Changes to Intestinal Transport Physiology and Carbonate Production at Various CO₂ Levels in a Marine Teleost, the Gulf Toadfish (Opsanus beta)

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ABSTRACT

Most marine teleosts defend blood pH during high CO₂ exposure by sustaining elevated levels of HCO₃⁻ in body fluids. In contrast to the gill, where measures are taken to achieve net base retention, elevated CO₂ 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 in marine teleosts. The main objective of this study was to characterize the intestinal transport physiology of the Gulf toadfish (Opsanus beta) when exposed to varied levels of CO₂: control, 5,000, 10,000, and 20,000 µ atm CO₂ (0.04, 0.5, 1, and 2 kPa, respectively). Results of this study suggest that intestinal apical anion exchange is highly responsive to hypercarbia, evidenced by a dose-dependent increase in intestinal luminal HCO₃⁻ (mmol L⁻¹) that was mirrored by a reduction in Cl⁻ (mmol L⁻¹). Despite activation of HCO₃⁻ transport pathways typically used during osmoregulation, fractional fluid absorption was only significantly lower at the highest level of CO₂. Although increased HCO₃⁻ excretion could provide more substrate for intestinally produced carbonates, carbonate production was not significantly increased during hypercarbia at the levels tested. This study is among the first to thoroughly characterize how compensation for elevated CO₂ affects transport physiology and carbonate production in the marine fish intestine. This deeper understanding may be particularly relevant when considering the impacts of future predicted ocean acidification, where prolonged base loss may alter the energetic cost of acid-base balance or osmoregulation in marine fish.

Keywords: hypercapnia, carbon dioxide, hypercarbia, anion exchange, fish, osmoregulation, acid-base balance, HCO₃⁻, temperature, carbonate.

Introduction

The typical acid-base regulatory response exhibited by fish exposed to elevated ambient CO₂ (hypercarbia) has been well characterized across a wide array of species and exposure levels (Heisler 1984; Larsen and Jensen 1997; Claiborne et al. 2002; Evans et al. 2005; Perry and Gilmour 2006; Brauner and Baker 2009). After experiencing an initial drop in blood plasma pH and increase in pCO₂ at the onset of CO₂ exposure, most fish respond by elevating and maintaining high levels of plasma HCO₃⁻. This compensation leads to an altered steady state, where pH is maintained at preexposure levels but both HCO₃⁻ and pCO₂ remain elevated (Toews et al. 1983; Larsen and Jensen 1997; Ishimatsu et al. 2004; Michaelidis et al. 2007; Perry et al. 2010; Esbaugh et al. 2012, 2015; Ern and Esbaugh 2016). The gill is the primary organ used to cope with elevated CO₂ and is estimated to account for ~90% of dynamic acid-base regulation in fishes (Claiborne et al. 2002; Perry and Gilmour 2006). In most instances, the accumulation of HCO₃⁻ in the plasma is usually associated with a corresponding and equimolar decrease in plasma Cl⁻ (Larsen and Jensen 1997; Claiborne et al. 2002; Brauner and Baker 2009).

In addition to the gill, the intestine may also contribute to whole-body acid-base balance (Wood et al. 1999; Perry and Gilmour 2006). Most evidence to date does not suggest that the intestine dynamically regulates acid-base disturbances (Perry et al. 2010). However, the intestine is known to be responsive to changes in plasma HCO₃⁻ (Taylor et al. 2010; Heuer et al. 2012) and appears to counteract measures employed at the gill to retain HCO₃⁻ during elevated CO₂, at least during short-term exposures (Perry et al. 2010; Heuer et al. 2012). 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; Scott et al. 2008; Sattin et al. 2010; Taylor et al. 2010; Guffey et al. 2010), and thus the contribution of the intestine to acid-base balance has received less attention.
2011; Gilmour et al. 2012), 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 (Na\(^+\) and Cl\(^-\)), which ultimately drives water from the lumen into the body (Smith 1930; Skadhauge 1974; Usher et al. 1991). In part, Cl\(^-\) absorption occurs in exchange for HCO\(_3\)^- on the apical membrane, connecting base secretion to water absorption (Grosell and Genz 2006; Grosell et al. 2009b). One source of HCO\(_3\)^- for this exchange results from Na\(^+\)-coupled HCO\(_3\)^- movement from the blood to the intracellular space via the basolateral Na\(^+\):HCO\(_3\)^- cotransporter NBC1 (Taylor et al. 2010). Elevated \(p\)CO\(_2\) also provides a source of HCO\(_3\)^- for apical anion exchange, since it is a substrate for hydration via intracellular carbonic anhydrase (CAc; Wilson et al. 1996; Grosell and Genz 2006; Sattin et al. 2010). Thus, elevated CO\(_2\) may increase HCO\(_3\)^- secretion through increased transepithelial HCO\(_3\)^- movement via NBC1 or through increased hydration of intracellular pCO\(_2\). Furthermore, changes to cellular Na\(^+\) gradients that occur as a result of an increase in transepithelial HCO\(_3\)^- movement during elevated CO\(_2\) could potentially alter the Na\(^+\)-K\(^+\) ATPase (NKA), leading to increased O\(_2\) consumption and therefore metabolic CO\(_2\) production, providing yet another substrate for anion exchange following intracellular hydration.

A short-term study (3 d) exposing toadfish to elevated CO\(_2\) levels relevant for global climate change scenarios predicted for year 2300 (1,900 \(\mu\)atm CO\(_2\); Meehl et al. 2007) showed a significant increase in rectal base secretion that corresponded to a significant decrease in intestinal Cl\(^-\) (mmol L\(^{-}\)) (Taylor et al. 2010). Elevated total CO\(_2\) in the intestinal fluid of plainfin midshipman (Porichthys notatus) exposed to 1,900 \(\mu\)atm CO\(_2\); however, it is unclear whether higher CO\(_2\) levels would alter the composition.

