classified, the next major contributions to the field studying impacts at low CO<sub>2</sub> levels, will be studies addressing the potential for acclimation and adaptation as well as studies delving into mechanistic physiology that explain why impacts are occurring. I feel certain that acclimation as well as adaptation to elevated CO<sub>2</sub> is and will be occurring but also that such responses may come at cost and will represent tradeoffs, as illustrated by my work.

### ***Environmental and adaptive considerations at low CO<sub>2</sub> levels***

One important, but largely unavoidable caveat to most fish ocean acidification studies is that abrupt and relatively short terms CO<sub>2</sub> exposures are inherently limiting in their ability to assess the adaptive capacity of a species. Despite this constraint, research on impacted mechanisms and pathways may show which traits are most susceptible to selective pressure (Kelly and Hofmann 2013). Multigenerational studies and/or assessment of standing variation are needed to more directly assess the scope for adaptation (Kelly and Hofmann 2013). One study examining fish living near natural highly acidic CO<sub>2</sub> seeps found behavioral defects similar to those noted in current acute studies, suggesting that this particular trait could be persistent across generations (Munday et al. 2014). Limited fish studies have aimed to quantify transgenerational effects with mixed results. Some effects are alleviated or dampened by parental exposure while others are unaffected (Allan et al. 2014; Miller et al. 2012), and at least two are not (Welch et al. 2014) making it unclear whether abrupt and short term exposures may be overestimating the impacts of CO<sub>2</sub> on fish for certain traits. General



considerations likely to be helpful in predicting adaptive capacity would be comparisons of current to historical rates of environmental change, general life history strategy, potential gene flow, background mutation rate, and existing genetic variation present in particular species (Carroll et al. 2007; Kelly and Hofmann 2013; Munday et al. 2012a; Pespeni et al. 2013; Sunday et al. 2011; Wittmann and Pörtner 2013). These are merely some of the factors discussed in recent literature regarding climate change and adaptation and is outside of the scope of the present dissertation. Readers are referred to many thorough reviews for a more detailed discussion on this topic (Crozier and Hutchings 2014; Helmuth 2009; Kelly and Hofmann 2013; Munday et al. 2012a; Somero 2010; Wittmann and Pörtner 2013).

Evaluation of both acclimation and adaptation potential should also be examined in the context of an organism's environment (Hofmann et al. 2011; Melzner et al. 2013), since large diel and seasonal variations have already been documented (Baumann et al. 2014; Godbold and Solan 2013; Hofmann et al. 2011; Reum et al. 2014; Shaw et al. 2013) and many areas of the world are already seeing levels of CO<sub>2</sub> that match global predictions beyond year 2100 (Hofmann et al. 2011; Kelly and Hofmann 2013; Thomsen et al. 2010). This occurs in coastal regions where acidification from CO<sub>2</sub> is exacerbated by nutrient runoff and hypoxia (Cai et al. 2011; Feely et al. 2010; Melzner et al. 2013; Reum et al. 2014), coastal upwelling areas (Feely et al. 2008; Hofmann et al. 2011; Kelly and Hofmann 2013), shallow coral reefs (Hofmann et al. 2011; Shaw et al. 2013), tidal marshes (Baumann et al. 2014), kelp forests (Frieder et al. 2012; Hofmann et al. 2011),

marine water in close proximity to natural CO<sub>2</sub> seeps (Fabricius et al. 2011; Hofmann et al. 2011; Munday et al. 2014), and in deeper relative to more shallow portions of the water column (McElhany and Busch 2013). In contrast, open ocean (Hofmann et al. 2011). Variability in exposure levels has generally not been included in most studies and provides another reason to be conservative when examining reported effects (Hofmann et al. 2011; McElhany and Busch 2013; Reum et al. 2014; Shaw et al. 2013), however more recent studies have begun to recognize the importance of including variability in experimental design (Ou et al. 2015).

The integration of many factors is needed to have a realistic assessment with predictive power of the effects of future projected ocean acidification on fish. To accomplish this goal, simultaneous consideration of adaptive capacity, synergistic stressors, acclimation, physiological mechanisms, community interactions, habitat type, and environmental variability would be necessary. Using acute CO<sub>2</sub> exposures is admittedly limited as a predictive tool since it does not encompass all of the aforementioned factors. However, knowing how, why, and the degree to which a certain physiological pathway is affected by a stressor may facilitate a more targeted approach in assessing the adaptive capacity of a species (Somero 2010) and provide clues to which costs of adaptation organisms may incur.

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