Although the gill is rightfully well studied as the dominant organ responsible for regulating acid-base disturbances during hypercarbia in marine teleosts, it is nonetheless surprising that the intestine has received relatively little attention, since the secretion of HCO\(_3\)^- through the intestine appears to be linked to digestion, osmoregulation, and acid-base balance in the multifunctional gut of marine teleosts. While some information is available on how the marine fish intestine responds to hypercarbia (Perry et al. 2010; Heuer et al. 2012; Tseng et al. 2013; Esbaugh et al. 2015; Tirsgaard et al. 2015), most in vivo work on transport mechanisms is restricted to two species that were investigated at two \(p\)CO\(_2\) tensions that differ by 25-fold. The abundant gulf toadfish has served as a model for study of the marine fish intestine and more specifically for investigation of bicarbonate secretory pathways, anchoring findings from this study to a rich body of previous literature. The functional significance of these pathways in the face of salinity challenge and during digestion has been explored and is present in other species, but mechanisms underlying intestinal responses to elevated ambient CO\(_2\) are not well documented. Understanding these responses is especially important, since it appears that in at least some circumstances, transport pathways impacted during exposure to high CO\(_2\) may be at odds with osmoregulatory needs. Although this particular study are beyond climate change–projected scenarios, results from this study could provide insight into potential physiological consequences to be investigated at lower CO\(_2\) levels, as even small sustained perturbations to elevated CO\(_2\) could affect energetic allocations that could manifest in population-level and ecological consequences. On this background, the overarching goal of this study was to better characterize intestinal transport mechanisms in response to hypercarbia and to explore these findings in the context of whole-body acid-base balance. To achieve these goals, we capitalized on the strength of a dose-dependent study design, examining intestinal responses in the gulf toadfish exposed to a...
range of CO₂ levels (control, 5,000, 10,000, and 20,000 μatm CO₂). Our first prediction was that increased plasma HCO₃⁻ and pCO₂ that occur in the blood plasma during hypercarbia would lead to an increase in HCO₃⁻ secretion in the intestine of the toadfish. This change in intestinal transport was also expected to alter the content of other ions involved in the HCO₃⁻ transport pathway and support the idea that apical anion exchange is impacted by hypercarbia. Given the important role of HCO₃⁻ secretion in osmoregulation, the activation of this same pathway during hypercarbia was expected to potentially alter fluid transport and increase carbonate production. These predictions were tested using rectal collection sacs to examine the effect of hypercarbia on the relationship between blood acid-base chemistry and intestinal base secretion (series 1), ion concentrations in plasma and intestinal fluid (series 2), and intestinal fluid movement, base secretion rates, and carbonate composition (series 3). 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 CO₂ exposure (series 4).

Methods

Animal Collection and Care

Gulf toadfish (Opsanus beta) were obtained as bycatch from shrimp fishermen in Biscayne Bay, Florida, 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 ppt). Polyvinylchloride tubing was provided as shelter, and all fish were acclimated to the lab for at least 2 wk before experimentation. Fish were fed squid biweekly, but food was withheld for at least 2 or 3 d before experimentation to prevent variation associated with specific dynamic action (Taylor and Grosell 2009). All protocols adhered to University of Miami animal care protocols (IACUC 13-3225).

Seawater CO₂ Manipulation

Desired pCO₂ levels were attained using a pH-stat CO₂ 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 pCO₂ levels after a standard curve was generated by measuring pH at known CO₂-air gas mixes and ambient seawater pH. For experiments, a pH setpoint corresponding to each desired pCO₂ level was set, and 100% CO₂ was dosed directly into flow-through aerated tanks to achieve the chosen pCO₂ level. The pH electrode and meter (Sentix H electrode, 3310 pH meter, WTW) corresponding to each experimental tank were connected to CapCTRL software that dosed CO₂ using solenoid valves controlled by a pCO₂/pH DAQ-M digital relay instrument (Loligo Systems). Desired pCO₂ levels remained stable using this system and typically stayed within 4%–10% of setpoint values. Independent pH₅⁰₅ (National Bureau of Standards) of experimental tanks was validated on most days during each experimental run using an independent electrode (PHC3005, Radiometer). A Corning 965 CO₂ Analyzer (Corning Diagnostics) was used to further validate that desired CO₂ levels were being achieved. Total CO₂ readings and pH₅⁰₅ measurements were put into CO2SYS software to define all the other parameters of the carbonate system (pCO₂ and TA; Pierrot et al. 2006). These results, along with measurements of temperature and salinity, are presented in Table A1.

Rectal Collection Sac and Blood-Sampling Protocols (Series 1–3)

Rectal collection sacs were utilized to examine the effect of hypercarbia on the relationship between blood acid-base chemistry and intestinal base secretion (series 1), ion concentrations in plasma and intestinal fluid (series 2), and intestinal fluid movement, base secretion rates, and carbonate composition (series 3). Before surgery, individual toadfish were placed into plastic containers with multiple openings to allow for water movement and submerged directly into experimental tank bottoms at their respective CO₂ level (control, ∼400, 5,000, 10,000, and 20,000 μatm CO₂) for a 3-d 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 ± 4.2 g; 5,000 μatm: 60.8 ± 7.6 g; 10,000 μatm: 57.2 ± 4.1 g; 20,000 μatm: 63.3 ± 4.9 g). Following this acclimation period, toadfish were anesthetized (0.2 g L⁻¹ MS-222 buffered with 0.3 g L⁻¹ NaHCO₃), 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 stitch 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 d.

Following a total 6-d exposure (3-d acclimation, 3-d post-surgery), toadfish were anesthetized with MS-222 in ∼1.5 L⁻¹ of their respective treatment seawater (0.2 g L⁻¹ MS-222 buffered with 0.3 g L⁻¹ NaHCO₃). This process took around 1 min. 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), total CO₂ (Corning 965, Corning Diagnostics), 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 the rectal collection sac and samples from the intestinal tract were combined into one sample (preweighed 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 min) 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$_2$, 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$_2$ 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 disrupter), and recenterfuged (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$^{-1}$ NaCl and sonicated a final time. This 150 mmol L$^{-1}$ NaCl solution was used to stabilize pH electrode readings. Before titration, the sample was gassed with N$_2$ for a period of 15 min, and an initial pH reading was determined (PHC 3005-8, Radiometer Analytical). N$_2$ gassing continued throughout the analysis. Samples were titrated with 0.2 mol L$^{-1}$ HCl until a stable pH reading was obtained below 3.8 and then returned to the initial pH using 0.02 mol L$^{-1}$ NaOH (British Drug Houses Standard for both acid and base, Anachemia Canada). 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$^+$, Mg$^{2+}$, Ca$^{2+}$), rectal fluid (Na$^+$, Mg$^{2+}$, Ca$^{2+}$), and rectal solid (Mg$^{2+}$, Ca$^{2+}$) carbonates were measured using flame atomic absorption spectrometry with an air/acetylene flame (Varian, Palo Alto, CA). For anions, HCO$_3^-$ in plasma and rectal fluid was estimated using methods described above, while Cl$^-$ (plasma and rectal fluid) and SO$_4^{2-}$ (rectal fluid) were analyzed using anion chromatography (Dionex 120, Sunnyvale, CA).

Total rectal base secretion rate ($\mu$mol kg$^{-1}$ h$^{-1}$; series 3) was determined by adding molar amounts of HCO$_3^-$ 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 h$^{-1}$ kg$^{-1}$) 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 µatm CO$_2$ for 6 d. 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 three fish from each 20-L tank were pooled for intestinal and tank bottom measurements. The average mass (g) per fish was 54.0 ± 2.0, 54.4 ± 2.0, and 53.7 ± 1.4 for control, 10,000, and 20,000 µatm 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°C–27°C). To test the effects of temperature on toadfish carbonate production rates, toadfish were exposed to target temperatures of 21°C, 25°C, 28°C, and 31°C (see fig. 7 for measured values) for a total of 4 or 5 d. The first portion of the exposure (2.5–3.5 d) served as an acclimation period. All carbonates were cleared from the tank bottom following acclimation, and the next 1.5 d (42.9 ± 0.3 h) 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 three to five per tank in this portion of the experiment when appropriate. The average mass (g) in each tank for each treatment was 43.4 ± 2.5, 46.7 ± 5.1, 41.7 ± 4.6, and 79.1 ± 20.3 for 21°C, 25°C, 28°C, and 31°C, respectively. All other analytical procedures were 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 mean ± SEM. Since all comparisons involved at least two treatment groups in addition to a control group, one-way ANOVAs were used for all statistical comparisons, followed by Holm-Sidak tests for multiple comparisons when appropriate. Data that were not normally distributed were tested using the nonparametric Kruskal-Wallis ANOVA on ranks test followed by Dunn’s method for multiple comparisons. For tem-
serum HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}) appeared to be nearly equimolar to the increase in plasma HCO\textsubscript{3}^– (22.9 mmol L\textsuperscript{−1}) when comparing control values to the highest CO\textsubscript{2} exposure level (20,000 μatm CO\textsubscript{2}). Hypercarbia did not significantly alter Na\textsuperscript{+}, Mg\textsuperscript{2+}, or Ca\textsuperscript{2+} plasma concentration (mmol L\textsuperscript{−1}; fig. 2; table A2). Plasma osmolality generally declined with increasing levels of hypercarbia (one-way ANOVA, \(P > 0.032\); table 1); however, post hoc tests revealed a significant decrease only at the 10,000 μatm CO\textsubscript{2} exposure level.

A significant increase in intestinal fluid HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}) at all CO\textsubscript{2} levels occurred with a corresponding decline in Cl\textsuperscript{−} (mmol L\textsuperscript{−1}; significant at 10,000 and 20,000 μatm CO\textsubscript{2}; fig. 3A; table A2). This change was not equimolar as seen for the plasma, and the ratio of HCO\textsubscript{3}^– increase to Cl\textsuperscript{−} decrease dropped as pCO\textsubscript{2} levels increased. Na\textsuperscript{+} (mmol L\textsuperscript{−1}) exhibited a slight increase with increasing exposure level that was not statistically significant (fig. 3A). Outside of a transient nonsignificant increase in SO\textsubscript{4}\textsuperscript{2−} at 5,000 μatm CO\textsubscript{2}, all three divalent ions showed a significant decrease with increasing levels of CO\textsubscript{2} exposure (fig. 3B). A trend for a decrease in intestinal fluid osmolality was also evident, although this change was not statistically significant (table 1).

**Series 3: Effects of Hypercarbia on Intestinal Fluid Movement, Base Secretion Rate, and Carbonate Composition**

Both the drinking rate and the rectal fluid excretion rate did not change with CO\textsubscript{2} exposure (fig. 4). In contrast, the fraction of fluid absorbed from imbibed seawater was significantly lower at 20,000 μatm CO\textsubscript{2} (fig. 4).

Figure 1. Relationship between CO\textsubscript{2} exposure level, plasma HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}), and intestinal fluid HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}). A, Plasma HCO\textsubscript{3}^– and intestinal fluid HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}) of gulf toadfish following 6 d of exposure to control (~400 μatm CO\textsubscript{2}), 5,000, 10,000, and 20,000 μatm CO\textsubscript{2} (n = 17, 13, 13, 14 for plasma and n = 13, 8, 9, 11 for intestinal fluid, respectively). The HCO\textsubscript{3}^– concentration (mmol L\textsuperscript{−1}) of intestinal fluid was determined from excretions collected in rectal sacs affixed on day 3 of the acclimation period and sampled on day 6. B, Intestinal HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}) as a function of plasma HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}). Values are mean ± SEM. An asterisk denotes a statistically significant difference from control values (\(P < 0.05\)).

**Series 2: Effects of Hypercarbia on Ion Concentrations in Plasma and Intestinal Fluid**

Figure 2. Plasma ion concentrations (mmol L\textsuperscript{−1}) of toadfish exposed to various CO\textsubscript{2} levels. Ion concentrations (mmol L\textsuperscript{−1}) in plasma from gulf toadfish following 6 d of exposure to control (~400 μatm CO\textsubscript{2}), 5,000, 10,000, and 20,000 μatm CO\textsubscript{2} (n = 18, 12, 12, 15 for Na\textsuperscript{+}, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+}, n = 17, 13, 13, 14 for HCO\textsubscript{3}^−, and n = 18, 9, 12, 14 for Cl\textsuperscript{−}, respectively). Samples were taken from fish that had a rectal collection sac affixed on day 3 of the exposure period. Note that HCO\textsubscript{3}^− (mmol L\textsuperscript{−1}) is reported in both figures 1 and 2. Values are mean ± SEM. An asterisk denotes a statistically significant difference from control values (\(P < 0.05\)). Values are also shown in table A2.

**Results**

**Series 1: Relationship between Blood Acid-Base Chemistry and Intestinal Base Secretion**

Plasma HCO\textsubscript{3}^– exhibited a steady increase as a function of increased CO\textsubscript{2} levels (linear fit, \(f = 0.0012x + 5.6152\), \(r^2 = 0.99\), while intestinal fluid HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}) started to show signs of saturation at higher levels of CO\textsubscript{2} (quadratic polynomial fit, \(f = 63.55 + 0.0055x - 1.16e-7x^2\), \(r^2 = 0.99\); fig. 1A). A fivefold increase in plasma HCO\textsubscript{3}^– from 400 to 20,000 μatm CO\textsubscript{2} translated to around a twofold increase in HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}) in intestinal fluids (fig. 1A). When intestinal fluid HCO\textsubscript{3}^– was expressed as a function of plasma HCO\textsubscript{3}^– (mmol L\textsuperscript{−1}; fig. 1B), a quadratic polynomial fit best described the relationship (\(f = 37.34 + 5.29x - 0.075x^2\), \(r^2 = 0.99\)).

perature data, any follow-up tests were conducted using pairwise comparisons. Significant difference was determined from controls for all tests at \(P < 0.05\).
Similar to findings at lower CO₂ levels, the fluid base secretion rate and the total (fluid combined with solid CaCO₃) base secretion rate were significantly increased with CO₂ exposure (fig. 5). The solid, or CaCO₃, base secretion did not change with CO₂ exposure. Consequently, the fluid component of the base secretion became an increasing proportion of the total base secretion at increasing levels of CO₂ exposure.

It is important to note that there was some variation in ambient water temperatures, generally ranging between 22°C and 27°C, throughout the rectal collection sac experiments. Since temperature has been previously demonstrated to affect CaCO₃ precipitation rate in the sheepshead minnow (Wilson et al. 2009), the relationship between temperature and CaCO₃ production was estimated for the toadfish in this study (see “Series 5: Effects of Temperature on Carbonate Production” for more details; linear fit, $y = 1.6455x - 26.986$, $r^2 = 0.96$). Accordingly, solid CaCO₃ base secretion rates were normalized to 25°C to account for effects of inadvertent temperature variation in rectal collection sac experiments.

Ion analysis of carbonates from rectal collection sacs revealed no difference in the proportion of the CaCO₃ as Mg²⁺ relative to total Mg²⁺ and Ca²⁺ ions (table 2), suggesting that there were no changes in carbonate composition. The proportion of total Ca²⁺ or Mg²⁺ in the rectal sac excretion present as solid CaCO₃ or the intestinal fluid did not change with CO₂ exposure (table 3). These results also support the finding that the ionic composition of carbonates was not altered.

Series 4: Effect of Hypercarbia on CaCO₃ 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, we collected carbonates directly from undisturbed toadfish exposed to control, 10,000, or 20,000 µatm CO₂ for 6 d. Similar to findings from rectal collection sacs, these spot collections from toadfish showed a trend for an increase in carbonate production with an increase in $\rho$CO₂; however, it was not statistically significant (fig. 6A).

In contrast to intestinal carbonate production, precipitates collected from the tank bottom were significantly reduced at 20,000 µatm CO₂ (fig. 6B).

Series 5: Effects of Temperature on Carbonate Production

To account for the effects of temperature on carbonate production, we collected rectal carbonates produced by toadfish over ~1.5 d (42.9 ± 0.3 h) from the tank bottom. Carbonate production rate (µmol kg⁻¹ h⁻¹) increased significantly with

Table 1: Osmolality and pH from intestinal fluid and plasma of gulf toadfish (Opsanus beta) exposed to various CO₂ levels

<table>
<thead>
<tr>
<th>CO₂ level (µatm CO₂)</th>
<th>Intestinal fluid</th>
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<th>Plasma</th>
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<tr>
<td></td>
<td>mOsm</td>
<td>pH</td>
<td>mOsm</td>
</tr>
<tr>
<td>Control</td>
<td>330.7 ± 5.8 (13)</td>
<td>8.50 ± .02 (13)</td>
<td>314.2 ± 2.5 (18)</td>
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<td>8.48 ± .02 (9)</td>
<td>304.3 ± 2.8 (13)*</td>
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<td>20,000</td>
<td>316.0 ± 4.4 (11)</td>
<td>8.31 ± .02 (11)*</td>
<td>307.5 ± 2.3 (15)</td>
</tr>
</tbody>
</table>

Note. Osmolality (mOsm) and pH in blood plasma of mixed venous nature and intestinal fluid of gulf toadfish exposed to control (~400 µatm CO₂), 5,000, 10,000, and 20,000 µatm CO₂ for 6 d. Measurements of intestinal fluid were determined from the fluid component of excretions collected in rectal sacs affixed on day 3 of the acclimation period and collected on day 6. Values are mean ± SEM, with n in parentheses.

* Statistical significance from control values ($P < 0.05$).

Figure 3. Intestinal fluid ion concentrations (mmol L⁻¹) of toadfish exposed to various CO₂ levels. Concentrations (mmol L⁻¹) of monovalent (A) and divalent (B) ions from intestinal fluid of gulf toadfish following 6 d of exposure to control (~400 µatm CO₂), 5,000, 10,000, and 20,000 µatm CO₂ (n = 13, 8, 11, respectively). Ion concentrations of intestinal fluid were determined from excretions collected in rectal sacs affixed on day 3 of the acclimation period and sampled on day 6. Note that HCO₃⁻ (mmol⁻¹) is presented in both figures 1 and 3. Values are mean ± SEM. An asterisk denotes a statistically significant difference from control values ($P < 0.05$). Values are also shown in table A3.
As expected, exposing gulf toadfish over a range of CO₂ levels allowed for a more thorough characterization of the intestinal response to hypercarbia. Results from this study demonstrated that increased plasma HCO₃⁻ activated HCO₃⁻ transport pathways in the intestine that have previously been implicated in osmoregulation. The resulting intestinal HCO₃⁻ loss countered adjustments to retain HCO₃⁻ in the body needed to maintain and defend blood plasma pH. The stimulation of HCO₃⁻ transport pathways led to dilution of divalent ions in intestinal fluids with increasing CO₂ levels, suggesting potential impacts of hypercarbia on intestinal fluid absorption. Finally, despite an expectation for increased intestinal carbonate production that would theoretically scale to the observed increase in HCO₃⁻ secretion, there were no differences in the production of carbonates with increasing CO₂ exposure.

Like many other species, toadfish accumulate HCO₃⁻ to correct pH during a CO₂-induced acidosis. This linear rise in plasma HCO₃⁻ was dose dependent and tightly regulated, suggesting that toadfish have the capacity to cope with even higher CO₂ levels (fig. 1A). In contrast to the other species, toadfish appear to compensate for a CO₂-induced acidosis through HCO₃⁻ uptake rather than acid excretion, as fish held in nominally HCO₃⁻-free seawater (182 μmol L⁻¹) were unable to effectively adjust pH (Esbaugh et al. 2012). While this mechanism was used by toadfish exposed to 1,900 μatm CO₂, it is unknown whether this strategy would shift to H⁺ excretion with increasing CO₂ exposure. Plasma pH was significantly increased in the 10,000 and 20,000 μatm CO₂ exposures compared to controls (table 1). This trend would be opposite if compensation were incomplete, as has been noted at high levels of CO₂ in certain species, where extracellular pH is partially compensated and intracellular pH is preferentially regulated (Brauner and Baker 2009). Overall, the plasma pH values were low compared to previous measurements from cannulated toadfish (Esbaugh et al. 2012; Koldkjaer et al. 2013), as would be expected for samples obtained by caudal puncture due to pCO₂ increases in anesthetized and air-exposed fish (Esbaugh et al. 2015). It is also possible that samples from fish exposed to higher CO₂ levels, where blood pCO₂ was higher relative to ambient air, could have experienced greater CO₂ off-gassing, raising pH. Indeed, calculated pCO₂ values for plasma samples approach pCO₂ levels of the water as pCO₂ increased and was lower than ambient in fish exposed to 20,000 μatm CO₂ (data not shown). Accordingly, caution should be applied when interpreting the apparent overshoot of pH compensation.

The possibility that plasma HCO₃⁻ levels were underestimated in samples obtained by caudal puncture due to CO₂ off-gassing, especially at high CO₂ levels, is not supported by the linear increase in plasma HCO₃⁻. Such HCO₃⁻ loss would be highest at high CO₂ concentration and would have resulted in loss of linearity at higher CO₂ levels. Though HCO₃⁻ accumulation in the plasma does not appear to be limited, HCO₃⁻ (mmol L⁻¹) in intestinal fluid may plateau (fig. 1A), demonstrating an upper limit to HCO₃⁻ secretion into the intestine. Interestingly, when intestinal HCO₃⁻ was plotted as a function of plasma HCO₃⁻, increasing temperature (fig. 7; linear fit, $y = 1.6455x - 26.986$, $r^2 = 0.96$).

**Discussion**

Figure 4. Drinking rate, rectal fluid excretion rate, and fraction fluid absorption of toadfish exposed to various CO₂ levels. Estimated drinking rate (ml kg⁻¹ h⁻¹), rectal fluid excretion rate (ml kg⁻¹ h⁻¹), and fractional fluid absorption of gulf toadfish following 6 d of exposure to control (~400 μatm CO₂), 5,000, 10,000, and 20,000 μatm CO₂ (n = 11, 8, 9, 10, respectively). Drinking rates were estimated using intestinal fluid Mg²⁺ and SO₄²⁻ concentrations (mmol⁻¹) 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 mean ± SEM. An asterisk denotes a statistically significant difference from control values ($P < 0.05$).

Figure 5. Rectal base secretion rates of toadfish exposed to various CO₂ levels. Rectal base excretion rates (μmol kg⁻¹ h⁻¹) of gulf toadfish following 6 d of exposure to control (~400 μatm CO₂), 5,000, 10,000, and 20,000 μatm CO₂ (n = 11, 8, 9, 10, respectively). Ion concentrations of intestinal fluid were determined from the fluid component of excretions collected in rectal sacs affixed on day 3 of the acclimation period and collected on day 6. Total rectal base excretion is the sum of solid (carbonate) and fluid base equivalents. Values are mean ± SEM. An asterisk denotes a statistically significant difference from control values ($P < 0.05$).
(fig. 1B), the calculated apparent $K_m$ (9.7 mmol L$^{-1}$) was similar to that noted during in vitro isolated tissue experiments performed in a combined Ussing/pH-stat system (10.2 mmol L$^{-1}$) and was also similar to the $K_m$ for $HCO_3$ uptake in isolated tfNBCe1 expressed in xenopus oocytes (8.5 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.

Similar to many previous studies (Toews et al. 1983; Larsen and Jensen 1997; Claiborne et al. 2002; Hayashi et al. 2004; Brauner and Baker 2009), the increase in plasma $HCO_3$ was paired with a nearly equimolar decline in plasma $Cl$. All other ions (Na$^+$, Mg$^{2+}$, and Ca$^{2+}$) showed no significant differences (fig. 3). In the intestinal fluid, increased $HCO_3$ (mmol L$^{-1}$) was mirrored by a reduction in $Cl$ (mmol L$^{-1}$), providing robust support to previous findings at lower CO$_2$ levels that exposure to CO$_2$ impacts apical anion exchange in the intestine (Heuer et al. 2012). This increase in $HCO_3$ loss from the body counteracts whole-body acid-base balance, since the retention and/or uptake of $HCO_3$ is necessary for compensation. Anion exchanger slc26a6 has been cloned in both the seawater-acclimated pufferfish (Kurita et al. 2008) and the toadfish (Grossell et al. 2009b) and is an electrogenic exchanger (nHCO$_3$/Cl$^-$). Results of this study comparing the concentrations of $HCO_3$ and $Cl$ support this assertion since exchange was not equimolar in control fish. Although it is clear that anion exchange increases drastically with CO$_2$ exposure in the intestine, it appears that the ratio of $\Delta HCO_3/\Delta Cl$ at each CO$_2$ level relative to control values declines as CO$_2$ exposure increases ($\Delta HCO_3/\Delta Cl$ 1.85, 1.66, and 1.33 at 5,000, 10,000, and 20,000 $\mu$atm CO$_2$, respectively). This change in ratios is not likely due to a shift in Cl$^-$ uptake from slc26a6 to apical NCC or NKCC1 cotransport, since Na$^+$ concentrations remained stable across CO$_2$ levels (figs. 3, 8). Alternatively, this reduction could theoretically reflect an increase in the incorporation of $HCO_3$ into 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 H$^+$ pump. The H$^+$ pump secretes H$^+$ into the intestinal lumen and titrates some of the secreted $HCO_3$, reducing $HCO_3$ 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. 8). Significantly lower pH in the intestinal fluid of fish exposed to the highest CO$_2$ level (20,000 $\mu$atm CO$_2$) also supports the idea of an increase in apical H$^+$ pump activity.

All three divalent ions (Mg$^{2+}$, SO$_4^{2-}$, Ca$^{2+}$) were significantly lower at higher CO$_2$ levels (fig. 3B). 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 Ca$^{2+}$ in the rectal excretion is incorporated into the precipitation of CaCO$_3$. In isolation, a lower concentration of Ca$^{2+}$ (mmol L$^{-1}$) at higher 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 (Mg$^{2+}$ and SO$_4^{2-}$) suggests that fluid transport is impacted by hypercarbia, at least at high levels of CO$_2$. Decreased fractional fluid absorption at 20,000 $\mu$atm CO$_2$ supports this assertion and is discussed below.

Neither the calculated rate of seawater ingestion (drinking rate) nor the rectal fluid excretion rate was significantly impacted by hypercarbia (fig. 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 Na$^+$ and Cl$^-$ have also been hypothesized to control drinking rate in the Japanese eel in a feedback system where Cl$^-$ inhibits drinking at higher luminal levels but at lower levels allows for Na$^+$ to stimulate drinking (Ando and Naga-shima 1996). If this is the case for marine teleosts in general, the lack of change in Na$^+$ could explain why drinking rate was not

### Table 2: Percentage of carbonates as Mg$^{2+}$

<table>
<thead>
<tr>
<th>CO$_2$ level ($\mu$atm CO$_2$)</th>
<th>%Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50.7 ± 1.3</td>
</tr>
<tr>
<td>5,000</td>
<td>52.6 ± 2.8</td>
</tr>
<tr>
<td>10,000</td>
<td>50.5 ± 1.5</td>
</tr>
<tr>
<td>20,000</td>
<td>46.8 ± 1.6</td>
</tr>
</tbody>
</table>

Note. Percentage of whole carbonate (Ca$^{2+}$ and Mg$^{2+}$) present as Mg$^{2+}$. Values are mean ± SEM. No statistically significant differences were noted ($P < 0.05$).

### Table 3: Relative percent of Mg$^{2+}$ and Ca$^{2+}$ in intestinal fluid and CaCO$_3$ (solid) portion of excretions from rectal collection sacs

<table>
<thead>
<tr>
<th>CO$_2$ level ($\mu$atm CO$_2$)</th>
<th>% in fluid</th>
<th>% in carbonate</th>
<th>% in fluid</th>
<th>% in carbonate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.0 ± 1.8</td>
<td>82.0 ± 1.8</td>
<td>91.5 ± 0.4</td>
<td>8.5 ± 0.4</td>
</tr>
<tr>
<td>5,000</td>
<td>19.4 ± 5.0</td>
<td>80.6 ± 5.0</td>
<td>90.6 ± 1.3</td>
<td>9.4 ± 1.3</td>
</tr>
<tr>
<td>10,000</td>
<td>12.5 ± 1.5</td>
<td>87.5 ± 1.5</td>
<td>89.4 ± 0.9</td>
<td>10.6 ± 0.9</td>
</tr>
<tr>
<td>20,000</td>
<td>13.0 ± 2.1</td>
<td>87.0 ± 2.1</td>
<td>89.3 ± 1.1</td>
<td>10.7 ± 1.1</td>
</tr>
</tbody>
</table>

Note. Percent of Ca$^{2+}$ and Mg$^{2+}$, respectively, as fluid and solid (CaCO$_3$) component of total excretion collected from gulf toadfish exposed to control ($\sim$400 $\mu$atm CO$_2$), 5,000, 10,000, and 20,000 $\mu$atm CO$_2$ for 6 d ($n = 11, 8, 9, 10$, respectively). Measurements of intestinal fluid were determined from the fluid component of excretions collected in rectal sacs affixed on day 3 of the acclimation period and collected on day 6. Values are mean ± SEM. No statistically significant differences were noted ($P < 0.05$).
influenced by hypercarbia despite dramatic reductions in luminal Cl\(^-\) (mmol L\(^{-1}\)). Considering both the drinking rate and the rectal fluid excretion rate, we can assume an estimate of imbibed fluid that is absorbed. At the highest CO\(_2\) level, fish did not change their drinking rate but absorbed significantly less of the imbibed fluid than control fish (fig. 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 (∼Δ6 mOsm) than what was observed in the intestinal fluid (∼Δ15 mOsm) at 20,000 μatm CO\(_2\).

Similar to previous findings (Heuer et al. 2012), the total and fluid rate of rectal base secretion increased with CO\(_2\) exposure, but there was no change in the solid CaCO\(_3\) base secretion rate (fig. 5), suggesting that carbonate production in the toadfish does not change even at high levels of hypercarbia. Midshipman show an increased TA of carbonates when exposed to 50,000 μatm CO\(_2\) for 48 h (Perry et al. 2010), so it is unclear whether the lack of difference in toadfish reflects a species-specific difference or is related to the level of CO\(_2\) exposure or ambient temperature (the midshipman 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\(^{2+}\) incorporation could occur with hypercarbia if already low levels of Ca\(^{2+}\) become limiting for precipitation with an increase in carbonate production, since Mg\(^{2+}\) 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\(^{2+}\) 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.

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, we spot-collected carbonates from undisturbed toadfish exposed to control, 10,000, or 20,000 μatm CO\(_2\), using the same exposure time as above (6 d). Carbonates collected using this alternative method also showed no significant increase in production although a trend was observed for increased presence of CaCO\(_3\) precipitates in the intestinal lumen at 20,000 μatm (fig. 6A). In contrast, collections of carbonates from the tank bottom indicated a significant dose-dependent decrease in carbonate production that was significant at 20,000 μatm CO\(_2\). This decrease is not due to a lack of production, since the intestinal lumen shows

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**Figure 6.** Carbonates found in the toadfish intestine compared to carbonate dissolution rate in tank bottom with varied CO\(_2\) exposure. Intestinal carbonate content in A was determined from carbonates residing in the intestine of fish sacrificed after 6 d of exposure to control (∼400 μatm CO\(_2\)), 10,000, and 20,000 μatm CO\(_2\) (n = 5 groups of 3 fish). B, Net intestinal carbonate appearance in tanks of gulf toadfish acclimated to control (∼400 μatm CO\(_2\)), 10,000, and 20,000 μatm CO\(_2\) (n = 5 groups of 3 fish) for 3 d. All carbonates were cleared from the tank bottom after the first 3 d, and carbonates were collected from day 3 to day 6 to determine the carbonate production rate. Values are mean ± SEM. An asterisk denotes a statistically significant difference from control values (P < 0.05).

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**Figure 7.** Effect of temperature on carbonate production rate in the gulf toadfish. Carbonate production rate (μmol kg\(^{-1}\) h\(^{-1}\)) of gulf toadfish as a function of temperature (°C) at ambient control CO\(_2\) (∼400 μatm CO\(_2\)). Carbonate production rates were determined by performing double endpoint titrations (Genz et al. 2008) on carbonates collected from the tank bottom following ∼1.5 d (42.9 ± 0.3 h) of exposure. Temperature acclimation n numbers were 6, 12, 12, and 4 at 21.2°C, 24.7°C, 24.8°C, and 31.1°C, respectively. Due to the small size of some fish, carbonates from more than one fish per tank were pooled for analysis. Values are mean ± SEM. Letters represent statistically significant differences using a one-way ANOVA, followed by a pairwise multiple comparison (Holm-Sidak, P < 0.05). Linear fit, y = 1.6453x - 26.986, r\(^2\) = 0.96.
constant or perhaps even elevated carbonate levels at higher ambient $pCO_2$, but rather to a product of dissolution that occurred in the tank bottom due to reduced seawater pH. The low value of carbonate production (fig. 6B) assessed by collection from the tank bottom compared to that found in the rectal collection sac even at control levels 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), meaning that carbonates are bathed in highly alkaline conditions before excretion. Since the solubility and structure of carbonates vary across species, the stability of carbonates after 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.

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*<sub>t</sub>) for this increase was 3.21, a value similar to previously reported *Q*<sub>t</sub> (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). CaCO₃ 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. It is also important to note that a higher average fish mass in the highest-temperature treatment could have influenced results.

**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 CO₂ levels (1,900–20,000 μatm CO₂ in the gulf toadfish, 50,000 μatm CO₂ in the plainfin midshipmen; Perry et al. 2010; Heuer et al. 2012). Increased gene expression of AE1α in the intestine of seawater-acclimated me-daka following hypercarbia exposure (7,000 μatm CO₂) 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 HCO₃⁻ retention and defend blood pH. The functional consequence of this sustained base loss is unclear but could result in a reallocation or increase in energetic demands on the intestine, since many transporters involved in HCO₃⁻ transport are secondarily active and depend on the gradients established by NKA (Grosell 2011a).

While impacts to anion exchange with respect to CO₂ are evident across all examined CO₂ levels, the intracellular source of this extra HCO₃⁻ could come from transepithelial HCO₃⁻ transport and/or increased intracellular CO₂ hydration (fig. 8). Interestingly, the transepithelial movement of HCO₃⁻ does not generate a proton. However, the hydration of intracellular CO₂ creates HCO₃⁻ 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⁻ absorption. For example, the seawater-acclimated trout obtains all of its HCO₃⁻ 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 HCO₃⁻ transport (Grosell et al. 2005; Grosell and Genz 2006).

Unlike anion exchange, carbonate production measured using two different methods (rectal collection sacs and spot sampling) does not appear to change dramatically under high CO₂, at least in the toadfish. A third method, utilizing collections from the tank bottom, illustrated that under high CO₂ 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 Mg²⁺ and Ca²⁺, exhibited no difference. Since Mg²⁺ 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 CO₂ exposure, although ocean acidification could increase dissolution of carbonates regardless of Mg²⁺ content. Unlike CO₂, temperature does affect carbonate production, which is not unexpected given that elevated temperature elevates metabolic rate. While fluid absorption was lower at the highest CO₂ level, drinking rate and rectal fluid excretion rate were not altered with CO₂, indicating that hypercarbia does not stimulate an increase in fluid ingestion.

Furthermore, it is interesting that both salinity and hypercarbia result in increased intestinal HCO₃⁻ secretion. Hypersalinity appears to increase the production of carbonates (Genz et al. 2008), while hypercarbia does not. During hypersalinity, the concentrations of both Ca²⁺ and Mg²⁺ are significantly increased in luminal fluids. Since Ca²⁺ and Mg²⁺ were lower although not completely depleted during exposure to CO₂, the lack of increased CaCO₃ 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 in 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 CO₂ 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, CO₂ levels occasionally meet or exceed these predictions (Feely et al. 2008; Thomsen et al. 2010; Melzner et al. 2013), and fish in high-density aquaculture settings regularly experience high levels of CO₂ (Tirsgaard et al. 2015). 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⁺ and Cl⁻ and/or HCO₃⁻ and H⁺. These findings pose interesting questions about bicarbonate transport pathways and their associated functional significance in the context of evolutionary processes. 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 CO₂ levels (Heuer and Grosell 2014), and further work in this area may aid interpretation of other downstream impacts. All investigated marine teleosts appear to secrete bicarbonate (Grosell et al. 2001; Grosell 2006; Perry...
et al. 2011), meaning that any potential impacts to baseline metabolism or energetic reallocations as a consequence of sustained higher CO₂ levels expected under future CO₂ scenarios could impact ecosystems. Finally, although not demonstrated in this study, even small increases to carbonate production or alterations to carbonate composition could alter the fish contribution to the global oceanic carbon cycle.

Acknowledgments

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APPENDIX

Table A1: Water chemistry parameters for CO₂ exposure levels and temperature experiments

<table>
<thead>
<tr>
<th>CO₂ level (µatm CO₂)</th>
<th>pH (NBS)</th>
<th>pCO₂ (µatm)</th>
<th>Titratable alkalinity (µmol kg⁻¹)</th>
<th>Total CO₂ (µmol kg⁻¹)</th>
<th>Salinity (ppt)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.14 ± .02</td>
<td>4.46 ± 19.9</td>
<td>2,387.1 ± 47.8</td>
<td>2,131.7 ± 40.2</td>
<td>34.9 ± .3</td>
<td>25.2 ± .8</td>
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<tr>
<td>5,000</td>
<td>7.21 ± .01</td>
<td>4.910.4 ± 70.0</td>
<td>2,410.6 ± 52.7</td>
<td>2,408.6 ± 54.7</td>
<td>34.8 ± .4</td>
<td>24.8 ± 1.1</td>
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<tr>
<td>10,000</td>
<td>6.92 ± .01</td>
<td>9,661.6 ± 243.2</td>
<td>2,403.1 ± 38.6</td>
<td>2,660.0 ± 46.1</td>
<td>34.7 ± .4</td>
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<tr>
<td>20,000</td>
<td>6.62 ± .02</td>
<td>19,953.3 ± 487.0</td>
<td>2,373.7 ± 33.4</td>
<td>2,935.1 ± 56.6</td>
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Series 4: CaCO₃ collected from intestinal tract and tank bottom

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<tr>
<th>CO₂ level (µatm CO₂)</th>
<th>Salinity (ppt)</th>
<th>Temperature (°C)</th>
<th>pH (NBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.0 ± .2</td>
<td>26.1 ± .2</td>
<td>8.20 ± .6</td>
</tr>
<tr>
<td>10,000</td>
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<td>26.2 ± .3</td>
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<tr>
<td>20,000</td>
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<td>26.0 ± .2</td>
<td>6.70 ± .5</td>
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Series 5: Effects of temperature on carbonate production

<table>
<thead>
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<th>Temperature (°C)</th>
<th>Salinity (ppt)</th>
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<tr>
<td>21.2 ± .5</td>
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<tr>
<td>24.7 ± .3</td>
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<tr>
<td>28.3 ± .3</td>
<td>34.5 ± .03</td>
</tr>
<tr>
<td>31.1 ± .4</td>
<td>34.2 ± .1</td>
</tr>
</tbody>
</table>

Note. Values are presented as mean ± SEM. NBS = National Bureau of Standards.

Table A2: Plasma ion concentrations (mmol L⁻¹) of toadfish exposed to various CO₂ levels

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5,000 (µatm CO₂)</th>
<th>10,000 (µatm CO₂)</th>
<th>20,000 (µatm CO₂)</th>
</tr>
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<tbody>
<tr>
<td>Na⁺</td>
<td>159.0 ± 2.7</td>
<td>153.1 ± 2.6</td>
<td>161.2 ± 5.0</td>
<td>156.3 ± 2.2</td>
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<tr>
<td>Cl⁻</td>
<td>131.6 ± 6.6</td>
<td>123.3 ± 9.8</td>
<td>106.0 ± 6.4*</td>
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<tr>
<td>HCO₃⁻</td>
<td>5.7 ± .3</td>
<td>11.9 ± .6*</td>
<td>17.0 ± .4*</td>
<td>28.6 ± .6*</td>
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<tr>
<td>Mg²⁺</td>
<td>2.7 ± .1</td>
<td>2.5 ± .2</td>
<td>2.4 ± .1</td>
<td>2.7 ± .2</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2.2 ± .2</td>
<td>2.0 ± .2</td>
<td>1.9 ± .2</td>
<td>2.0 ± .2</td>
</tr>
</tbody>
</table>

Note. Ion concentrations (mmol L⁻¹) in plasma from gulf toadfish following 6 d of exposure to control (~400 µatm CO₂), 5,000, 10,000, and 20,000 µatm CO₂ represented in figure 2. Values are mean ± SEM. * Statistically significant difference from control values (P < 0.05).
Table A3: Intestinal fluid ion concentrations (mmol L\(^{-1}\)) of toadfish exposed to various CO\(_2\) levels

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5,000 (μatm CO(_2))</th>
<th>10,000 (μatm CO(_2))</th>
<th>20,000 (μatm CO(_2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>28.2 ± 1.6</td>
<td>33.6 ± 4.0</td>
<td>33.6 ± 2.8</td>
<td>36.8 ± 3.8</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>79.0 ± 4.8</td>
<td>67.2 ± 8.9</td>
<td>55.3 ± 5.9*</td>
<td>33.3 ± 4.7*</td>
</tr>
<tr>
<td>HCO(_3)^-</td>
<td>65.4 ± 3.0</td>
<td>88.9 ± 3.8*</td>
<td>106.5 ± 2.8*</td>
<td>127.2 ± 2.9*</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>172.6 ± 7.1</td>
<td>157.3 ± 5.1</td>
<td>143.3 ± 7.0*</td>
<td>119.8 ± 6.3*</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>3.3 ± 3.0</td>
<td>2.9 ± 3.0</td>
<td>2.2 ± 3.0*</td>
<td>2.1 ± 1.0*</td>
</tr>
<tr>
<td>SO(_4)^2-</td>
<td>85.6 ± 6.6</td>
<td>93.8 ± 12.1</td>
<td>86.5 ± 6.2</td>
<td>58.9 ± 6.9</td>
</tr>
</tbody>
</table>

Note. Ion concentrations (mmol L\(^{-1}\)) in intestinal fluid from gulf toadfish following 6 d of exposure to control (~400 μatm CO\(_2\)), 5,000, 10,000, and 20,000 μatm CO\(_2\) represented in figure 3. Ion concentrations of intestinal fluid were determined from excretions collected in rectal sacs affixed on day 3 of the acclimation period and sampled on day 6. Values are mean ± SEM.

* Statistically significant difference from control values (P < 0.05).

Literature Cited


